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The Effect of Heat Treatment on Sugar, HMF and Antioxidant Properties of Honey Obtained from Different Regions in Turkey

Türkiye'de Farklı Bölgelerden Elde Edilen Balların Şeker, HMF ve Antioksidan Özelliklerine Isıl Uygulamanın Etkisi

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Abstract

The aim of this study was to investigate the thermal effect on 5-hydroxymethylfurfuraldehyde (HMF), glucose, fructose, maltose contents and changes in the antioxidant potential of honeys threated at different temperatures (25, 35, 50 and 65 °C for 30 min). Three types of honey (flower, pine, chestnut) samples were collected from eight different cities located at five different regions in Turkey and analyzed.

The HMF and sugars constituents were also determined by HPLC. Changes in the antioxidant capacity of honeys were measured as a percentage of free radical ($ABTS^{\cdot+}$and $DPPH^{\cdot}$) scavenging ability. Total polyphenols contents (TPC) were determined as equivalents of gallic acid mg/100 g of honey. The expose of honey samples of at 65°C for 30 min, resulted a significant increase in the concentration of HMF (2,531 mg/kg). In this study, the sum of glucose, fructose and maltose varied from 50.27% to 74.44% and the average value was 62.35%.

Keywords: Antioxidant activity, HMF, Honey, Sugar, Thermal processing

Özet

Bu çalışmanın amacı farklı sıcaklık (25, 35, 50 ve 65 °C, 30 dakika) uygulanan balların 5-hidroksimetilfurfuraldehit (HMF), glikoz, fruktoz, maltoz içerikleri ve antioksidan potansiyelindeki değişim üzerine ısıl etkiyi araştırmaktır. Türkiye'nin beş farklı bölgesinde bulunan sekiz farklı ilden üç çeşit bal (çiçek, çam, kestane) örneği toplanmış ve analiz edilmiştir. HMF ve şeker bileşenleri de HPLC ile belirlenmiştir.

Balların antioksidan kapasitesindeki değişimler serbest radikal ($ABTS^{\cdot+}$ve $DPPH^{\cdot}$) süpürme yeteneğinin bir yüzdesi olarak ölçülmüştür. Toplam polifenol içerikleri (TPC), mg gallik asit / 100 g balın eşdeğerleri olarak belirlenmiştir. Bal örneklerinin 65°C'de 30 dakika boyunca maruz kalması HMF konsantrasyonunda (2,531 mg/kg) önemli bir artışa neden olmuştur. Bu çalışmada, glukoz, fruktoz ve maltoz toplamı % 50,27'den % 74,44'e, ortalama değer % 62.35'e çıkmıştır.

Anahtar kelimeler: Antioksidan aktivite, HMF, Bal, Şeker, Isıl işlerm

Abbreviations: HMF, 5-hydroxymethylfurfural.

1. INTRODUCTION

Honey is a sweet food material which is produced by the honey bees from the nectar found in the flowers of plants or the sweet secretions of insects living on some plants (Tuzen, Silici, Mendil, & Soylak, 2007; Tutkun, 2006). The most important factor affecting the composition of honey is the source of the plant, as well as the seasonal and climatic conditions also determine the honey composition (Shelear Hussein Hasan, 2013). According to its source, honey is classified as flower honey and honeydew honey (Turhan, I., Tetik, N., Karhan, M., Gurel, F., Tavukcuoglu, 2008). In addition to being a concentrated mixture of glucose and fructose, honey also contains various minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other physico-chemical components in its structure (Bertoncelj, Dobersek, Jamnik, & Golob, 2007; Karadal, Yildirim, 2012). The production of unifloral and multifloral honey is very common in Turkey. Different processing methods affect the quality of honey. Storage conditions, storage time, harvesting, filtration and heating operations can change the structure of honey (Silici, 2004).

Honey's most important physical quality criterion is the crystallization. Crystallization occurs as a result of the separation of glucose from the water in honey and its crystal precipitation with other particles of honey. This is a natural process and there is no difference in nutritional value between crystallized honey and liquid honey. Natural crystallization is undesired by consumers as it causes deterioration in honey textures (Bath & Singh, 1999; Fallico, Zappalà, Arena, & Verzera, 2004 ; Khalil, Sulaiman, & Gan, 2010; Castro-Vázquez, Díaz-Maroto, González-Viñas, De La Fuente, & Pérez-Coello, 2008). Many factors like moisture and dextrin content, water activity, presence of microcrystals, storage temperature and applied heat treatments influence the crystallization of honey. Particles and air bubbles remaining in the honey during filtration are factors that increase the crystallization (Tosi et al., 2004). Glucose/water ratio and fructose/glucose ratios also affect crystallization. When the fructose/glucose ratio is between 1.0 and 1.2, the crystallization occurs quickly, whereas if the ratio is 1.3 or more, the crystallization is delayed (Ruoff et al., 2006).

During the processing of honey, heat treatment is required to destroy foreign microorganisms (Turkmen et al., 2006), to prevent crystallization and fermentation, to facilitate extraction and filtration and to increase the viscosity of honey. Heat treatment, causes the loss of honey enzymes and increases the amount of HMF (hydroxymethylfurfural), thus causing the honey to lose its freshness. For this reason, the duration and temperature of the heat treatment must be controlled (Castro-Vázquez et al., 2008; Tosi et al., 2004; Khalil et al., 2010). Deepening of honey during storage can result from Maillard reaction, fructose caramelization and polyphenol reactions. The degree of deepening varies depending on the temperature and/or storage time (Bertoncelj et al., 2007).

Honey has usually heat treated in two different ways, either by keeping it in air-cooled rooms at 45-50 °C for 4-7 days, or keeping it in hot water. HMF is a product resulting from the heat treatment applied to food during cooking or sterilization, resulting from the non-enzymatic browning (Maillard) reaction of the reducing sugars with amino acids, or dehydration of acid in the hexoses. It is not known that these reaction products are mutagenic or antimutagenic. Maillard reaction products (MRPs) are believed to have antioxidant properties (Turkmen et al., 2006). Honey composition and antioxidant properties can be different according to region, climatic factors, transport and storage (Al-Mamary et al., 2002; Wang et al., 2004).

The HMF is a compound that is released as a result of the breakdown of fructose in the honey composition, and its level is a real benchmark for the quality of honey. Because, after the post-harvest heat treatment, fructose degradation is accelerated and the amount of HMF is increased. Therefore, the increase in the amount of HMF is an indication of the decrease in the quality of honey. The HMF should not exceed 40 mg/kg (Tosi et al., 2002; Tutkun, 2006; Turhan, 2009). The amount of HMF is restricted by regulations to prevent excessive heat application in many food products containing carbohydrates. (Khalil et al., 2010) (Turhan, I., Tetik, N., 2004) (Rizelio et al., 2012, Karhan, M., Gurel, F., Tavukcuoglu, 2008, Turkmen et al., 2006). Honey provides very favorable conditions for HMF formation due to high amounts of simple sugars (glucose and

fructose) and many acid contents (Khalil et al., 2010). The degree of heat treatment, the duration, the storage conditions (such as exposure to light) and the storage in metal cups effect the amount of HMF in the honey (Khalil et al., 2010; Alvarez-Suarez et al., 2010). It is suggested that initial heat application accelerates the formation of acidity and triggers an increase in the amount of HMF throughout the storage period (Samborska and Czelejewska, 2014). For example, in a study conducted, the level of HMF increased by 1.10 over a period of 6 months in honeys maintained at 15 to 20 °C (Sahinler and Gul, 1988).

Natural and synthetic antioxidants have been used as food preservatives for many years. Antioxidants include both enzymes (catalase, glucose oxidase) as well as nonenzymatic substances (e.g. organic acids, Maillard reaction products, amino acids, proteins, flavonoids, phenolics, α-tocopherol, flavonols, catechins, ascorbic acid and carotenoids) (Meda et al., 2005). The amount of these compounds varies depending on the flora and geographic origin of the honey. The antioxidant capacity of honey is also dependent on the amount of botanical polyphenolic compounds flavonoids and vitamin C. Studies have shown that dark colored honeys have a high amount of phenolic and antioxidant content (Tutkun, 2006; Bertoncelj et al., 2007). In recent years, the use of antioxidant properties of honey has concentrated on studies such as anti aging, degenerative diseases of the heart and nervous system and protection from oxidation of foods (Tutkun, 2006). The aim of this study was to investigate the effects of heat treatment on some physicochemical properties of honey samples collected from eight different regions of Turkey.

2. MATERIALS AND METHODS

2.1. Materials

Honey samples were collected from four different regions in Anatolia namely; Malatya (MA), Adiyaman (AD), Gumushane (GM), Istanbul (IS), Aydin (AY), Trabzon (TR), Tekirdag (TK) and Erzurum (ER) (Fig. 1). Until analysis, samples of honey were kept at 25ºC in the dark.

The honey samples used in this study were collected from different cities in Eastern Anatolia, South Eastern Anatolia, Aegean, Black Sea and Marmara regions where the beekeeping is intense.

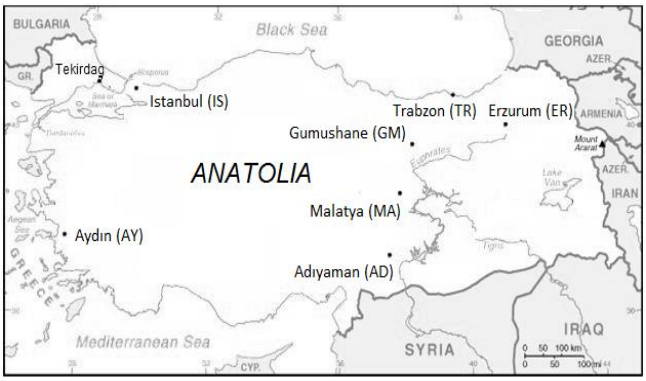

Figure 1. The locations of the honey samples

For the analysis, 8 different honeys (chestnut, keven, thyme, sunflower, blackberry, lime, forest rose, thunderbird, clover, acacia, vetch, spiked flower honeys and pine honeys are known) which were produced in 2016 (Table 1). Honey specimens were obtained from beekeepers that directly produce honey in the specified areas of Turkey. These samples were collected in 500 g glass jars and stored at room temperature until analysis.

Table 1. Locations and types of honey samples

Sample	Locations	Honey Type
1-MA	Malatya	Flower
2-AD	Adiyaman	Flower
3-GM	Gumushane	Flower
4-IS	Istanbul	Flower
5-AY	Aydin	Pine
6-TR	Trabzon	Chestnut
7-TK	Tekirdag	Flower
8-ER	Erzurum	Flower

2.1.1. Chemicals and Instruments

Calibration standards for 5-HMF analysis were prepared using 5-Hydroxymethyl-2-furaldehyde (99%, Aldrich, Germany) at concentrations 0.0, 1.0, 5.0, 10.0 and 15.0 mg/L with ultra pure water (LiChrosolv® for liquid chromatograpy). Methanol (LiChrosolv® for liquid chromatography) (Merck, Darmstadt, Germany), acetic acid, (Glacial Emprove®) (Merck, Darmstadt, Germany) was used as the mobile phase. As the purifying reagent, solutions of Carrez-I (Clarification Reagent Kit, MAK-191A) (Sigma-Aldrich, Germany) and Carrez-II (Clarification Reagent Kit, MAK-191B) (Sigma-Aldrich, Germany) were used. The standard solutions for the sugar analysis were prepared with

D-(-)-Fructose (Merck, Darmstadt, Germany), D-(+)-Glucose (Sigma, Germany) and Maltose Monohydrate (Merck, Darmstadt, Germany), Sucrose (Sigma-Aldrich, Germany) at concentrations of 0.2%, 0.5%, 1.0% and 2.0% using ultra pure water (LiChrosolv® for liquid chromatograpy) (Merck, Darmstadt, Germany). A thermostatic water bath (Microtest, Turkey) was used to keep the temperature constant. A Shimadzu 2000S Model UV / VIS spectrophotometer (Kyoto, Japan) was used for absorbance measurements. Folin&Ciocaltue phenol reagent; 2,20-azino-bis-(3-ethylbenzthiazoline-6- sulfonic acid (ABTS), potassium peroxodisulfate, sodium carbonate, glacial acetic acid, hydrochloric acid 30% was purchased from Merck (Darmstdt, Germany), ascorbic acid from Panreac (Barcelona, Spain) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was acquired from Sigma Chemical Co. (St. Louis, MO).

2.2. Methods

2.2.1. Heat treatment

The heat treatment was carried out for 30 minutes at four different temperatures (25, 35, 50, 65°C) after weighing 50 g from each honey sample.

2.2.2. Instrumentation and Chromatographic Conditions

Waters High Pressure Liquid Chromatography (HPLC) instrument equipped with, Waters 600 pump, Rheodyne injector and diode array detection (DAD, model 2996) was used for HMF analysis. Sugars were detected with Waters 2414 Refractive Index Detector. XBridge C18, 10 µM, 3.9x300 mm column was used with column oven (Jet Stream, VDS Optilab, Berlin, Germany) at 15°C for HMF and 23°C for sugar, after performing studies at temperature range of 5–40°C for seperations.

2.2.3. Sample Preperation of HMF Analysis

The analysis of hydroxymethylfurfural was carried out using an isocratic elution with a mobile phase mixture, water-methanol-acetic acid (90:9.5:0.5, v/v). The mobile phase was filtered through the 0.45 µm filter and degassed by ultrasonication before use. The flow rate was determined to be 0.7 ml/min and the injection volume to be 20 µL. The optimum wavelength for hydroxymethylfurfural was set to 285 nm and the analysis time was set to 25 minutes (Švecová et al., 2015) (Khalil et al., 2010).

2.2.4. Sample Preperation of Sugar Analysis

For determination of sugars (glucose, fructose and maltoz) water: acetonitrile (75:25, v/v) was used as the mobile phase, which was filtered through the 0.45 mm filter and degassed by ultrasonication before use. The flow rate was set to 1.4 ml/min and isocratic elution was used. A volume of 10 µL of sample or solution of standards was injected into the instrument with a column temperature of 23°C. The detection wavelength was 200–450 nm with specific monitoring at 285 nm (Švecová, Bordovská, Kalvachová, & Hájek, 2015)(Khalil et al., 2010).

2.2.5. Folin–Ciocalteu (FC) Determination for Total Phenolic Content

Total phenol content was determined by making some modifications in the Folin-Ciocalteu method. Each honey sample (1 g) obtained by applying the different temperature was dissolved in 4 mL of methanol using a vortex-mixer and the solution was filtered though Whatman No. 1. Then, 50 µl of the sample was quickly mixed with 250 µl of Folin-Ciocalteu reactant and allowed to incubate for 5 minutes.

After incubation, 2 mL 2% sodium carbonate was added. After 2 h incubation at ambient temperature, the resulting absorbance was measured with UV-VIS spectrophotometer at 755 nm against a methanol blank. The total phenolic content was determined using a standard curve with gallic acid (0–1 mg/ml) as the standard. The phenolic content is expressed as mg of gallik asit equivalent/g of honey sample (Mathew & Abraham, 2006; Singleton & Rossi Jr, 1965).

2.2.6. ABTS· Radical Cation Decolorization Assay

The 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) assay was performed as previously described by Meda et al.(2005). The $ABTS^{\cdot+}$ cation was generated in dark at room temperature by reacting a 7 mM solution of ABTS (30 mg) with 2.46 mM

potassium persulphate ($K_2S_2O_8$) for 12-16 h incubation. The honey sample, 10 µl, was reacted with 1 ml of $ABTS^{\cdot+}$ solution and absorbance (A) measured at 734 nm after 1 min. Results were expressed as Trolox equivalent antioxidant capacity per gram of DMAE. Trolox equivalency of the samples was calculated by comparing with a standard curve prepared with Trolox ($R^2 = 0.98$). The necessary dilution procedures were applied to honeys. To 96 well plate wells were added 200 µL $ABTS^{\cdot+}$ radical solution and 15 µL honey and incubated for 30 minutes. After incubation, the absorbance value at 734 nm was determined on BioTek Eon Eliza Microplate spectrophotometer. % ABTS radical cation decolorization was calculated from the absorbance values obtained (Re et al., 1999).

2.2.7. Determination of Radical Scavenging Activity

The scavenging activity of honey samples for the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described by Gyamfi, et al. (1999), with some modifications. Honey sample (1 g) was dissolved in 4 mL methanol using a vortex-mixer and the solution was filtered though Whatman No. 1. Aliquots of 50 µl of honey samples were mixed with 450 µl Tris–HCl and 1000 µl of 6x10-5 M DPPH in methanol. The mixtures were left for 2 h at room temperature in the dark and the absorbances at 517 nm measured using a spectrophotometer using methanol as blank.

2.2.8. HMF Analysis

HMF level of honey samples determined using two different methods. The honey samples (5 g each) were dissolved in 25 ml of water and transferred into a 50 ml volumetric flask. Then, 0.5 ml of Carrez-I solution [consisting of 15 g of potassium hexacyanoferrate(II), $K_4Fe(CN)_63H_2O$ in 100 ml of water] and 0.5 ml of Carrez-II solution [consisting of 30 g of zinc acetate, $Zn(CH_3COO)_{22}.H_2O$ in 100 ml of water] were added. And the mixture was made up to 50 ml with deionized water. The solution was filtered using filter paper after rejecting the first 10 ml of the filtrate and injected to HPLC. HMF content was determined by making some modifications in the method (Khalil et al., 2010).

The second method for HMF analysis in honey samples is a direct method. Briefly, the honey samples (2 g each) were diluted to 10 ml with deionized water, filtered using a 0.45µm membrane filter and injected (20 µl) into an HPLC system (Waters 2695, Milford, MA, USA) equipped with a Diode Array Detector (Waters 2996).

2.2.9. Sugar Analysis

HPLC was used to identify and quantify the main sugar profile (fructose, glucose and maltose) in honey samples, following standard methods. Three sugars in the pure phase and HPLC grade (Sigma chemical Co.) were used as standards, fructose, glucose and maltose. For each sugar a standard 4.0 g/L solution was prepared using deionized water. Honey samples (0.25 g) were dissolved in 5 ml of deionized water. Three replicates were prepared for each honey sample. Samples were cleaned by passing them through a Sep-Pak plus C18 cartridge (Waters Corporation) and filtered using a 0.45 µm filter and injected (20 µl) into an HPLC system. The samples were freshly prepared and immediately analysed (Shahidi, Naczk, & Myhara, 1990; Bogdanov, S.; Baumann, 1988).

HMF and sugar content of the sample were calculated by comparing the corresponding peak areas of the sample and those of the standard solutions of HMF and sugar after correcting for the honey's dilution. There was a linear relationship between the concentration and the area of the HMF and sugar peaks, respectively (results are expressed in mg/kg and sugar %).

3. RESULTS AND DISCUSSION

3.1. Effect of Heating on HMF Contents of Honey Samples

The heat treatment is used to facilitate loading and retention of the crystallization process. Speed crystallization of honey depends on temperature oscillations inside the room in which the honey is located. Hydroxymethylfurfural (HMF) is considered the most important degradation product of heating honey (E. Tosi, Ciappini, Ré, & Lucero, 2002; E. A. Tosi, Ré, Lucero, & Bulacio, 2004).

The results showed that HMF concentrations were at different levels at different temperatures.

For this reason, the analyses of the honey samples exposed to heat at different temperatures and was performed. As previously noted, the HMF concentration were investigated in samples exposed to temperatures of 25, 35, 50 and 65 °C for 30 minutes.

HMF is the result of heating or storing carbohydrates in unsuitable and unhealthy environments. Storing the honey in improved conditions and the heat treatment can result in the formation of HMF due to the link between sugars and amino acids in the honey (Assia and Ali, 2015) (Spano et al., 2006) When the honey samples are heated to 77 °C suddenly and left at that temperature for 5 minutes, the fermentation and honey crystallization are significantly delayed, resulting in a disadvantageous increase in the content of 5-HMF in the honey (Dogaroglu, 1999). Since heating affects the flavour of honey in a negative way, other methods than heating such as ultrasonic, freezing and chemical inhibitor use have been tried to prevent crystallization in the honey (R. Subramanian, 2007).

The initial concentration of HMF in honey samples is low. The increase in HMF concentration as a result of heating processes at four different temperatures (25, 35, 50, 65 ºC) is shown in Figure 2. During the 2nd and 3rd process (conventional heating), increases in the HMF contents in the wells were observed. The results direct analysis showed that the HMF concentration varied between 0.05 and 2.531 mg/kg. Among the honey samples, Gumushane (GM) honey has the lowest (0.05 mg/kg) HMF value at 25 °C and the highest (2.531 mg/kg) HMF value in the Tekirdag (TK) honey sample at 65 °C. (Figure 2-3).

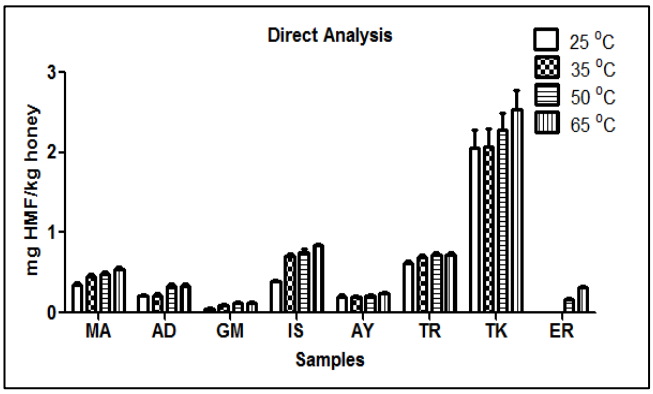

Figure 2. HMF levels with HPLC of honey samples exposed to different temperatures with direct analysis.

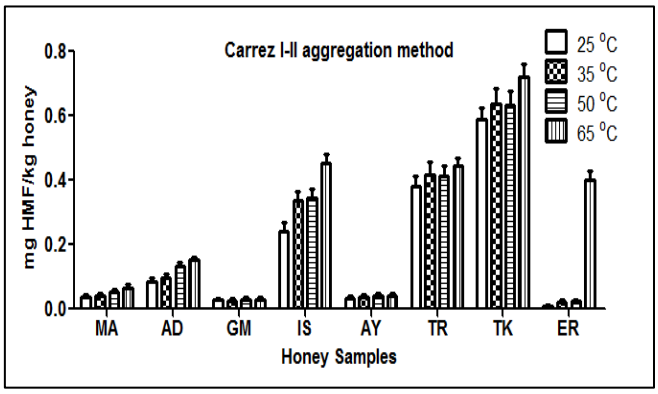

Figure 3. HMF levels with HPLC after operation with Carrez I-II of honey samples exposed to different temperatures

The use of thermal treatments for technological purposes, such as the elimination of crystallisation or pasteurisation, can increase the HMF content. Data reported by Tosi al. (Tosi et al., 2004;Tosi et al., 2002) for HMF showed that, a treatment of 90 seconds at 130 °C is produced approximately the same HMF increase in 30 seconds 150 °C treatment. Therefore, treatment time at a given temperature must be as short as possible. If temperatures are higher than 130 °C, even for short times, HMF increase reaches values above these accepted by international standards. But, HMF increases reach significantly higher values at high temperatures for longer times. All tests started with standard initial temperature of 25°C (Tosi et al., 2002). The variation of the content of HMF in the honey samples during heating with respect to the four heating programs showed similar behavior and the HMF concentration continuously increased during the heat treatment. The HMF content increased from 5.20 to 6.06 at the end of the highest temperature (65°C) applied compared to the initial value (S. Nuray and G. Aziz, 2004). The European Union (EU Directive 110/2001) has established a maximum HMF limit of 40 mg/kg in honey samples (Fallico et al., 2004). The HMF concentration on this value assumes that honey is stored in a warm environment (R. Krell, 1996) and is in a legally unacceptable condition. In our study, as the temperature increased, it was determined that there was a linear increase in HMF of 8 samples of honey. Regardless of the initial value, the mean HMF values of each honey group increased significantly when HMF was treated for 1 hour at 65 ºC. However, it has been determined that the increase in these HMF values does not exceed the limit values. ($P<0.05$) (Figure 2-3).

Karabournioti and Zervalaki (2001) reported an increased HMF content in orange honeys with the values of 3.45, 3.75, 4.35 and 19.00 mg/g in heat treated at 35 °C, 45 °C, 55 °C and 65 °C for 24 hours, respectively. It has also been shown that mild temperatures up to 55 ºC don't affect the increase in HMF regardless of exposure duration. The study has reported that the amount of HMF in unheated control honey increased from 1.20 mg/g to 43.40 mg/kg in pine honey. The HMF contents of pine honey was recorded as 1.95 at 35 °C, 2.25 at 45 °C, 4.80 at 55 °C, 12.40 at 65 °C, 43.40 mg/kg at 75 °C (Karabournioti, S. and Zervalaki, 2001).

In the previous studies, HMF content was found to be approximately 0.32–1.8 mg/kg (Merin et al., 1998); 3.2-27.6 mg/kg (Fallico et al., 2004); 10.7 mg/kg (Sahinler, N. Sahinler, S., and Gul, 2004); 3.916 mg/kg (Shelear Hussein Hasan, 2013b).
Honey is a convenient base for the formation of 5-hydroxymethylfurfuraldehyde (HMF), as it contains high concentrations of sugar. Fresh honey has a low content of HMF. However, its concentration increases when honey is heated.

According to our results, changes in HMF levels of honey samples were parallel with increasing the heating temperature (Figure 2-3). These results demonstrate that chemical composition and antioxidant properties of honey may be affected from storage conditions and heat-treatment. Especially, HMF levels of honey samples were affected from heat treatment. As results showed in Figure 2-3, it was found that HMF contents in the eight honey samples were lower than 40 mg/kg, as recommended by European Union Commission (EUC) (1996).

3.2. Effect of Heating on Sugars Contents of Honey Samples

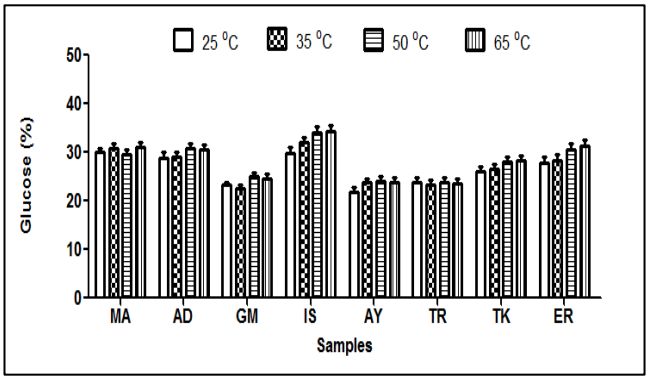

Figure 4. Glucose levels of honey samples exposed to different temperatures.

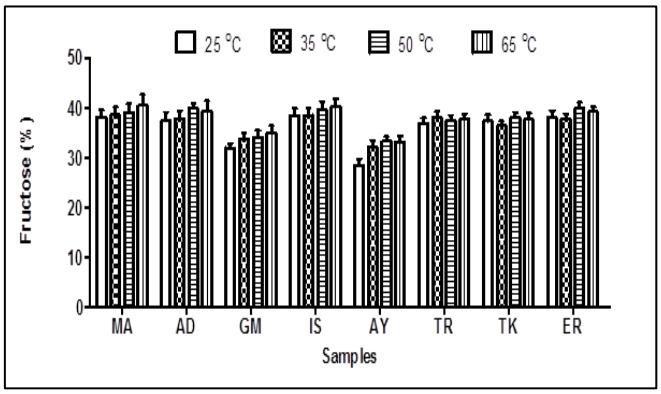

Figure 5. Fructose levels of honey samples exposed to different temperatures.

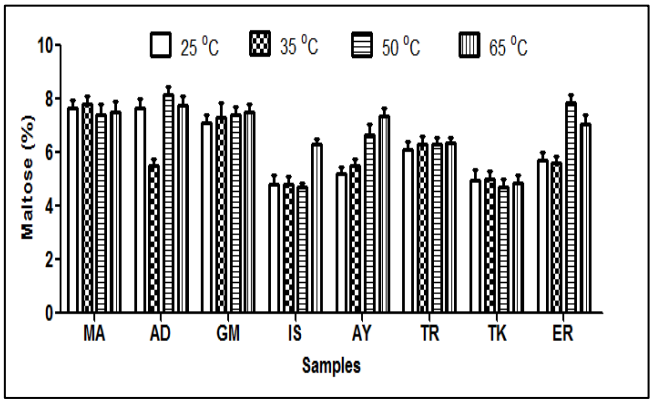

Figure 6. Maltose levels of honey samples exposed to different temperatures.

Because honey contains simple and complex sugars, it can also be used as a natural sweetener (Molan 1996). It also has a lower glycemic index (GI) than sucrose (Samanta, Burden, & Jones, 1985). Almost all honey varieties have a large amount of fructose and glucose (Finola et al., 2005).
The presence of glucose, fructose and maltose in the honey samples were detected by HPLC and the results were given as glucose, fructose and maltose, invert sugar (glucose+fructose) and F/G (%).

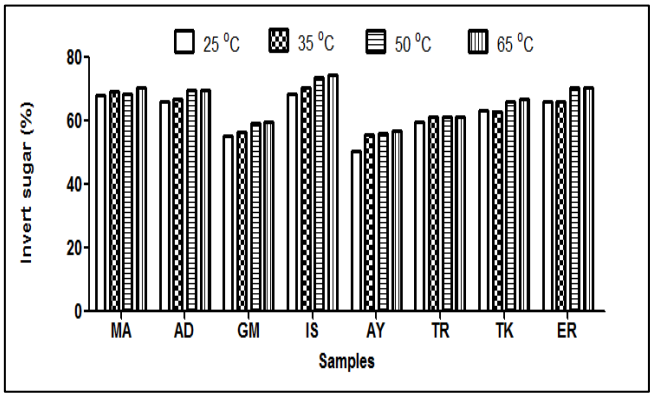

Figure 7. Variation of invert sugar of honey samples exposed different temperatures

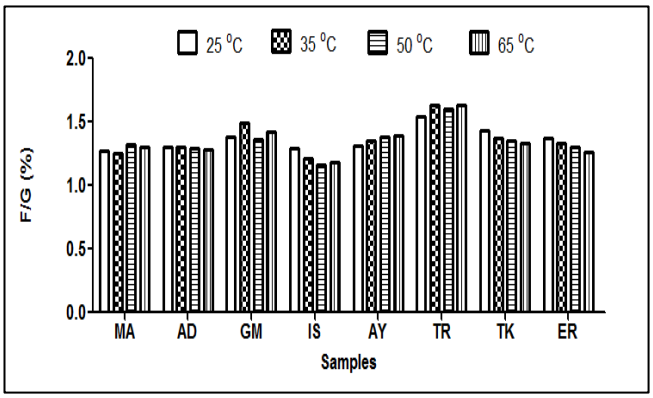

Figure 8. Variation of F/G of honey samples exposed different temperatures

It was determined that the percentage amount of fructose in honey samples taken from different locations was changed between 28.48% and 38.42% and on average 33.45% in the 25 ºC. When heated to temperatures of 65ºC (30 min), especially; Fructose ratios ranged from 34.06% to 40.60% with an average of 37.33% (Figure 5). It was determined that the glucose ratios in the honey samples were changed between 21.79% and 34.20%, on average 27.99% and the maltose ratios in the honeys ranged from 4.83% to 7.65%, on average 6.24%. It was determined that the glucose ratios in the wells kept at 65ºC (30 min) ranged from 23.15% to 34.20% and the average was 28.67%. Maltose ratios were found to vary between 4.86% and 7.78% with an average of 6.32% (Figure 4, 6). The lowest value in terms of fructose content was found at 25 ºC in Aydın (AY) pine honey sample while it was observed in Gumushane (GM), Trabzon (TR), Tekirdag (TK), Adiyaman (AD), Malatya (MA), Erzurum followed by respectively. The highest fructose value was found in Istanbul (IS) flower honey ($P<0.05$). The lowest statistically significant value in terms of glucose content was found at 25°C in Aydın (AY) honey. Trabzon (TR), Gumushane (GM), Tekirdag (TK), Erzurum (ER), Adiyaman (AD) and Istanbul (IS) were followed by honeys respectively. Malatya (MA) honey showed the highest glucose value ($P<0.05$).

The honey samples from the AY, GM and TR regions do not comply with the standards, while the other six honeys have a total of more than 60g/100 g of glucose and fructose content (invert sugar) (Figure 4-6). These values respect the requirements of the EU Directive Codex standard (2001).

Glucose, fructose and maltose are the major constituents of honey. In this study shows that the combined levels of these sugars varied from 50.27% to 74.44% and the average was 62.35%. 3 of samples were lower than the permitted levels, which they are AY, GM and TR honey samples (Figure 7). Our results have approximately similarity with the results of (Muhammet Furkan Yardibi & Tuncay Gumus, 2010; Yilmaz, H., & Yavuz, 1999; Yilmaz, H., & Kufrevioglu, 2000; Przybylowski, P., & Wilczynska, 2001; Turhan, I.; Tetik, N.; Karhan, M.; Gurel, F.; Tavukcuoglu, 2008; Merin, Bernstein, & Rosenthal, 1998; Sahinler, N.,Sahinler,S., and Gul, 2004; Sahinler & Gul, 1988).

The thermal degradation of sugars can occur via two different major reaction pathways: the Maillard reaction in the presence of amino acids and the caramelization by heating simple sugars at high temperatures. Caramelization is the common name for a group of reactions that occur when carbohydrates are exposed to high temperatures without amino groups. This reaction is influenced by pH and sugar concentrations (Eggleston G, 2000; Quintas M, Brandão TRS, 2007). These results show that the sugar composition of the honey was not affected even at high heat treatment (65ºC).

Fructose and glucose values of the honey depend on the source of the nectar. The sum of these carbohydrates must be a minimum of 60% and Fructose⁄glucose (F⁄G) ratio should be between 0.90 and 1.40. F⁄G is a good indicator for explaining the structure and crystallisation ratio of honey. These criteria very important for determination of honey quality. Fructose / glucose values of honey samples from different regions were given (Figure 8). According to this; MA has the lowest F/G value. This is followed by IS, AD, AY, ER, GM, TK and TR honeys.

The study showed that fructose / glucose ratios of honey samples taken from different regions ranged from 1.18% to 1.63%, while the average F/G ratio was 1.41%.

According to Turkey Food Codex, acceptable range for F/G ratio was 0.9-1.4, and only two samples were determined to have higher than F/G ratio the standard limit. The range determined for the fructose / glucose ratio in the Turkish Food Codex Honey Notification is 0.9-1.4 and it is seen that this ratio is 1,41 for Gumushane (GM) and

1,66 for Trabzon (TR). While the honey samples from these regions don't comply with the standards, the other six honeys used in the study are suitable. Furthermore, it was determined that the changes in F / G values due to temperature increase were not statistically significant after exposure to heat. Our results showed approximately similarity with the results of (Finola, M.S., Lasagno, M.C. & Marioli, 2007; Merin et al., 1998).

3.3. Effect of Heat Treatment on Total Phenolic Content

Table 2 shows the determined content of total phenolic compounds both thermally treated and untreated honey samples. The results were expressed as mg of gallic acid equivalent (GAE)/g of honey. The total phenolic contents of honey samples were between 0.054–0.216 mg GAE/g honey. The chestnut honey from the TR region exhibited the highest amount of TPC (0.215 mg GAE/g honey), while the sample from the AD regions contained the lowest (0.054 mg GAE/g honey). These results are consistent with the work of (Perna, A., Simonetti, A., Intaglietta, I., Sofo, A., Gambacorta, 2012), (Aygul, I., Karahalil, F.Y., Supuran, 2016) and (Kıvrak, Ş., Kıvrak, I., Karababa, 2017) who reported that chestnut honey possessed the highest polyphenol content. The differences in the values of the total phenolic content among the honey samples were probably because of diversity of flower sources. It is interesting to note that there are uneven changes in the total phenolic content among each honey samples after heating, i.e. in samples GM, IS and TK, the total phenolic content increased after heating, while in others it decreased. Heat treatment might be contributed to the increased extractability of phenolic compounds because of the disruption of honey samples during high treatment. On the other hand, some phenolic compounds could most likely be degraded by heat treatment. There are several reports about increases or decreases of total phenolic content after thermal processing. Our results are consistent with the work of (Saric, G., Markovic, K., Vukucevic, D., Lez, E., Hruskar, M., Vahcic, 2013). Analysis of variance results implied that a significant ($P<0.05$) variability among the different types of honey. However, heat treatment had a significant ($P<0.05$) effect on TPC for only IS sample. IS sample displayed significant ($P<0.05$) difference when it was heated at 35°C, 50°C and 65°C for 30 min, resulting in increased TPC values compared with the unheated sample. From the results obtained, we commented that level of phenolic compounds of honey extracts did not greatly affected from heat-treatment in this study. In addition, the results indicate that the phenolic compounds in honey samples differ depending on the species. For this reason, we can say that effective processing steps may be different for the liberate of phenolic compounds from various honey samples.

Table 2. The total phenolic content of honey samples exposed to different temperatures

Sample	25 ºC	35 ºC	50 ºC	65 ºC
MA	0.084 ± 0.007^{f}	0.074 ± 0.002^{f}	0.095 ± 0.003^{f}	0.091 ± 0.002^{f}
AD	0.054 ± 0.004^{g}	0.063 ± 0.009^{g}	0.057 ± 0.001^{g}	0.063 ± 0.009^{g}
GM	0.153 ± 0.03^{b}	0.160 ± 0.01^{b}	0.158 ± 0.007^{b}	0.164 ± 0.008^{b}
IS	$0.081\pm0.006^{f,A}$	$0.122\pm0.003^{c,B}$	$0.136\pm0.007^{c,C}$	$0.135\pm0.01^{c,D}$
AY	0.102 ± 0.008^{e}	0.086 ± 0.01^{e}	0.106 ± 0.01^{e}	0.098 ± 0.01^{e}
TR	0.215 ± 0.03^{a}	0.215 ± 0.02^{a}	0.211 ± 0.01^{a}	0.216 ± 0.01^{a}
TK	0.112 ± 0.01^{d}	0.107 ± 0.01^{d}	0.122 ± 0.01^{d}	0.127 ± 0.001^{d}
ER	0.138 ± 0.01^{c}	0.128 ± 0.004^{c}	0.134 ± 0.002^{c}	0.136 ± 0.004^{c}

Data are presented as the mean ± standard deviation from three independent experiments (n=3). Within rows and columns, means followed by the same letters are significantly different at $P < 0.05$

3.4. Effect of Heat Treatment on Antioxidant Activity of Honey Samples

According to the chemical composition, honey is a natural product that contains natural antioxidants. Many plants synthesize phytochemical substances with antioxidant activity, which are very important for the defence of the human body against radicals.

The effects of heat treatment on the antioxidant activity of honey samples determined using the DPPH and ABTS radical scavenging assays are shown in Tables 3. The radical scavenging activities of the honey samples were expressed as mg Trolox equivalents (TE) per g of honey sample. The results of DPPH radical scavenging activity showed that the honey samples from TR exhibited a significantly higher antioxidant activity in comparison to other samples at the same temperature, followed by GM and IS honey samples. On the other hand, the lowest radical scavenging activity was reported for honey sample from AD. In the ABTS reaction system, radical scavenging activity of honeys was much higher than in the DPPH and varied between 4.782 and 46.367 mg trolox/g honey in the 25^{o}C (Table 3).

The lowest radical scavenging activity was determined for sample AD, and the highest one for sample TR. The statistically significant difference ($P<0.05$) was observed in honey when DPPH and ABTS assay was used. The radical scavenging activity of honey samples is completely consistent with their total phenolic content. Overall, from our results we can infer that the total phenolic content highly correlated with the antioxidant activity. Nevertheless, the obtained results showed that there was no correlation with the antioxidant activities of the samples depend on increasing of temperature. The heat treatment can influence the antioxidant activity when published articles are examined. The changes in the antioxidant activity generally depend on chemical composition of the investigated sample and temperature and duration of heat treatment (Saric, G., Markovic, K., Vukucevic, D., Lez, E., Hruskar, M., Vahcic, 2013). Most likely the antioxidant activity didn't change because the temperature of heat treatment procedure was relatively low. Moreover, present research revealed that the short time heat treatment did not affect significantly total phenolic content and antioxidant activity.

3.5. Correlation Between Total Phenolic Contents, Antioxidant Activity and Total Sugar

We plotted the values for the total phenolic contents of honeys against their antioxidant power to determine the correlation between total phenolic content and antioxidant power, as shown in Table 4. We also determined whether the total phenolic content in the samples was related to the total amount of sugars measured or not. As seen in Table 4, the total phenolic contents of honey samples exhibited a good correlation with antioxidant properties for each temperature. We found that the correlation between TPC/DPPH and TPC/ABTS are above 0.9507 and 0.9698, respectively, in 35, 50 and 65 ^{o}C. Furthermore, we found highly significant the relation between DPPH and ABTS radical scavenging activities for each temperature (R^2=0.9463-0.9832). These results prove the accuracy of our measurement method. The strongest correlations between two methods of measuring antioxidant capacity and the total phenolic content indicated that antioxidant activities in honey samples are directly proportional to the concentration of total phenolic. These results are supported by (Küçük, M., Baek, Y., Kim, Y.J., Baik, M.Y., Kim, D.O., Lee, 2015; Herken, E.N., Erel, O., Guzel, S., Celik, H., Ibanoglu, 2010 and Sant'Ana, L.D, Ferreira, A.B.B., Lorenzon, M.C.A., Berbara, R.L.L, Castro, 2014), who obtained significant correlation. In addition, the total phenolic content of honey samples is not affected from their total sugar amount (R^2=0.2889-0.5098).

4. CONCLUSIONS

All over the world there is a growing demand for food safety or control of production and feeding with natural foods. Honey is the most commonly produced natural food. However, some of the product processing techniques at the stages of honey consumption (harvest, filtration, storage, filling and packing) can lead to some problems especially regarding healthy consumption and quality of honey. The chemical composition and variability (especially the 5-HMF, antioxidant activity, phenolic compounds) must be analyzed and revealed in order to know the contents of these marketed products.

Table 3. Changes in antioxidant activities of honey samples

Sample	DPPH				ABTS			
	25ºC	35 ºC	50 ºC	65 ºC	25 ºC	35 ºC	50 ºC	65 ºC
MA	0.013±0.001^{d}	0.012±0.001^{d}	0.013±0.001^{e}	0.014±0.001^{d}	4.782±0.09^{f}	4.405±0.48^{e}	5.064±1.54^{d}	4.876±0.19^{e}
AD	0.005±0.001^{e}	0.008±0.001^{e}	0.010±0.001^{f}	0.010±0.001^{e}	ND	ND	ND	ND
GM	0.030±0.002^{b}	0.032±0.004^{b}	0.031±0.001^{b}	0.033±0.002^{b}	34.419±1.54^{b}	30.749±1.25^{b}	33.384±3.76^{b}	31.784±5.98^{b}
IS	0.021±0.002^{c}	0.021±0.003^{c}	0.023±0.002^{c}	0.022±0.002^{c}	22.470±0.86^{c}	18.989±0.38^{c}	20.494±0.57^{c}	22.564±1.15^{c}
AY	0.013±0.001^{d}	0.014±0.002^{d}	0.017±0.001^{d}	0.014±0.001^{d}	10.615±1.06^{e}	9.486±3.57^{d}	13.155±0.19^{c}	13.061±0.28^{d}
TR	0.037±0.001^{a}	0.040±0.002^{a}	0.038±0.001^{a}	0.043±0.001^{a}	46.367±0.28^{a}	49.190±1.06^{a}	48.908±0.19^{a}	47.402±1.83^{a}
TK	0.015±0.003^{d}	0.018±0.001^{c}	0.020±0.002^{c}	0.020±0.001^{c}	15.319±0.28^{d}	16.260±0.48^{c}	15.884±0.57^{c}	16.542±0.96^{d}
ER	0.019±0.002^{c}	0.019±0.002^{c}	0.021±0.001^{c}	0.021±0.001^{c}	15.978±0.67^{d}	16.354±0.09^{c}	17.013±0.28^{c}	14.849±0.96^{d}

Radical Scavenging Activities (DPPH and ABTS) were given as mg Trolox equivalent/g honey. Data are presented as the mean ± standard deviation from three independent experiments (n=3). Means with different letters within a column are significantly different ($p<0.05$). ND=Not Determined.

Table 4. Correlation among total phenolic content, antioxidant capacity and total sugar

Temperature (ºC)	Correlation coefficient (R^2)			
	TPC/DPPH	TPC/ABTS	DPPH/ABTS	TPC/Total Sugar
25	0.8170	0.7367	0.9762	0.4037
35	0.9718	0.9776	0.9802	0.2889
50	0.9507	0.9698	0.9832	0.4566
65	0.9625	0.9428	0.9463	0.5098

We determined content of total phenolic compounds, antioxidant activity, total sugar and HMF of both thermally treated and untreated honey samples collected from eight different city of four different localities in Anatolia namely of Turkey.

The results of this study indicated that honey samples that are available commercially can differ in quality on account of various factors like seasons, packaging, heat treatment and processing conditions, floral source, geographical origin, and storage period. It is important that the essential precautions should be taken to ensure standardisation and rationalisation of beekeeping techniques, manufacturing procedures and storing processes to improve honey quality.

In conclusion, we found that the correlation coefficients between TPC/DPPH and TPC/ABTS are above 0.9507 and 0.9698, respectively, in 35, 50 and 65^{o}C. Furthermore, we found highly significant the relation between DPPH and ABTS radical scavenging activities for each temperature. The radical scavenging activity of honey samples is completely consistent with their total phenolic content.

Overall, from our results we can infer that the total phenolic content highly correlated with the antioxidant activity. Moreover, present research revealed that the short time heat treatment didn't affect significantly total penolic content and antioxidant activity. Some samples displayed significant ($P < 0.05$) difference when they were heated at 35°C, 50°C and 65°C for 30 min, resulting in increased HMF, TPC and radical scavenging activity values compared with the unheated samples.

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