Menara Perkebunan 2026, 94(1), 1-13
p-ISSN: 0125-9318/ e-ISSN: 1858-3768
http://dx.doi.org/10.22302/iribb.jur.mp.v94i1.704
Accreditation Number: 177/E/KPT/2024

Impact of pasteurization on tetracycline residues, physicochemical, microbiological, and antioxidant properties of acacia honey

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Received 14 Jan 2026/ Revised 12 Mar 2026/ Accepted 30 Mar 2026

Abstract

Honey is recognized for its high nutritional and functional value, yet it remains vulnerable to contamination by antibiotic residues such as tetracycline when beekeeping practices are not properly managed. Post-harvest pasteurization is widely utilized to improve honey safety and quality. This study examined the effects of pasteurizing acacia (*Apis mellifera*) honey at 60 °C for 10, 20, or 30 minutes on tetracycline and oxytetracycline residue levels, as well as on its physicochemical, microbiological, and antioxidant properties. Honey samples were artificially contaminated with antibiotics at 1 µg g^{-1} and analyzed using high-performance liquid chromatography (HPLC). Pasteurization at 60 °C significantly reduced oxytetracycline residues by approximately 33% ($P<0.01$), with comparable reductions observed at 10, 20, and 30 minutes, indicating that longer pasteurization did not provide additional significant benefits in residue degradation. Tetracycline residues decreased slightly but not significantly. Pasteurization also decreased moisture content and diastase enzyme activity, while acidity increased with longer pasteurization durations. Hydroxymethylfurfural levels remained very low (<1 mg kg^{-1}), indicating minimal thermal damage. Reducing sugar and sucrose levels were largely unchanged, total plate count (TPC) decreased slightly but not significantly, and yeasts and molds were undetectable after 10 minutes of pasteurization. Antioxidant activity showed a slight, non-significant reduction, whereas flavonoid content declined substantially due to heat exposure. In summary, pasteurization at 60 °C for 10 minutes effectively reduces antibiotic residues and microbial presence in honey, but also affects certain quality parameters, particularly diastase enzyme activity, acidity, and flavonoid content.

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[Keywords: antibiotic degradation, diastase enzyme, flavonoids, hydroxymethyl-furfural, reducing sugar]

Introduction

Honey is a natural product derived from floral nectar collected by bees and is widely recognized for its nutritional value and health-promoting properties. It contains diverse nutrients and biologically active compounds, such as flavonoids, phenolic substances, and enzymes, which play a significant role in its antioxidant and anti-inflammatory activities (Al-Farsi et al., 2018). The primary components of honey are simple sugars—predominantly fructose and glucose—along with small amounts of proteins, enzymes, vitamins, minerals, organic acids, and phenolic compounds that contribute to its antioxidant and antimicrobial properties (Da Silva et al., 2016). Beyond serving as a natural sweetener, honey is widely used in the food, pharmaceutical, and cosmetic industries.

Honey quality and safety can be affected by microbiological contamination and antibiotic residues. Antibiotics are commonly applied in apiculture to control brood diseases, which may result in residues in honey and affect its quality and safety (Wang et al., 2022). Tetracycline antibiotics, such as tetracycline and oxytetracycline, help control bacterial diseases in bee colonies. For example, oxytetracycline is used to treat foulbrood disease in the US, Canada, and the UK (Wang et al., 2022). Many countries and organizations have set Maximum Residue Levels (MRLs) for tetracycline antibiotics in honey. Japan's MRL is 0.1 mg kg^{-1}. The UK and Switzerland have MRLs of 0.02–0.05 mg kg^{-1}, while Codex Alimentarius sets MRLs of 0.1–1.2 mg kg^{-1}. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) use 0–0.03 mg kg^{-1} (Khosrokhavar et al., 2021). In Indonesia, the National Standardization Agency (BSN) set MRLs

Menara Perkebunan is DOAJ indexed Journal and accredited as Sinta 2 Journal (https://sinta.kemdikbud.go.id/journals/profile/3125)
How to Cite: Al Farisi, F., Radiati, L. E., Manab, A., Amalia, F., Pinilih, W.D., Metrizal & Dimawarnita, F. (2026). Impact of pasteurization on tetracycline residues, physicochemical, microbiological, and antioxidant properties of acacia honey. *Menara Perkebunan, 94*(1), 1-13.
http://dx.doi.org/10.22302/iribb.jur.mp.v93i1.704

for tetracycline in animal foods at 0.1 mg kg^{-1} for chicken meat and 0.05 mg kg^{-1} for eggs and milk (BSN, 2000). Tetracycline residues can disrupt gut bacterial balance and increase bacterial resistance, thereby reducing the effectiveness of antibiotics (Khosrokhavar et al., 2021).

Pasteurization is often used after harvest to stabilize honey, reduce moisture, and extend shelf life. Ramly et al. (2021) describe pasteurization as pasteurizing honey to 40-80 °C for a set time. This process lowers moisture, stops fermentation, and helps honey last longer. Molino et al. (2011) found that pasteurizing Spanish multifloral honey at 78 °C for 1 minute reduced tetracycline residues by 10–18%. Kellnerová et al. (2015) found that pasteurizing fresh cow's milk at 85 °C for 3 seconds reduced oxytetracycline and tetracycline by 40% and 30%, respectively. However, pasteurization can also change honey's physical and bioactive qualities and increase hydroxymethylfurfural (HMF) levels, which shows sugar breakdown. It can also lower diastase enzyme activity and change honey's acidity and pH (Kędzierska-Matysek et al., 2016; Ribeiro et al., 2018). Because of these effects, it is important to study how pasteurization affects tetracycline residues and honey quality. This balance is needed for food safety and product quality.

The objective of this study is to evaluate the effects of pasteurizing honey at 60 °C for different durations on tetracycline and oxytetracycline residues, as well as on the physicochemical, microbiological, and bioactive properties of acacia honey. The results are intended to inform optimal pasteurization conditions that ensure honey safety while maintaining honey quality and functional properties.

Material and Methods

Materials

Pure *Apis mellifera* (Acacia) blossom honey was obtained from PT Kembang Joyo Beekeeping, Malang. Chemicals and reagents utilized throughout this research included tetracycline and oxytetracycline standards (Sigma-Aldrich), Milli-Q water, acetonitrile p.a., methanol p.a., oxalic acid, acetonitrile (HPLC grade), methanol (HPLC grade), NaOH 0.05 M, HCl, $NaHSO_3$, Carrez I and II, NaCl, iodine solution, Luff solution, $(NH_4)_2HPO_4$, KI 20%, H_2SO_4 25%, sodium thiosulfate 0.1 N, starch solution 0.5%, Buffered Peptone Water (BPW), Dichloran 18% glycerol agar (DG-18) medium, DPPH (2,2-diphenyl-1-picrylhydrazyl), $AlCl_3$, HMF standard and quercetin standard (Sigma-Aldrich).

The instruments used in this study comprised an analytical balance (Ohaus, USA), a water bath model WNB14 and oven (Memmert GmbH, Schwabach, Germany), a digital thermometer, an open-air shaker, and an HPLC UV-Vis Detector model L-2420 (Hitachi High-Technologies, Tokyo, Japan). Centrifugation was performed using Eppendorf Centrifuge 5810R and Centrifuge 5430 (Eppendorf AG, Hamburg, Germany). Additional equipment included a vortex mixer, a rotary evaporator (Heidolph Instruments GmbH, Germany), a refractometer, and a hot plate type 220 (Thermo Scientific, USA). Thermal treatments were conducted using a furnace (Carbolite Gero Ltd, UK), while mixing was facilitated by a magnetic stirrer. Spectrophotometric analysis was performed using a UV-Vis spectrometer (model T80+; PG Instruments Ltd, UK). Other laboratory apparatus included porcelain crucibles, Petri dishes, burettes, a pH meter model HI 3512 (Hanna Instruments, USA), measuring cylinders, Erlenmeyer flasks, pipettes, a colony counter, a stomacher, filters, and Whatman No. 1 filter paper.

Sample preparation and pasteurization treatment

Honey samples (100 g) were weighed into 300 mL glass bottles using an analytical balance. Prior to artificial contamination, the samples were analyzed to confirm the absence of tetracycline and oxytetracycline residues. Artificial contamination was performed by spiking each sample with 1 mL of tetracycline and oxytetracycline standard solutions (100 µg mL^{-1}), resulting in a final concentration of 1 µg g^{-1} in the honey matrix. This concentration level was selected to simulate contamination scenarios exceeding the residue levels typically reported in honey. The spiked samples were homogenized using an open-air shaker at 195 rpm for 30 minutes to ensure uniform distribution of the antibiotics. Notably, Bonerba et al. (2021) reported that no maximum residue limits (MRLs) for tetracycline antibiotics have been established for honey in either the European Union or Codex Alimentarius, and the presence of antibiotic residues in apicultural products is generally considered unacceptable.

Pasteurization was carried out by placing honey samples in sealed glass bottles in a thermostatically controlled water bath (Memmert WNB14). The water bath was set at 65 °C to ensure that the internal honey temperature reached and was maintained at 60 ± 1 °C, which was continuously monitored using a calibrated digital thermometer. Pasteurization durations were applied according to the treatments (10, 20, and 30 minutes), with three replicates for each condition. After pasteurization, samples were rapidly cooled in an ice bath and stored at 10 ± 2 °C under controlled refrigeration until further analysis. The experimental treatments consisted of different pasteurization durations at 60 °C: P0 (without pasteurization), P1 (10 minutes), P2 (20 minutes), and P3 (30 minutes).

Tetracycline residue analysis using HPLC

Confirmation testing for tetracycline-class antibiotic residues was conducted before treatment to ensure that the honey samples were free from tetracycline residues, and after pasteurization treatment using a modified protocol described by Molino et al. (2011). One gram of honey was combined with 6 mL of McIlvain buffer (pH 4), homogenized with a vortex mixer for 15 minutes, and centrifuged at 4000 rpm for 30 minutes using an Eppendorf 5810R centrifuge. The resulting supernatant was filtered through Whatman No. 1 paper and subsequently passed through a C-18 cartridge that had been preconditioned with 5 mL acetonitrile (p.a.), 5 mL oxalic acid (pH 3), and 3 mL saturated EDTA. The cartridge was rinsed with 10 mL Milli-Q water, then eluted with 12 mL ethyl acetate:methanol (90:10 v/v). The eluate was evaporated to dryness under vacuum at 40 °C using a Heidolph rotary evaporator. The residue was then reconstituted in 1 mL of mobile phase containing 0.01 M oxalic acid and acetonitrile:methanol (3:5 v/v), and transferred to a 2 mL vial.

Analysis was performed using a Hitachi HPLC UV-Vis Detector L-2420 equipped with a UV-Vis Detector L-2420 and an ACE C18 column (250 × 4.1 mm, 5 µm). Detection was carried out at 365 nm, with a flow rate of 1 mL min^{-1}, column temperature maintained at 25 °C, and a total run time of 10 minutes. The retention times (RT) were 5.68 minutes for oxytetracycline and 6.44 minutes for tetracycline.

Moisture content

Moisture content determination followed SNI 8664:2018 by measuring the refractive index of honey using a refractometer at 20 °C (or corrected to 20 °C if measured at another temperature). The refractive index values were compared with standard tables to determine the honey's moisture content.

Ash content

Ash content was determined in accordance with SNI 8664:2018, based on SNI 2892:1992. Three grams of honey were weighed into a pre-weighed porcelain crucible using an Ohaus balance. The sample was evaporated on a water bath until dry, charred on a Thermo Scientific hot plate type 220, and incinerated in a Carbolite furnace at a maximum temperature of 550 °C until complete ashing was achieved. The crucible was cooled in a desiccator and reweighed repeatedly until a constant mass was obtained. The percentage of ash was calculated using the following equation:

$$Ash\ content\ (\%) = \frac{(W_1 - W_0)}{W}\ x\ 100\% \qquad (1)$$

where W is the initial sample weight, W_0 is the weight of the empty crucible, and W_1 is the weight of the crucible with ash.

Water-insoluble solids

Determination of water-insoluble solids was performed according to SNI 8664:2018, referring to SNI 2892:1992. Approximately 20 g of honey was dissolved in 200 mL of hot water, and the insoluble portion was collected on Whatman No. 1 filter paper that had been pre-dried and pre-weighed. The beaker and filter paper were rinsed with hot water, then dried in an oven at 105 °C for 2 hours until constant weight. Water-insoluble solid content was calculated using the formula:

$$Water - insoluble\ solids\ (\%) = \frac{(W_1 - W_2)}{W}\ x\ 100\% \qquad (2)$$

where W = weight of sample, W_1 = weight of filter paper with residue, and W2 = weight of empty filter paper.

Acidity

Acidity determination followed SNI 8664:2018. A 10 g portion of honey was dissolved in 75 mL of CO_2-free water and mixed using a magnetic stirrer. The initial pH was recorded using a pH meter, and the solution was titrated with 0.05 M NaOH until the pH reached 8.50. Subsequently, 10 mL of 0.05 M NaOH was subjected to back-titration with 0.05 M HCl until the pH decreased to 8.30. A blank test was performed by titrating 75 mL CO_2-free water with NaOH until the pH reached 8.50. Total acidity was calculated as the sum of free acid and lactone using the following formulas:

$$\text{Free acid} = \frac{(V_{NaOH} - V_{blank}) \times N_{NaOH} \times 1000}{\text{weight sample}} \qquad (3)$$

$$\text{Lactone} = \frac{(10 - V_{HCl}) \times N_{HCl} \times 1000}{\text{weight sample}} \qquad (4)$$

Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural (HMF) was determined using UV-VIS HPLC following the AOAC Official Methods of Analysis (1990), with modifications as described by Kukurova et al. (2006). A 10 g portion of honey was diluted to 50 mL with Milli-Q water. The solution was clarified using Carez I and II reagents, filtered through a 0.45 µm nylon membrane, and subsequently injected into the HPLC system. The chromatographic separation was performed on a Nucleosil C18-RP column (250 mm × 4 mm, particle size 5 µm). The HPLC operating conditions were as follows: isocratic mobile phase consisting of 90% deionized water and 10% acetonitrile; flow rate of 1.0 mL/min; injection volume of 20 µL. All solvents used were of HPLC grade. HMF was identified by comparing the

chromatographic peak of honey samples with that of a standard HMF solution (purity > 95.0%, Sigma–Aldrich), and by spectral comparison between the standard and sample. Quantification was carried out using an external calibration curve, with absorbance measured at 280 nm. The eluent was degassed prior to use in an ultrasonic bath. The total analysis time was approximately 15 minutes.

Diastase enzyme activity

Diastase activity was determined according to SNI 8664:2018 by incubating a honey solution with starch and measuring the time to the endpoint photometrically. Five grams of the honey sample was dissolved in distilled water and acetate buffer, then NaCl solution was added to a 25 mL volumetric flask, and the absorbance was measured after mixing with starch solution. The mixture was incubated in a water bath at 40 °C for 15 minutes. Aliquots were collected at 5-minute intervals, combined with iodine solution, and their absorbance was measured at 660 nm until the value decreased below 0.235. Diastase activity (DN) was calculated using the formula:

$$DN = {}^{300}/_{t} \quad (5)$$

where t represents the time (minutes) required to reach the absorbance threshold.

Reducing sugar (glucose)

Reducing sugar content was determined using the Luff-Schoorl method in accordance with SNI 8664:2018, referring to SNI 2892:1992. A 2 g portion of honey was accurately weighed, dissolved in water, and treated with half-basic lead acetate and $(NH_4)_2HPO_4$ solution to precipitate interfering substances. The mixture was filtered, and 10 mL of the filtrate was transferred into an Erlenmeyer flask, diluted with distilled water, and combined with the Luff solution and boiling stones. The solution was heated for 10 minutes, then cooled in an ice bath. Subsequently, 20% KI and 25% H_2SO_4 were added, and the mixture was titrated with 0.1 N sodium thiosulfate using 0.5% starch solution as an indicator. The titration value of the sample was compared with the blank, and the reducing sugar content before inversion was calculated using the formula:

$$\%\ \text{Reducing sugar} = \frac{(W_1 \text{ x fp} \times 100\%}{\text{W}} \quad (6)$$

where W_1 = mass of glucose corresponding to the volume of thiosulfate used, fp = dilution factor, and W = sample weight.

Sucrose

Sucrose content was determined using the Luff-Schoorl method in accordance with SNI 8664:2018, referring to SNI 2892:1992. 50 mL of filtrate from the reducing sugar test was pipetted, mixed with 25 mL of 25% HCl, and hydrolyzed at 58–70 °C for 10 minutes. The hydrolyzed solution was cooled, neutralized with 30% NaOH using phenolphthalein as an indicator, and diluted to a final volume of 100 mL in a volumetric flask. Then, 10 mL of the solution was pipetted into an Erlenmeyer flask, diluted with distilled water, and mixed with Luff solution and boiling stones. The mixture was boiled for 3 minutes, held for 10 minutes, cooled in ice, and treated with 20% KI and 25% H_2SO_4. Titration was performed using 0.1 N sodium thiosulfate with 0.5% starch solution as an indicator. The difference in sugar content before and after inversion was multiplied by a correction factor of 0.95 to determine sucrose content, calculated as:

$$\%\ Sucrose = 0.95\ x\ (\%\ sugar\ after\ inversion - \%\ sugar\ before\ inversion) \quad (7)$$

Total plate count (TPC)

The determination of TPC was performed according to SNI 2897:2008. For sample preparation, 25 g or 25 mL of honey was aseptically transferred into a sterile container. To obtain a 10^{-1} dilution, 225 mL of sterile Buffered Peptone Water (BPW) was added to a sterile bag containing the sample. Serial dilutions were prepared by transferring 1 mL of the 10^{-1} suspension into 9 mL of sterile BPW to produce a 10^{-2} dilution; subsequent dilutions were prepared as needed. From each dilution, 1 mL was inoculated in duplicate into sterile petri dishes, followed by the addition of 15–20 mL of Plate Count Agar (PCA) cooled to 45 ± 1°C. The mixture was homogenized using the plate-rotation method and then allowed to solidify. Plates were incubated in an inverted position at 34–36 °C for 48 hours. Colony counts were performed on plates containing 25–250 colonies, excluding plates with spreader colonies (BSN, 2008).

Yeast and mold assay

The yeast and mold count procedure followed SNI ISO 21527-2:2012. Dichloran 18% glycerol agar (DG-18) medium and BPW diluent were prepared. Samples were weighed at a ratio of one test portion to nine volumes of BPW to obtain the first dilution (10^{-1}) or a single decimal dilution series (10^0), followed by homogenization using a stomacher at 230 rpm for 1 minute, A second dilution (10^{-2}) was prepared by transferring 1 mL of the first dilution into 9 mL of BPW, and further serial dilutions were prepared as necessary. Inoculation was performed by transferring 0.1 mL of each dilution into petri dishes containing DG-18 medium using sterile pipettes. The inoculum was spread evenly over the medium surface with a sterile

spreader until fully absorbed. Plates were incubated aerobically at 25 ± 1 °C for 5–7 days. Colony counting was performed between day 3 and day 5, with an additional 48-hour incubation if no growth was observed. Plates containing fewer than 150 colonies were selected for enumeration. Yeast colonies were characterized as round, matt or glossy, and pink (on DRBC medium) or whitish-yellow (on PDA or DG-18 medium). Mold colonies were identified by irregular shapes and margins, with spore structures resembling fine, cotton-like filaments, and a colony color ranging from green to yellowish-green, dark brown, and black (BSN, 2012). The yeast and mold counts were calculated using the formula:

$$N = \sum a \,/\, (Vx1.1xd) \quad (8)$$

where Σa = total number of yeast colonies counted on successive dilution plates, V = volume of inoculum plated in each petri dish, and d = dilution factor of the lowest dilution enumerated.

Antioxidant activity

The antioxidant activity of honey was assessed using the DPPH method with UV-Vis spectrophotometry. This method relies on the ability of antioxidant compounds to neutralize DPPH free radicals, which is indicated by a color change from purple to yellow (Al-Farsi et al., 2018). Free radical scavenging activity was expressed as the percentage reduction in DPPH absorbance, calculated using the following equation:

$$\%\ inhibition = \frac{(A_{control} - A_{sample})}{A_{control}}\ x\ 100\% \quad (9)$$

where $A_{control}$ = absorbance of the DPPH solution without honey, and A_{sample} = absorbance of the DPPH solution containing honey. The half-maximal inhibitory concentration (IC_{50}) was defined as the sample concentration required to inhibit 50% of DPPH radicals. IC_{50} values were obtained by plotting a linear regression curve between sample concentration (x-axis) and percentage inhibition (y-axis). The IC_{50} was calculated using the equation:

$$IC_{50} = (50 - a)/b \quad (10)$$

where a and b represent the intercept and slope of the regression line, respectively.

Flavonoid content

The quantification of total flavonoids in honey was carried out using the Dowd method, as described by Hernandez-Fuentes et al. (2021). A 1 g portion of honey was extracted with 10 mL of pure methanol, then stirred and centrifuged at 4000 rpm for 30 minutes. From the resulting supernatant, 2 mL was combined with 2 mL of a 2% aluminum chloride ($AlCl_3$) solution and incubated in the dark for 20 minutes. Absorbance was then recorded at 415 nm using a UV/Vis spectrophotometer. A calibration curve was constructed with quercetin standards at concentrations ranging from 0 to 100 mg per 100 g. The flavonoid content of honey samples was expressed as milligrams of quercetin equivalents (QE) per 100 g of sample.

Statistical analysis

A Completely Randomized Design (CRD) was applied, consisting of four pasteurization time treatments (including a control) with three replications per treatment. The experimental data were subjected to Analysis of Variance (ANOVA) to evaluate the presence of significant differences among treatments. When statistical significance was detected at the 5% probability level ($P < 0.05$), Duncan's Multiple Range Test (DMRT) was employed to distinguish further treatment groups showing significant variation.

Results and Discussion

Tetracycline and oxytetracycline residues

Figure 1 shows the retention times of oxytetracycline and tetracycline at 5.683 and 6.443 minutes, respectively. As presented in Table 1, the concentrations of tetracycline and oxytetracycline in the control samples without pasteurization (P0) were 0.7543 ± 0.0885 µg g^{-1} and 0.8633 ± 0.0639 µg g^{-1}, respectively. These values were slightly lower than the theoretical concentrations, indicating incomplete recovery during sample preparation. Such discrepancies may arise from extraction inefficiencies and matrix effects during chromatographic analysis. Honey is a complex food matrix rich in sugars and other co-extracted constituents, including proteins and phenolic compounds, which may interfere with analyte recovery and detector response, potentially causing signal suppression or enhancement and affecting quantification accuracy. Therefore, evaluating extraction efficiency and matrix effects is an essential step in analytical method validation to ensure reliable quantification of target compounds in complex food matrices (Cortese et al., 2020; Steiner et al., 2020). Rodrigues et al. (2025) also reported that the extraction of antibiotic residues from honey remains analytically challenging due to its complex composition, the predominance of sugars, the presence of diverse bioactive compounds, and the typically low concentration levels of antibiotic residues.

Pasteurization at 60 °C significantly influenced the levels of tetracycline and oxytetracycline residues in honey (Table 1). Moderate pasteurization

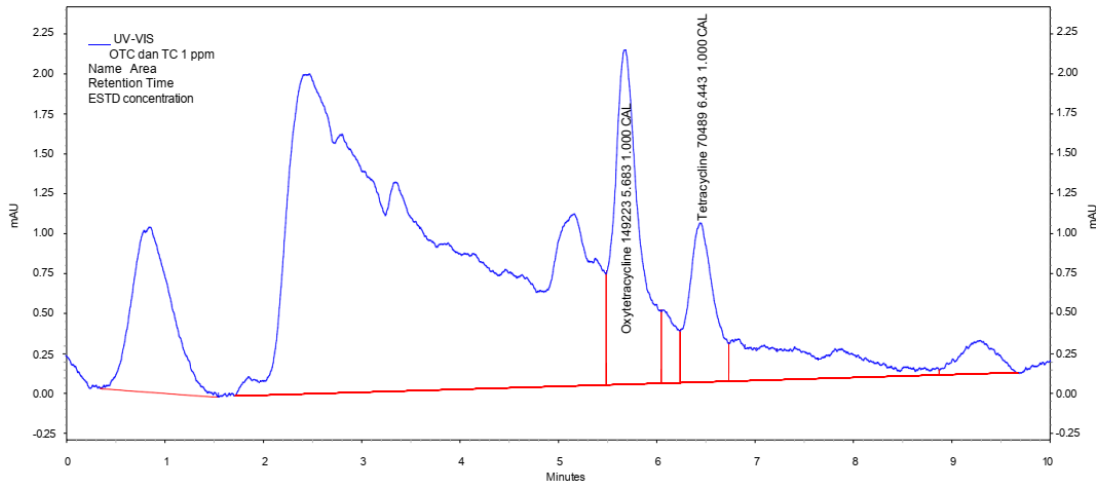

Figure 1. Chromatogram of oxytetracycline and tetracycline at 1 µg/g concentration using HPLC

Table 1. Tetracycline and oxytetracycline residues after different pasteurization durations (0, 10, 20, and 30 minutes)

Treatments	Oxytetracycline (µg g^{-1}) **	Tetracycline (µg g^{-1})
P0 (control without pasteurization)	0.7543 ± 0.0885[a]	0.8633 ± 0.0639
P1 (pasteurization at 60 °C, 10 min)	0.5043 ± 0.0386[b]	0.6853 ± 0.0635
P2 (pasteurization at 60 °C, 20 min)	0.5193 ± 0.0567[b]	0.7253 ± 0.0813
P3 (pasteurization at 60 °C, 30 min)	0.5167 ± 0.0424[b]	0.7193 ± 0.0766

Note **: Different superscript letters within the same column indicate significant differences ($P < 0.01$). Values are expressed as mean ± standard deviation (n = 3).

significantly reduced oxytetracycline residues ($P < 0.01$), with the greatest reduction observed after 10 minutes (0.5043 ± 0.0386 µg g^{-1}), corresponding to a 33.14% decrease compared with the control. In contrast, tetracycline residues decreased non-significantly ($P > 0.05$), with the largest reduction also observed after 10 min (0.6853 ± 0.0635 µg g^{-1}), equivalent to a 20.62% decrease relative to the control. These reductions were higher than those reported by Molino et al. (2011), who found that pasteurization at 78 °C for 1 minutes reduced tetracycline residues by 18.7% and oxytetracycline residues by 10.2%. The greater reduction observed in the present study may be attributed to several methodological and matrix-related factors. Pasteurization duration is a critical parameter influencing antibiotic degradation, as prolonged thermal exposure can accelerate the breakdown of tetracycline molecules beyond levels observed in shorter treatments. In addition, the physicochemical properties of honey may influence degradation kinetics. Variations in moisture content can affect thermal conductivity and facilitate hydrolytic reactions during pasteurization, potentially accelerating the degradation of tetracycline residues (Buzia et al., 2019). Methodological differences in extraction and analytical procedures may also contribute to discrepancies among studies, since incomplete extraction recovery or matrix effects during chromatographic analysis can influence the apparent concentration of antibiotic residues (Cortese et al., 2020; Steiner et al., 2020). Collectively, these factors may explain the greater reduction observed in this study compared with previous reports.

Similar effects of heat treatment on tetracycline residues have also been reported in dairy products. Pasteurization has been shown to reduce tetracycline and oxytetracycline residues in cow's milk (Kellnerová et al., 2015; Khaskheli et al., 2021). Tian et al. (2017) reported that thermal processing can reduce antibiotic residues in food, with the extent of degradation depending on temperature, time, and the physicochemical properties of the compounds. De Freitas et al. (2024) explained that upon pasteurization, tetracycline initially undergoes dehydration (when water is present), followed by melting or direct decomposition, and subsequently degrades stepwise into gaseous products such as H_2O, CO_2, ammonia, HCl/SO_2, methane, and dimethylamine before leaving carbonaceous residues that may further oxidize.

Overall, pasteurization reduces tetracycline-class antibiotic residues in honey but does not eliminate them completely. Therefore, effective control of antibiotic residues must be implemented at the beekeeping stage rather than relying solely on honey processing.

Moisture content

The data in Table 2 show that the moisture content of honey before and after pasteurization at 60 °C for up to 30 minutes remained within the limits established by the Indonesian honey quality standard (SNI 8664:2018). According to this regulation, the maximum moisture content permitted in honey is 22% for both domesticated and wild bee honey (BSN, 2018). Pasteurization at 60 °C significantly influenced the moisture content of the samples ($P < 0.01$), with the lowest value recorded after 30 minutes of pasteurization (20%). In general, extending the pasteurization time led to a gradual decline in moisture levels. Nevertheless, treatments P1, P2, and P3 shared the same superscript letters, indicating that the reductions observed after 10, 20, and 30 minutes were not statistically different. This result suggests that a large portion of the easily removable water may have evaporated during the early stage of pasteurization, leaving only a small fraction of moisture available for further removal. Honey behaves as a highly concentrated sugar solution in which water molecules interact strongly with glucose and fructose through hydrogen bonding, restricting their mobility within the matrix (Da Silva et al., 2016). Consequently, once the free water fraction has largely evaporated, the remaining water becomes increasingly associated with the sugar network, which limits further evaporation during prolonged pasteurization. Comparable findings have been reported by Jaya et al. (2022), who observed that thermal treatment generally causes only a modest reduction in honey moisture content compared with fresh honey.

From a physicochemical standpoint, the decrease in moisture during pasteurization is primarily driven by evaporation and internal mass transfer. When temperature increases, the vapor pressure of water also rises, creating a stronger gradient that promotes moisture loss from the honey surface to the surrounding environment (Da Silva et al., 2016). At the same time, higher temperatures lower honey's viscosity, enhancing molecular mobility and facilitating the diffusion of water molecules toward the surface, where evaporation occurs (Ramly et al., 2021). However, because honey contains very high concentrations of sugars, the remaining water molecules become increasingly associated with the sugar matrix, slowing diffusion and reducing the efficiency of further evaporation during extended pasteurization. Moisture content is also closely linked to water activity (aw), an important parameter that determines the microbiological stability of honey during storage (Chen et al., 2019). When moisture levels increase, water activity tends to rise, providing favorable conditions for osmophilic yeasts that may trigger fermentation and lead to quality deterioration (Polatidou et al., 2025). For this reason, maintaining appropriate moisture levels through controlled processing conditions, such as pasteurization, is essential to preserving honey quality and prolonging shelf life.

Ash content

Mineral content in honey is defined as ash content. Pasteurization at 60 °C did not significantly affect ash content (Table 2). There is no significant difference observed between unheated honey and honey pasteurized at 60 °C ($P > 0.05$). Ash content reflects mineral composition, which is generally stable under moderate heat treatment at 60 °C. These findings are consistent with those of Kadri et al. (2017), who reported that ash content in honey remains relatively stable because minerals are not readily degraded by heat. Rani et al. (2024) also noted that mineral content in honey is relatively stable under both heat treatment and storage, with ash levels more strongly influenced by botanical origin and environmental conditions. The average ash content in this study was 0.9171%, exceeding the Indonesian National Standard, which sets a maximum ash content of 0.50% (BSN, 2018). Elevated ash levels indicate the influence of nectar sources and environmental factors during production and storage. Santos-Buelga and González-Paramás (2025) emphasized that honey's chemical composition, including mineral content, depends primarily on geographical and botanical origin rather than heat treatment.

Table 2. Moisture content, ash content, and water-insoluble solids after different pasteurization durations (0, 10, 20, and 30 minutes)

Treatments	Moisture content (%)**	Ash content (%)	Water-insoluble solids (%)
P0 (control without pasteurization)	21.0833 ± 0.1443[a]	0.9194 ± 0.0015	0.1093 ± 0.0133
P1 (pasteurization at 60 °C, 10 min)	20.2500 ± 0.0000[b]	0.9160 ± 0.0041	0.0966 ± 0.0277
P2 (pasteurization at 60 °C, 20 min)	20.1167 ± 0.1258[b]	0.9111 ± 0.0069	0.1104 ± 0.0437
P3 (pasteurization at 60 °C, 30 min)	20.0000 ± 0.0000[b]	0.9218 ± 0.0054	0.0991 ± 0.0143

Note **: Different superscript letters within the same column indicate significant differences ($P < 0.01$). Values are expressed as mean ± standard deviation ($n = 3$).

Water-insoluble solids

Water-insoluble solids in honey consist of bee pollen, fragments of honeycomb, debris, and bee parts (Ameliya et al., 2023). Albu et al. (2021) noted that the presence of insoluble solids in honey can be attributed to both organic and inorganic materials. These include wax residues, fragments of bee bodies or larvae, plant-derived particles such as wood, propolis, and pollen, and environmental contaminants such as soil and dust. No significant variations in water-insoluble solids were observed among unpasteurized honey (control) and honey pasteurized at 60 °C for 10, 20, and 30 minutes (Table 2) ($P > 0.05$), indicating that increasing pasteurization duration did not influence the formation or precipitation of insoluble compounds. The average content of water-insoluble solids was 0.102%, which complies with the Indonesian National Standard that sets a maximum of 0.50% (BSN, 2018). This result suggests that pasteurizing honey at 60 °C for 10, 20, and 30 minutes remains within safe limits for maintaining honey clarity.

Hydroxymethylfurfural (HMF)

The HMF content is an indicator of sugar degradation and excessive pasteurization in honey. These results indicate that the honey samples were fresh and had not undergone significant thermal or storage-related degradation. Table 3 shows that HMF values remained very low (<1 mg kg^{-1}) in honey pasteurized at 60 °C for 10, 20, and 30 minutes, indicating that moderate thermal treatment did not cause significant sugar degradation. Ramly et al. (2021) reported that freshly harvested honey has nearly zero HMF values, which increase with processing and storage, making HMF a useful parameter for assessing honey freshness and quality. The HMF results in Table 3 show a tendency to increase with longer pasteurization durations, although the differences were not statistically significant ($P > 0.05$). The highest value was observed at 60 °C for 20 minutes, with a value of 0.64 ± 0.01 mg kg^{-1}. These findings are consistent with Eshete and Eshete (2019), who noted that HMF content increases while diastase activity decreases when honey is overheated, aged, or improperly stored. Jaya et al. (2022) also reported that HMF levels tend to increase during processing or aging, depending on temperature and pasteurization duration.

Diastase enzyme

Diastase activity is widely used as an indicator of honey freshness and thermal history because this enzyme is highly sensitive to heat treatment. As shown in Table 3, pasteurization at 60 °C for 10, 20, and 30 minutes significantly affected diastase activity ($P < 0.01$). In general, increasing pasteurization duration reduced enzyme activity, with the lowest value observed after 20 minutes of pasteurization (9.043 ± 2.08 DN), indicating the sensitivity of diastase enzymes to thermal treatment. Diastase mainly consists of α- and β-amylases, which are responsible for starch hydrolysis, and their catalytic activity decreases at elevated temperatures, which induce conformational changes and protein denaturation (Kędzierska-Matysek et al., 2016). Similar results were reported by Jaya et al. (2022), who observed a reduction in diastase activity after pasteurization. Thermal inactivation of enzymes generally follows first-order degradation kinetics, in which enzyme activity progressively declines with increasing temperature and exposure time.

Interestingly, the diastase activity measured after 30 minutes (19.870 ± 4.16 DN) was higher than that observed after 20 minutes (9.043 ± 2.08 DN), which deviates from the expected decreasing trend. This anomaly may be attributed to the biological heterogeneity of the honey matrix and analytical variability during diastase number (DN) determination. Honey is a complex biological product, and enzyme distribution may vary depending on botanical origin and bee-derived secretions (Seraglio et al., 2021). In addition, DN determination by starch hydrolysis can be influenced by experimental conditions and instrumental sensitivity, leading to fluctuations in measured values as enzyme activity declines (Tsavea et al., 2022). Therefore, the apparent increase in diastase activity after 30 minutes is likely related to experimental variability rather than an actual recovery of enzyme activity during prolonged pasteurization.

Acidity

Table 3 also shows that pasteurization at 60 °C for 10, 20, and 30 minutes had a highly significant effect ($P < 0.01$) on honey acidity. The results revealed a tendency for acidity to increase with longer pasteurization durations, with the highest value observed after 30 minutes (57.59 ± 0.85 mL NaOH kg^{-1}). This suggests that pasteurization duration plays an important role in altering honey's chemical properties, particularly through the accumulation of acidic compounds. Ribeiro et al. (2018) reported that pasteurization at 65 °C reduced honey pH, while different maturation temperatures influenced moisture content, water activity, pH, and alcohol production. Song et al. (2025) also observed a slight, non-significant decrease in pH in cocoa honey subjected to thermal pasteurization and high-pressure processing, attributed to microbial metabolism producing organic acids.

Table 3. Hydroxymethylfurfural (HMF) content, diastase enzyme activity, and acidity after different pasteurization durations (0, 10, 20, and 30 minutes)

Treatments	HMF (mg kg^{-1})	Diastase enzyme (DN)**	Acidity (mL NaOH kg^{-1}) **
P0 (control without pasteurization)	0.51 ± 0.16	46.997 ± 1.74[a]	54.47 ± 0.37[b]
P1 (pasteurization at 60 °C, 10 min)	0.59 ± 0.01	19.366 ± 1.86[b]	54.26 ± 1.22[b]
P2 (pasteurization at 60 °C, 20 min)	0.64 ± 0.01	9.043 ± 2.08[c]	53.89 ± 0.59[b]
P3 (pasteurization at 60 °C, 30 min)	0.62 ± 0.01	19.870 ± 4.16[b]	57.59 ± 0.85[a]

Note: **: Different superscript letters within the same column indicate significant differences ($P < 0.01$). Values are expressed as mean ± standard deviation (n = 3).

In this study, the average acidity was 55.05 mL NaOH kg^{-1}, exceeding the Indonesian National Standard, which sets a maximum acidity of 50 mL NaOH kg^{-1} for domesticated honey (BSN, 2018). Acidity reflects the amount of organic acids present in honey (Jaya et al., 2022). Higher acidity can enhance honey's antimicrobial properties and extend shelf life, but excessive acidity may negatively affect sensory characteristics, making the taste more sour and less acceptable to consumers.

Reducing sugar (glucose) and sucrose

The reducing sugar content ranged between 56.47–57.87%, indicating that all honey samples remained within the Indonesian National Standard limits, which specify a maximum of 65% for domesticated and wild bee honey (BSN, 2018). The results presented in Table 4 show no significant differences ($P > 0.05$) in reducing sugar (glucose) content between unheated honey and honey pasteurized at 60 °C for 10, 20, and 30 minutes. This suggests that the primary carbohydrate structure of honey is relatively stable under moderate pasteurization at 60 °C for durations 10, 20, and 30 minutes. Ribeiro et al. (2018) reported that mild heat treatment does not significantly alter glucose and fructose levels, as degradation reactions of simple carbohydrates generally occur only at higher temperatures or with prolonged pasteurization.

Sucrose represents one of the principal soluble sugars in honey, together with fructose and glucose (Song et al., 2025). In the present study, sucrose levels in all samples ranged from 5.44% to 6.49%, exceeding the 5% threshold established by both the Codex Alimentarius and the Indonesian National Standard (BSN, 2018). Elevated sucrose levels may indicate incomplete honey maturation, premature harvesting, or adulteration, such as the addition of cane sugar or refined beet sugar. Jaya et al. (2022) explained that high sucrose levels may suggest adulteration, whereas low sucrose levels indicate the absence of invert syrup adulteration. The results in Table 4 also show that pasteurization at 60 °C for 10, 20, and 30 minutes did not significantly affect sucrose content ($P > 0.05$), suggesting that sucrose, as a carbohydrate component of honey, is relatively stable under moderate heat treatment. Da Silva et al. (2016) reported that pasteurization can inactivate enzymes, thereby maintaining sucrose stability, although other quality attributes, including diastase activity and HMF, may be affected.

Total plate count (TPC), yeasts, and molds

Table 5 shows that TPC values decreased during pasteurization at 60 °C for 10, 20, and 30 minutes, although the differences were not statistically significant ($P > 0.05$). Longer pasteurization durations tended to reduce microbial counts, with the lowest TPC observed after 30 minutes of pasteurization ($1.73 \times 10^2 \pm 4.2 \times 10^1$ CFU g^{-1}). This indicates that honey naturally exhibits antimicrobial activity, which is attributed to its high osmotic pressure, acidic pH, and diverse bioactive constituents. Scepankova et al. (2024) similarly reported that pasteurization at 78 °C for 6 minutes effectively eliminated microorganisms in honey, although it reduced physicochemical quality.

The results in Table 5 also show that pasteurization at 60 °C inactivated yeasts and molds in honey, although the effect was not statistically significant ($P > 0.05$). Yeasts and molds were not detected in honey pasteurized at 60 °C for 10 to 30 minutes. These findings are consistent with those of Nascimento et al. (2025), who reported that yeasts and molds were not detected in honey pasteurized at 65 °C for 30 minutes and stored for 28 days, confirming that pasteurization is highly effective at inactivating these microorganisms.

Antioxidant activity and flavonoid content

Table 6 shows that pasteurization at 60 °C for 10, 20, and 30 minutes slightly reduced the antioxidant activity of honey, although the differences were not statistically significant ($P > 0.05$). A decreasing trend in antioxidant activity was observed with increasing pasteurization duration, with the lowest value recorded after 30 minutes of pasteurization (14.91 ± 0.17 mg mL^{-1} IC_{50}). The IC_{50} value is the concentration required to inhibit 50% of DPPH radicals ; lower IC_{50} values indicate stronger

Table 4. Reducing sugar (glucose) and sucrose content after different pasteurization durations (0, 10, 20, and 30 minutes)

Treatments	Reducing sugar (glucose) (%)	Sucrose (%)
P0 (control without pasteurization)	57.87 ± 1.15	5.44 ± 2.16
P1 (pasteurization at 60 °C, 10 min)	56.47 ± 1.27	6.14 ± 2.59
P2 (pasteurization at 60 °C, 20 min)	57.49 ± 2.37	6.49 ± 0.83
P3 (pasteurization at 60 °C, 30 min)	56.90 ± 1.83	5.82 ± 0.45

Note: Values are expressed as mean ± standard deviation (n = 3).

Table 5. Total plate count (TPC), yeast, and mold counts after different pasteurization durations (0, 10, 20, and 30 minutes)

Treatments	TPC (CFU g^{-1})	Yeasts and Molds (CFU g^{-1})
P0 (control without pasteurization)	$2.20 \times 10^2 \pm 3.9 \times 10^1$	1.2×10^2
P1 (pasteurization at 60 °C, 10 min)	$2.34 \times 10^2 \pm 1.0 \times 10^1$	Not detected
P2 (pasteurization at 60 °C, 20 min)	$2.08 \times 10^2 \pm 7.6 \times 10^1$	Not detected
P3 (pasteurization at 60 °C, 30 min)	$1.73 \times 10^2 \pm 4.2 \times 10^1$	Not detected

Note: Values are expressed as mean ± standard deviation (n = 3).

Table 6. Antioxidant activity and flavonoid content after different pasteurization durations (0, 10, 20, and 30 minutes)

Treatments	Antioxidant activity (IC_{50} mg mL^{-1})	Flavonoid content (mg QE 100 g^{-1}) **
P0 (control without pasteurization)	16.55 ± 1.02	21.10 ± 2.55^a
P1 (pasteurization at 60 °C, 10 min)	15.17 ± 0.56	20.65 ± 2.21^a
P2 (pasteurization at 60 °C, 20 min)	15.11 ± 2.32	12.22 ± 1.78^b
P3 (pasteurization at 60 °C, 30 min)	14.91 ± 0.17	11.55 ± 0.00^b

Note **: Different superscript letters within the same column indicate significant differences ($P < 0.01$). Values are expressed as mean ± standard deviation (n = 3).

antioxidant activity (Akullo et al., 2023). According to commonly used classifications, antioxidant activity is categorized as very strong when IC_{50} < 50 ppm, strong at 50–100 ppm, moderate at 100–250 ppm, and weak when IC_{50} exceeds 250 ppm (Yuniarto et al., 2025). Based on this classification, the antioxidant activity of the honey samples in this study (14,910 ppm) is relatively weak. However, this classification should be interpreted cautiously, as honey is a complex natural matrix in which antioxidant capacity arises from the synergistic effects of multiple bioactive compounds rather than a single highly active antioxidant molecule (Becerril-Sánchez et al., 2021).

In contrast, flavonoid content (Table 6) significantly decreased ($P < 0.01$) with increasing pasteurization duration (10, 20, and 30 minutes), demonstrating a clear time-dependent degradation pattern. The lowest value was observed after 30 minutes (11.55 ± 0.00 mg QE 100 g^{-1}), suggesting that prolonged thermal exposure accelerates the degradation of phenolic compounds. This reduction suggests that phenolic compounds, particularly flavonoids, are susceptible to thermal degradation during honey processing. Heat exposure can promote oxidation and structural modification of phenolic molecules, leading to a decline in flavonoid concentrations. Similar findings have been reported by Song et al. (2025), who observed that thermal pasteurization decreases the levels of phenolic and flavonoid compounds in honey, while Yalçin (2021) reported that elevated temperatures and environmental factors, such as light, can reduce antioxidant-related phenolic compounds.

Despite the significant decrease in flavonoid content, the overall antioxidant activity remained relatively stable. This indicates that antioxidant capacity in honey is not determined solely by flavonoids but also by other antioxidant constituents such as phenolic acids, enzymes, and organic acids (Becerril-Sánchez et al., 2021). In addition, mild pasteurization may promote Maillard reactions between reducing sugars and amino acids, generating intermediate compounds with antioxidant

properties that can partially compensate for the loss of natural phenolics (Mahani et al., 2024). Consequently, moderate pasteurization conditions may preserve the overall antioxidant capacity of honey even when certain phenolic compounds decrease.

Conclusion

Pasteurization at 60 °C effectively reduced oxytetracycline residues by up to 33.14% and improved the microbiological safety of acacia honey, although it also affected certain quality parameters, such as diastase activity, acidity, and flavonoid content. Moisture content decreased slightly, while HMF levels remained low, indicating minimal thermal damage. Overall, pasteurization at 60 °C for 10 minutes appears to provide the best balance between reducing antibiotic residues and preserving key honey quality attributes. However, effective control of antibiotic residues should primarily be implemented during beekeeping rather than relying solely on post-harvest processing.

Acknowledgements

The authors gratefully acknowledge the Biotechnology Laboratory of Animal Product Quality Testing and Certification Center (*Balai Pengujian Mutu dan Sertifikasi Produk Hewan*), Ministry of Agriculture, Republic of Indonesia, for providing laboratory facilities and technical support.

Conflict of Interest

The authors have declared that no competing interests exist.

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