Egypt. J. Chem. **Vol. 65,** No. SI:13B pp. 427-434 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Evaluation of vitamin C, proline, enzymes and hydroxymethylfurfural levels in clover honey at different storage conditions

Helmy Abdou Ghoniemy [1], Abdel Haleem Mishref Esmail [1], Awad Abd El-Tawab Mahmoud [2] and Ahmed Mohamed Saleh Mohamed [3*]

[1] *Plant Protection Department, Faculty of Agriculture, Fayoum University*

[2] *Food Science Department, Faculty of Agriculture, Fayoum University*

[3] *Teacher - Atfih Educational Administration - Ministry of Education*

Abstract

Honey is a popular sweetener throughout the world. It is consumed since the most remote times. In this study, the two groups of clover honey (natural honey and adulterated honey produced by the supplementary feeding of bees with sucrose syrup with ratio 2:1) through the nectar flow season were used for study the effect of different storage conditions on honey properties. The obtained honey samples were stored for different periods (3, 6, 9 and 12 months) at room temperature (25º C), refrigerator (-4 ºC) and freezer (-20º C) in the different package (white and black glasses). The levels of vitamin C, proline, enzymes (glucosidase and daistase) and hydroxymethylfurfural in the stored honey were determined. The result noticed that the levels of diastase activity, glucosidase activity and ascorbic acid content were decreased after storage for 12 months at room temperature as compared with those stored in the refrigerator and freezer. Also, the highest losses of diastase activity, glucosidase activity and ascorbic acid content were recorded for honey samples stored in the white glass than those stored in the black glass. Meanwhile, the proline content of the honey was stable during the first 6 months of storage, then decreased after the next 6 months of storage. On the other hand, levels of hydroxymethylfurfural (HMF) were increased with increasing the storage period at room temperature especially in the white glass. Meanwhile, no significant differences occurred between the levels of HMF in stored honey samples under cooling conditions. It can be concluded that, the honey stored in black glass at low temperatures (refrigerator and freezer) maintained properly the quality of the honey.

Keywords: Clover honey, storage, vitamin C, proline, enzymes, hydroxymethylfurfural

1. Introduction

Honey is a popular sweetener throughout the world. It is consumed since the most remote times. It has multifarious utility as well as honored place in most religions. It is mentioned in Paranoiac papyri as an ingredient in medicines and in all holy books. It was described as a remedy for man and as food in paradise in "The Quran". Therefore, it occupied a great respect position among different food. It was used early as preferred food for kings and nobles class. Floral honey is the sweet, viscose substance elaborated by the honeybee from the nectar of plants. The bee harvested, transported, and processed the nectar to honey, and packaged and stored it in the comb [1].

Honey is considered to be good for health, so potential methods of honey adulteration should be disclosed, as well as to confirm sanitary conditions for honey manipulation and storage. The most common forms of honey tampering are the addition of cheap sweeteners (such as cane sugar or refined beet sugar, corn syrup, high fructose or maltose syrup) and honeybees fed with sucrose [2]. Hydroxymethylfurfural (HMF) is present in honey naturally from analysis of reducing sugars under the action of normal honey acidity. HMF is recognized as a marker of honey freshness and quality deterioration. The HMF content increases during processing and prolonged storage [3, 4].

The proline is the most abundant amino acid in honey and pollen as 50–85% of total protein. Proline produces from the secretions of the salivary glands of honeybees during the conversion of nectar into honey [5]. Proline has been used as an indicator to evaluate the quality and the maturation of honey, and in some cases, adulteration with sugar [4]. Diastase is an enzyme found naturally in honey and its amount varies depending on the origin of the flora, and is also an indicator of the applied heat because that has the best resistance to heat, so it is

*Corresponding author e-mail: ben_saleh25@yahoo.com.; (Mr. Ahmed Mohamed Saleh Mohamed).

Receive Date: 29 March 2022, Revise Date: 27 April 2022, Accept Date: 04 July 2022

DOI: 10.21608/EJCHEM.2022.130362.5747

widely used as an indicator of honey freshness [6, 7].

The most important vitamin of honey is vitamin C, which has an antioxidant effect. Vitamin C is an unstable indicator because it is very vulnerable to chemical and enzymatic oxidation and has an accelerated rate of change due to various factors such as oxygen, light or heat [8]. Goncalves et al. [9] mentioned glucoseoxidase is secreted from the hypopharyngeal glands of bees. The levels of glucoseoxidase in honey can vary according to the floral origin and geographical location. It catalyzes the conversion of glucose to gluconic acid producing hydrogen peroxide (H_2O_2) in the process which is related to honey antibacterial activity. This work aims to study the optimum storage condition for clover honey through evaluation of vitamin C stability, proline content and enzymes (glucosidase and daistase) as well as formation of hydroxymethylfurfural in the stored honey different conditions such as temperature (room temperature refrigerator and freezer), period (3, 6, 9 and 12 months) and package type (white and black glasses).

2. Materials and methods

2.1. Honey bee colonies

Honey samples were taken from colonies during the nectar flow period from Giza governorate which is rich in nectar-producing plant clover.

2.2. Sugar source

The industrial sugar used in the study is sucrose which purchased from the local market.

2.3. Syrup level and preparation

The sugar solution was used for the first time as a bee food supplement during the main nectar flow period in this study. Syrup was prepared daily, mixed often, left for period and were finally given to the colonies. Concentrate of syrup (2 sugar: 1 water) with rate 0.5 L/colony through the week.

2.4. Honey harvest and honey sample preparation

Honey was harvested by centrifugation at an apiary located in region Atfeeh, southern of Giza governorate of Egypt during Alfalfa flowering season. Honey samples were taken from six colonies which were chosen randomly from all of the available groups. Clover honey samples were collected and filtered, stored in dark and white glass containers and kept in appropriate conditions until analyses.

2.5. Determination the effects of storage condition on different properties of the tested honeys

Effects of three factors of storage conditions (storage period, temperature and container type) on properties of the stored tested honeys were studied over 12 months of storage as follow:

Two types of containers (white glass and black glass) were used for storing the samples of the tested honeys. The tested containers were cleaned, dried and filled with the tested honeys. Each honey sample was represented by 4 groups of each containers; each group consists of three replicates (250 g. each). The first group was stored for 3 months, while the second, third, and fourth were stored for 6, 9 and 12 months, respectively. Also, the effects of different temperatures (room temperature, refrigerator storage at 4°C and freezer storage at -20°C) were evaluated. At the end of storage period of each group, their honeys were subjected to physical and chemical evaluation.

2.6. Determination of diastase activity

Diastase activity was measured photometrically through the procedures of the method described by Bogdanov [10] which modified By Vit and Pulcini [11] and diastase activity was expressed as diastase number. One ml of 2 % starch solution was pipette into several 50 ml graduated cylinder containing l0 ml of diluted iodine solution (0.007 N) and mixed. The quantity of water necessary for dilution was determined to produce an absorbency value of 0.76. Five grams of honey samples dissolved in l0 ml water plus 2.5 ml acetate buffer solution at pH 5.3 were added to 1.5 ml NaCl solution (0.5 N) in a 25 ml volumetric flask and brought to volume by water. Five ml of starch solution and l0 ml of each sample were placed in a flask and kept in a water bath for 15 minute at 40. One ml was added to I0 ml of Iodine solution after 5 minute. Absorbency values were determined using Spectronic-20 (Bausch & Lomb) at 660 nm in I cm cells. Aliquots of 1 ml were taken continuously at intervals until the absorbency values reach less than 0.235. Diastase number (DN) was obtained by dividing 300 by the time taken to reach the absorbency value of 0.235.

2.7. Determination of glucoseoxidase activity

Glucoseoxidase activity in honey was determined by the horseradish peroxidase/o-dianisidine method as previously described by Flanjak et al. [12]. Glucoseoxidase catalyzes the oxidation of D-glucose to D-gluconolactone and subsequent transformation to gluconic acid and hydrogen peroxide (H_2O_2). H_2O_2 is reduced to water by peroxidase utilizing o-dianisidine known as a cosubstrate. The maximum absorption of the formed colored product was observed at 400 nm using a spectrophotometer (Shanghai Mapada Instruments Co., China). The quantification was performed using H_2O_2 as the standard (10–100

μmol/L) with peroxidase and o-dianisidine. The results were expressed as μg H_2O_2/h g honey.

2.8. Determination of ascorbic acid (vitamin C) according to AOAC methods [13].

Preparation of extracting solution: Metaphosporic acid – acetic acid (HPO_3 – HOAC) was prepared by weighing 15g of HPO_3 sticks and dissolving in 40 ml of HOAC and 200 ml of distilled water. The resulting solution was diluted to 500 ml with distilled water, filtered and stored in the dark for use.

Preparation of Indophenols Solution: Fifty mg of 2, 6-dichlorophenol indophenols powder were dissolved in 50 ml of distilled water containing 42 mg of $NaHCO_3$. The resulting solution was diluted to 200 ml with distilled water, filtered and stored in the dark for use.

Preparation of standard ascorbic acid solution: One mg/ml of standard ascorbic acid solution was prepared by dissolving 50 mg of ascorbic acid in 40 ml of HPO_3 - HOAC solution and made up to 50 ml in a volumetric flask.

Standardization of indophenols solution with standard ascorbic acid solution: Three aliquots ascorbic acid solution (2 ml) were transferred into three conical flasks (50 ml) for each. The content of each of the conical flask was titrated against indophenols from the burette until a distinct rose color persisted for about 5 seconds. Blank titrations were also carried out using 7 ml of HPO_3 – HOAC solution against indophenols.

Extraction of ascorbic acid from the samples: Five g of each of the powdered samples (A, B, C, D) were weighed and transferred into conical flasks. 50 ml of the extracting solution was added to each sample and triturated to form a suspension and then allowed to stand for 30 minutes. The volume obtained was noted and designated as V ml. Warm extracting solution (50°C) was used for sample A as it froths in cold extracting solution. Sample aliquots (7 ml each) were obtained by filtering the suspension. The filtrate obtained from sample D was decolorized with activated charcoal.

Titration of extracted solution against indophenols: Seven ml of sample aliquots (A, B, C and D) were measured into conical flasks and titrated against indophenols from the burette until a distinct pink to rose color persisted for 5 seconds. The titration was repeated three times for each sample aliquot and average titer values obtained.

Calculation: the concentration of ascorbic acid in each sample (Z mg/g of powdered sample) was calculated using the following formula:

Where:

$$Zmg/g = (X - B)x\frac{F}{E}x\frac{V}{Y}$$

X = Average titer value obtained from sample titration

B = Average titer value obtained from blank titration.

F = Mg of ascorbic acid equivalent to 1ml of indophenols solution.

E = No. of grams of powdered fruit sample assayed.

V = Volume of initial assay solution

Y = Volume of sample aliquot titrated

2.9. Determination of proline content in honey samples

The proline content was measured according to Association of Official Analytical Chemists [13].

2.10. Determination of hydroxymethylfurfural content (HMF)

Honey samples were clarified with Carrez solution, and sodium bisulfate was added, then absorbance of the tested sample (1cm quartz cuvette) was determined through using ultra violet spectrophotometer (Labomed, Inc) at 284 and 336 nm according to methodology described in AOAC [13].

3. Results and Discussion

3.1. Diastase activity.

Diastase enzyme is resistant to heat, so it is widely used as an indicator of honey freshness. Data in Table (1) showed that the reduction rate of diastase activity was increased after storage for 12 months at room temperature as compared with those stored in the refrigerator. On the other hand, no change occurred in honey samples stored in freezer. But, change occurred in the diastase activity between white and black containers, where the highest loss of diastase activity was recorded for honey samples stored in white glass as compare with those stored in black glass.

These results are close to the results obtained by Huidobro et al. [14] who reported that diastase loses ranged from 10 to 33 % of its activity after the first year of storage. Also, Nour [15], Bonvehi and Coll [16] and Moussa [17] found that diastase activity was declined during storage. Also, the decrease in diastase activity was also noticed by White and Subers [18] and Qamer et al. [19]. The decrease of diastase activity depends on many factors such as storage temperature, moisture content, pH of honey and storage periods [20].

In this study the results of honey samples obtained from natural feeding were in agreement with criteria of the Egyptian and international standards which mentioned that diastase activity of the honey should not decrease than 3 and 8 (Goethe's Scale), respectively. On contrary, the results of honey samples obtained from artificial feeding were less than international acceptable limit. But, in view point of the Egyptian Standards was acceptable.

TABLE 1. Effect of different condition (feeding, temperature, package and storage) on daistase activity (u/kg) in honey samples.

Feeding type	Storage condition	Package type	Storage period			
			3 months	6 months	9 months	12 months
Natural feeding	Room temperature	White glass	10.16	9.67	8.91	8.18
		Black glass	10.52	10.13	9.79	9.25
	Refrigerator temperature	White glass	10.64	10.47	10.34	10.11
		Black glass	11.73	11.59	11.41	11.13
	Freezer temperature	White glass	11.77	11.74	11.65	11.46
		Black glass	11.81	11.78	11.73	11.57
Artificial feeding	Room temperature	White glass	4.92	4.17	3.67	3.21
		Black glass	5.70	5.43	5.03	4.60
	Refrigerator temperature	White glass	5.83	5.71	5.30	4.83
		Black glass	5.94	5.80	5.52	5.24
	Freezer temperature	White glass	6.11	5.97	5.85	5.73
		Black glass	6.14	6.04	5.94	5.87

3.2. Glucoseoxidase

Glucoseoxidase is mainly produced in the hypopharyngeal glands of the bees (animal origin). In the present study the activity of glucoseoxidase enzyme decreased during storage at room temperature as shown in Table (2) especially in the white glass container as compare with the black glass container. On contrary, no changes significantly occurred for activity of glucoseoxidase enzyme in honey samples stored under cooling conditions. The glucoseoxidase enzyme is activated by unripe and a dilution of honey generates hydrogen peroxide have an antimicrobial effect, and is inactivated by heating and by exposure to light as reported by Molan [21] and Bogdanov et al. [22].

3.3. Ascorbic acid (Vit. C)

Ascorbic acid (Vit. C) is found in almost all types of honey and has been evaluated mainly due to its antioxidant effect. Results in Table (3) indicated that the contents of ascorbic acid of the stored honey samples at room temperature were decreased greatly with ratios ranged between 63-72% and 64-74% for honey samples obtained from natural and artificial feeding respectively, in comparison with the fresh honey.

But honey samples stored at 4°C decreased with ratio ranged between 11-26% and 38-48% for honey samples obtained natural and artificial feeding, respectively. The determination of vitamin C is an unstable indicator because it is very sensitive to chemical oxidation and enzymatic oxidation and has a quick rate of change due to various factors such as light, oxygen and heat Leon-Ruiz et al. [8]. Also, the filtration process of honey may cause a lowering in vitamin content due to the almost complete removal of pollen. Another factor that causes the loss of vitamins in honey is the oxidation of ascorbic acid by the hydrogen peroxide produced by glucoseoxidase and catalase, Ball [23] and Ciulu et al. [24].

TABLE 2. Effect of different condition (feeding, temperature, package and storage) on glucoseoxidase (u/kg) content in honey samples.

Feeding type	Storage condition	Package type	Storage period			
			3 months	6 months	9 months	12 months
Natural feeding	Room temperature	White glass	22.37	16.82	14.04	11.70
		Black glass	22.82	18.50	16.72	14.13
	Refrigerator temperature	White glass	25.82	25.55	24.94	24.39
		Black glass	25.90	25.73	25.34	25.26
	Freezer temperature	White glass	25.91	25.83	25.71	25.60
		Black glass	26.00	25.99	25.92	25.85
Artificial feeding	Room temperature	White glass	16.31	12.14	9.18	7.05
		Black glass	17.90	16.61	15.23	13.61

	Refrigerator temperature	**White glass**	18.52	18.00	17.32	16.82
		Black glass	18.82	18.50	18.11	17.50
	Freezer temperature	**White glass**	19.13	18.93	18.74	18.38
		Black glass	19.24	19.02	18.86	18.53

TABLE 3. Effect of different condition (Feeding, temperature, package and storage) on vitamin C (mg/100 g) content in honey samples.

Feeding type	Storage condition	Package type	Storage period			
			3 months	6 months	9 months	12 months
Natural feeding	**Room temperature**	**White glass**	1.76	1.32	1.03	0.83
		Black glass	2.30	1.80	1.40	1.11
	Refrigerator temperature	**White glass**	2.81	2.67	2.45	1.90
		Black glass	2.90	2.86	2.81	2.65
	Freezer temperature	**White glass**	2.94	2.88	2.85	2.77
		Black glass	2.96	2.92	2.89	2.84
Artificial feeding	**Room temperature**	**White glass**	0.83	0.60	0.46	0.39
		Black glass	0.96	0.79	0.68	0.51
	Refrigerator temperature	**White glass**	1.13	0.98	0.87	0.77
		Black glass	1.21	1.08	1.02	0.91
	Freezer temperature	**White glass**	1.32	1.22	1.15	0.99
		Black glass	1.38	1.30	1.25	1.16

3.4. Proline content

The proline is the most abundant amino acid in honey and pollen as 50–85% of total protein. Proline produces from the secretions of the salivary glands of honeybees during the conversion of nectar into honey [4, 5]. Proline has been used as an indicator to evaluate the quality and the maturation of honey, and in some cases, adulteration with sugar. A minimum value of 180 mg/kg of proline is accepted as the limit value of pure honey [25, 26]. Values found in the present study as shown in Table (4) revealed that proline content of the honey samples obtained from natural feeding was stable during the first 6 months of storage but, decreased after the next 6 months of storage at room temperature, refrigerator and freezer temperature with ratios of 2.55-3.10%, 1.65-2.17% and 0.64-0.86% respectively.

Despite the proline content of the honey samples obtained from artificial feeding were less than the acceptable limit (180 mg/kg), but had the same line the honey samples obtained from natural feeding which decreased after 6 months of storage at room temperature, refrigerator and freezer temperature with ratios 2.59-2.97%, 1.88-2.08% and 0.29-0.36% respectively. Perhaps, attributed the increase in proline content through the first 6 months of storage to the presence of some enzymes in honey such as protease and peptidase. While the decrease of proline after 6 months of storage may be due to the reaction among the carboxylic group at the end of reducing sugars and the free amino groups in amino acids and proteins (Maillard reaction), because the source of these amino acids is pollen grains.

These results are in an agreement with the results of other authors. Also, Qamer et al. [27] mentioned that despite the increase in Proline content during the initial eight months, there was a decline of 13.4% in the proline content of honey stored for more than one year. Iglesias et al. [5] found that the content of proline increased during the first 6 months of storage then decreased after 6 months of storage.

3.5. Hydroxymethylfurfural (HMF) content

HMF is present in honey naturally from analysis of reducing sugars under the action of normal honey acidity. HMF is recognized as a marker of honey freshness and quality deterioration. The HMF content increased during processing and prolonged storage. Many factors influence the content of HMF such as temperature, heating process, storage conditions, pH and floral source, thus it provides an indication of overheating and storage in poor conditions as reported by Habib et al. [3], Fallico et al. [28] and Truzzi et al. [4]. In the present study, the values of HMF content are shown in Table (5). All samples of honey were less than the national and international legal limit (≤ 40 mg/kg) indicating

the freshness of the honey and good storage condition.

TABLE 4. Effect of different condition (Feeding, temperature, package and storage) on proline (mg/100 g) content in honey samples.

Feeding type	Storage condition	Package type	Storage period			
			3 months	6 months	9 months	12 months
Natural feeding	Room temperature	White glass	345.29	345.31	339.18	334.44
		Black glass	345.36	345.38	340.24	336.31
	Refrigerator temperature	White glass	345.25	345.27	342.49	337.62
		Black glass	345.26	345.29	343.37	339.45
	Freezer temperature	White glass	345.18	345.20	344.59	342.17
		Black glass	345.21	345.23	344.88	342.93
Artificial feeding	Room temperature	White glass	167.33	167.43	165.62	162.25
		Black glass	167.41	167.52	165.74	162.87
	Refrigerator temperature	White glass	167.25	167.31	166.61	163.81
		Black glass	167.27	167.34	166.69	164.07
	Freezer temperature	White glass	167.23	167.25	166.92	166.61
		Black glass	167.26	167.27	166.98	166.74

TABLE 5. Effect of different condition (Feeding, temperature, package and storage) on HMF (mg/kg) content in honey samples.

Feeding type	Storage condition	Package type	Storage period			
			3 months	6 months	9 months	12 months
Natural feeding	Room temperature	White glass	4.12	5.42	7.72	9.13
		Black glass	3.90	4.82	6.17	8.78
	Refrigerator temperature	White glass	3.75	3.88	3.99	4.43
		Black glass	3.71	3.83	3.90	4.30
	Freezer temperature	White glass	3.69	3.72	3.74	3.77
		Black glass	3.67	3.68	3.69	3.72
Artificial feeding	Room temperature	White glass	7.53	8.96	9.63	12.77
		Black glass	7.30	8.80	9.29	12.54
	Refrigerator temperature	White glass	7.12	7.69	8.00	8.45
		Black glass	7.05	7.60	7.85	8.40
	Freezer temperature	White glass	6.83	6.89	7.12	7.21
		Black glass	6.82	6.86	7.02	7.08

Regarding honey obtained from natural feeding, the honey samples stored at room temperature recorded the highest levels of HMF which ranged from 8.78 to 9.13 mg/kg after storage one year. Meanwhile, the level of HMF in honey samples obtained from artificial feeding was increased and ranged between 12.54 and 12.77 mg/kg. Meanwhile, the honey samples stored in the refrigerator recorded a little increase in their HMF levels and ranged from 4.30 to 4.43 mg/kg and from 8.40 to 8.45 mg/kg for honey samples obtained from natural and artificial feeding, respectively.

In contrast, no significant increase in HMF content of the stored honey samples under the storage of freezer condition within 12 months of storage. The level of HMF in honey samples stored in black glass was lower than those recorded in honey samples stored in white glass. These results are similar to those obtained, by other authors such as Turhan [29] and Khalil et al. [30] who reported that the level of HMF increased in honey stored for 12-24 months, especially by changes in pH and acidity of the honey.

Also, Castro-Vazquez et al. [31] indicated that an increase in HMF during 12 months of storage of Turkish floral honey at constant temperature 25 ºC (from 3.3 to 19.1 mg/kg) and in citrus honey at 20 ºC (from 10.2 to 30.4 mg/kg). Also, Cervantes et al. [32] revealed that the HMF rises up to concentrations around 30 mg/kg and 16 mg/kg of Tahonal and Dzidzilche honey samples, respectively stored at 26 ± 2º C for 23 weeks. Islam et al. [33] postulated that Bangladeshi honey samples with low pH levels (pH

less than 4.6) and low moisture content may also contribute to the low HMF formation.

Conclusion

The honey obtained from natural feeding had the best properties as compare with those obtained from artificial feeding. The fresh honey had high properties of qualities which can be conserve for a limit period of time, but their properties undergoes to degradation with increase the storage period. Honey stored at low temperatures (refrigerator and freezer) with black bottles maintained properly the quality of the honey, as compare with those stored at room temperature with light bottles.

References

[1]. White J. W. (1978). Honey. Advances in food research, 24: 287-374.

[2]. Puscas, A.; Hosu, A. and Cimpoiu, C. (2013). Application of a newly developed and validated high-performance thin-layer chromatographic method to control honey adulteration. Journal of Chromatography A, 1272: 132–135.

[3]. Habib, H. M.; Al Meqbali, F. T.; Kamal, H.; Souka, U. D. and Ibrahim, W. H. (2014). Physicochemical and biochemical properties of honeys from arid regions. Food Chemistry, 153: 35-43.

[4]. Truzzi, C.; Annibaldi, A.; Illuminati, S.; Finale, C. and Scarponi, G. (2014). Determination of proline in honey: comparison between official methods, optimization and validation of the analytical methodology. Food chemistry, 150: 477-481.

[5]. Iglesias, M. T.; Martín-Álvarez; P. J., Polo, M. C.; de Lorenzo, C.; González, M. and Pueyo, E. (2006). Changes in the free amino acid contents of honeys during storage at ambient temperature. Journal of agricultural and food chemistry, 54 (24): 9099-9104.

[6]. El-Ansary, O. M; Gadelhak, G. G.; El-Zoghby F. and Gomaa, W. M. K. (2004). Physical and chemical studies on the Egyptian honey compared with standard honey specification. J. Pest Cont. & Ewiron. Sci., I2 (I/2): II3-I33.

[7]. Gomes, S.; Dias, L. G.; Moreira, L. L.; Rodrigues, P. and Estevinho, L. (2010). Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. Food and Chemical Toxicology, 48 (2): 544-548.

[8]. Leon-Ruiz, V.; Vera, S.; González-Porto, A. V. and San Andrés, M. P. (2013). Analysis of water-soluble vitamins in honey by isocratic RP-HPLC. Food Analytical Methods, 6 (2): 488-496.

[9]. Goncalves, J.; Ribeiro, I.; Marçalo, J.; Rijo, P.; Faustino, C. and Pinheiro, L. (2018). Physicochemical, antioxidant and antimicrobial properties of selected Portuguese commercial monofloral honeys. Journal of Food and Nutrition Research, 6 (10): 645-654.

[10]. Bogdanov, A. (1984). Red star: The first Bolshevik utopia. Indiana University Press.

[11]. Vit, P. and Pulcini, P. (1996). Diastase and invertase activities in Meliponini and Trigonini honeys from Venezuela. Journal of Apicultural Research, 35 (2): 57-62.

[12]. Flanjak, I.; Strelec, I.; Kenjeric, D. and Primorac, L. (2016). Croatian ´produced unifloral honey characterized according to the protein and proline content and enzyme activities, Journal of Apicultural Science, 60 (1): 39-48.

[13]. A.O.A.C (1990). Official Methods of Analysis. Sugars and Sugar Products. In: Horwitz, W. (Ed.). Official Methods of Analysis of A.O.A.C International. Maryland, U.S.A. 2 (44): 22 -33.

[14]. Huidobro, J. F.; Santana, F. J.; Sanchez, M. P.; Sancho, M. T.; Muniategui, S. and Simal-Lozano, J. (1995). Diastase, invertase and β-glucosidase activities in fresh honey from north-west Spain. Journal of Apicultural Research, 34 (1): 39-44.

[15]. Nour, M. E. (1988). Some factors affecting quality of Egyptian honey. Ph. D. Thesis, Fac. Agric., Cairo Univ., 252.

[16]. Bonvehi, S. J. and Coll, V. F. (1995). Characterization of citrus honey (Citrus spp.) produced in Spain. Journal of Agricultural and food chemistry, 43 (8): 2053-2057. Bonvehi and Coll

[17]. Moussa, A. S. (2005): Physiochemical and technological studies on some types of Egyptian honey. M.Sc. Agric. Thesis in food science, Fac. of Agric. Assiut Univ., pp.122.

[18]. White J. W. and Subers, M. H. (1963). Studies on honey inhibine. 2. A chemical assay. Journal of Apicultural Research, 2 (2): 93-100.

[19]. Qamer, S.; Muzaffar, N.; Ali, S. S. and Shakoori, A. R. (2009). Effect of storage on various honey quality parameters of unifloral sidder honey from Pakistan. Pakistan Journal of Zoology, 41 (4): 313-316.

[20]. Hassan, M., I. M. (1985). Studied on food: effect of storage on some physical and chemicals characteristics of bee honey. M.Sc. Faculty of Agriculture. Alex. University.

[21]. Molan, P. C. (1992). The antibacterial activity of honey: 1. The nature of the antibacterial activity. Bee world, 73 (1): 5-28.

[22]. Bogdanov, S.; Martin, P. and Lullmann, C. (1997). Harmonised methods of the European honey commission. Apidologie (France), 1-59 (extra issue).

[23]. Ball, D. W. (2007). The chemical composition of honey. Journal of chemical education, 84 (10): 1643-1646.
[24]. Ciulu, M.; Solinas, S.; Floris, I.; Panzanelli, A.; Pilo, M. I.; Piu, P. C.; Spano, N. and Sanna, G. (2011). RP-HPLC determination of water-soluble vitamins in honey. Talanta, 83 (3): 924-929.
[25]. Hermosin, I.; Chicon, R. M. and Cabezudo, M. D. (2003). Free amino acid composition and botanical origin of honey. Food Chemistry, 83 (2): 263-268.
[26]. Manzanares, A. B.; Garcia, Z. H.; Galdon, B. R.; Rodriguez, E. R. and Romero, C. D. (2014). Physicochemical characteristics of minor monofloral honeys from Tenerife, Spain. LWT-Food Science and technology, 55 (2): 572-578.
[27]. Qamer, S.; Ahamed, F.; Ali, S. S. and Shakoori, A. R. (2013). Effect of storage on various honey quality parameters of Apis dorsata honey from Nepal. Pakistan Journal of Zoology, 45 (3): 741-747.
[28]. Fallico, B.; Arena, E.; Verzera, A. and Zappalà, M. (2006). The European Food Legislation and its impact on honey sector. Accreditation and Quality Assurance, 11 (1): 49-54.
[29]. Turhan, K. (2009). Effects of thermal treatment and storage on hydroxymethylfurfural (HMF) content and diastase activity of honeys collected from middle Anatolia in Turkey. In Innovations in chemical biology (pp. 233-239). Springer, Dordrecht.
[30]. Khalil, M. I.; Sulaiman, S. A. and Gan, S. H. (2010). High 5-hydroxymethylfurfural concentrations are found in Malaysian honey samples stored for more than one year. Food and chemical toxicology, 48 (8-9): 2388-2392.
[31]. Castro-Vazquez, L.; Díaz-Maroto, M. C.; González-Viñas, M. A.; De La Fuente, E. and Pérez-Coello, M. S. (2008). Influence of storage conditions on chemical composition and sensory properties of citrus honey. Journal of Agricultural and Food Chemistry, 56 (6): 1999-2006.
[32]. Cervantes, M. R.; Novelo, S. G. and Duch, E. S. (2000). Effect of the temporary thermic treatment of honey on variation of the quality of the same during storage. Apiacta, 35 (4): 162-70.
[33]. Islam, A.; Khalil, I.; Islam, N.; Moniruzzaman, M.; Mottalib, A.; Sulaiman, S. A. and Gan, S. H. (2012). Physicochemical and antioxidant properties of Bangladeshi honeys stored for more than one year. BMC complementary and alternative medicine, 12 (1): 1-10.