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Effects of crystallization on antioxidant property of honey

Duran Özkök[1], Sibel Silici[2]

[1]Safiye Cikrikcioglu Vocational School, Erciyes University, Kayseri 38039, Turkey

[2]Department of Agricultural Biotechnology, Agriculture Faculty, Erciyes University, Kayseri 38039, Turkey

ABSTRACT

Melissopalynological and biochemical analyses of 50 honey samples (chestnut, citrus, clover, cotton, and sunflower) were performed in this study. The total phenolic contents (TPC) with the Folin–Ciocalteu, antioxidant activities with the phosphomolybdenum, and free radical scavenging activities with the 2,2-diphenyl-1-picrylhydrazyl methods of honey samples were determined. The fructose (F) content of honey samples varied between 31.44% and 35.16%, glucose (G) contents between 22.46% and 32.71%, and sucrose contents between 0.37% and 1.94%. Moreover, the G + F contents of clover, citrus, sunflower, chestnut, and cotton honey were respectively observed as 67.87%, 66.21%, 67.39%, 53.90%, and 66.26%. The F/G ratios of the honey samples varied between 1.07 and 1.40 and the glucose/water (G/W) ratios between 1.36 and 1.94. Furthermore, biological analyses performed in every 6 months throughout the 18 months of storage revealed decreasing TPCs, antioxidant, and antiradical activities over time in the honey samples ($p < 0.05$). However, such decreases were attributed to the negative impacts of storage on honey rather than crystallization.

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Introduction

Honey contains several substances, mainly carbohydrates, water, vitamins, minerals, proteins, free amino acids, enzymes, organic acids, flavonoids, phenolic acids, and other phytochemicals [1]. Honey is valuable for the treatment of several diseases. Because its therapeutic actions include antioxidant and antimicrobial properties, anti-inflammatory, and wound healing activities [2]. Honey includes enzymatic and non-enzymatic antioxidants, including glucose oxidase, catalase, ascorbic acid (AA), carotenoid derivatives, organic acids, Maillard reaction products, amino acids, and proteins [3]. The chemical composition of honey depends on the floral sources, seasonal and environmental factors, and processing methods [1].

Crystallization is a significant parameter for the market value of honey. In temperate climates, honey can crystallize even under normal storage temperatures and crystallization negatively influences consumer preferences. The majority of honeys are supersaturated solutions with glucose and this glucose can spontaneously crystallize into glucose-monohydride at room temperature [4]. Supersaturation is not a thermodynamically stable state. An increase in the amount of solids over saturation level creates a tendency towards crystallization and reduces water activity [5]. In general, fructose (F) is dominant in most honeys. However, the glucose ratio is higher than fructose in sunflower, alfalfa, cotton, rape, and dandelion honeys [6]. Not every honey is crystallized at the same time. The crystallization trend of honey from different botanical origins is closely related to some physical and chemical parameters, some of these parameters are glucose, glucose/water (G/W), G-W/F, F/G ratios, and melezitose content. Specifically, honey crystallizes faster when the glucose content is >28%–30%, G/W ratio is ≥2.1, F/G ratio is <1.14, and melezitose ratio is over 10% [7]. Besides these parameters, the existence of dust, pollen, comb, and propolis particles in honey also influences the crystallization of

Contact Sibel Silici ✉ sibelsilici@gmail.com Department of Agricultural Biotechnology, Agriculture Faculty, Erciyes University, Kayseri 38039, Turkey

honey [8,9]. Furthermore, botanical origin, processing conditions, storage conditions, storage temperature, relative humidity, and the container in which the honey is kept also influence the crystallization of honey [5].

Wang et al. [10] observed the reduced antioxidant activity for processed honey and also indicated that high water content in honey resulted in fermentation and brewing and the crystallized honey was fermented more easily and spoiled irreversibly. Additionally, it is investigated the water activity and moisture content of honey samples obtained from two different harvest seasons of two regions with different climates and assessed the relationships among harvest year, storage duration, and crystallization [11]. The same researchers also reported significant changes in the water content of honey samples with time. In a study, it is found out that the F/G ratio of honey collected from Canada as 1.12 and indicated that all honey samples were saturated with glucose (glucose > 30%) [12]. Manikis and Thrasivoulou [13] investigated the relationships among the physico-chemical characteristics of honey samples and assessed the sensitivity parameters against crystallization. The researchers used 50 honey samples with different botanical origins (*Anchusa officinalis*, *Echium vulgaris*, and *Eucalyptus camaldulensis*) and stored them for 1–12 months. They also reported that 16 samples with a glucose ratio of less than 34% crystallized at a slower rate. The researchers also indicated that glucose was a good indicator for granulation of honey. It was difficult to estimate crystallization in the samples with medium glucose levels and the G/W ratio could also be used in crystallization estimations. Gleiter et al. [14] analyzed the water activity of 249 honey samples produced in Germany to investigate the effects of crystallization type and status on the water activity of honey. Initially, honey samples were identified based on palynological and physico-chemical characteristics. The water content was determined with a refractometer at 20°C and it was reported that the water content of crystallized honey samples had higher moisture contents than that of liquid honey. Several researchers investigated the relationships between the factors effective in crystallization and the chemical structure of honey. However, the number of studies investigating the effects of crystallization with time on the bioactive characteristics of honey is very limited. Therefore, in this study, the effects of crystallization on the bioactive characteristics of honey crystallized at different times and stored for 18 months were investigated.

Materials and Methods

Honey samples

The monofloral honey samples [10 samples for clover (*Trifolium* spp.), citrus (*Citrus* spp.), sunflower (*Helianthus annuus*), chestnut (*Castanea sativa*), and cotton (*Gossypium hirsutum*) honey] were directly supplied by beekeepers from different regions of Turkey where they are produced (Table 1). Samples were collected in accordance with the method specified by the Turkish Food Codex Regulation [15] and labeled with harvest date, botanical, and geographical origin.

Melissopalynological analysis

Honey samples were classified on the basis of melissopalynological characterization according to their specific botanical variety [16]. The pollen types present in the honey samples were identified, counted, and classified according to their percentages, as dominant pollen (45% or more), secondary pollen (16%–44%), important minor pollen (3%–15%), and minor pollen (<3%). Counts were expressed as a percentage after counting a minimum of 1,000 pollen grains on four slides from each sample.

Chemicals and biochemical analysis of honey samples

The 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3,4,5-trihydroxybenzoic acid (gallic acid; GA), Folin–Ciocalteu reagent, AA, and ethanol were obtained from Sigma Chemical Co. (St. Louis, MO). The sulfuric acid (H_2SO_4), sodium phosphate (Na_3PO_4),

Table 1. Geographical, botanical origins, and pollen frequency of honey samples.

Honey samples	Geographical origin	Botanical origin	Pollen frequency (%)
Chestnut	Bursa/Turkey	*Castanea sativa*	93.60–88.90
Citrus	Mersin/Turkey	*Citrus* spp.	71.50–42.80
Clover	Adıyaman/Turkey	*Trifolium* spp.	76.80–72.60
Cotton	Adana/Turkey	*Gossypium hirsutum*	62.50–42.60
Sunflower	Edirne/Turkey	*Helianthus annuus*	78.60–52.70

ammonium molybdate ($(NH_4)_2MoO_4$), sodium carbonate (Na_2CO_3), and methanol (MeOH) were obtained from Merck (Darmstadt, Germany).

Some physico-chemical characteristics of the honeys were in agreement with those of the European Union [17]. The color value of the honey was determined using a Hunter spectrometer (CR-400, Minolta, Osaka, Japan). Moisture content was measured using a refractometer (Atago, Tokyo, Japan), electrical conductivities with a conductometer (WTW inoLab Cond/720, Germany), and optical activity or rotation with a polarimeter (Beta PPP7, England). Sugar analysis of the samples was performed using a refractive detector with high performance liquid chromatography (Elite LaChrom, Hitachi, Japan) and a reverse phase–amide column (200/4.6 Nucleosil 100-5 NH2). Quantitative and qualitative sugar analyses were performed using the method described before [18]. The calibration curves of all analyzed sugars were between 0.994 and 1.000.

Determination of total phenolic content

The Folin–Ciocalteu method was used to determine total phenolic contents (TPCs) with some modifications [19]. Each honey sample (1 g) was dissolved in 4 ml of methanol using a vortex mixer and the solution was filtered through Whatman No. 1 filter paper. This solution (40 µl) was mixed with 2.4 ml water, 200 µl non-diluted Folin–Ciocalteu reagent, and 600 µl Na_2CO_3 (20%) and then 760 µl water was added. After incubation at room temperature in the dark for 2 hours, the absorbance of the reaction mixture was measured at 765 nm against a methanol blank and the results were calculated as milligram GA equivalents (GAE/100 g of sample) [20].

Determination of antioxidant activity

The honey samples were evaluated by the phosphomolybdenum method for antioxidant activity [21] and expressed relative to that of AA. Briefly, a 0.4 ml aliquot of the sample in methanol was mixed with 4 ml of the reagent solution. Methanol was used as blank instead of the honey solution. The reaction mixture was vortexed and left to stand in a water bath at 95°C for 90 minutes. Absorbance was measured at 695 nm and results were calculated as AA equivalents (mg AAE/1 g of sample).

Radical-scavenging effect on DPPH

The honey samples were evaluated by the DPPH assay with some modifications for antiradical activity [22]. The honey sample (1 g) was dissolved in 4 ml methanol using a vortex mixer and the solution was filtered through Whatman No. 1 filter paper. Then, a 100 µl aliquot of the honey sample was mixed with 450 µl Tris–HCl and 1,000 µl of 6×10^{-5} M DPPH in methanol. The mixtures were left in the dark for 2 hours at room temperature and the absorbances at 517 nm were measured using a spectrophotometer with methanol as blank. The antiradical activities of the samples were calculated according to formula and results were shown as % inhibition.

Statistical analysis

The data obtained from normal (non-crystallized) and crystallized (at different levels) honey samples were subjected to one-way variance analysis. Duncan's multiple range test was used to identify the differences in group means. SPSS for Windows 13.0 software was used in statistical analyses.

Results

The pollen ratios and geographical origins of the monofloral honey samples are provided in Table 1. Chestnut pollens were represented by 88.90%–93.60% (over-represented).

Physico-chemical analyses revealed that the color values of the honey samples varied based on the Pfund scale between 19.51 and 46.72 (Table 2). Based on this scale, citrus honey had the lightest color and chestnut honey had the darkest color. The water contents of honey samples (moisture content) were below 18% and varied between 16.20% and 17.02%. Cotton honey had the lowest moisture content and it was followed by clover, sunflower, citrus, and chestnut honey. With regard to acidity and conductivity, chestnut honey had the highest values. The diastase activity of the honey samples varied between 11.80 and 18.20. Moisture, G/W, F/G, and F + G values have significant influences on the crystallization of honey. G and F are the most abundant sugars in honey. The fructose content of honey samples varied between 34.91% and 37.90%, the glucose contents varied between 29.35% and 31.68%, and sucrose contents varied between 0.37% and 1.94%. Considering the sugar analyses results of the honey samples, the greatest F and G contents were observed in chestnut honey and the lowest values were seen in cotton honey. Such values comply with the values specified in both the Turkish Food Codex Regulation [15] and the Codex Alimentarius [23]. The F + G content of

Table 2. Physico-chemical properties of honey samples.

Parameters	Honey samples				
	Chestnut	Citrus	Clover	Cotton	Sunflower
Color (Pfund scale)	46.72 ± 19.97^{c}*	19.51 ± 8.63^{a}	20.20 ± 1.99^{a}	38.72 ± 7.65^{b}	37.27 ± 23.74^{b}
Moisture (%)	16.50 ± 3.93^{a}	16.83 ± 3.77^{a}	17.02 ± 0.25^{b}	16.20 ± 3.96^{a}	16.62 ± 3.94^{a}
Diastase number (DIN)	13.75 ± 4.32^{b}	11.80 ± 6.73^{a}	18.25 ± 1.78^{c}	13.96 ± 3.14^{b}	13.63 ± 4.24^{b}
Acidity (mq. g/kg)	20.88 ± 7.78^{c}	15.51 ± 3.82^{a}	16.20 ± 1.47^{ab}	19.75 ± 4.77^{bc}	18.97 ± 7.80^{b}
Electrical conductivity (20°C)	0.42 ± 0.41^{c}	0.19 ± 0.07^{a}	0.18 ± 0.01^{a}	0.39 ± 0.12^{c}	0.29 ± 0.14^{b}
F (%)	31.44. ± 2.64^{a}	34.49 ± 7.47^{b}	35.16 ± 8.37^{c}	34.91 ± 8.27^{b}	35.14 ± 8.79^{c}
G (%)	22.46 ± 2.19^{b}	31.72 ± 6.25^{a}	32.71 ± 7.32^{a}	31.35 ± 7.27^{a}	32.25 ± 7.98^{a}
Sucrose (%)	0.37 ± 0.13^{a}	1.94 ± 1.91^{e}	1.07 ± 0.34^{c}	0.86 ± 0.32^{b}	1.39 ± 0.63^{d}
F + G (%) (DIN)	53.90 ± 4.83^{a}	66.21 ± 13.72^{b}	67.87 ± 15.46^{b}	66.26 ± 15.51^{b}	67.39 ± 16.76^{b}
F/G (DIN)	1.40 ± 0.01^{c}	1.08 ± 0.25^{a}	1.07 ± 0.31^{a}	1.11 ± 0.26^{b}	1.09 ± 0.25^{ab}
G/W (DIN)	1.36 ± 0.14^{a}	1.88 ± 0.38^{b}	1.92 ± 0.45^{c}	1.93 ± 0.43^{c}	1.94 ± 0.45^{c}

*Values are mean ± standard deviation. Different letters in the same column indicate significant differences ($P < 0.05$).

clover, citrus, sunflower, chestnut, and cotton honey was respectively observed as 67.87%, 66.21%, 67.39%, 53.90%, and 66.26%. The F/G ratios varied between 1.07 and 1.40 and G/W ratios varied between 1.36 and 1.94. Chestnut honey had the lowest G/W ratio.

Biological analyses carried out at the beginning and every 6-month in the 18-month period. The highest pre-storage TPC was observed in chestnut honey and it was followed by citrus, sunflower, cotton, and clover honey. As was the case in other biological analyses, a decrease was observed in the TPC of all honey samples throughout the 18-month storage period. The TPC of honey samples at 0, 6, 12, and 18 months varied respectively between 38.34–156.86, 23.34–153.92, 8.51–97.23, and 7.96–30.92 mg GAE/100 g honey and exhibited a decreasing trend with time (Table 3). Current analyses revealed that chestnut honey had the highest TPC, antioxidant activity, and free radical scavenging activity (FRSA) before the storage. As was the case in chestnut honey, a decrease was observed in the biological activities of all samples throughout the 18-month storage period.

According to analyses carried out at every 6-month in the 18-month period revealed decreasing antioxidant activity with time. The antioxidant activity of honey samples varied between 130.79 and 139.95 mg AAE/g honey throughout the initial 6 months. The values decreased to 112.79–131.89 mg AAE/g honey in analyses in the sixth month and finally went down to 56.59–100.78 mg AAE/g honey in the 18th month (Table 3).

The highest pre-storage antiradical activity was observed in chestnut honey and it was followed by sunflower, citrus, cotton, and clover honey. Similar to antioxidant activity, a decreasing trend was also observed in the FRSAs of honey samples. The pre-storage FRSA of the samples varied between 4.73% and 56.18% and the values at the end of 18-month storage varied between 2.81% and 34.39% (Table 3). All results of every 6-month analyses revealed a decrease in all samples.

Discussion

There are more than 100 monofloral types of honey in Europe [24]. Turkey with quite a rich flora has a great potential to produce several monofloral honeys. Sunflower, citrus, clover, chestnut, linden, heather, thyme, vetch, and honeydew honeys are among some of these monofloral honeys. Sugars, the primary components of honey, depend more on botanical and geographical origins and less on climate, processing, and storage conditions [25,26]. The crystallization of honey is a significant parameter for consumer preference and thus, the market value of honey. Crystallization not only depends on chemical composition but also on seed crystals, pollen, and wax particles within the honey. Crude honey (unheated and unfiltered) contains slight amounts of wax and pollen residues and crystallizes faster [6].

Moisture, G/W, F/G, and F + G values play a significant role in the crystallization of honey. In the present study, the least moisture content was observed in cotton and chestnut honey and the greatest moisture content was observed in clover honey. G and F are the most abundant sugars in honey. Fructose was dominant in all the monofloral honey samples in the present study. While chestnut honey had the least glucose content, clover and sunflower honey had higher glucose content than the others. Escuredo et al. [27] reported significantly higher glucose contents for sunflower and rape honey than for the other honeys. Except for chestnut honey,

Table 3. Bioactive properties of honey samples (changing in the 18-month).

Honey samples	Time (month)			
	0	**6**	12	18
	TPCs of honey samples (mg GAE/100 g sample)			
Chestnut	156.86 ± 3.82^{d*}	153.92 ± 7.37^{c}	97.23 ± 6.01^{d}	74.17 ± 8.92^{c}
Citrus	94.45 ± 2.57^{c}	29.99 ± 1.97^{a}	25.54 ± 2.32^{b}	21.04 ± 2.49^{ab}
Clover	38.34 ± 1.77^{a}	23.34 ± 1.77^{a}	8.51 ± 0.56^{a}	7.96 ± 0.62^{a}
Cotton	38.99 ± 5.08^{a}	36.99 ± 5.08^{a}	35.96 ± 3.52^{b}	25.05 ± 2.79^{b}
Sunflower	69.08 ± 3.52^{b}	63.08 ± 3.52^{b}	57.60 ± 2.84^{c}	30.92 ± 2.78^{b}
	Antioxidant activities of honey samples (mg AAE/g sample)			
Chestnut	130.79 ± 3.49^{a*}	112.79 ± 3.49^{a}	96.63 ± 3.08^{a}	74.57 ± 2.62^{b}
Citrus	136.89 ± 2.79^{a}	131.89 ± 2.79^{b}	125.10 ± 4.23^{b}	56.59 ± 5.81^{a}
Clover	158.93 ± 3.96^{b}	129.93 ± 3.96^{b}	99.51 ± 11.26^{a}	97.49 ± 2.66^{c}
Cotton	139.95 ± 4.52^{a}	118.95 ± 4.52^{ab}	107.59 ± 9.01^{ab}	100.78 ± 6.74^{c}
Sunflower	130.68 ± 4.33^{a}	110.68 ± 4.33^{a}	88.35 ± 5.35^{a}	64.88 ± 4.35^{ab}
	Antiradical activities of honey samples (% inhibition)			
Chestnut	71.19 ± 3.98^{d*}	42.05 ± 2.83^{d}	37.65 ± 1.06^{d}	34.39 ± 1.69^{c}
Citrus	27.07 ± 1.72^{b}	17.25 ± 2.34^{b}	8.13 ± 0.36^{bc}	3.57 ± 0.21^{a}
Clover	4.73 ± 0.51^{a}	3.73 ± 0.51^{a}	3.26 ± 0.57^{a}	2.81 ± 0.28^{a}
Cotton	6.67 ± 0.42^{a}	5.67 ± 0.42^{a}	5.48 ± 1.84^{ab}	5.04 ± 0.30^{a}
Sunflower	56.18 ± 2.88^{c}	33.18 ± 2.88^{c}	10.79 ± 1.35^{c}	10.08 ± 1.03^{b}

*Different letters in the same properties and the same column indicate significant differences ($P < 0.05$). Values are mean ± standard deviation.

the glucose content of the other honey samples was over 30%. Thus, honey with a glucose content of less than 30% has slower crystallization rates [13]. Escuredo et al. [27] indicated that honeydew, chestnut, heather, bramble, and acacia honey had slow crystallization rates while sunflower, linden, and rape honey had fast crystallization rates. Citrus honey had the greatest sucrose content in this study. In another study, the highest sucrose content was reported for acacia honey (2.30%) and the least sucrose content was reported for chestnut and eucalyptus honey (0.2%). Similarly, it is indicated that the rapid crystallization of sunflower honey was due to its high glucose content and the long-lasting feature of the liquid form of chestnut honey due to its high fructose and low glucose contents [24]. Except for chestnut honey (53.90%), the G + F values of other honey samples varied between 60% and 70%. While multifloral honey generally had an F + G value over 60%, honeydew honey had a value below 60% [27]. Such findings comply with the results of the present study. As indicated in Turkish Food Codex Regulation and the Codex Alimentarius, this parameter is used in the separation of flower and honeydew honey [15,23]. It is also indicated that rape and sunflower honey had the highest F + G value (over 75%) [27].

The F/G ratio is a parameter recommended to assess the crystallization of honey since glucose is less water soluble than fructose and thus, it was proposed as the parameter for the best estimation of crystallization [28]. When the F/G ratio of the honey is 1.14 or less, then the honey crystallizes quickly; the honey with an F/G ratio over 1.58 does not have a crystallization tendency [29] and honey with an F/G ratio of 1.3 crystallizes slowly [25]. While the F/G ratio for chestnut honey in the present study was 1.40, the F/G ratios of the other honey samples varied between 1.07 and 1.11. In another study, the F/G ratios of sunflower, rape, and linden honey were respectively observed as 1.02, 1.13, and 1.17 [27].

The G/W ratios of the honey samples in the present study varied between 1.36 and 1.94. According to the National Honey Board [30], the crystallization time of honey mostly depends on the F/G and G/W ratios. Thus, moisture is an important factor which takes the production season and meteorological factors of the production region into consideration [27]. Moisture content affects not only the physical characteristics (viscosity, crystallization, and rheological behavior) of honey but also the appearance, color, taste, specific gravity, water solubility, storage, and commercial value of the honey. It was reported the highest moisture content (22.4%) for sunflower honey [27]. The researchers reported the average G/W ratio as 1.5 for chestnut and honeydew honey, and as 2.0 for rape honey. Some researchers stated the G/W ratio was a good indicator for the crystallization of honey [13,25]. Honey with a high glucose and low moisture content crystallizes quickly. According to the previous literature, crystallization

is slower or even zero when the G/W ratio of honey is less than 1.7, and the crystallization is faster and complete at G/W ratios over 2.0 [25]. Also, it was reported close F + G and G/W ratios for rape, sunflower, and linden honey and indicated that these honey crystallized fast [27].

Previous research studies revealed that botanical origin and sugar content had great impacts on the crystallization of honey. Fructose, glucose, moisture content, and sugar ratios (F + G, F/G, and G/W ratios) are the best indicators to estimate the crystallization phenomenon of honey. It was reported that rape and sunflower honey had the least reduced sugar (F + G) content and these honeys with high glucose and low F/G ratios crystallized quickly [27]. The researchers also reported high F/G ratios (less than 30% glucose) and slow crystallization rates for blackberry, chestnut, eucalyptus, heather, acacia, and honeydew honey. Hamdan [6] reported fast crystallization rates for clover, sunflower, and cotton honey, slow crystallization rates for chestnut, thyme, and citrus honey and very slow crystallization rates for vetch honey.

In conclusion, the crystallization of honey is not well understood by consumers, as yet. Some consumers may consider crystallized honey as a fraudulent or non-natural product. However, crystallization is a natural process in honey and occurs spontaneously. Pure, crude, or unheated honeys generally crystallize as a natural phenomenon. It is well known that crystallization does not influence the structure of the honey, but influences only the color and appearance [6]. The present results revealed fast crystallization rates for clover, citrus, sunflower, and cotton honey and a slow crystallization rate for chestnut honey.

Total phenolic and flavonoid contents of honey were variable and depended greatly on the floral source [31]. In our study, chestnut honey had the greatest TPC and antioxidant activity. It was also observed in this study that regardless of rapid or slow crystallization rates, long storage times reduced over the TPC, antioxidant activity, and FRSA of honey samples.

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Conflict of Interest

The authors declare no conflict of interest.

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