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Diastase Enzyme activity and Hydroxymethylfurfural production during thermal processing of honey

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Abstract

The thermal treatment of honey is used to prevent honey crystallization which is one of the main problems that face beekeepers. In this study three samples of honey were heated at 40, 60, 80 and 100 °C, as a function of time in hours (h). Heating the samples at 40 °C did not show any significant effect on hydroxymethylfurfural (HMF) production or diastase enzyme activity up to 95 hours. Heating to higher temperatures 60, 80 and 100 °C resulted in a regular increase in HMF content and drop-off in diastase activity as a function of time. The HMF content reached 40 mg/kg after 80, 12.5 and 6 hours. For the three samples at 60, 80 and 100 °C respectively. Conversely, diastase enzyme activity reached close to 8 IU after 96, 12.5 and 5 hours. For the following temperatures 60, 80 and 100 °C respectively. The results showed that heating temperatures up to 40 °C is safe for long-term storage, while heating at 60 °C could be used but for a shorter treatment time. Results showed that the must temperature does not exceed 60 °C for 5 hours or 80 °C for one hour to preserve the honey's quality. The reaction rate constants and activation energy Ea of HMF formation in three samples were found 83.07, 91.79 and 89.57 kJ.mol-1 respectively. Therefore, honey can be preserved in this way, while at the same time the HMF remains below the permissible values, and the enzymatic activity remains at its highest level.

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Introduction

According to the Codex Alimentarius Commission and Harmonised methods of the International Honey Commission (IHC), honey is a natural sweet nutrient collected by honeybees from plants or from the secretions of living parts of plants. After collecting, honeybees dehydrate and store the honey until it becomes ripe (Bogdanov *et al.*, 2002). The type of honey depends on the sources that the bee collected the honeydew and nectars from. Honey contains many substances, carbohydrates between 70-83% the predominant sugars are glucose and fructose which represent more than 70% of the total carbohydrate content, and moisture content between 14-23%. Additionally, polyphenols, flavonoids and organic acids are also found. Proteins and some enzymes produced by the hypo-pharyngeal glands of bees such as diastase which degrades polysaccharides such as starch to simpler sugars, and invertase, which hydrolyze the disaccharide sucrose to monosaccharides glucose and fructose *(*Kek *et al.*, 2016; Simpson *et al.*, 1968). Glucose oxidase which oxidizes glucose to gluconic acid is among honey bee enzymes (Uddin *et al.*, 2022). Other compounds that exist in smaller ratios are amino acids, vitamin C and some B vitamins and metals representing 0.001- 0.1% of the total honey composition (Bogdanov *et al.*, 1997).

Honey has biological activity as anti-inflammatory, antifungal, antibacterial and antioxidant (Ilia *et al.,* 2021), as a result of this, it is used to treat several injuries and diseases (Cianciosi *et al.,* 2018; Bogdanov *et al.,* 2008). The criteria for honey quality as stated by the international standards for honey are acidity, pH, electric conductivity, water content, ash content, sugars (monosaccharide fructose, glucose and disaccharide sucrose), and activity of diastase and proline amino acid (Bogdanov *et al.,* 1999; White, 1994). The quality of honey is a reflection of the plants that the bee feeds on, the geographical area, its topography and the climate. Many honey types crystallize during storage, which is sometimes unacceptable to consumers. Many methods are therefore employed to resolve this issue; the simplest is heating honey to high temperatures. Other methods include using trehalose to prevent honey crystallization (Amariei *et al.,* 2020), ultrasonic process (Scripcă and Amariei, 2021) and the addition of some chemicals to prevent crystallization, such as isobutyric acid and sorbic acid. However, heat treatment is the most common approach due to its effectiveness and simplicity in stopping or delaying crystallization and facilitates the filling process by lowering viscosity (Bath and Singh, 1999; Tosi *et al.,* 2002).

The heat processing leads to the formation of some undesired byproducts such as hydroxyl methylfurfurals (HMF). HMF is formed as a result of the Maillard reaction. The Maillard reaction is a series of chemical reactions that occur when food is heated or stored at room temperature for an extended period (Caballero *et al.,* 2015; Nagai *et al.,* 2018). whereby heating honey that contains sugar and amino acids causes a change in texture, taste, and color during the manufacture and storage of honey (Blidi *et al.,* 2017). Similarly, the heating procedure has an impact on the diastase activity over time because the enzyme activity declines depending on the storage temperature over time (Schade *et al.,* 1958; Tosi *et al.,* 2008).

The goal of the study was to determine the lowest temperature that could be used to prevent honey from crystallizing and to determine the optimum temperature that honey can tolerate and achieve without compromising honey quality, this study examined the effects of different temperatures as a function of time on honey by monitoring HMF production and diastase activity.

Materials and methods

Three different multi-floral honey samples from different geographical and botanical origins in the Kurdistan region, all chemicals and reagents came from Chem-Lab from Belgian and met analytical requirements. For experiments requiring the determination of UV-visible absorbance, Thermo Fisher's EMC-61PC UV-spectrophotometer (Germany) was utilized along with Thermo Fisher HPLC with a Vanquish detector and an ERC RefrectoMax 520 for measuring refractive index with assistance from Chromeleon software used (Germany).

Initial quality evaluation of honey samples

Three separate samples of honey, each coming from a different region, were obtained in the north of Iraq. Several biochemical parameters were evaluated for three samples including pH, acidity (meq/kg), conductivity, total and individual sugars including (sucrose, glucose, and fructose), in order to detect and evaluate the quality of honey samples (Bogdanov *et al.,* 1999).

Hydroxymethylfurfural (HMF)

HMF contents were assessed according to the method by White and Bogdanov (Bogdanov *et al.,* 1997; White, 1979), Accordingly, 5 g of honey was dissolved in roughly 30 mL of water to create the solution, which was then mixed with 0.5 ml from each of the Carrez I and II solutions, and then the volume was completed to 50 ml. Preparation of the reference solution, which was made by mixing 5 ml of matabisulfite ($Na_2S_2O_5$) (0.02N) with 5 ml of water-in a test tube, then the 5 ml sample solution was added to 5 ml of water, and the absorbance of the sample was assessed at 284 and 336 nm to the reference solution's blank readings. The following equation was used to determine HMF:

HMF in mg/kg = (A284 - A336) x 149.7 x 5 x D/W ………………… (2)

Where, W is the weight of the sample and D dilution factor. 149.7 constant (White, 1979).

Diastase activity

A stock solution for the Phadebas method was prepared by dissolving 1g of honey in 100 ml of acetate buffer (pH 5.2). After equilibrating in a water bath at 40 $^{\circ}$C for 5 min, a Phadebas tablet was dissolved in 5 ml of this solution. After 30 minutes, the reaction was terminated by adding 1 mL of 2% NaOH. Within 2 minutes, the solution was filtered, and the absorbance was measured at 620 nm against a blank (Bogdanov *et al.,* 1999; Oddo and Pulcini, 1999). Diastase activity was calculated by using equation (3).

Diastase activity (IU) = (28.2*(Abs-0.008)) + 2.64……………….. (3)

Results and Discussion

Three different multi-floral honey samples from different geographical and botanical origins in the Kurdistan region-Iraq were used in this research.

Iraqi Kurdistan region produces more than 5000 tons of honey per year and depends on the environment and weather. The predominant honey type in the region is multi floral and highly valued for its quality and nutritional value(Mohammad *et al.,* 2023). Unfortunately, this high-value product usually crystallizes during the storage period before marketing, especially for beekeepers that produce massive quantities of honey, resulting in decreased demand due to the change in its appearance. Customer preferences can be influenced by secondary physical characteristics such as taste, appearance, aroma, flavor, and color.

The qualities of honey are certainly reflected in its chemical and physical components, among these properties are acidity, pH, moisture, 5-Hydroxy methyl furfural (HMF), total sugars content, and the diastase enzyme activity(El Sohaimy *et al.,* 2015). These biochemical properties are due to the aforementioned sources in nature (Jandric *et al.,* 2015). One of the biggest marketing problems that beekeepers and honey manufacturers suffer from is honey crystallization. The tendency of honey to crystallize is a consequence of several biochemical variables, including the kind and source of the honey, the amount and type of the sugar (most honey types exhibit crystallization based on the ratio fructose/glucose), acidity, texture, remaining wax or pollen, or even the temperature and humidity during storage (Amariei *et al.,* 2020; Ghorab *et al., 2021*; Schade *et al.,* 1958).

This research explored the effects of different temperatures (40, 60, 80 and 100 $^{\circ}$C) as a function of time (1- 95 hr.) However, the samples need to be well-thought-out when one of the HMF parameters exceeds 40 mg/kg or the diastase activity falls below 8 IU, Therefore, according to the International Honey Commission (IHC), the end point is when the HMF reaches 40 mg/kg or diastase activity reaches less than 8 IU the thermal process of honey must be finished when HMF and diastase reach these values standards (Bogdanov *et al.,* 1997). HMF is produced from fructose, glucose and other monosaccharides through the Maillard reaction, this compound has significant negative effects on human health due to its carcinogenic effect and toxicity (Shapla *et al.,* 2018).

The observed data revealed that the diastase activity for the three samples was between 24-41IU/kg (Table 1), which indicates the honey samples are fresh, unheated or unprocessed, and their quality is acceptable. The HMF values of the honey samples were 5, 11 and 15 mg/kg, which implies that the honeys are fresh and not temperature treated. These HMF levels are less than the Iraqi standard 1982 with its Amendments 2020 limit and IHC (40 mg/kg). The conductive index ranged from 0.215 to 0.456 ms/m, and the mean value of acidity of the samples was 4.3meq/kg. While the mean levels of water, sucrose, glucose, and fructose were 13.4, 3.26, 25.98, and 39.08 %, respectively, as shown in (figure 1).

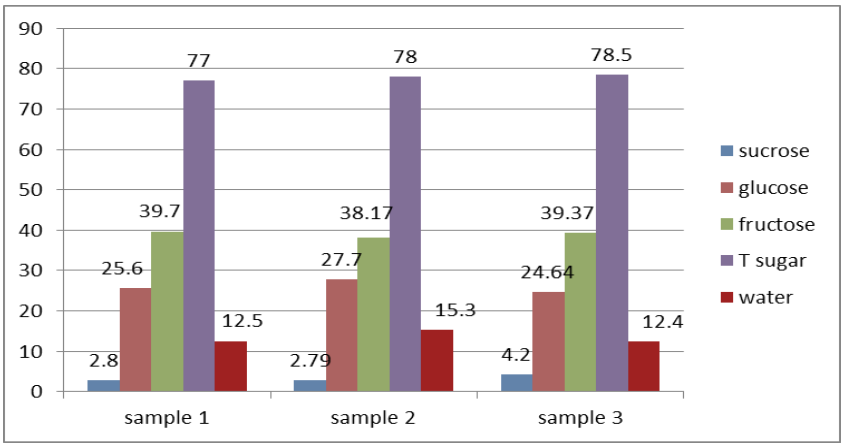

Figure 1. The percentage of sucrose, glucose, fructose, total sugars and water

The above mentioned parameters are within the normal values and are consistent with the international values for honey (Bogdanov *et al.*, 1999; Cozzolino *et al.*, 2011; Mohammad *et al.*, 2023; Thrasyvoulou, 1986).

Table 1. The biochemical parameters of Honey treatments

Sample no.	HMF mg/kg	acid meq/kg	water g%	total sugar g%	conductance mS/m	Diastase activity IU/kg	sucrose g%	glucose g%	fructose g%
1	11.0	18	12.5	77.0	0.456	41.0	2.8	25.6	39.7
2	13.0	11	15.3	78.0	0.241	23.6	2.8	27.7	38.2
3	5.0	14	12.4	78.5	0.420	31.8	4.2	24.6	39.4
max	13.0	18	15.3	78.5	0.456	41.0	4.2	27.7	39.7
min	5.0	11	12.4	77.0	0.241	23.6	2.8	24.6	38.2
average	9.7	14	13.4	77.8	0.372	32.2	3.3	26.0	39.1

Most beekeepers, as well as factories that produce honey, work to heat the honey randomly to avoid crystallization. The viscosity of honey is reduced when it is heated during processing, which may prevent fermentation or crystallization.

Thus, honey samples were heated during preset times of 1-95hr. at 40C. After withdrawal from the heating, the samples were immediately cooled in order to stop more heat effects.

The generation of HMF in honey at temperature 40 did not change over the course of 95 hours, but a sample's concentration grew by 1 mg/kg, and the activity of the diastase enzyme did not alter at this temperature (figure 2) this result close to the results reported by Esciche (Escriche et al., 2008) but disagree with Khan’s work

(Khan *et al.,* 2015). While, at the temperatures 60, 80 and 100 °C, it was found that HMF gradually increases to above 40 mg/kg at the 96th, 14th, and 6th h. respectively as shown in (Figure 2) these results agree with those of Tosi et al. (2002). The rate constant at different temperatures and activation energies for the formation of HMF measured for the three samples are (83.07, 91.79 and 89.57 kJ.mol-1) respectively; see Table 2.

Table 2. The HMF formation rate constant (k_o) at different temperatures (T), activation Energy (Ea) and correlation coefficients (R^2)

samples	T (k)	K_o ($hr.^{-1}$)	R^2	Ea ($kJ.mole^{-1}$)
1	313	0.04	0.27	83.07
	333	0.41	0.98	
	353	2.50	0.87	
	373	6.43	0.89	
2	313	0.03	0.13	91.79
	333	0.44	0.99	
	353	3.73	0.91	
	373	7.02	0.95	
3	313	0.03	0.11	89.57
	333	0.27	1.00	
	353	2.59	0.82	
	373	5.74	0.93	

These results line up with numerous studies on HMF formation in honey, but when compared to the foods or fruits which need higher activation energy to form HMF, this slows down the formation of HMF in some fruits for a period. This is because honey contains the ingredients needed for HMF production, according to the maillard reaction (Aslanova *et al.,* 2010; Nagai *et al.,* 2018).

Meanwhile, dastase activity was investigated in relation to temperature and time. It discovered diastase activity stayed stable at 40 °C, while at 60, 80 and 100 °C were approached to less than 8 IU at the following times 95, 13 and 6 hr. respectively. A great deal of research has been conducted on various types of honey and from various geographical regions, and the same investigation discovered that enzymatic activity decreases with an increase in temperature above 50°C (figure 3).

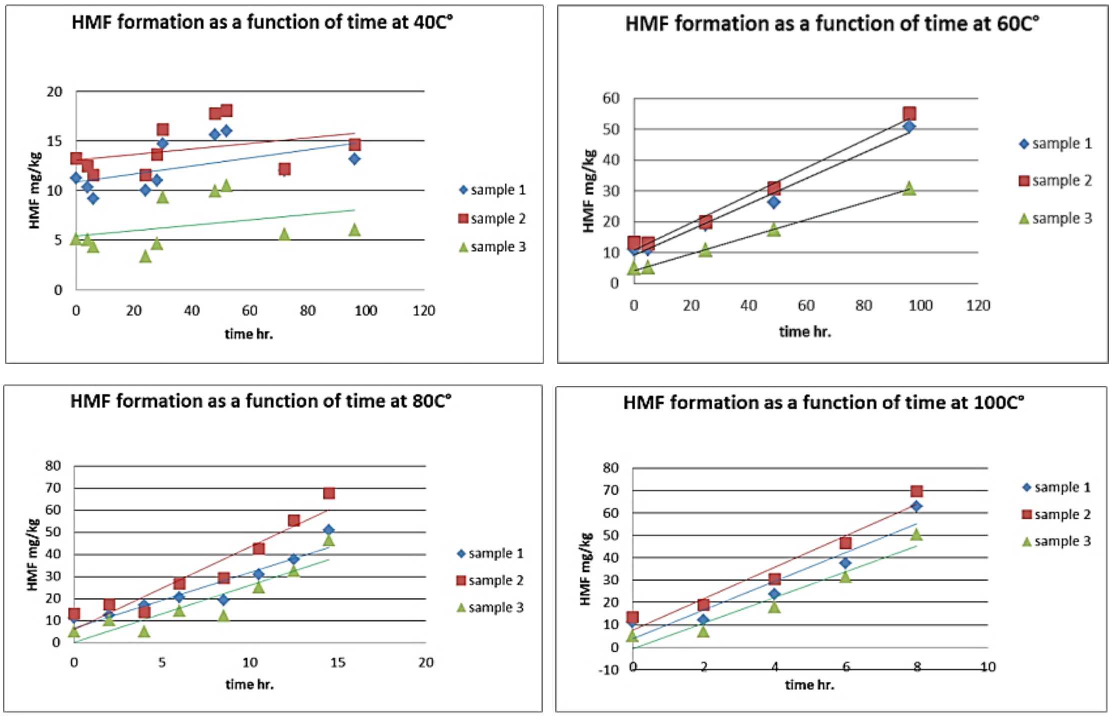

Figure 2. HMF formation as a function of time at 40, 60, 80 and 100°C

The results obtained were very close to other studies on heating honey for specific periods (Huang *et al.*, 2019; Wesołowska and Dżugan, 2017). This process leads to changing chemical properties through increasing acidity from converting glucose to gloconic acid then the formation of HMF and decreasing diastase activity by heating (which is one of the most important properties of honey) and affect the quality of honey (Cozzolino *et al.*, 2011; Dżugan *et al.*, 2020; Thrasyvoulou, 1986).

When temperatures rise above a certain point, activated enzymes become inactive or decrease in activity because they are so sensitive to heat (Tosi *et al.*, 2002). Unfortunately, the high temperatures used to prevent honey crystallization will cause a significant percentage of the active enzyme ingredients to become inactive. Therefore, it is important to investigate how different heating scenarios affect the enzyme activity in honey (Escuredo *et al.*, 2014). Thus, the diastase index is negatively affected by increasing temperature and changes in pH through gluconic acid formation (Blidi *et al.*, 2017; Villacres-Granda *et al.*, 2021). There are numerous methods to prevent honey from crystallizing for more information, microwaves (MW) are used to kill yeast cells through a thermal process (Bucekova *et al.*, 2018). Crystallization is prevented by chemical compounds such as isobutyric and sorbic acid, as well as other chemical additives (Amariei *et al.*, 2020), and some investigations used ultrasonic devices to achieve the same goal (Scripcă and Amariei, 2021). But the easiest and best method is the thermal process, provided the heat does not reach the destruction of the components of honey and the preservation of honey to the least extent possible at HMF levels.

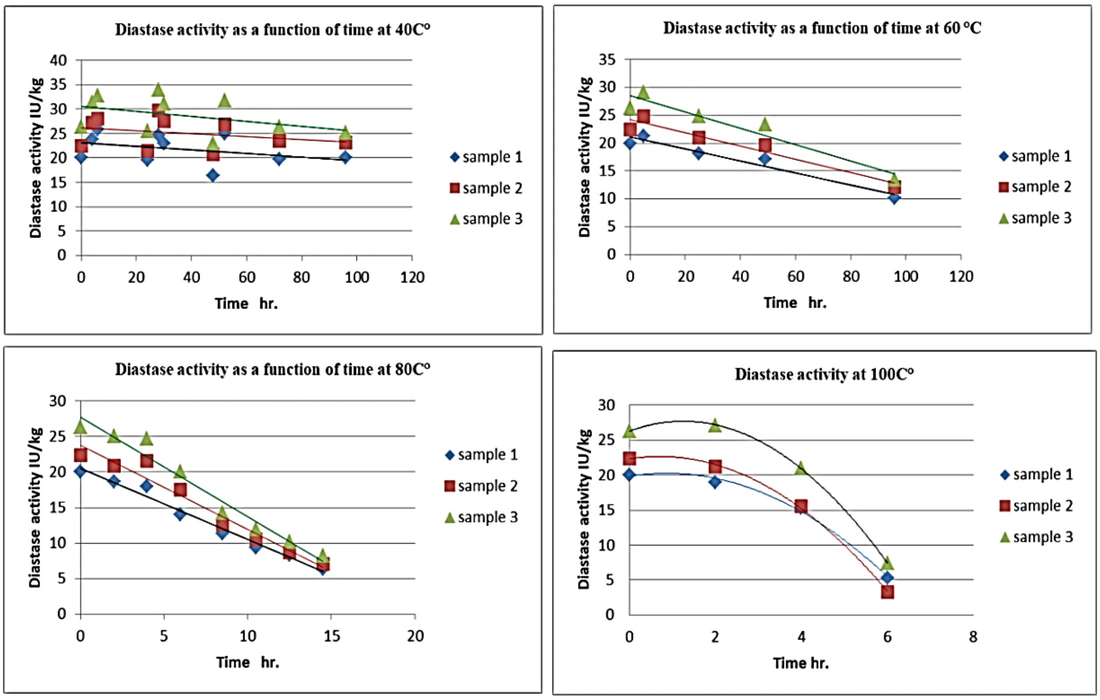

Figure 3. Diastase activity as a function of time at 40, 60, 80 and 100 °C

Conclusion

The best process is used at 40 °C which does not affect honey for a period of time, therefore it could be used as storage temperature, the maximum allowable temperature is between 60-65 C to pasteurize honey and at the same time prevent its crystallization, provided that it is not heated for longer than 30 minutes, because it affects and destroys other beneficial substances in honey. Meanwhile, the usage of 80 and 100 °C is not recommended this high temperature affect honey components and quality as well as HMF and diastase activity, even if used for a short-time.

Conflict of interest

Regarding the publication of this manuscript, the authors declare that there are no conflicts of interest.

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