

Evaluation of Honey Quality with Stored Time and Temperatures

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Abstract Honey from two sources, *Bidens pilosa* and *Dimocarpus longan* were stored at 35, 25, or 4°C under dark or light for 3-24 months. They were evaluated for the nutrients, antioxidant activities and quality parameters required in Codex Standard. Diastase activity of examined honey was reduced to less than 8 schade unit soon after storage for 3-6 months, and its acidity was increased with long storage time at high temperature. Storage of honey at 4°C significantly diminished the loss of diastase activity and maintained the proper acidity. Hydroxymethylfurfural (HMF) content retained low level as honey was stored up to two years at either 4 or 25°C, while its content was quickly increased at 35°C. More phenolic content but less flavonoid content was found in honey after long storage time. Along with longer deposit time and higher temperature, less antioxidant activities were detected in honey, including scavenging activity of DPPH and superoxide radicals. In contrast, the reducing power was increased. Storage of honey at 35°C caused the worst impact on its quality parameters of Codex Standard, while 4°C was the best storage condition to maintain most quality parameters, nutrients and antioxidant functions. No apparent difference was found between the storage conditions under dark and light.

Keywords: honey, storage, hydroxymethylfurfural, antioxidant

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1. Introduction

Since the ancient times, honey has been extensively applied in food and medical product. The floral nectar, the exudates of trees and honeydew were collected and regurgitated to produce honey by bees [1]. Honey is well known for its nutrient value, and comprises carbohydrates and water mainly. It also contains minerals, free amino acids, vitamins, proteins, and various substances including pigments, flavonoids and antibacterial factors [2,3]. At least 200 natural substances were found in honey [4]. The composition of floral honey is associated with the floral sources, along with external factors, environment, climate, season and processing [5].

Honey has showed the therapeutic potential for heart disease, cancer, cataracts, and several inflammatory diseases [6]. It is attributed to its antioxidant capacity, antimicrobial properties, and wound healing and anti-inflammatory activities [7]. Many studies showed that its antioxidant activity is related to the floral source [8]. Both enzymatic and non-enzymatic antioxidants have been found in honey, including glucose oxidase, catalase, ascorbic acid, carotenoid derivatives, organic acids, Maillard reaction products (MRP), phenolic compounds, and free amino acids [9,10]. Among them, the phenolic

compounds are primarily responsible for the antioxidant capacity [11,12]. And honey flavonoids are originally from nectar, pollen or propolis. The identified flavonoids include apigenin (belongs to flavone), such as kaempferol (a flavanol), hesperetin (a flavavone) and diverse phenolic acids.

Honey is often preserved until use. To keep the freshness and antioxidant function of stored honey is critical for the retail and consumers. According to Codex Standard [13], all retail honey should have the following compositional and quality parameters: moisture content, ≤ 20 g/100 g; free acidity, ≤ 50 mequiv/ kg; diastase activity, ≥ 8 schade unit; hydroxymethylfurfural (HMF) content, ≤ 40 mg/kg (or ≤ 80 mg/Kg for honey from tropical ambient temperatures).

Honey has an acidic pH value, ranging from 3.5 to 4.5, owing to its organic acids. The acidity of honey is correlated with its flavor and stability against microorganisms [14]. Nevertheless, microbial alterations in honey may cause total acidity over the legal limitation [15,16,17,18]. Diastase in honey catalyzes starch into short-chain sugars, and its protein conformation may be destroyed after heating. Maillard reaction (MR), a non-enzymic browning reaction between sugars and free amino acids, occurs during prolonged storage and heating [19]. HMF is one of the major intermediate products of MR. Less diastase activity and more HMF was detected in honey after prolonged storage and heating [20,21,22,23,24].

Herein, we studied the effect of storage conditions on honeys from two floral sources, *Bidens pilosa* and *Dimocarpus longan* in Taiwan. *D. longan* is the main floral source of honey bee, and its fruits are edible and popular in Taiwan. *B. pilosa* is used as ingredient of herbal tea, as well as a folk medicine [25]. The climate in the plains of Taiwan is often between 25 and 35°C. Thus, the storage conditions of honey at 35, 25, or 4°C under dark or light for 3-24 months were evaluated. The quality parameters of honeys were examined and discussed, as well as antioxidant activities, total flavonoids and phenolic compound contents.

2. Materials and Methods

2.1. Honey Samples

B. pilosa and *D. longan* honey, produced by *A. mellifera* bees were prepared and provided by Honey Bee Town Company (Hualien, Taiwan). Each honey was divided into six equal parts, and subjected to different storage conditions, dark and light at temperature (35, 25, 4°C), followed by analyses of the physicochemical parameters and antioxidant activities.

2.2. Diastase Activity Assay

The diastase activity was measured using the Phadebas amylase test tablets purchased from Magle (Lund, Sweden), according to the International Honey Commission [26]. Diastase Activity was referred to as diastase number in the Schade scale, corresponding to the Gothe scale number, or hydrolyzed starch (g) /100 g of honey/hour at 40°C.

2.3. Total Acidity

5.0 g of examined honey was dissolved in 100 ml distilled water, and then subjected to determination of the total acidity, according to method of AOAC method 962.19 [27]. A pH meter (Mettler Toledo SevenEasy digital) was applied to measure pH value. The total acidity was then calculated and expressed as meq/kg.

2.4. Determination of HMF Content in Honey

5 g of honey sample each was diluted to 50 mL, and then filtered through a membrane (0.45 µm). Afterward, HMF content was determined by high-performance liquid chromatography (HPLC) under OD₂₈₅ detection, according to AOAC method 980.23 [28]. HPLC analysis was performed using a Waters 1525 pumping system, a Water 2489 detector, an RP-18 GP250 column (4.6 mm), and a Waters 717plus autosampler. And the isocratic mobile phase comprised 90% water, and 10% methanol at a flow rate of 0.7 mL/min. HMF content of the sample was calculated, according to the corresponding peak of HMF standard solutions. A linear relationship ($R^2=0.9981$) between the concentration and the area of HMF peak was achieved. Each sample was performed three times, and the mean of HMF content was expressed as mg/kg.

2.5. Determination of Total Polyphenol and Total Flavonoid Contents in Honey

The total polyphenolic content of honey was measured by Folin-Ciocalteu colorimetric method using gallic acid as a calibration standard [8]. An aliquot of the honey sample solution (0.1 mL) was diluted to 5 mL with water, followed by adding Folin-Ciocalteu reagent (0.5 mL). After mixing for 3 min, 1 mL of an aqueous Na₂CO₃ solution (35 g/L) was added and mixed thoroughly by vortex. After placing the mixture at room temperature for 1 h, the absorbance of the mixture was measured at 725 nm against a blank.

The total flavonoid content was determined using an aluminum chloride colorimetric method [8]. 0.5 ml of honey sample was mixed with 1.5 ml of 95% alcohol, 0.1 ml of 1 M potassium acetate, 0.1 ml of 10% aluminum chloride hexahydrate, and 2.8 ml of deionized water. After incubation at room temperature for 30 min, the absorbance of reaction mixture was measured at 415 nm. Quercetin was applied to obtain a standard curve (0–100 µg/ml). The total flavonoid content in each honey sample was determined in triplicate.

2.6. Radical Scavenging Effect on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radicals

DPPH assay was performed based on the method of Liu et al. [8]. 0.3 mL of honey sample was mixed with 2.4 mL of ethanol (99%) and 0.3 mL of 1.0 mM DPPH radical solution. The mixture was shaken vigorously and left to stand for 30 min in the dark, before the absorbance was measured at 517 nm. Ascorbic acid (0.1 mM and 1.0 mM) were used as positive controls. The capability of the test material to scavenge DPPH radicals was calculated as (%) = $1 - (\text{OD}_{517} \text{ of the sample}) / (\text{OD}_{517} \text{ of the control}) \times 100$.

2.7. Radical Scavenging Effect on Superoxide Radicals

The scavenging of superoxide anion radicals was estimated according to the method of Robak and Gryglewski [29]. The honey sample solution (500 µL) was mixed with an equal volume of 80 µM PMS, 624 µM NADH and 200µM NBT each. The mixture was shaken vigorously and left to stand for 5 min. The absorbance of reaction mixture was measured at 560 nm. Ascorbic acid (0.1 mM, 1.0 mM and 10.0 mM) were used as positive controls. The inhibition ratio was calculated as (%) = $(\text{OD}_{560} \text{ of the control} - \text{OD}_{560} \text{ of the sample}) / (\text{OD}_{560} \text{ of the control}) \times 100$.

2.8. Reducing Power

The reducing power of honey was determined according to the method of Oyaizu [30]. The honey sample solution (2.5 mL) was mixed with an equal volume of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Afterward, an equal volume of 1%

trichloroacetic acid was added to the mixture, which was then centrifuged at 1400g for 10 min. The upper layer was mixed with distilled water and 0.1% ferric chloride at a ratio of 1:1:2, and the absorbance of the incident radiation by the solution was measured at 700 nm. The absorbance was proportional to the reducing power, and dibutyl hydroxytoluene (BHT) was used as a reference for comparison.

2.9. Statistical Analysis

All results were analyzed using the general linear model procedure available from Statistical Analysis System software (Statistical Analysis System Institute, Cary, NC). Duncan's multiple range test [31] was used to detect differences between means of the treatments. Each experiment was conducted in triplicate. Differences between means at the 95% ($p < 0.05$) confidence level were considered as statistical significance.

3. Results and Discussion

3.1. Total Acidity Contents

The acidity of honeys under different storage conditions were examined, including 35, 25, or 4°C for 3-24 months under dark or light. As shown in Figure 1, the acidity of honeys from *B. pilosa* and *D. longan* increased with the duration of storage. Storage at high temperature would increase total acidity in honeys significantly. The initial acidity of *B. pilosa* honey was 43.06 meq./kg. After stored for 9 months at 35°C, its acidity was higher than legal limit, 50 mequiv/kg. After stored for 18 months at 25°C, its acidity was out of legal limit as well. *B. pilosa* honey kept at 4°C maintained its acidity in legal value up to 24 months. The initial acidity of *D. longan* honey was 18 meq./kg, less than *B. pilosa* honey. The increasing rate of acidity of honey from *D. longan* was faster than *B. pilosa*. After stored for 12 months at 35 or 25°C, its acidity was higher than legal limit. And the acidity of *D. longan* honey stored for 18 months at 4°C was out of legal limit. Moreover, there was no significantly difference between the conditions under dark and light.

According to the results, the storage of honey at 4°C is recommended in order to hold its proper acidity. The stored temperature and time had considerable effect on acidity, which is coincided with the studied results by Castro-Vázquez [32]. Among different honey types, the variation in acidity may attribute to the variation of organic and inorganic acids contents [33]. Nevertheless, the compounds affecting acidity of honeys from *B. pilosa* and *D. longan* remain further study.

3.2. Diastase Activity in Honey

The initial diastase activity was similar in both examined honeys, as shown in Figure 2. Prolonged storage under high temperature accelerated the loss of diastase activity in honeys. After storage for 3 months under our test conditions, diastase activity in the honeys was less than legal limit, 8 schade unit, except for *D. longan* honey stored at 4°C. Stored at 4°C may significantly diminish the loss of diastase activity, particularly for *B. pilosa* honey. There was no apparently difference between dark storage and light storage.

The diastase activity in honeys are more or less diverse, depending on the sugar content of honeys, the floral and geographical origins of honeys, the period of nectar collected, the age of bees, and the bee colony [34]. Its activity has been as an indicator to monitor whether the honeys had been kept for too long time, or its product was overheated, > 60°C [35,36].

3.3. HMF Contents

Both honeys from *B. pilosa* and *D. longan* contained a low initial HMF content (1.21 mg/kg and 2.13 mg/kg, respectively) (Figure 3). HMF was quickly generated in these honeys after storage at 35°C. HMF contents in *B. pilosa* honey stored at 35°C for 6 months was reached to the legal limit, 80 mg/Kg for honey from tropical ambient temperatures. After storage at 35°C for 9 months, HMF contents in *B. pilosa* honey was exceeding the legal limit; while HMF contents in *D. longan* honey was reached to the legal limit. HMF contents in both honeys, stored at 4 and 25°C for 24 months, remain quite low levels that were less than 10 mg/ml.

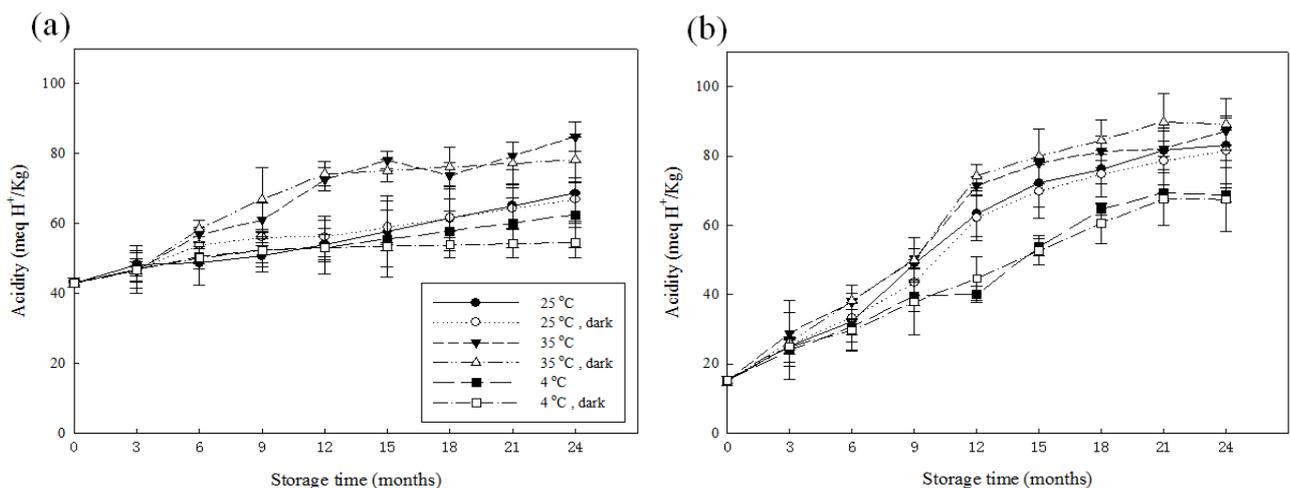


Figure 1. Acidity of honeys under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey; Each point is expressed as mean \pm S.D. (n=3)

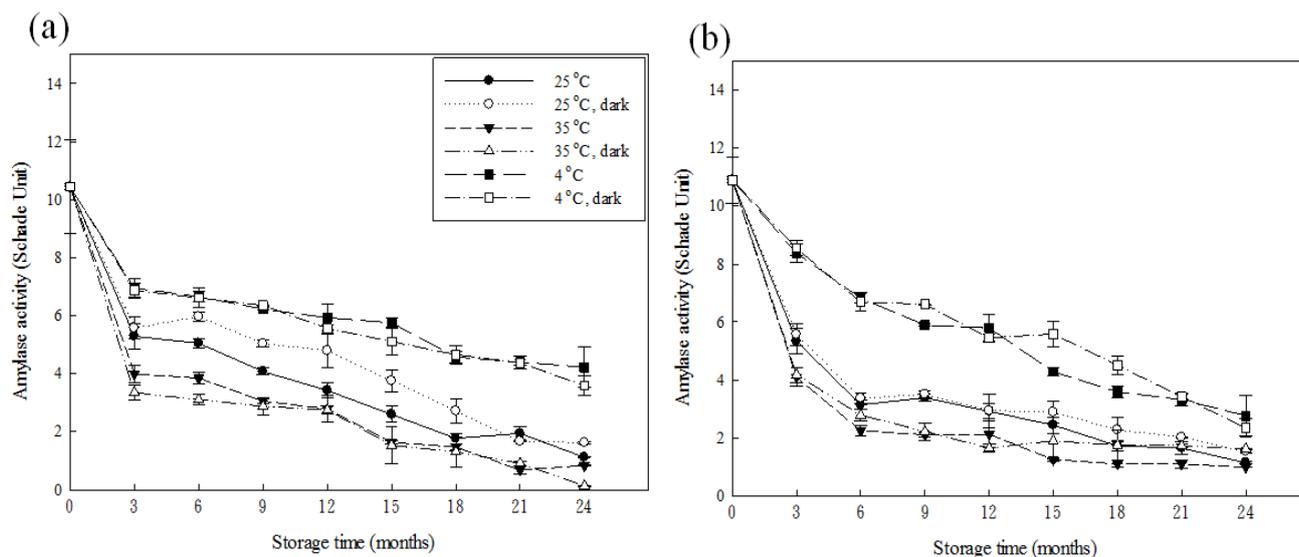


Figure 2. Diastase activity of honeys under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey

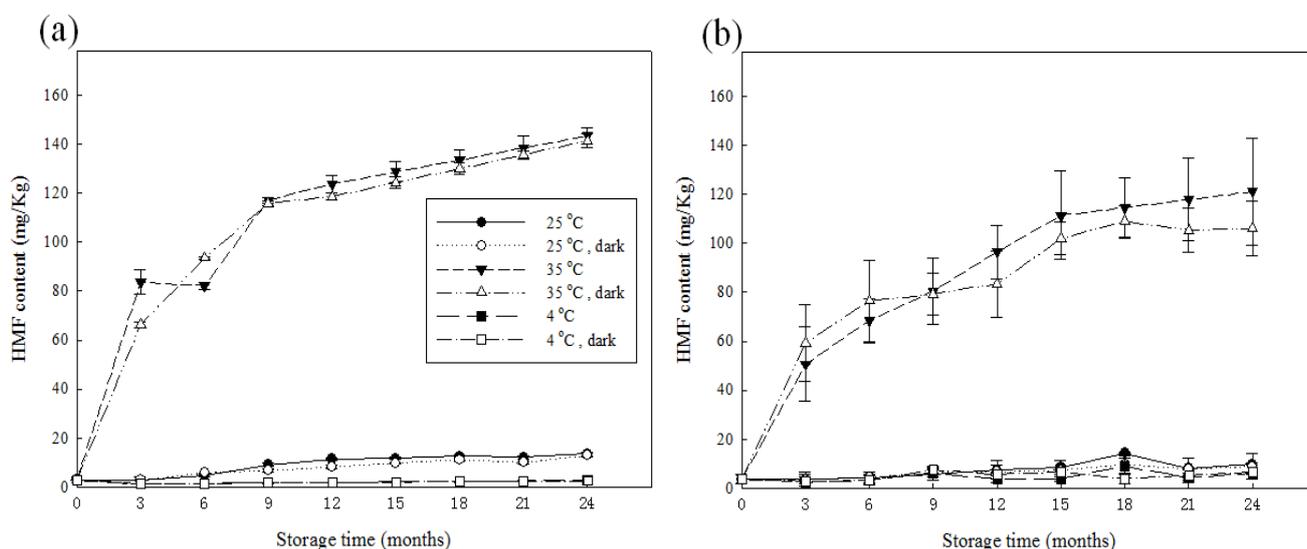


Figure 3. HMF content of honeys under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey; Each point is expressed as mean \pm S.D. (n=3)

HMF was reported to have genotoxic effects and mutagenic potential [37]. Some studies indicated that heat treatment or storage at room temperature caused a considerable increase in HMF contents of honeys [38,39,40]. Based on our results with HMF contents, it is suggested to store honeys at 4-25°C, or consume honeys within 6 months.

3.4. Total Phenolic and Total Flavonoid Contents in Honey

The influence of storage condition on total phenolic and total flavonoid contents of *B. pilosa* honey and *D. longan* honey was examined and shown in Figure 4 and Figure 5. Total phenolic content of *B. pilosa* honey was increased to 2-3 folds after storage for 12 months. Higher temperature would more or less increase its phenolic content. Compared with *B. pilosa* honey, *D. longan* honey had less total phenolic compounds; however, its total phenolic

content was increased to 10-18 folds after storage for 12 months. The effect of heating on phenolic content of *D. longan* honey was clearly observed. More phenolic content of *D. longan* honey was detected as stored at higher temperature.

B. pilosa honey was rich in total flavonoid compounds (initially 180 μ g/ml). After storage for 3 months, around half of its total flavonoid contents were reduced. Honey from *D. longan* had less total flavonoid compounds (initially 55 μ g/ml) than *B. pilosa* in our previous and current studies [8]. After storage for 9 months, around half of total flavonoid contents of *D. longan* honey were reduced. The decay rate of total flavonoid contents from *B. pilosa* was much faster than *D. longan* in the first three months. In general, storage at 4°C under dark would be better for honeys to retain total flavonoid contents. Honeys stored under dark had slightly less change in total phenolic and total flavonoid contents, comparing to under light.

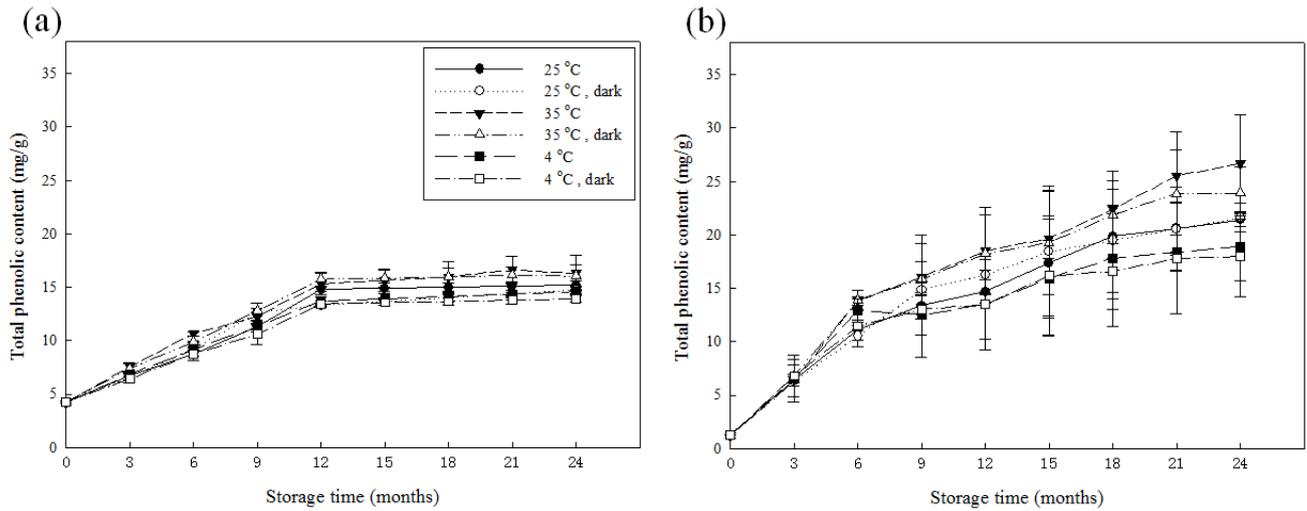


Figure 4. The total phenolic content of honeys under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey; Each point is expressed as mean \pm S.D. (n=3)

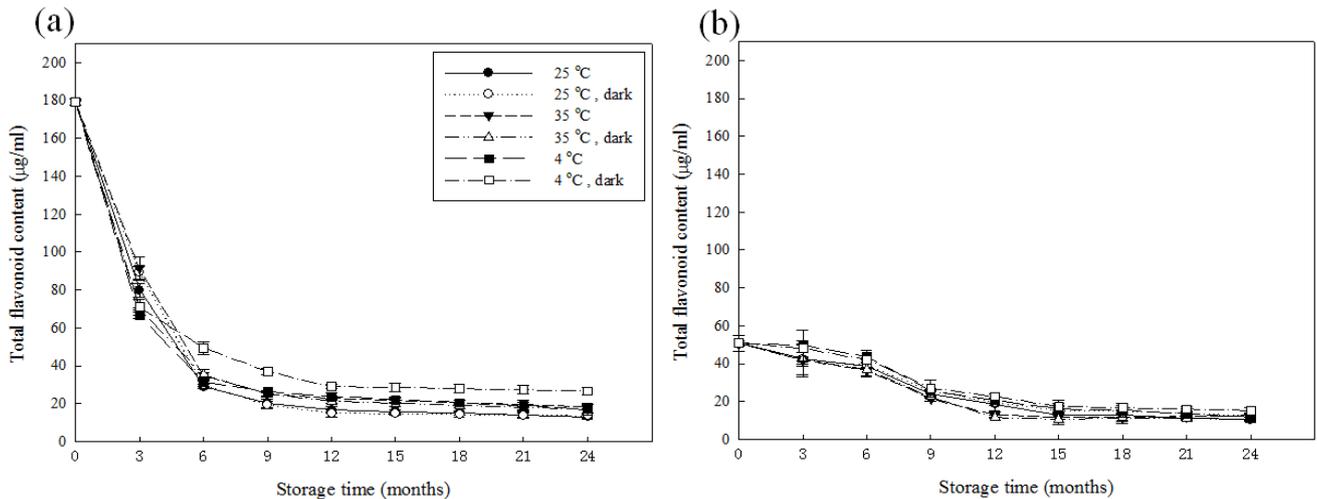


Figure 5. The total flavonoid content of honey under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey; Each point is expressed as mean \pm S.D. (n=3)

3.5. Scavenging Effect of Honey upon DPPH Radicals

DPPH with stable radicals is often applied to demonstrate the radical scavenging ability of natural compounds [8]. The antioxidant potential of honey was directly proportional to the amount of phenolic acids and flavonoids present [7]. The higher DPPH radical-scavenging activity may be attributed to its higher phenolic and flavonoid content [8]. Compared to *D. longan* honey, *B. pilosa* honey initially contained more phenolic compound and flavonoid, and exhibited better DPPH radical-scavenging activity.

As shown in Figure 6, *B. pilosa* honey and *D. longan* honey lost DPPH radical-scavenging activity with the duration and temperature of storage. In the beginning, *B. pilosa* honey had better DPPH radical-scavenging activity than *D. longan* honey. *B. pilosa* honey still had better activity than *D. longan* honey after storage under the same condition for two years. At 25°C, *B. pilosa* honey lost 50% of activity within ~18 months; while *D. longan* honey lost ~50% of activity after storage for 9 months. When honeys were stored at 4°C, their decline rates of DPPH radical scavenging activity were retarded.

No apparent difference was found between the storage conditions under dark and light.

3.6. Radical Scavenging Effect upon Superoxide Radicals

Superoxide radical damaging the cell and tissues could be produced via autoxidation and enzymatic reactions in our bodies [8]. NBT method was applied to examine the scavenging rate of superoxide radicals by honeys. Honeys from *B. pilosa* and *D. longan* could inhibit superoxide radical formation with an inhibition rate of 60-75% initially, but lost such ability during storage (Figure 7). *B. pilosa* honey lost $\geq 50\%$ of the superoxide radical-scavenging ability soon after 3 months storage, while *D. longan* honey lost 15-40% of the ability. Unlike *B. pilosa* honey, such ability in *D. longan* honey gradually decreased with the prolong storage and high temperature. According to superoxide radical scavenging rate, it is suggested to store *D. longan* honey at 4°C. Regarding to prolong storage for more than 12 months, *B. pilosa* honey stored at 35°C under light had unusually better superoxide radical scavenging ability than those stored at other conditions.

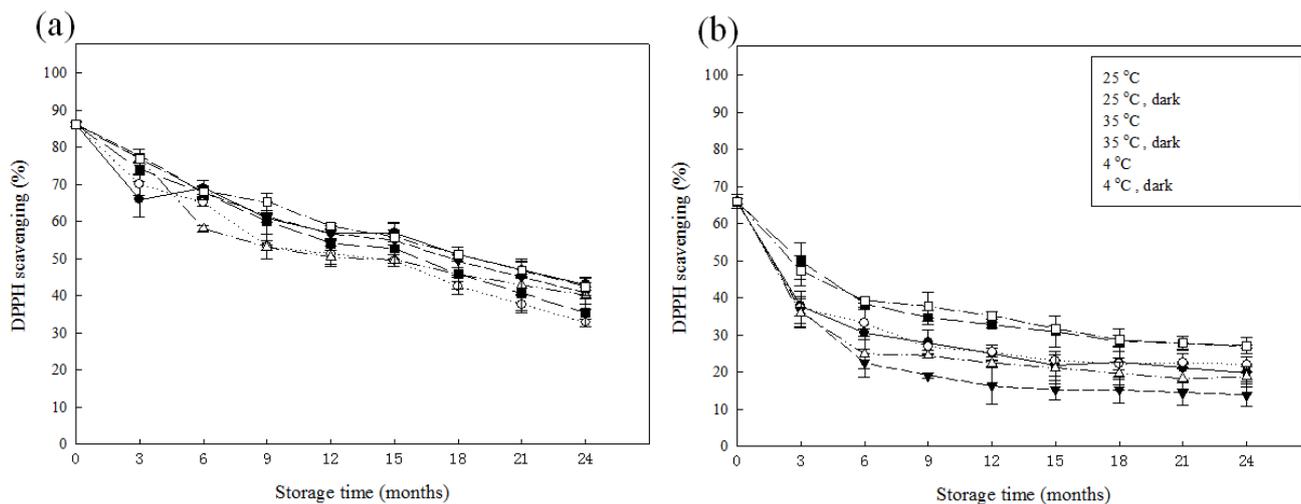


Figure 6. The DPPH scavenging rate of honey under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey; Each point is expressed as mean \pm S.D. (n=3)

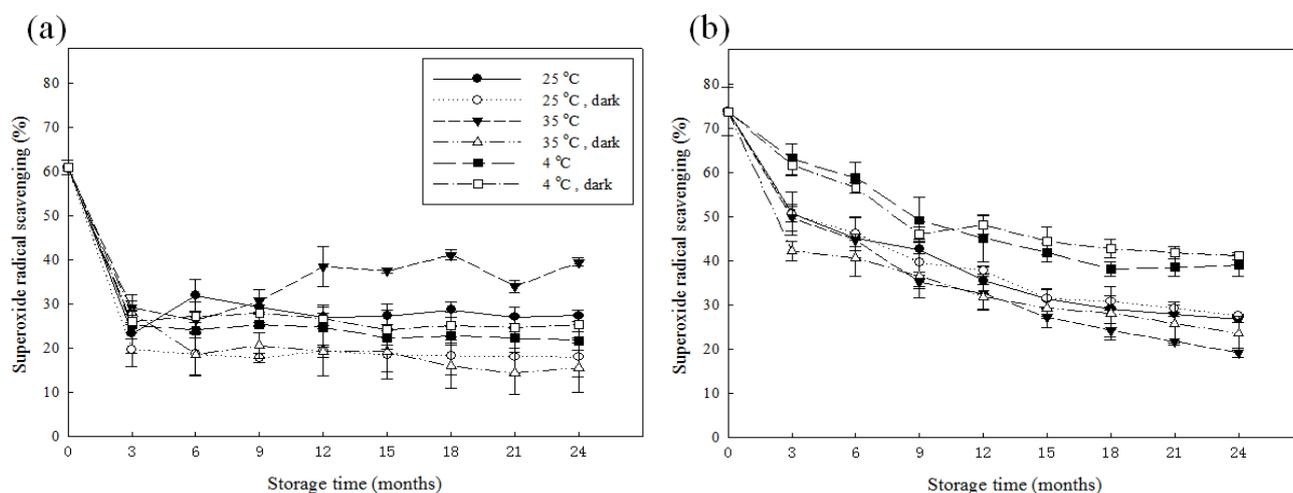


Figure 7. The superoxide radical scavenging rate of honey under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey; Each point is expressed as mean \pm S.D. (n=3)

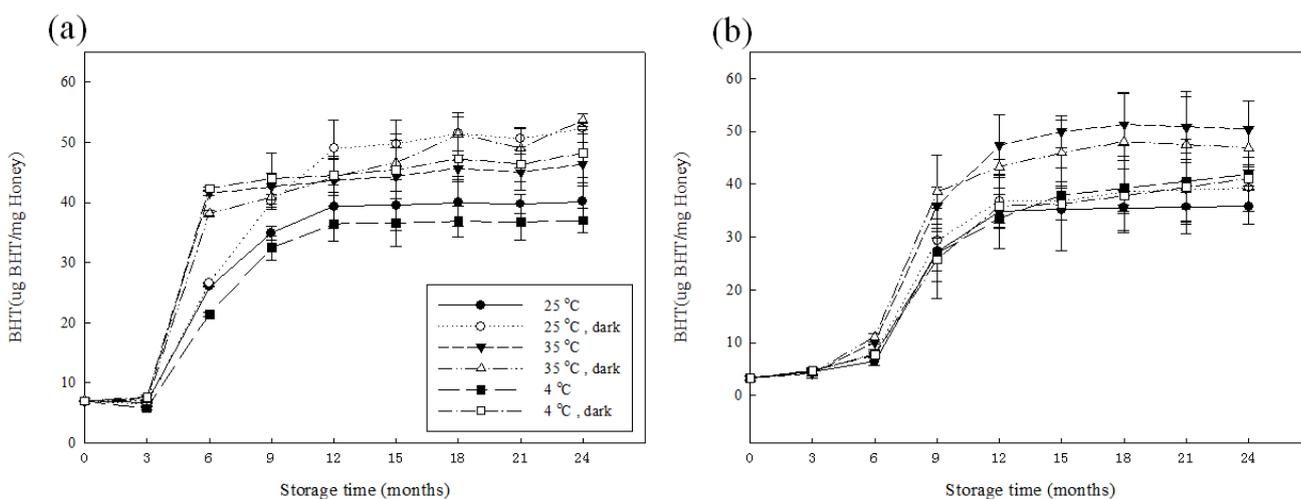


Figure 8. The reducing power of honeys under different storage conditions, at 35, 25 or 4°C for 3-24 months under dark and light. (a) *Bidens pilosa* honey; (b) *D. longan* honey; Each point is expressed as mean \pm S.D. (n=3)

3.7. Reducing Power of Honey

The reducing power is mainly associated with the phenolic contents in honey. The reducing power of honey increased after prolong storage (Figure 8). It may

due to the amount of total phenolic contents increased in both *D. longan* and *B. pilosa* honey (Figure 4). Similar results were reported that there was good correlation between reducing power and polyphenolic contents in honey [8].

Table 1. The correlation coefficient between physicochemical properties, antioxidant capacity and storage conditions in *Bidens pilosa* honey

	Storage condition					
	25°C	25°C/ dark	35°C	35°C/ dark	4°C	4°C/dark
Acidity	0.473	0.591	0.987	0.758	0.105	0.245
HMF content	0.483	0.329	0.866	0.793	0.116	0.102
DPPH scavenging activity	-0.927	-0.902	-0.926	-0.996	-0.881	-0.840
Superoxide radical scavenging activity	-0.501	-0.385	-0.698	-0.501	-0.379	-0.446
Total phenolic content	0.919	0.974	0.997	0.958	0.825	0.910
Total flavonoid content	-0.860	-0.730	-0.980	-0.970	-0.705	-0.743
Reducing power	0.960	0.940	0.972	0.988	0.817	0.764

Table 2. The correlation coefficient between physicochemical properties, antioxidant capacity and storage conditions in *D. longan* honey

	Storage condition					
	25°C	25°C/dark	35°C	35°C/dark	4°C	4°C/dark
Acidity	0.839	0.940	0.953	0.966	0.581	0.362
HMF content	0.173	0.104	0.961	0.910	0.010	-0.021
DPPH scavenging activity	-0.882	-0.959	-0.953	-0.923	-0.804	-0.839
Superoxide radical scavenging activity	-0.890	-0.800	-0.927	-0.921	-0.824	-0.721
Total phenolic content	0.873	0.857	0.937	0.840	0.713	0.788
Total flavonoid content	-0.904	-0.896	-0.940	-0.998	-0.846	-0.784
Reducing power	0.873	0.852	0.892	0.975	0.800	0.862

3.8. Correlation Analysis

The relationships between storage condition and the parameter analyzed in this study (total phenolic content, total flavonoid content, antioxidant activity, and physicochemical properties) are presented in Table 1 and Table 2.

As shown in Table 1, the correlation coefficient has a great difference of acidity and HMF content at different temperatures, it's clearly indicated that HMF content in honey samples significantly correlated with storage duration, and total acidity. Storage duration showed a very strong correlation with HMF level, indicating that time and temperature are the most important factors that affect HMF formation. The other physicochemical parameters such as total acidity showed strong correlations with HMF formation. Since both experimental data and statistical analysis indicated that storage duration and total acidity levels significantly correlated with HMF formation, conducting additional tests such as measuring free acids and total acidity may provide ways to quickly assess honey quality.

A positive correlation between the reducing power, phenolic content, and storage condition (Table 1) was observed. The high correlation coefficient indicates that phenolics are one of the main components responsible for the reducing behaviour of honey but not storage conditions. A negative correlation between the DPPH scavenging activity, superoxide radical scavenging activity, total flavonoid content, and storage condition (Table 1) was observed.

The correlation coefficient indicates that flavonoids are one of the main components responsible for the proton radicals scavenging ability of honey but not storage conditions. Gheldof et al. [11] addressed that the antioxidant activity of honey contributed by phenolic compounds significantly, but in spite of this, it seems that antioxidant activity appears to be a result of the combined activity of honey phenolics, peptides, organic acids, enzymes and Maillard reaction products. The correlation coefficient of total phenolic content, total flavonoid content and antioxidant capacity showed insignificant

difference with different temperature after two years storage, which may indicate the storage time was one reason of the parameters' change.

Table 2 showed similar result with Table 1, suggesting that long term storage has similar influence on *B. pilosa* and *D. longan* honey.

4. Conclusion

Long term storage under high temperature affected the quality of honeys from both sources. Upon storage for longer time and higher temperature, acidity was increased, more total flavonoid contents and radical scavenging ability was loss in honey. *B. pilosa* honey had around 3.5 folds more flavonoid contents than *D. longan* honey initially. *B. pilosa* honey lost lots of flavonoid, and exhibited almost the same flavonoid contents as *D. longan* honey after one year storage. HMF content was quickly increased at 35°C, while it retained low level up to two years at either 4 or 25°C. According to our study, 4°C was the best storage condition to maintain most quality parameters, nutrients and antioxidant functions. It is suggested to store honey at lower temperature less than one year. Considering HMF content, honey should not be stored at temperature higher than 35°C.

Abbreviations

HMF: Hydroxymethylfurfural; DPPH: 1, 1-diphenyl-2-picryl-hydrazyl; MRP: Maillard reaction products; HPLC: high-performance liquid chromatography; NBT: nitroblue tetrazolium chloride; BHT: dibutyl hydroxytoluene;

Data Availability

All data used in the manuscript are already included in the manuscript.

Conflicts of Interest

The authors report no potential conflicts of interest.

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