Original Article

Journal of Food Safety and Hygiene

Journal homepage: http://jfsh.tums.ac.ir

Evaluating the antioxidant activity and the level of 5-hydroxymethylfurfural in honey

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ARTICLE INFO

Article history:
Received 26 Aug. 2023
Received in revised form
02 Dec. 2023
Accepted 09 Dec. 2023

Keywords:
Honey;
Antioxidant;
Antiradical;
5-hydroxymethylfurfural;
Ferric reducing antioxidant power;
1, 1-diphenyl-2-picrylhydrazyl

ABSTRACT

Honey is one of the richest sources of antioxidants due to a variety of phenolic acids and flavonoids. Another prime chemical indicator of assessing honey quality is 5-hydroxymethylfurfural (HMF) which indicates the freshness of honey as well as exposure to heat. Increasing HMF above the permissible limit could cause cytotoxic and mutagenic effects. In this study, we evaluate the antioxidant activity and radical scavenging capacity of forty samples of natural and commercial Iranian honey. Also, we determine the HMF content in these samples. This aim was achieved through ferric reducing antioxidant power (FRAP), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, and white methods. Results of DPPH (in the range of 3.13% to 66.61%) revealed there was no significant difference between natural and commercial honey in terms of radical scavenging activity ($p>0.05$). On the other hand, the results of FRAP showed that natural honey had a significantly higher total antioxidant capacity than commercial honey ($p<0.05$). There was a significant difference between honey samples from different plants, too. Furthermore, there was a significant difference between natural and commercial honey in terms of radical scavenging activity and HMF levels ($p<0.05$). The result of HMF content was in the range of 8.64 to 98.48 mg/kg. Eventually, it could be concluded that climate, plant source, process of production and storage have an impact on these parameters. Also, some honey samples have high HMF content due to heat-treated processes or dated products.

Citation: Nobari Moghaddam H, Alaeepajouh Sh, Behzad M, Hajimahmoodi M, Sadeghi N. **Evaluating the antioxidant activity and the level of 5-hydroxymethylfurfural in honey.** J food safe & hyg 2023; 9 (4): 241-251. DOI: 10.18502/jfsh.v9i4.15000

1. Introduction

According to the Codex Alimentarius, "honey is a natural sweetener produced by *Apis melifera* bees from the nectar plants (1)". Honey is a complicated food matrix that is used as a natural sweetener and (2) it is economically critical (3).

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Honey has been considered an effective medicinal substance since ancient times because of its application in the treatment of various diseases. Antimicrobial and wound-healing properties of honey have been well-known throughout centuries (4). It is also known to be worthwhile for the treatment of malnutrition in children (5), digestion problems (6), pregnancy nausea

(6), pregnancy constipation (6), cough (6), peptic ulcers (7), and vaginal inflammation (8).

The chemical composition of honey consists of more than 90% sugars (including glucose and fructose), water (15-17%), and proteins (0.1-0.4%) which are mainly honey enzymes, and ash (0.2%). Also, honey consists of amino acids, phenolic compounds, organic acids, vitamins, minerals (mostly potassium) (9,10), volatile compounds (including aldehydes, alcohols, ketones, esters, benzene, and its derivatives, furan, pyran, norisoprenoids, terpenes and its derivatives, sulfur, and cyclic compounds) in small quantities.

Volatile compounds are responsible for the aroma and partly radical scavenging activity of honey (11). Furthermore, honey has neutral lipids such as hydrocarbons, waxes, cholesterol esters, fatty acids, and sterols in very small amounts (12). These components are added by bees or directly from plant extracts (13,14).

The physicochemical properties of honey markedly depend on the floral and geographical origin of honey, climate, and age of the bees through the effects of the enzymes involved in honey production (15).

Oxidative stress and the production of free radicals have been increased as a result of modern lifestyles such as the consumption of fast foods and tobacco, excessive stress, and industrial pollution. Antioxidants have a crucial role in the conservation of people against oxidative stress and its relevant diseases such as cancer, inflammatory diseases, aging, and coronary and cardiovascular disorders (16,17). It is demonstrated honey is one of the richest sources of natural antioxidants and radical scavengers. The responsible components for antioxidant and radical scavenging activity of honey consist of flavonoids (chrysin, pinocembrin, pinobanksin, quercetin, kaempferol, luteolin, galangin, apigenin, hesperetin, and myricetin), phenolic acids (caffeic, coumaric, ferulic, ellagic, chlorogenic), ascorbic acid, catalase, peroxidase, and carotenoids (18). Some notable therapeutic properties of honey are wound healing acceleration (6), tissue regeneration (6), healing of gastrointestinal ulcers (6), and gingivitis (19) which are strongly correlated to antioxidant and radical scavenging activity (20).

Numerous analytical methods are being employed for assessment of the antioxidant capacity of honey; for instance ferric reducing antioxidant power (FRAP) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, oxygen radical absorbance capacity (ORAC), superoxide radical-scavenging activity, and Trolox equivalent antioxidant activity (TEAC). Additionally, there is a critical chemical indicator of honey quality called 5-hydroxymethylfurfural (HMF). HMF is a heterocyclic product of the Millard reaction which is a chemical reaction between amino acids and reducing sugars (mainly fructose) by the impact of heat (21). The amount of HMF is commonly low in fresh honey but its concentration tends to be rocketed during prolonged storage and exposure to excessive heat. The European quality standard allows a maximum of 40 mg/kg of HMF in honey. However, this limit is 80 mg/kg in tropical regions (22). Negative effects of HMF have been reported in previous studies, for instance, cytotoxicity toward mucous membranes, skin, and upper respiratory tract; mutagenicity; chromosomal aberrations; and carcinogenicity in humans and animals (23,24). On the other hand, some positive effects also have been reported recently such as anti-allergic (25), anti-inflammatory (26), anti-hypoxic (27), and anti-hyperuricemic effects (28). Furthermore, in a

work done by Afshari et al., the amount of HMF was assessed in honey samples of Khorasan Province and results showed that 16% of samples had higher HMF levels than the legally permitted maximum level. They revealed that heating may cause the formation of HMF which does not naturally exist in fresh honey and might be harmful to human health (29). In another study done by Ghorbani et al. the amount of HMF in honey samples of South-West and North-West of Iran were compared. The results showed higher HMF content in kinds of honey produced in South-West Iran (30). Today, post-marketing quality control is so highlighted all over the world. Some physicochemical markers could be relied on to decide the quality of honey, for instance, DPPH, FRAP and HMF. In this study, the antioxidant activity and radical scavenging capacity of forty samples of natural and commercial Iranian honey were evaluated. Also, the HMF content in these samples was determined. This goal was achieved via ferric reducing antioxidant power (FRAP), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay and white methods.

2. Materials and Methods

In this study, the antioxidant capacity and radical scavenging activity of forty natural and commercial honey samples on the market were appraised by employing the FRAP assay and DPPH method. Also, the HMF content of these samples was evaluated via the White method.

2.1. Chemicals and instruments

All of the provided chemicals and reagents had an analytical grade. DPPH, TPTZ, methanol, sodium bisulfite, HCl 37%, sodium acetate, glacial acetic acid, ferric chloride, ferrous sulfate, potassium ferrocyanide, and zinc acetate were obtained from Merck. Double distilled water was used for the preparation of aqueous solutions. A UV–Vis spectrophotometer GBC CINTRA 40 was used for absorbance measurements.

2.2. Samples

Twenty natural honey samples were purchased from beekeepers in various regions of Iran and twenty commercial honey samples were purchased from local markets. The samples were from distinct floral origins including thyme, milkvetch, cedar, camelthorn, citrus, fennel flower, saffron, Pennyroyal, Jujube, and multi-floral. All forty samples were stored at room temperature until analysis time.

2.3. DPPH assay

DPPH is a stable free radical that can accept an electron from antioxidants. If there is no antioxidant compound in the DPPH methanolic solution, the color of this solution remains dark purple. Otherwise, discoloration from purple to yellow and a change in light absorbance at 517 nm occurs which could be measured as the ability of radical scavengers.

The determination of DPPH radical scavenging activity went through the following sample preparation: 75 mg of each honey sample was dissolved in 1 ml of methanol and stirred for 5 min with a magnet stirrer (500 rpm). A 0.02 mg/ml methanolic solution of DPPH was made ready daily by dissolving 2 mg DPPH in 100 ml methanol. 0.75 ml of each honey solution was blended with 1.5 ml of DPPH solution and kept in the dark for 30 min at room temperature. The light absorbance of each sample was read at 517 nm with methanol as blank. 0.75 ml methanol mixed with 1.5 ml DPPH was used as the control solution (31).

DOI: 10.18502/jfsh.v9i4.15000

The radical scavenging activity was calculated via the below equation:

$$(\%)\text{DPPH radical scavenging activity} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

2.4. FRAP assay

The aim of employing FRAP assay was an assessment of the ability of ferric (Fe^{3+}) reduction to ferrous (Fe^{2+}) in honey samples. Ferrous forms a blue complex with TPTZ which has light absorbance in 593 nm. The determination of antioxidant capacity required the following sample preparation:

100 mg of each honey sample was dissolved in 1 ml double distilled water and stirred for 5 min with a magnet stirrer (500 rpm). The FRAP reagent was made ready daily by mixing 10 ml of an aqueous 10 mm TPTZ solution in 40 mm HCl, 10 ml of a 20 mm $FeCl_3$ solution, and 100 ml of a 0.3 M acetate buffer (pH = 3.6). The reaction was performed by mixing 0.5 ml of aqueous honey solutions (100 mg/ml) with 4.5 ml of the FRAP reagent. Samples were incubated at *37*°C for 10 min. The light absorbance of each sample was read at 593 nm with a mixture of 0.5 ml double distilled water and 4.5 ml of FRAP reagent as blank. The results of aqueous standard solutions of $FeSO_4$ $7H_2O$ (100–1000 μm) were expressed as the FRAP value (μmol Fe (II)/100 g) for the calibration curve (18).

2.5. Determination of HMF

The White method was operated to determine the HMF content of honey samples to evaluate the impact of heat, storage time and condition. The determination of HMF required the following sample preparation:

Five g of each honey sample was dissolved in 25 ml of double-distilled water and stirred for 5 min with a magnet stirrer. The solutions were transferred to 50 ml volumetric flasks. Carrez solution 1 was prepared by dissolving 15 g potassium ferrocyanide in double-distilled water and diluted to 100 ml. Carrez solution 2 was prepared by dissolving 30 g zinc acetate in double-distilled water and diluted to 100 ml. Sodium bisulfite 0.2% solution was composed of 0.2 mg sodium bisulfite dissolved in 100 ml of double-distilled water. Carrez solution 1 (0.5 ml) was added to the honey solutions and mixed. Carrez solution 2 (0.5 ml) was added to the honey solutions and mixed. The volumetric flasks were filled with double distilled water and filtered through paper rejecting the first 10 ml of filtrate. Each filtrate (0.5 ml) was pipetted into two groups of test tubes. Five ml of double-distilled water was poured into the first group of test tubes (sample) and 5 ml of sodium bisulfite 0.2% solution was added to the second group (reference). All tubes were mixed using a vortex mixer (33). The absorbance of each sample was read at 284 and 336 nm. HMF content of each sample was calculated by the below equation:

$$\text{HMF}\left(\frac{\text{mg}}{\text{kg}}\right) = (\text{Abs in 284 nm} - \text{Abs in 336 nm}) * 149.7$$

2.6. Statistical analysis

All analyses were done three times, and data were shown as mean±SD. One-way analysis of variance (One-way ANOVA) was employed to analyze the data. All analyses were done by Simplot 12 software.

3. Results

3.1. DPPH assay

The results of the evaluation of radical scavenging activity of honey samples in 517 nm are demonstrated

and compared in Table 1. Statistical analysis showed there was no significant difference between natural and commercial honey samples in terms of radical scavenging activity ($p>0.05$). However, there was a statistically significant difference between the samples of each category ($p<0.05$). In the natural category, sample H20 exhibited the highest DPPH inhibition percentage of 66.612±2.339, and sample H29 exhibited the lowest DPPH inhibition percentage of 3.139±1.329. Further, sample H13 exhibited the highest DPPH inhibition percentage of 63.075±1.669, and sample H37 exhibited the lowest DPPH inhibition percentage of 5.236±0.983 among the commercial category.

3.2. FRAP assay

The calibration curve obtained from accessing the total antioxidant capacity of standard $FeSO_4.7H_2O$ solutions within the concentration range of 100-1000 µm in 593 nm is demonstrated in Fig. 1. The results of the evaluation of the total antioxidant capacity of honey samples are demonstrated and compared in Table 1 based on their FRAP value in 593 nm. Statistical analysis revealed there was a statistically significant difference between natural and commercial honey samples in terms of total antioxidant capacity ($p<0.05$). There was a statistically significant difference between the samples of each category ($p<0.05$). In the natural category, sample H29 exhibited the highest FRAP value of 1215.8376±23.3780 and sample H21 exhibited the lowest FRAP value of 153.1966±2.7311. Additionally, sample H27 exhibited the highest FRAP value of 306.2094±7.6530 and sample H24 exhibited the lowest FRAP value of 137.8675±4.4469 among the commercial category.

3.3. Determination of HMF

The results of the evaluation of the HMF content of honey samples via the White method are demonstrated and compared in Table 1. The results showed there was a statistically significant difference between natural and commercial honey samples in terms of HMF content ($p<0.05$). There was a statistically significant difference between the samples of each category ($p<0.05$). In the natural category, sample H20 had the highest HMF content of 98.145±4.539 and sample H8 had the lowest HMF content of 8.647±0.564. Sample H36 had the highest HMF content of 98.487±2.599, and sample H17 had the lowest HMF content of 11.455±1.456 among the commercial category. Exceeding the limit of 40 mg/kg which is mentioned in the Codex Alimentarius, could be a result of inappropriate storage conditions such as exposure to excessive heat. It could also be as a result of harvest a long time ago and honey is outdated.

DOI: 10.18502/jfsh.v9i4.15000

Table 1. Radical scavenging activity, total antioxidant capacity and HMF content of natural and commercial honey samples

Natural/commercial	Sample code	Scientific name of plants	Radical scavenging activity (mean inhibition (%))	Total antioxidant capacity (mean Frap value (µmol Fe(Ⅱ)/100g))	HMF content (mean HMF (mg/kg))
Natural	H1	Thyme	48.740±1.181	433.085±12.427	17.702±0.375
	H2	Jujube	30.088±1.653	344.675±0.862	12.608±0.691
	H3	Multi-floral	34.853±2.595	368.551±11.251	35.974±0.490
	H4	Astragalus	55.868±2.065	348.611±7.886	29.214±0.431
	H5	Multi-floral	53.667±2.026	233.307±6.839	70.487±0.648
	H6	Alhagi maurorum	12.505±0.379	467.953±21.710	23.008±0.319
	H7	Citrus bigardia Duh	23.110±0.123	170.649±8.104	23.453±0.718
	H8	Jujube	19.677±2.082	513.042±18.557	8.647±0.564
	H9	Astragalus	54.618±1.232	216.363±9.938	31.513±0.666
	H10	Thyme	47.808±0.808	263.743±12.349	86.563±1.086
	H19	Nigella Sativa	54.405±1.684	272.333±4.247	55.552±3.091
	H20	Ziziphus jujuba	66.612±2.339	376.474±7.965	98.145±4.539
	H21	Multi-floral	46.347±1.353	153.196±2.731	74.083±3.057
	H22	Multi-floral	43.460±2.893	207.628±6.908	78.361±3.501
	H23	Multi-floral	57.194±1.082	226.222±7.240	62.612±1.664
	H29	Multi-floral	3.139±1.329	1215.837±23.370	26.781±0.726
	H32	Astragalus	37.064±2.998	165.923±3.675	37.172±3.083
	H33	Crocus sativus	48.903±0.819	169.098±2.493	51.435±2.879
	H39	Thyme	57.897±2.060	304.756±8.741	74.296±2.556
	H40	Mentha pulegium	59.511±2.351	295.534±4.886	70.789±1.710
Commercial	H11	Multi-floral	44.494±1.587	234.021±9.893	11.561±1.037
	H12	Multi-floral	33.096±1.763	158.230±5.866	15.627±0.866
	H13	Multi-floral	63.075±1.669	230.217±11.200	89.249±1.831
	H14	Multi-floral	34.834±3.230	166.803±1.805	16.922±1.396
	H15	Multi-floral	36.094±2.008	160.440±4.619	11.836±1.362
	H16	Multi-floral	51.004±0.654	217.316±6.791	19.026±0.909
	H17	Multi-floral	45.067±1.254	192.581±1.052	11.455±1.456
	H18	Multi-floral	41.739±2.841	175.192±2.764	32.005±2.609
	H24	Multi-floral	52.875±2.180	137.867±4.446	21.962±1.018
	H25	Multi-floral	51.830±1.108	230.427±9.907	19.923±1.279
	H26	Multi-floral	36.022±1.072	259.081±6.119	21.104±1.951
	H27	Astragalus	38.588±3.398	306.209±7.653	22.548±1.104

H28	Multi-floral	45.620±2.773	204.645±8.399	60.498±2.697
H30	Multi-floral	38.224±2.986	181.803±6.318	70.887±1.747
H31	Multi-floral	7.544±0.066	198.871±4.768	46.293±2.818
H34	Multi-floral	58.707±3.162	174.854±4.132	30.755±2.282
H35	Multi-floral	37.803±3.836	162.444±5.427	24.412±1.972
H36	Multi-floral	25.357±0.440	169.457±1.513	98.487±2.599
H37	Multi-floral	5.236±0.983	147.517±7.896	19.941±2.543
H38	Multi-floral	23.317±2.055	159.906±6.678	23.571±0.974

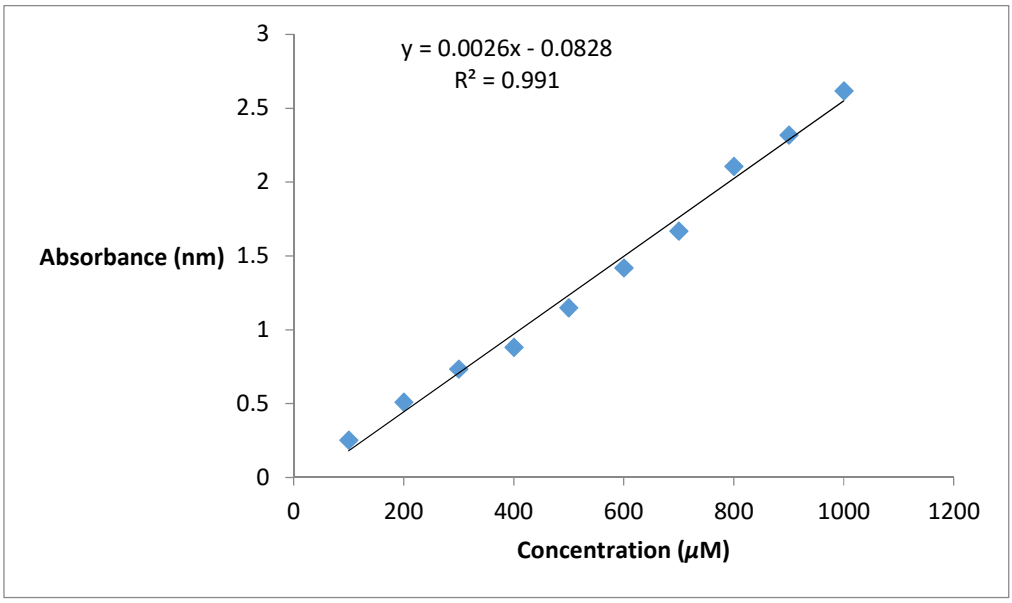

Figure 1. Calibration curve standard $FeSo_4.7H_2O$ solutions

DOI: 10.18502/jfsh.v9i4.15000

4. Discussion

4.1 DPPH assay

In the study done by Azad et al., the range of DPPH was mentioned as 22.40±2.49% to 92.50±0.20% for monofloral honey (33).

Recently, Alshammari et al. evaluated the honey samples of Saudi Arabia through an assessment of biological compounds. It was found out the DPPH range of the samples was between 3.4–66.4% (34).

In our study, there was a range from 3.139±1.329 to 66.612±2.339 for honey samples. Remarkably, there is a wide range for DPPH. It is so obvious the amount of DPPH depends on the content and physicochemical properties of honey. The floral origin is an influential factor in the radical scavenging activity of honey. The results of accessing the effect of floral origin on the radical scavenging activity of honey are demonstrated and compared in Table 2. Statistical analysis showed a significant difference between honey samples from different floral origins ($p<0.05$). Jujube honey exhibited the highest DPPH inhibition percentage of 66.6115±2.3400 since jujube is a rich source of cafeic acid and rutin and Camelthorn honey exhibited the lowest DPPH inhibition percentage of 12.5054±0.3791.

4.2 FRAP assay

The range of FRAP value was reported from 9.30±0.26 to 20.72±0.44 in the work done by Azad et al. It was concluded geographical and climatic characteristics of different areas were responsible for antioxidant contents, hence, a variety of FRAP values could be seen (33). Also, in a work done by Ibrahimi and Hajdari, it was stated that the FRAP activity varies from 3.65 ± 1.96 to 22.39 ± 12.86 mg TE/100 g honey (35).

Recently, it was declared the FRAP value range varied between 72.78 and 164.88 μmol $FeSO_4.7H_2O$/100 g as a part of a study done by Shamsudin et al. (36). The floral origin greatly affects the total antioxidant capacity of honey samples because of its flavonoids and phenolic acids. The results of accessing the effect of floral origin on the total antioxidant capacity of honey are demonstrated and compared in Table 3. The statistical analysis explained a significant difference between honey samples from different floral origins ($p<0.05$). Camelthorn honey exhibited the highest FRAP value of 467.952±21.710 (μmol Fe (II)/100 g) due to the presence of flavonoids, fatty acids, coumarins, sterols, alkaloids, and triterpenoids such as Lupeol. Saffron honey exhibited the lowest FRAP value of 169.098±2.493 (μmol Fe (II)/100 g).

Table 2. Radical scavenging activity of honey samples from different floral origins

Plant	Mean DPPH inhibition (%)
Thyme	51.481±5.036
Cedar	24.882±5.945
Multifloral	36.753±16.669
Milkvetch	46.534±9.383
Camelthorn	12.505±0.379
Citrus	23.110±0.123
Fennel flower	54.405±1.684
Jujube	66.611±2.340
Saffron	48.903±0.819
Pennyroyal	59.510±2.351

Table 3. Total antioxidant capacity of honey samples from different floral origins

Plant	Mean Frap value (µmol Fe(Ⅱ/100 g)
Thyme	333.861±77.131
Cedar	428.858±92.964
Multi floral	238.8758±128.407
Milkvetch	259.276±75.4895
Camelthorn	467.952±21.710
Citrus	170.649±8.104
Fennel flower	272.333±4.247
Jujube	376.474±7.965
Saffron	169.098±2.493
Pennyroyal	295.534±4.886

Table 4. Mean HMF content of honey samples from different floral origins

Plant	Mean HMF (mg/kg)
Thyme	59.520±31.840
Cedar	10.627±2.241
Multifloral	33.615±25.509
Milkvetch	30.112±5.658
Camelthorn	23.008±0.319
Citrus	23.453±0.718
Fennel flower	55.552±3.091
Jujube	98.145±4.539
Saffron	51.435±2.878
Pennyroyal	70.789±1.710

4.3 Determination of HMF

HMF was stated for the assessment of honey freshness, therefore it should be presented in trace amounts for fresh honey. HMF is known as a result of the acid-catalyzed dehydration of hexose sugars with fructose. As mentioned above, the amount of HMF increase during the storage and prolonged heating. The HMF content is not allowed more than 40 mg/kg according to the codex. In the work done by Abselami et al., the HMF content of all the samples was within the allowed range. It could be concluded the variety in the amount of HMF is due to the various methods adopted by the farmers for the extraction and storage of honey (37). Recently, in a work done by Kuliçi et al., it was found out there was a range of 0.833-9.547 mg/kg and 17.22-67.9 mg/kg for traditional and market honey samples, respectively (38). The heat resistance is different according to the floral origin of the honey due to the variety of pH and acidity. The results of accessing the effect of floral origin on the HMF content of honey samples are demonstrated in Table 4 (as seen in supplementary). Statistical analysis revealed a conspicuous difference between honey samples from different floral origins ($p<0.05$). Jujube honey had the highest HMF content of 98.145±4.539, therefore it was the least resistant sample to heat. On the other hand, Cedar honey was the most resistant sample to heat as a result of the lowest HMF content of 10.627±2.241. Overall, it could be stated one part of the distribution chain did not have acceptable performance, consequently high amount of HMF was seen in most samples.

5. Conclusion

Due to the special therapeutic properties of honey which are because of high radical scavenging activity and antioxidant capacity, employing appropriate methods to evaluate these variables is vital. FRAP and DPPH assay were applied as simple, reproducible, and rapid methods to appraise the antioxidant and radical scavenging activity of honey samples. The results demonstrated antioxidant and radical scavenging activities of honey are seriously influenced by the floral

DOI: 10.18502/jfsh.v9i4.15000

origin, climate, and harvesting region of honey. In the second part of this study, the HMF content of honey samples was assessed as a chief indicator of honey freshness and heat treatment. The White method was employed as an easy and reliable method to determine the HMF content of honey samples. Exceeding the limit of 40 mg/kg defines the sample as exposed to heat or outdated.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

This work was a student thesis and supported by a grant (No: 99-3-104-50859), from Tehran University of Medical Science, Tehran, Iran.

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DOI: 10.18502/jfsh.v9i4.15000