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To investigate the effect of long-term storage on the physicochemical properties of honey

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Abstract

The freshness of food is of great importance to the consumer. To investigate the effect of long-term storage on the physicochemical properties of honey, 6 polyfloral samples were stored at ambient temperature for 12 months and their chemical properties including, hydroxymethylfurfural, diastase number, sugar content (glucose, fructose, and sucrose), and proline were determined periodically (3 month interval). The hydroxymethylfurfural and sugars content were determined by high performance liquid chromatography. Results showed that dramatic changes were observed after 9 and 12 months storage in the chemical and biochemical characteristics of honey samples. The average values of determined specifications of 6 honey samples after 12 months were as follows: hydroxymethylfurfural 74.2±0.1 to 753.5±1.2 mg/kg, diastase 3.2±0.5 to 18.1±0.2, Proline 219.3±1.6 to 507.0±2.2 mg/kg, glucose from 15.6±1.2 to 25.4±1.2 (%), fructose 18.3±1.7 to 27.6±1.3 (%), and sucrose 0.4±0.3 to 4.2±0.5 (%).

The current study revealed that hydroxymethylfurfural values in all of the samples were higher than standard limit after one year storage. Although sugars, proline, and diastase showed a wide range of variation in entire polyfloral honey samples during long-term storage, some of them were still in accordance with the standard range recommended by the Codex Alimentarius.

Therefore these specifications cannot be used to determine the freshness of honey since: 1) their initial levels in honey samples are very different (depends on the type of honey and other factors), 2) their changing behavior during storage is not consistent; which make them unreliable parameters for determining the freshness of polyfloral honey.

Keywords Honey, Diastase number, Long-term storage, Proline, sugars, hydroxymethylfurfural (HMF)

Introduction

Honey, one of the natural precious foodstuffs, is a complex mixture of water, sugars, acetic acid, lactone, nitrogenous compounds, minerals and vitamins which contains many nutritional and therapeutic properties [1-2]. However, composition of honey depends on the plant source (from which the bees have fed), season, processing and storage conditions [3-4]. Honey sugars are made up of about 70% mono saccharides (fructose and glucose) and 10–15% disaccharides [5]. Honey is essentially a highly concentrated water solution of fructose (38.2%) and glucose (31.3%) with small amounts of at least 22 other more complex sugars. Honey's sugar accounts for 95 to 99% of honey dry matter [1].

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Honey quality can undergo some changes with duration and temperature of storage, which leads essentially to destroy some of its nutrient compounds, decrease of enzymes (such as diastase) and formation of hydroxymethylfurfural (HMF). HMF, a cyclic aldehyde, is produced by degradation of sugars. HMF, is absent in fresh honey (or exists in very low concentration) therefore, presence of excessive amounts of HMF in honey is considered as evidence of overheating and implies loss of freshness [6-7].

HMF is easily absorbed from food through the gastrointestinal tract and, upon being metabolized into different derivatives, is excreted via urine. In addition, it is converted to a non-excretable, genotoxic compound called 5-sulfoxymethylfurfural, is beneficial to human health. Therefore, HMF is a neo-forming contaminant that draws great attention from scientists [8].

Diastase is one of the most important enzymes in honey, which is produced in saliva of bee and makes honey easily digestible. Diastase degrades depending on the thermal processing. However, enzyme activity varies within considerably wide limits, even in fresh honeys [9]. In addition to improper thermal processing, long-term storage is another factor that leads to reduce diastase in honey. Therefore HMF along with diastase are used as the key indicators to determine the freshness of honey. Based on international standards HMF must be lower than 80 mg kg^{-1} in tropical areas and lower than 40 mg kg^{-1} in other countries, while DN must be more than 8 Gothe [10]. However, there are some researches showing high HMF content of honey. Makawi reported that 18 out of the 28 honey samples from Sudan were contaminated with HMF levels ranging from 5 to 922 mg kg^{-1} [11]. The HMF content of 50 Portuguese honey samples ranged from 1.7 ± 0.0 to 471 ± 15.6 mg/kg [12].

In a related study, the HMF content of 16 Iranian honey samples was determined. The results showed that all the samples (100%) contained HMF ranging from 20.55 up to 928.96 mg kg^{-1} [13].

According to Kıvrak and co-workers [14], the floral honey contains about 1.0–1.5% protein and proline is its predominant component (about 50–85% of the total amino acids). Besides proline, the honey contains 26 amino acids which their amount depends on the origin of honey (nectar or honeydew). Previous study showed that acacia, rape, sunflower and coriander honeys can be clearly distinguished from other honey types by their proline content [15]. Proline content is an indication of honey ripeness and, in some cases, sugar adulteration.

From the consumer's point of view, the freshness of the product is very important, while expire date for honey is not specified in the international standards and sometimes honey samples are marketed long-term after their production. Therefore, it is important to determine changes in honey properties after storage. The aim of the current study was to investigate the effect of long-term storage (one year) on the honey key properties including HMF, diastase, sugars, and proline.

2. Materials and methods

2.1. Materials

HPLC-grade water was obtained from a water purifier (Elga, Marlow, and Buckinghamshire, UK). The potassium hexacyanoferrate (II) (K4Fe(CN) 6.3H2O), zinc acetate (ZnCH3COO)2.2H2O and the HMF standard were purchased from Merck (Darmstadt, Germany). While Membrane filter paper (Whatman, 0.45 µm) was prepared from Schleicher & Schuell Microscience GmbH (Dassel, Germany). Potassium hexacyanoferrate and zinc acetate were used to prepare Carrez solutions I and II.

2.2. Samples

To carry out the current study, six polyfloral honey samples (S01, S02, S03, S04, S05, and S06) were prepared from 6 different factories immediately after production. Then they were stored at ambient conditions for one year and their chemical properties including, HMF, diastase, sugars content and proline were determined periodically (with a 3-month interval).

2.3. Chemical analysis

Refractive index was determined with an Abbe refractometer (Abbe™ 2 WAJ, China) at 20 °C, the corresponding moisture content (%) was calculated using the Wedmore Table [16]. Ash, pH, acidity, and prolin were determined according to the harmonised methods of the international honey commission [17]. The DN of honey samples was determined using a UV-visible spectrophotometer (Jenway 6305, UK), according to the method recommended by IHC [17]. All the chemicals used were analytical grade. Entire experiments were repeated in triplicate and results were presented as mean±SD.

2.4. HMF Determination

The honey samples were prepared for HMF determination based on described method by the DIN 10751-3 [18]. In brief, 10 g of each honey sample was diluted with distilled water. Then, 1 mL of Carrez I solution (potassium hexacyanoferrate) and 1 mL of Carrez II solution (zinc acetate dehydrate) were added. The solution was filtered first through a Whatman filter paper (No. 41) and then through a 0.45 µm nylon membrane filter. The applied HPLC was Varian model 9010 HPLC (Creek, California, USA) equipped with a variable wavelength UV-VIS

detector (Varian 9050, Creek, California, USA) and a Knauer degasser (Berlin, Germany). The HPLC column was from Agilent Bondesil, RP-C18 (4.6 mm, 5 µm, and 25cm). The mobile phase consisted of water and methanol (95:5 v/v) with the flow rate of 1.0 mL min^{-1}. Fifty µL of the filtered sample elutes was injected into HPLC. The calibration curve was used to determine the concentration of HMF in all the samples. The HMF content was calculated by comparing the corresponding peak areas of the sample and those of the standard calibration curve of HMF. The HMF content of all the treatments was measured in triplicate.

2.5. Method validation

To evaluate the linearity, two calibration curves of HMF at different concentration (2, 5, 10, 20, 50, mg L^{-1}) and (50, 100, 200, 400, 600 mg L^{-1}) were constructed, applying the linear least squares regression procedure of the peak area versus the concentration. To determine the recovery of the method, the HMF-free samples were spiked with 50.0 mg kg^{-1} (n=3). The repeatability was conducted using the samples spiked with 10 mg kg^{-1} HMF within a one-month period (with six-day time intervals) and was expressed as relative standard deviation (RSD). The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the signal-to-noise approach (S/N), defined as the concentration resulting in a signal-to noise ratio of approximately 3:1 and 10:1 for LOD and LOQ, respectively usinh following Eqs.

$$\mathbf{LOD} = \frac{\mathbf{lowest\ spiked\ level}}{\mathbf{average\ of\ S/N}} \times \mathbf{3}$$

$$\mathbf{LOQ} = \frac{\mathbf{lowest\ spiked\ level}}{\mathbf{average\ of\ \ S/N}} \times \mathbf{10}$$

2.6. Determination of sugars

The sugars content (fructose, glucose, sucrose) were determined by HPLC based on the method published by Sesta [19] with some modification. In brief, 25 g of honey sample was mixed with 40 ml of pure water in a flask. Then 25 ml of methanol was added, mixed thoroughly and made up to 100 ml with distilled water. Then the mixture was filtered through a Whatman filter paper. Ten µl of the filtered sample elute was injected into the HPLC for sugar analysis. The HPLC apparatus (Varian 9010, Creek, California, USA), was equipped with a refractive index detector (Varian RI-4). The HPLC column was an Agilent Bondesil, RP-C18, (4.6 mm, 5 µm, 25cm). The mixture of acetonitrile: water (80:20 v/v) was used as the mobile phase, at a flow rate of 1.2 mL/min. The quantitative determination of sugars was based on the injection of an external standard, prepared as follows: 25 mL of methanol and 40 ml of pure water solution was added to a volumetric flask. Then 2 g of fructose, 1.5 g of glucose, 0.25 g of sucrose was added and mixed thoroughly. Results were expressed as a weight/weight percentage (g/100 g) of each sugar.

2.7. Statistical analysis

To investigate the effect of storage on the chemical properties of honey (HMF and DN, sugar content, proline), analysis of variance (ANOVA) followed by the Post Hoc Tukey's test was performed. A value of $p<0.05$ was applied to estimate the statistically significant differences between honey samples for each parameter. Data analysis was carried out using statistical package Minitab v.17 (Minitab Inc., PA, State College, USA).

3. Results and discussion

3.1. Physicochemical analyses

Table 1 shows the physicochemical properties (moisture content, pH, acidity, electrical conductivity, and ash) of 6 honey samples expressed as mean (±SD). Moisture content ranged from 15.4±1.1 to 19.6±0.7% which were within the acceptable range specified by Codex Alimentarius [20]. The pH value of entire samples was more than 3.5 (the minimum level specified in codex standard).

Table 1. Physicochemical properties of different honey samples (mean±SD)

Sample No.	Moisture content (%)	Electrical conductivity (mS/cm)	Ash (g/100g)	pH	Acidity (meq/kg)
1	17.7±1.2	0.8±0.0	0.5±0.0	3.7	25.5±0.1
2	19.3±0.8	0.6±0.0	0.4±0.0	3.9	32.4±0.5
3	15.4±1.1	0.8±0.1	0.4±0.1	4.3	29.8±0.3
4	18.3±0.7	0.7±0.1	0.4±0.1	4.1	28.4±0.8
5	17.8±0.5	0.7±0.0	0.5±0.1	4.4	33.5±0.9
6	19.6±0.7	0.7±0.0	0.5±0.1	3.8	38.4±0.4

In general, honey is acidic in nature irrespective of its geographical origin, due to the constituent acids, mainly gluconic acid and minerals [21]. Total acidity value of the samples was within limits (lower than 50 meq/kg), indicating absence of undesirable fermentation. The values obtained for electrical conductivity and ash were also in agreement with the criteria defined by the Codex Alimentarius, which are 0.8 mS/cm and 0.6 % (w/w) respecively [20].

3.2. Method validation for determination of HMF

The calibration curve obtained by least-squares' linear regression was linear with the correlation coefficient of more than 0.999 (Fig 1). Moreover, the mean recovery value was 93±4.3% and the RSD was found to be 5.1%. The results also revealed LOD and LOQ were 0.03 and 0.09 mg kg^{-1}, respectively.

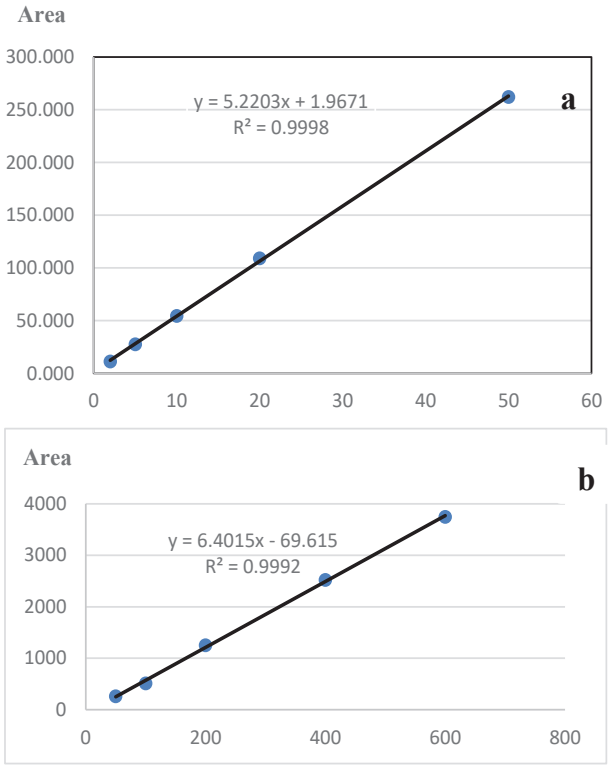

Figure 1. Calibration curves of HMF at different concentration from 2-50 mgL^{-1} (a); and from 50-600 mgL^{-1} (b)

Table 2. HMF content (mg/kg) of 6 honey samples during storage for one year (mean±SD)

Sample No	*Storage period (month)*				
	0	3	6	9	12
1	4.3±0.6^{a}	5.4±0.9^{a}	14.2±0. 4^{b}	23.5±0.4^{c}	87.9±0.9^{d}
2	6.9±0.1^{a}	8.4±0.9^{b}	15.99±0.23^{b}	18.5±1.6^{b}	99.5±0.5^{c}
3	23.5±1.5^{a}	25.10±0.15^{a}	38.3±0.03^{b}	80.6±2.1^{c}	201.9±0. 9^{d}
4	29.3±1.9^{a}	33.20±1.7^{a}	34.8±0.3^{a}	58.3±0.3^{a}	74.2±0.1^{b}
5	144.2±2.2^{a}	190.40±0.7^{a}	274.7±0.7^{b}	443.9±2. 5^{c}	753.5±1.2^{d}
6	136.0±3.1^{a}	161.06±0.4^{a}	236.5±0.8	436.6±0.6^{a}	543.7±0.8^{d}

^{a,b,c,d} Significant ($p<0.05$) differences in the same row

3.3. Effect of storage on the HMF content of honey

The results obtained for the HMF immediately after production and during storage are presented in Table 2. The low amount of HMF in samples S01 and S02 (4.34±0.6 and 6.9±0.1 mg L^{-1} respectively) indicated their appropriate thermal process and high degree of freshness. The S03 and S04 have an average HMF content ranged between 23.5±1.5 and 29.3±0.9 mg/kg. These values were lower than the specified maximum limit of 40 mg/kg as recommended by the Codex Alimentarius [20]. However, high amount of HMF was found in commercial samples S05 and S06 (144.2±2.2 and 136.0±3.1 mg/mL, respectively) showing overheating during honey processing. The results from ANOVA showed that after 3 months, no significant difference was observed between the HMF values in the samples while, after sixth month significant increases of HMF was observed in entire samples. However, their values (exclude S05 and S06) were still in accordance with the Codex standard range (lower than 40 mg/kg). After 9 months only samples S01 and S02 were also in compliance with the standard limit, while after 12 months, HMF value of all samples was higher than standard limit, ranging from 74.2±0.1 to 753.5±1.2 mg/kg. The highest level of HMF observed in the sample S05 and S06 after storage can be related to their high initial HMF content.

Judging by this feature, the best recommend time for consumption of honey is before one year since production date. The results of the current study are in agreement with those reported by Khalili and co-workers, who observed that the HMF content of fresh Malaysian honey samples stored for 3–6 months (2.80–24.87 mg kg^{-1}) was within the internationally recommended value (80 mg kg^{-1} for tropical honeys). However, honey samples stored for longer periods (12–24 months) contained much higher HMF concentrations (128.19–1131.76 mg kg^{-1}) [22]. In a related study, Mouhoubi-Tafinine [7] reported that when the honey samples were stored at 35 °C, a significant increase of HMF content was observed. After 9 months, HMF content varied from 100.84 to 353.09 mg/Kg and exceeded largely the allowed limit (40 mg/Kg). Samples of 4 year old honey contains on average 52.44% higher HMF than fresh honey samples. These results clearly show that longer storage of honey increases the concentration of HMF [23].

However, our results were different from those reported by Cherchi et al. [24]. They did not observe significant changes in HMF in three types of honey even after a storage period of 24 months at refrigeration and ambient temperature. Korkmaz and Kuplulu [9] reported that average HMF values of the honey samples stored at 10 °C and 22 °C for one year did not exceed 40 mg/kg, while it rapidly increased in the samples stored at 35 °C, and was determined to be over 40 mg/kg as of 6th month onwards.

3.4. Effect of storage on the diastase content of honey

Quality characteristics of honey can be evaluated in terms of some of its biochemical characteristics which are quite unique to honey (such as diastase enzyme activity) and not easy to be adulterated [25]. The results obtained for diastase number degradation during one year is demonstrated in Table 3. At the first day of the experiment, the DN values were ranged from 7.6±0.2 to 38.7±0.5 Schade units. The lowest DN content was obtained in S06 that was lower than the limit (8 Schade units) specified by

codex alimentarious [20], which probably indicates overheating during honey processing (regard to its high level of HMF content).

Table 3. Diastase number of 6 honey samples during storage for one year (mean±SD)

Sample No.	Storage period (month)				
	0	3	6	9	12
1	38.7±0.5^{ba}	37.9±0.4^{a}	27.3±0.6^{a}	22.6±0.3^{b}	18.1±0.2^{b}
2	34.6±0.4^{a}	32.3±0.2^{a}	28.6±0.1^{b}	23.3±0.5^{c}	16.6±0.3^{d}
3	26.7±0.3^{b}	25.4±0.2^{a}	24.8±0.1^{a}	18.6±0.4^{b}	14.1±0.2^{c}
4	22.8±0.2^{a}	21.8±0.2^{a}	17.6±0.5^{b}	14.6±0.5^{c}	9.2±0.4^{d}
5	10.4±0.4^{ed}	11.4±0.4^{a}	9.2±0.1^{b}	4.2±0.3^{c}	3.2±0.5^{c}
6	7.6±0.2^{f}	7.0±0.2^{a}	5.2±0.1^{b}	4.8±0.2^{b}	3.3±0.2^{b}

^{a,b,c,d} Significant ($p<0.05$) differences in the same row

The results revealed that DN is decreased by increasing the storage time in all entire samples. However significant reduction of DN was observed in none of the samples after 3 mounts (ranging from 2.1 to 9.6%). In the 6th month, the DN reduction was ranged between 7.1 to 31.6% (in S03 and S06 respectively). After 12 months in almost all samples the diastase level was reduced to half of the initial level or less (from 47.2% in S03 to 69.2% in S05). However, in the samples with higher initial level of DN (S01 to S04), the final DN (after storage for 12 months) was also higher than minimum permitted level (8 Schade units). Therefore the amount of DN in honey, during storage, depends on its initial diastase level, which in turn depends on the source of nectar, the region, and age of bees.

Our results were not in agreement with those reported by Hasan [26]. He investigated the effect of heating and storage conditions on the diastase number in three types of Iraqi honey samples and reported that during the first three months of the storage, the diastase activity decreased ranging from 19.5% to 22%, while after 6 months, a dramatic DN reduction (up to 79%) was observed in entire samples [26]. However, we didn't find any noteworthy reduction after 6 months. Our results were somewhat similar to those reported by White and coworkers. Their study showed that diastase values of unheated honey declined during storage at room temperature (23–28° C), with diastase showing a half-life of 17 months under these conditions of storage [27]. In a related study conducted by Sahinler [28], the diastase number and HMF content of honey samples were significantly affected by heating and storage time. The significant ($p < 0.01$) decrease of diastase activity (24.4 %) in honey stored at room temperature compared to fresh honey was observed by Kędzierska-Matysek and co-workers [29]. They also reported that honey samples stored in freezer (at −20 °C) for 18 months also showed an insignificant decrease in diastase activity (7.3 %). Average diastase activities of the samples at 10±2 °C and 22±2 °C did not drop below the limit value of 8 in TFC, though it was determined to be below 8 Schade units as of 6th month onwards, for the flower honey stored at 35±2 °C [9].

3.5. Effect of storage on the sugar content of honey

At the first day of experiment, glucose and fructose content ranged between 25.4±0.5 to 38.2±1.1 and 33.3±1.6 to 42.3±1.1 g/100g honey, showing significant variation between them. In the entire samples (exclude S06), the sucrose content was ranging from 2.1±0.3 to 4.9±0.6 g/100g honey which was in line with the legislated levels described by the Codex Alimentarius (which is less than 5 g/100g) [20]. These variations may be due to the variation of invertase activity of the honeybee species. In agreement with this idea, Wakhle reported that the invertase activity of some honeybee species is higher than the others [30]. The sucrose content of S06 (9.2±0.7 g/100g) was higher than Codex standard limit (5 g/100g), showing that probably the honey has been adulterated by sucrose. As has been demonstrated in Table 4, glucose and fructose content decreased significantly ($P < 0.05$) after 6–12 months of storage, showing that the sugar spectrum of polyfloral honey is not static; rather, it does change during storage, however there were no noticeable differences during the first six months.

Table 4. Sugar (fructose, glucose, sucrose) content (%) of 6 samples during storage for one year (n=3).

Sample NO	*Sugar*	*Storage period (month)*				
		0	3	6	9	12
1	Glucose	32.3±1.2^{a}	31.2±0.7^{a}	25.6±0.6^{b}	21.3±0.3^{c}	16.3±1.1^{d}
	Fructose	36.8±0.5^{a}	36.1±0.4^{a}	35.4±0.9^{a}	29.5±0.7^{b}	24.3±0.8^{c}
	Sucrose	4.7±.6^{a}	4.5±0.9^{a}	3.7±1.2^{b}	3.5±0.5b^{c}	2.1±0.7^{c}
2	Glucose	34.8±2.4^{a}	31.3±2.1^{a}	23.7±1.2^{b}	24.4±1.7^{b}	21.5±1.1^{c}
	Fructose	35.3±1.7^{a}	33.4±2.1^{a}	25.5±1.5^{b}	24.7±2.3^{b}	18.3±1.7^{c}
	Sucrose	4.8±1.2^{a}	4.5±0.9^{a}	3.4±1.2^{ab}	1.8±1.1b^{c}	1.5±2.3^{c}
3	Glucose	38.2±1.1^{a}	35.3±0.5a^{b}	33.1±0.5^{b}	25.3±0.7^{c}	18.4±0.3^{d}
	Fructose	42.2±0.8^{a}	41.4±1.2^{a}	39.3±1.3^{a}	31.1±0.5^{b}	26.7±1.2^{c}
	Sucrose	2.1±0.3^{a}	1.9±0.5^{a}	1.1±0.7^{b}	0.8±0.2^{bc}	0.5±0.1^{c}
4	Glucose	31.8±1.1^{a}	29.4±0.6^{a}	24.3±1.1^{b}	23.1±0.8^{b}	19.9±0.6^{c}
	Fructose	35.2±0.9^{a}	31.3±1.5^{a}	29.7±1.2^{a}	23.5±0.8^{b}	22.5±0.9^{b}
	Sucrose	4.5±0.3^{a}	4.3±0.7^{a}	4.5±0.9^{a}	2.3±0.7^{b}	1.5±0.5^{b}
5	Glucose	37.6±1.3^{a}	36.3±0.9^{a}	35.3±1.2^{a}	27.4±1.1^{b}	25.4±1.2^{b}
	Fructose	42.3±1.1^{a}	41.7±0.9^{a}	40.4±1.2^{a}	35.3±1.2^{b}	27.6±1.3^{c}
	Sucrose	4.9±0.6^{a}	4.2±0.4^{a}	4.4±0.5^{b}	0.9±0.5^{c}	0.4±0.3^{d}
6	Glucose	25.4±0.5^{a}	24.3±0.8^{a}	18.6±1.1^{b}	17.4±0.9^{b}	15.6±1.2^{b}
	Fructose	33.3±1.6^{a}	32.1±0.7^{a}	30.4±1.1^{a}	24.2±0.8^{b}	22.6±1.7^{b}
	Sucrose	9.2±0.7^{a}	9.1±0.5^{a}	8.9±.7^{a}	6.3±0.4^{b}	4.2±0.5^{c}

^{a,b,c,d} Significant ($p<0.05$) differences in the same row

A slight decrease in the quantity of fructose (4.5 to 27.8%) and glucose (6.1 to 26.8%) occurred after 6 months, while the sucrose reduction ranged between 0.5 to 47.2%. After 12 months a significant reduction was observed in the sugar content ranging from 32.5 to 51.8% for fructose, 32.1 to 48.2% for glucose and 55.3 to 91.8% for sucrose. Our results were in agreement with those obtained by Al-Ghamdi and coworkers [31]. They reported that a slight decrease (<15%) in the quantity of fructose and glucose occured after 6 months, due to the acid catalyzed the formation of maltose and other reducing disaccharides.

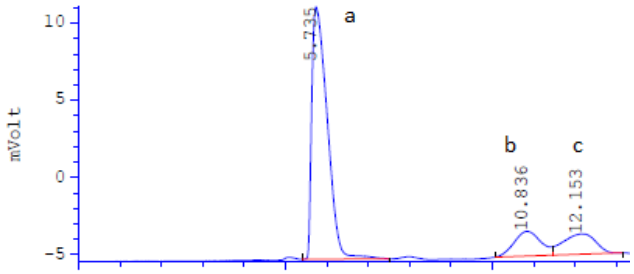

Figure 2. Chromatogram of sugars oh honey sample, a: fructose, b: glucose, c: sucrose

Although sample S06 was initially incompatible with the Codex standard for sucrose content (more than 9), it decreased after 12 months storage and fell into the standard Codex range. This changing behavior makes sucrose an unreliable parameter to determine if honey has been subjected to long-term storage.

A chromatogram of sugars of honey sample obtained from HPLC is presented in Fig 2.

3.6. Effect of storage on the proline content of honey

Proline, one of the non-essential and one of the most important amino acids, is the main and predominant amino acid found in honey. It was proved from the analysis of sugar-fed honey that proline came from the salivary secretion of honey bees during nectar conversion [32]. The proline content of entire honey samples varied from 225.7±2.5to 574±1.5 mg/kg, showing a wide range of changes in polyfloral honey (Table 5). According to Marcucci et al., proline in honey is derived from the bee itself, but it has been difficult to explain why such a variation exists in its content in honeys [33]. The prolin content of entire samples was in line with the legislated level (>180 mg/kg) described by the Codex Alimentarius [20]. Proline value below 180 mg/kg means that the honey is probably adulterated by sugar addition [17]. The level of proline content in the current study is in good consistency with those were reported in previous studies for floral honey including, 300-860 mg/kg for honey produced in turkey [34] and 315.9-770 mg/kg for honey samples from Morocco [35].

Table 5. Proline content (mg kg^{-1}) of 6 honey samples during storage for one year (mean±SD)

Sample No	Storage period (month)				
	0	3	6	9	12
1	275.7±1.5^{a}	279.7±2.1^{ab}	271.0±3.1^{b}	272±1.1^{b}	261.3±1.5^{c}
2	344.0±1.3^{a}	350.3±2.2^{b}	345.0±2.0^{b}	343.7±1.5^{b}	332.7±2.1^{c}
3	574.0±1.5^{a}	573.3±1.7^{a}	567.7±2.5^{a}	530.6±3.1^{b}	507.0±2.2^{c}
4	431.4±1.5^{a}	429.6±2.1^{a}	436.9±2.0^{b}	437.3±1.5^{b}	422.0±2.7^{c}
5	308.0±2.0^{a}	301.7±1.5^{ab}	298.3±2.5^{bc}	293.7±3.1^{c}	275.3±2.4^{d}
6	225.7±2.5^{a}	223.9±3.1^{ab}	220.4±2.1^{ab}	218.2±1.7^{ab}	219.3±1.6^{b}

^{a,b,c,d} Significant ($p<0.05$) differences in the same row

The effect of long-term storage (1 year) on proline content is presented in Table 5. Results showed that proline decreased significantly after 1 year storage at ambient temperature however, its value was still higher than the minimum permitted level legislated by Codex Alimentarius (180 mg/kg). Changes in proline content over the time (1 year) did not follow a consistent behavior which makes proline content an uncertain parameter to determine the freshness of honey. The current study also showed that proline has not changed dramatically over the course of a year (ranging from 3 to 12% reduction). Moreover, its range of variation is very wide in different kind of honey, and therefore it cannot be used as an indicator to determine the freshness of honey. Our results were in contrast to those reported by Von der Ohe [36], who demonstrated that proline content of honey constantly decreases during storage therefore the proline might be an indicator of honey ripeness [36]. In a related study, Nepalese honey samples produced by Apis dorsata, were collected from four different forests which include Shahagunj, Dhakeri, Narayanpur, Perari Forests. Then the proline content of the samples was determined during 16 months. Results showed that proline was increased during first 8 months ranging from 74.5 to 92.9% and decreased during the next eight month up to 55% [37].

4. Conclusion

The results of this study showed the most of honey samples were of good quality when compared with Codex Alimentarius standard. All of the investigated variables (HMF, DN, sugars, and proline) showed significant differences after storage for 1 year. However, regard to the fact that the variation range of sugar, proline and diastase in polyfloral honey samples is wide (depends on the type of honey and other factors), and their changing behavior during storage is not consistent, they cannot be considered as reliable parameters for determining the degree of honey freshness. Moreover, in some of the samples these features were also within standard limits, even after long-term storage. According to the findings of this study, the only factor that will increase over time (one year) and go beyond the standard limit is the HMF value. However, this high level of HMF may be due to improper thermal processing.

Judging by this feature, the best recommend time for consumption of honey is before one year since production date.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: May. 2023 Accepted: Jun. 2023
Published online: Jun. 2023

DOI: 10.22034/ASAS.2023.390055.1021

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