Journal of Food Engineering and Technology 2025;14(1):21-27
https://doi.org/10.32732/jfet.2025.14.1.21

# Evaluation of the Antioxidant Activity of Honey

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Received: 22 March 2025; Accepted: 3 May 2025; Available online: 20 June 2025

**Abstract:** Antioxidant activity of honey highly correlates with its total phenol content. The antioxidant capacity is attributable to various components, and there are several measurement methods for evaluating all of these components; hence, integration of measurement methods is important. Therefore, the aim of this study was to evaluate the antioxidant activity of honey by examining correlations among the total polyphenol content, color values, melanoidin content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, and iron-reducing capacity. Fourteen honey samples were collected from different nectar sources. Total polyphenol content was measured using the Folin–Ciocalteu method. Color values and melanoidin content were measured using the absorbance values of samples. The antioxidant capacity was evaluated by measuring DPPH radical-scavenging activity and iron-reducing ability using the ferric-reducing antioxidant power method. Substantial correlations were observed between the total polyphenol content and iron-reducing capacity, total polyphenol content and color value, color value and iron-reducing capacity, melanoidin content and total polyphenol content, melanoidin content and iron-reducing capacity, and melanoidin content and color value. Among all honey samples, buckwheat honey had the highest values for all parameters. These findings indicate that the antioxidant effect of honey should be evaluated based on not only its total polyphenol content and DPPH radical-scavenging activity but also its iron-reducing capacity, color value, and melanoidin content.
**Keywords:** Antioxidant capacity; Flavonoid; Honey; Iron-reducing capacity; Polyphenol.

## 1. Introduction

Honey is a functional food with various bioactivities, including antimicrobial, antioxidant, and enzyme-inhibitory activities [1,2]. It has various applications owing to its antioxidant properties, including healing of chronic ulcers in humans; removal of toxic reactive oxygen species [3]; and inhibition of lipid oxidation in meats, salad dressings, and mackerel meat [4–6]. Flavonoids (such as quercetin and rutin) and phenolic acids (such as chlorogenic acid and caffeic acid) are the major antioxidants present in honey [7,8], and they belong to the polyphenol group. Considering that the flavonoids are derived from polyphenols present in pollen [9], their content varies depending on the nectar source [10]. The antioxidant activity of honey is highly correlated with the total phenol content in pollen [9,11] and the color, antioxidant capacity, and total polyphenol (TP) content of honey [12,13]. Therefore, the antioxidant activity of honey may be considerably influenced by its polyphenols and pigment components.

The most commonly used method for evaluating antioxidant activity is the measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, whereas the reducing activity of foods is evaluated based on iron-reducing capacity [14]. Given that the components contributing to the antioxidant capacity of foods are diverse and the same measurement methods are not suitable for all components, combining the results of various evaluation approaches based on different principles is important [15]. Moreover, to the best of my knowledge, no study has compared various methods used to measure antioxidant activity and examine factors affecting antioxidant activity in honey. Therefore, the aim of this study was to evaluate the antioxidant activity of honey by initially measuring the total polyphenol content, color value, and melanoidin content, which are most likely to influence the antioxidant activity. Subsequently, the DPPH radical-scavenging activity and iron-reducing capacity were measured to assess the antioxidant activity. Finally, the correlation among the factors involved in antioxidant activity was confirmed to comprehensively evaluate the antioxidant activity of honey. To the best of my knowledge, this is the first study on the antioxidant activity of honey through initial measurements of total polyphenol content, color value, and melanoidin content. I hypothesize that the antioxidant activity of honey can be effectively evaluated by integrating measurements of total polyphenol content, color values, melanoidin content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, and iron-reducing capacity. This study could reveal correlations among these parameters; honey with higher total polyphenol content, color values, melanoidin content, DPPH radical-scavenging activity, and iron-reducing capacity might exhibit stronger antioxidant properties.

## 2. Materials and Methods

Fourteen types of honey were obtained; namely, three types from *Acacia* sp. (Ou Apiary, Akita, Japan), two from *Aesculus hippocastanum* L. (horse chestnut; Ou Apiary), two from *Hovenia dulcis* Thunb. (Japanese raisin tree; Ou Apiary), one from *Nelumbo nucifera* Gaertn. (lotus) root (Kato Brothers Honey Co., Ltd., Tokyo, Japan), one from *Fagopyrum esculentum* Moench (buckwheat; Ou Apiary), and five types of hundred-flower honey (Ou Apiary). The honey samples and the Folin–Ciocalteu reagent were individually diluted two-fold in distilled water.

Each honey sample was diluted two-fold (w/w) in 1% HCl-methanol solution. Thereafter, the diluted Folin–Ciocalteu reagent (500 µL) was added to 125 µL of this solution, which was stirred and allowed to stand for 3 min at 25°C. Subsequently, 325 µL of 1% sodium carbonate solution was added, the samples were stirred, and their absorbance was measured at 750 nm (iMark micro plate reader; Bio-Rad, Hercules, CA, USA). Total polyphenol content was determined using the Folin–Ciocalteu method as described by Hashimoto and Seki [16]. Chlorogenic acid was used as a standard and the total polyphenol content is expressed as chlorogenic acid equivalents.

Antioxidant activity was evaluated using the DPPH radical-scavenging activity as described by Nakatani and Seki [17]. Briefly, 1.8 mL of 160 mg/L DPPH-50% ethanol solution was added to 0.2 mL of honey, the mixture was stirred and allowed to stand for 30 min at 25°C, and its absorbance was measured at 540 nm (B). The absorbance of a sample solution to which distilled water was added as a control (C) and a sample to which ethanol was added instead of DPPH as a blank (A) was measured after 30 min.

DPPH radical-scavenging activity was calculated by substituting the absorbance values in the following formula (Equation 1):

$$\text{DPPH radical-scavenging activity (\%)} = \{C - (A - B)\} / C \times 100 \quad (1)$$

where A is the absorbance of honey, B is the absorbance of the blank, and C is the absorbance of the control.

Iron-reducing capacity was evaluated using the ferric-reducing antioxidant power (FRAP) method described by Seki and Nakanishi (2020). To prepare FRAP reagent, 10 mmol/L 2,4,6-Tris(2-pyridyl)-s-triazine dissolved in acetate buffer (pH 3.6), 40 mmol/L HCl, and 20 mmol/L $FeCl_3$ were mixed at a ratio of 10:1:1. Briefly, 1 mL of FRAP reagent was added to 100 µL of honey, incubated for 4 min at 37°C, and the absorbance was measured at 570 nm. The FRAP is expressed as the amount of $Fe^{2+}$ produced (µmol/L) using $FeSO_4.7H_2O$ as a control.

For color determination and melanoidin content measurement, the absorbance of samples was measured at 450, 570, and 750 nm, as described by Aazza et al. [12]. The color values and melanoidin content were calculated using the following equations (Equations 2 and 3):

$$\text{Color value} = \text{absorbance at 570 nm} - \text{absorbance at 750 nm} \quad (2)$$

$$\text{Melanoidin content} = \text{absorbance at 450 nm} - \text{absorbance at 750 nm} \quad (3)$$

All values are presented as mean ± standard deviation.

The correlation coefficients were calculated by plotting the total polyphenol content, color value, melanoidin value, antioxidant capacity, and iron-reducing capacity on the vertical and horizontal axes.

## 3. Results and Discussion

Buckwheat honey had the highest total polyphenol content (27.3 mg/g) and *Acacia* honey B had the lowest total polyphenol content (0.482 mg/g) (Table 1). Polyphenols were absent in *Acacia* honey C (0.00 mg/g). Buckwheat honey had the highest total polyphenol content and *Acacia* honey C had the lowest (Table 1). In 60 honey samples from 19 plant sources collected from various regions of Turkey, the total polyphenol content was 1051.58 mg/kg in thyme honey samples and 240.75 mg/kg in *Acacia* sp. honey samples [18]. Hence, the amounts of polyphenolic compounds in honey (such as phenolic acids and flavonoids) may vary according to the plant source [19]. Similarly, in the present study, the polyphenol content differed depending on the nectar source.

**Table 1.** TP content in each honey sample

| Type of honey | TP (mg/g) |
|---|---|
| *Acacia* A | 0.671 ± 0.00411 |
| *Acacia* B | 0.482 ± 0.00681 |
| *Acacia* C | 0.00 (0.00) |
| Horse chestnut A | 0.664 ± 0.00591 |
| Horse chestnut B | 0.497 ± 0.01270 |
| Japanese raisin tree A | 2.84 ± 0.00877 |
| Japanese raisin tree B | 2.32 ± 0.04100 |
| Lotus | 0.933 ± 0.00850 |
| Buckwheat | 27.3 ± 0.26900 |
| Hundred-flower honey A | 1.19 ± 0.00993 |
| Hundred-flower honey B | 2.64 ± 0.02790 |
| Hundred-flower honey C | 2.15 ± 0.00435 |
| Hundred-flower honey D | 1.06 ± 0.04510 |
| Hundred-flower honey E | 2.01 ± 0.00874 |

* Polyphenol content was calculated as chlorogenic acid equivalents (mg/g).
** Measurements were performed in triplicate. Values are presented as mean ± standard deviation. TP, total polyphenol.

Table 2 presents the antioxidant capacity of each honey type. The highest values were obtained for horse chestnut B, Japanese raisin tree, Buckwheat, and Hundred-nectar B honey samples (100%), and the lowest value (3.04%) was obtained for *Acacia* honey C. The antioxidant capacity ranged from 3.04% to 100% in the tested samples and greatly varied depending on the type of honey (Table 2). In previous studies, the antioxidant capacities ranged from 12.98% to 94.79% [20] for 60 different honey samples and 32.6% to 82.8% [21] for 11 Mexican honey samples. The presence of phenolic compounds and flavonoids is considered to play a major role in antioxidant activity; however, their mechanisms of action have not yet been elucidated [21]. In a previous study, the addition of various ratios of grape polyphenols, dibutylhydroxytoluene, tertiary butylhydroquinone, and ascorbic acid to lard, peanut, sunflower seed, rapeseed, soybean, and sesame oils resulted in different oxidation rates depending on the specific ratios used [22]. Therefore, the bioactive substances in honey that exhibit antioxidant activity most likely interact in a complex manner, leading to varying levels of antioxidant capacity.

**Table 2.** Antioxidant capacity of the honey samples

| Honey sample | Antioxidant capacity (%) |
|---|---|
| *Acacia* A | 26.1 ± 0.00681 |
| *Acacia* B | 31.3 ± 0.01420 |
| *Acacia* C | 3.04 ± 0.01230 |
| Horse chestnut A | 37.2 ± 0.04610 |
| Horse chestnut B | 100 ± 0.03120 |
| Japanese raisin tree A | 78.5 ± 0.02460 |
| Japanese raisin tree B | 11.9 ± 0.02010 |
| Lotus | 16.9 ± 0.00872 |
| Buckwheat | 100 ± 0.03210 |
| Hundred-flower honey A | 27.7 ± 0.00917 |
| Hundred-flower honey B | 100 ± 0.02490 |
| Hundred-flower honey C | 58.6 ± 0.04920 |
| Hundred-flower honey D | 34.3 ± 0.01760 |
| Hundred-flower honey E | 67.4 ± 0.0124 |

* Measurements were performed in triplicate. Values are presented as mean ± standard deviation.

The highest and lowest iron-reducing capacity values were obtained for buckwheat honey (30.6 µmol/L) and *Acacia* honey C (2.83 µmol/L), respectively (Table 3). Among several Polish honeys, buckwheat honey had the highest iron-reducing capacity, which was at least twice as high as that of honeys from other flowers [23]. The FRAP assay is used to investigate the electron-donating capacity of antioxidants [24], with rutin reportedly having the highest capacity [25]. Buckwheat honey has a 1.2–16-fold greater rutin content than other honeys [9,26], explaining the high iron-reducing capacity observed in the present study.

**Table 3.** Iron-reducing capacity of the honey samples

| Honey sample | $Fe^{2+}$ production (μmol/L) |
|---|---|
| *Acacia* A | 4.11 ± 0.00208 |
| *Acacia* B | 4.34 ± 0.01550 |
| *Acacia* C | 2.83 ± 0.01170 |
| Horse chestnut A | 4.78 ± 0.16900 |
| Horse chestnut B | 3.88 ± 0.02540 |
| Japanese raisin tree A | 11.1 ± 0.03020 |
| Japanese raisin tree B | 4.53 ± 0.00586 |
| Lotus | 6.37 ± 0.02750 |
| Buckwheat | 30.6 ± 0.22600 |
| Hundred-flower honey A | 7.31 ± 0.01310 |
| Hundred-flower honey B | 10.5 ± 0.01350 |
| Hundred-flower honey C | 9.18 ± 0.04200 |
| Hundred-flower honey D | 7.01 ± 0.00751 |
| Hundred-flower honey E | 9.90 ± 0.01880 |

* Measurements were performed in triplicate. Values are presented as mean ± standard deviation.

Buckwheat honey had the highest color value (0.839) and melanoidin content (3.678), whereas *Acacia* honey C had the lowest color value (0.0260) and hundred-flower honey C had the lowest melanoidin value (−0.0250) (Table 4). Honey compounds with antioxidant activity are integral to melanoidin (a brown pigment formed during the Maillard reaction) generation [27]. The mineral content of honey (including Ca, Mg, Mn, and Zn content) correlates with a darker color and stronger flavor [28]. A previous study on several honeys revealed that Buckwheat honey contains 1,535 ± 186 mg/kg Ca, which is higher than that in other honeys [28]. Similarly, purple-black-colored rice has a higher Ca content than non-colored Koshihikari rice [29]. Thus, the high Ca content in buckwheat honey likely contributes to its high color values.

**Table 4.** Color values and melanoidin content of honey samples

| Honey sample | Color value | Melanoidin value |
|---|---|---|
| *Acacia* A | 0.0287 ± 0.00115 | 0.0897 ± 0.00208 |
| *Acacia* B | 0.0347 ± 0.00231 | 0.0990 ± 0.00520 |
| *Acacia* C | 0.0260 ± 0.00100 | 0.0710 ± 0.00361 |
| Horse chestnut A | 0.0417 ± 0.00208 | 0.128 ± 0.00265 |
| Horse chestnut B | 0.0413 ± 0.00252 | 0.114 ± 0.00608 |
| Japanese raisin tree A | 0.0700 ± 0.00557 | −0.0917 ± 0.29200 |
| Japanese raisin tree B | 0.0310 ± 0.00624 | 0.117 ± 0.00321 |
| Lotus | 0.0517 ± 0.00153 | 0.343 ± 0.45000 |
| Buckwheat | 0.839 ± 0.0303 | 3.678 ± 0.0150 |
| Hundred-flower honey A | 0.0607 ± 0.00666 | 0.219 ± 0.00850 |
| Hundred-flower honey B | 0.0883 ± 0.00666 | 0.294 ± 0.01700 |
| Hundred-flower honey C | 0.0727 ± 0.00404 | −0.250 ± 0.01300 |
| Hundred-flower honey D | 0.0547 ± 0.00153 | 0.171 ± 0.00608 |
| Hundred-flower honey E | 0.0737 ± 0.00551 | 0.227 ± 0.01540 |

* Measurements were performed in triplicate. Values are presented as mean ± standard deviation.

Table 5 presents the correlations among the different factors. The correlation coefficients ($R^2$) of the physical parameters investigated in this study were as follows: 0.857 for total polyphenol content and iron-reducing capacity, 0.994 for total polyphenol content and color value, 0.855 for color value and iron-reducing capacity, 0.852 for melanoidin and total polyphenol content, 0.870 for melanoidin content and iron-reducing capacity, 0.870 for melanoidin and total polyphenol content, and 0.990 for melanoidin and color value. These values indicate relatively high correlations among the parameters. In contrast, the correlations between melanoidin content and other parameters were low. However, high correlations were observed between several pairs of variables: total polyphenol content and iron-reducing capacity, total polyphenol content and color value, color value and iron-reducing capacity, melanoidin value and total polyphenol content, melanoidin content and iron-reducing capacity, and melanoidin content and color value (Table 5). Similarly, high correlations have been observed in studies on Polish honey [30,31] and honeys from different regions [13,30,12]. Therefore, the polyphenol content in honey considerably affects its iron-reducing capacity, and the color value increases with the polyphenol content. Additionally, chlorogenic acid (a polyphenol involved in the formation of brown pigments in honey) [7,32] and quercetin (a pale-yellow flavonoid) [33] may be associated with the color value.

**Table 5.** Correlations among different factors in honey samples

| | Total polyphenol content | Antioxidant activity | Iron-reducing capacity | Color values | Melanoidin values |
|---|---|---|---|---|---|
| Total polyphenol content | 1 | | | | |
| Antioxidant activity | 0.360 | 1 | | | |
| Iron-reducing capacity | 0.857 | 0.419 | 1 | | |
| Color values | 0.994 | 0.229 | 0.855 | 1 | |
| Melanoidin values | 0.993 | 0.226 | 0.8566 | 0.9995 | 1 |

The melanoidin content correlated with the total polyphenol content in honey obtained from the southern region of Morocco [12], and similar results were obtained in the present study. Melanoidin is a brown, high-molecular-weight, nitrogen-containing compound [34]. It reported that melanoidins and its components showed strong reducing power, mainly because melanoidins has the property of negative charge, which makes it able to chelate with transition metals such as ferrous ion [35]. Therefore, an increase in melanoidin content may increase the color value and iron-reduction capacity.

In the present study, the antioxidant capacity did not correlate well with any of the parameters. The antioxidant capacity and iron-reducing capacity are affected by the type of polyphenols present. The combination of phenolic acids and flavonoids tends to be antagonistic in terms of antioxidant activity, but synergistic in terms of iron-reducing capacity [36]. Considering that honey contains chlorogenic acid and quercetin, these compounds may affect the antioxidant capacity measurement, thereby yielding results that differ from those of the iron-reducing capacity.

A limitation of the present study is the variability in honey color and composition, which heavily depends on the nectar source and seasonal fluctuations influenced by the health and foraging behavior of bees. This variability complicates the task of obtaining uniform honey samples for research purposes. An approach for future studies to address this challenge is meticulous monitoring bee colonies and seasonal nectar availability, coupled with the use of standardized sampling protocols, to obtain honey samples with more consistent quality across different conditions and regions.

## 4. Conclusions

The antioxidant activity of honey was evaluated in this study. Initially, the total polyphenol content, color value, and melanoidin content, which are most likely to influence the antioxidant activity, were measured. Among all honey samples, buckwheat honey had the highest values for all parameters. Subsequently, DPPH radical-scavenging activity and iron-reducing capacity were measured to assess antioxidant activities. DPPH radical-scavenging activity was the highest for horse chestnut B honey, Japanese honey, buckwheat honey, and hundred-flower honey B samples, and the iron-reducing capacity was the highest for buckwheat honey. Finally, strong correlations were observed between the total polyphenol content and iron-reducing capacity, total polyphenol content and color value, color value and iron-reducing capacity, melanoidin content and total polyphenol content, melanoidin content and iron-reducing capacity, and melanoidin content and color value. These findings indicate that the total polyphenol content, iron-reducing capacity, color value, and melanoidin content of honey have a significant effect on iron-reducing capacity, but little effect on antioxidant activity. As the effects of the total polyphenol content and other factors on antioxidant and iron-reducing capacities differed, future research should focus on clarifying the specific honey components that cause these effects to provide a comprehensive understanding of the antioxidant effect.

## 5. Acknowledgments

This research was partially supported by the Research Center for the Future of Food and Agriculture, Tokyo University of Technology. I express my deep gratitude to Ms. Sugimoto R. and Ms. Katsumata M. of the Department of Advanced Food and Agricultural Sciences, Faculty of Agriculture, Tamagawa University for their cooperation in conducting this study.

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