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Risk in some honey-based instant bee product mixtures

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Abstract

Lemon juice concentrate is one of the components included in the product recipe in addition to fractions such as propolis, royal jelly, pollen, bee bread, ginger, cinnamon, cloves, black pepper, ginkgo, and ginseng in the commercial sale of honey-based beekeeping products in Turkey. The present work aimed to examine the changes in some physicochemical properties of honey by adding lemon juice concentrate at different ratios (0, 1, 2, and 3%) to pine, flower, and chestnut honey during six-month storage period. Changes in the total phenolic content, antioxidant activity, and HMF levels of the samples stored at 25, 35, and 45°C were monitored. The degree of reaction, reaction rate constants, and Arrhenius coefficients were also determined by examining the HMF formation kinetics. HMF levels at the end of 24 months, the general shelf life of commercial mixtures, were calculated from the modelled kinetic data. Brix (78.8 - 82.0°), pH (3.00 - 4.46), free acidity (8.5 - 72.3 meq kg^{-1}), reducing sugar (87.5 - 105.1%), total sugar (93.7 - 111.1%), sucrose (4.0 - 7.7 g/100 g), total phenolic content (4.1 - 218.7 mg GAE/100 g), antioxidant activity (1.0 - 16.1 mg GAE/100 g), and HMF (12.4 - 7646.5 mg kg^{-1}) levels of the samples were measured. Adding lemon juice concentrate dramatically increased HMF levels. The estimated HMF levels of the samples at the end of 24 months were between 49.7 - 30038.7 mg kg^{-1}. The threshold energies of the HMF formation reactions were 126.2 - 219.2 kJ mol^{-1}.

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Introduction

According to Codex Alimentarius and Turkish Food Codex, honey is defined as “the natural product that can be crystallised by nature, which are the nectars of plants, the secretions of the living parts of plants, or the secretions of the plant-sucking insects living on the living parts of plants, are collected by the honey bee, and modified by combining them with their specific substances, lowering the water content, and maturing by storing them in the honeycomb”. Based on the nectar collected by bees, honey can be divided into two– flower honey and secretion honey. According to the Turkish Standards Institute, flower honey is defined as "honey made by bees from the nectars of plant flowers", and secretion honey is defined as "honey obtained from the secretions of living parts of plants or from the secretions of plant-sucking insects (Hemiptera) living on living parts of plants". Examples of flower honey include linden, clover, citrus, thyme, acacia, and chestnut, while pine, oak, and fir are secretion honey. Honey is widely consumed all over the world as a nutritious natural sweetener and nutraceutical. It contains glucose, fructose, organic acids, lactones, amino acids, minerals, vitamins, enzymes, pollen, wax, and pigments that have positive effects on health such as antimicrobial, antioxidant, and anti-inflammatory activities (Misirlioglu *et al*., 2003; Fallico *et al*., 2004; TSE, 2010; TGK, 2020; Mulugeta and Belay, 2022; Yan *et al*., 2022).

Lemon (*Citrus limonia* L.), a member of the Rutaceae family, is an important citrus variety. It is usually consumed directly, but in the form of sour lemon juice concentrate (LJC), it is also used as an additive in jams, salad dressings, cakes, or cookies. This concentrated food product, produced by the evaporation of lemon juice, and is sour, is produced without adding any sugar or other additives. It has been reported that it is effective in lipid metabolism,

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reducing oxidative damage, and preventing different types of cancer, cardiovascular diseases, and obesity, thanks to its vitamin C, minerals, dietary fibres, essential oils, organic acids, carotenoids, and flavonoids. In addition, flavanone, hesperidin, and eriocytrin are known to be the most important characteristic components of lemon juice (Abeysinghe *et al*., 2007; Gattuso *et al*., 2007; Adibelli *et al*., 2009; Bermejo *et al*., 2011; Ucan *et al*., 2016; Çavdir *et al*., 2020).

The use of nutraceuticals, which are known to have positive effects on health, and/or their addition as ingredients in foods, has increased in popularity in recent years, especially amidst the global COVID-19 pandemic. In this context, the marketing of bee products, including honey, propolis, royal jelly, pollen, and bee bread, alongside herbal mixtures such as ginger, cinnamon, cloves, black pepper, ginkgo, ginseng, and LJC, with the promise of enhanced health, elicit a significant response from society. The incorporation of LJC into honey serves multiple purposes, namely augmenting its taste, nutritional value, and possible health advantages. LJC may also serve as a flavour enhancer in drinks, sauces, dressings, and marinades. It serves as a natural preservative in fruit salads and sauces due to its elevated acidity and antioxidant properties. Its acidity activates baking powder, hence aiding in the leavening of baked goods. It is utilised in marinades and glazes for meat and seafood to enhance their taste and softness.

HMF (hydroxymethylfurfural) level is an important criterion used to evaluate the quality of honey. It is not usually found in fresh honey, and increases during heat treatment/storage. The level of HMF in honey, which is formed as a result of hexose dehydration and Maillard reaction, should not exceed 40 mg/kg as suggested by the World Health Organization and European Union Codex Alimentarius. HMF is readily absorbed in the gastrointestinal tract, and may degrade into a genotoxic compound, which cannot be eliminated from the body, called 5-sulfoxymethylfurfural. However, harmful effects of HMF such as mutagenicity, genotoxicity, organotoxicity, and enzyme inhibition are also known (Shapla *et al*., 2018).

The HMF formation in honey is markedly affected by several circumstances, including the incorporation of acidic agents like lemon juice. HMF is a chemical compound generated by the Maillard process, which occurs when reducing sugars are exposed to heat and acidic conditions. Acids, including those in lemon juice, can accelerate sugar breakdown, resulting in elevated HMF levels in honey (Shapla *et al*., 2018; Yang *et al*., 2019).

Studies have demonstrated that the acidity from lemon juice might provide favourable conditions for HMF production. Frizzera *et al*. (2020) revealed that acidified sugar solutions can result in markedly elevated amounts of HMF when exposed to extended heating, especially at low pH values. Yang *et al*. (2019) validated that acidic circumstances facilitate the dehydration of hexoses, a precursor to HMF. The temperature and length of heating are crucial parameters that intensify HMF generation in honey, as demonstrated by previous research (Vijayakumar *et al*., 2021; İçli, 2022).

The formation of HMF is a notable issue in several food systems beyond honey. It signifies heat-induced deterioration, and may affect food quality and safety. In baked products, HMF formation is affected by cooking parameters and the kind of sugar utilised (Nguyen *et al*., 2016). Fried potatoes (Miao *et al*., 2014) and heated dairy products (Francisquini *et al*., 2019) also exhibit significant HMF formation.

The present work was aimed to examine the effect of using LJC mixed with honey on the formation of HMF. In this context, some physicochemical properties such as Brix, pH, free acidity, reducing sugar, total sugar, and sucrose content of different honey samples (pine, flower, and chestnut) were determined. In addition, LJC was added to honey samples at different ratios (0, 1, 2, and 3%), and stored for six months at three different temperatures (25, 35, and 45°C). HMF levels, antioxidant activities (AA), and total phenolic content (TPC) of the mixtures were monitored during storage. The degree of reaction, reaction rate constants, and Arrhenius coefficients were determined by examining the HMF formation kinetics. It was hypothesised that increasing LJC concentration and storage temperatures would significantly increase the formation of HMF in honey samples.

Materials and methods

The chemicals such as sodium hydroxide, DPPH (2,2-Diphenyl-1-picrylhydrazyl), potassium sodium tartrate, copper (II) sulphate, gallic acid, Folin-Ciocalteu reagent, Fehling's reagent, methylene blue, sucrose, Carrez I, and Carrez II used in the

analyses were obtained from Sigma or Merck. Honey samples were obtained from commercial honey producers in the Bursa region. Lemon juices purchased from the market were used in the experiments as LJC after the Brix was adjusted to 65 using the BUCHI/R-3 evaporator.

Preparation of honey samples

LJC was added to three different honey types (pine, flower, and chestnut) at four different ratios (0, 1, 2, and 3%, w/w). Since LJC is used as approximately 2% in the product recipe in commercial preparations, these ratios were used in the trial design. The honey mixture samples were stored at three different temperatures (25, 35, and 45°C) for six months.

Determination of Brix, pH, free acidity, and total sugar

Brix analysis was performed following the refractometric method. For this, the Kyoto KEM/RA-600 (Tokyo, Japan) brand refractometer device was used. The pH was determined after calibrating the OHAUS ST3100 (Parsippany, New Jersey, USA) pH meter with buffer solutions. The free acidity level was determined by the titrimetric method. Total sugar determination was performed following the Lane-Eynon method.

Determination of total phenolic content (TPC)

In brief, 0.25 mL of a diluted sample (10%, w/v) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent using a vortex mixer. The mixture was allowed to stand at room temperature for 4 min. Then, 2 mL of 7.5% (w/v) sodium carbonate was added and vortexed. The prepared mixture was then incubated for 2 h in the dark, and absorbance values were read using a spectrophotometer (Thermoscientific, Evolution 201, USA) at a wavelength of 760 nm. The standard curve was prepared using a gallic acid solution. The results were expressed as milligrams of gallic acid equivalents (GAE) per 100 grams of dry-weight honey samples (Guldas *et al*., 2022).

Determination of antioxidant activity (AA)

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of honey samples was measured according to Wesołowska and Dżugan (2017) with some modifications. After 0.4 mL of the diluted sample (10%, w/v) was taken into a 5 mL Falcon tube, 3.6 mL of DPPH was added and vortexed. The prepared mixture was left to incubate for 30 min, and then absorbance values were read using a spectrophotometer at a wavelength of 517 nm. The standard curve of gallic acid (0 - 20 mg L^{-1}) was prepared in the same manner. The results were expressed as milligrams GAE per 100 grams of dry-weight honey samples.

Determination of HMF

Approximately 5 g of honey samples were diluted to 50 mL with distilled water, and passed through a 0.45 µm syringe filter. The prepared sample vials were stored at -18°C until HMF analysis. HPLC analysis was performed according to Zappala *et al*. (2005), with some modifications. HMF analysis was performed using an HPLC (AGILENT-Infinity 1260, USA) device equipped with a C18 column (100 × 4.6 mm, 2.7 µm) and diode array detector. The flow rate, injection volume, column temperature, and wavelength were 0.6 mL/min, 5 µL, 40°C, and 285 nm, respectively. Ultrapure water with 1% acetic acid (A) and methanol (B) was used as the mobile phase. Isocratic elution using a mixture of 90% A and 10% B was performed. HMF standard solutions (1-5-10-25-50-100 mg L^{-1}) were used to obtain the calibration curve (R^2 = 0.9999). HMF levels in honey samples were determined by comparing with the calibration curve, and results were expressed in mg HMF kg^{-1} (on a dry basis).

Kinetic study of HMF formation

In the HMF formation kinetic modelling, the zero-order reaction kinetics were used because a higher coefficient of determination (R^2) and a more linear HMF formation with time were obtained compared to the first and second-order reactions. The kinetics of HMF formation in honey are commonly classified as zero-order (Grainger *et al*., 2017; Turkut *et al*., 2018; Yap and Chin, 2020), using Eq. 1:

$$[A]_t - [A]_0 = k\,t \qquad \text{(Eq. 1)}$$

where, $[A]_0$: initial concentration of HMF (mg kg^{-1}); k: reaction rate constant (mg kg^{-1} mth^{-1}); $[A]_t$: HMF concentration after t month storage at the temperature (mg kg^{-1}); and t: storage time of honey samples (mth).

The variation of the reaction rate constant with temperature is given by the Arrhenius equation. The coefficients of the equation were calculated using Eq. 2 (Oral *et al*., 2012):

$$k = \mathrm{A}\exp\left(-\frac{\mathrm{E}_a}{\mathrm{RT}}\right) \quad \text{(Eq. 2)}$$

where, A: pre-exponential (or frequency) factor (mth^{-1}); E_a: activation energy of the reaction (kJ mol^{-1}); R: Universal gas constant (0.008314 kJ mol^{-1} K^{-1}); and T: temperature (K).

Since the shelf life of the commercially available honey-based instant mixes is determined as two years, the predicted HMF values of the samples at the end of the 24th month were calculated based on the kinetic data.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using RStudio software version 2021.09.1. The analysis of variance (ANOVA) was used to analyse the data, and differences between means were evaluated with Duncan's new multiple range test at a 0.05 significance level (Table 1). Pearson's correlation analysis was used to correlate each dependent variable.

Table 1. Summary of ANOVA table *p*-values for each measurement.

Measurement	Factor						
	P1	**P2**	**P3**	**P1:P2**	**P1:P3**	**P2:P3**	**P1:P2:P3**
pH	< .001	< .001	-	< .001	-	-	-
Brix	< .001	< .001	-	< .001	-	-	-
Free acidity	< .001	< .001	-	< .001	-	-	-
Reducing sugar	< .001	< .001	-	< .001	-	-	-
Total sugar	< .001	< .001	-	< .001	-	-	-
Sucrose	< .001	< .001	-	< .001	-	-	-
HMF at 0 mth	< .001	< .001	1	< .001	1	1	1
HMF at 3 mth	< .001	< .001	< .001	< .001	< .001	< .001	< .001
HMF at 6 mth	< .001	< .001	< .001	< .001	< .001	< .001	< .001
TPC at 0 mth	< .001	< .001	1	< .001	1	1	1
TPC at 3 mth	< .001	< .001	< .001	< .001	< .001	< .001	< .001
TPC at 6 mth	< .001	< .001	< .001	< .001	< .001	< .001	< .001
AA at 0 mth	< .001	< .001	1	< .001	1	1	1
AA at 3 mth	< .001	< .001	< .001	< .001	< .001	< .001	< .001
AA at 6 mth	< .001	< .001	< .001	< .001	< .001	< .001	< .001

HMF: hydroxymethylfurfural; TPC: total phenolic content; AA: antioxidant activity; P1: type of honey; P2: lemon juice concentrate; and P3: temperature.

Results and discussion

Brix, pH, free acidity, and total sugar determination

The physicochemical properties of the honey samples are given in Table 2. When only the control groups of pine, flower, and chestnut honey were considered, the Brix values of pine and chestnut honey were higher than flower honey ($p < 0.05$). The Brix values of all honey samples were determined in the range of 78.83 - 82.00%. The highest Brix value was seen in pine honey in the control group, and the lowest Brix value was seen in flower honey with 3% LJC added. It was observed that as the proportion of LJC, which had a lower Brix value compared to honey samples, increased, the Brix values of the samples generally decreased. Anupama *et al*. (2003) reported the Brix values of 11 Indian floral honey to be between 76.00 and 81.50%. Water is the second most common ingredient in honey after sugars. The amount of water in the honey may vary depending on the region where the honeybees collect the nectar, the maturity level of the honey, the processing methods of the honey, and the storage conditions (Silva *et al*., 2016; Al-Farsi *et al*., 2018).

The average pH values of honey samples were found in the range of 3.00 - 4.46 (Table 2). In the control groups, pine honey had the highest pH value, while chestnut honey had the lowest pH value ($p < 0.05$). Among all samples, the lowest pH value was observed in chestnut honey to which 3% LJC was added. As expected, it can be said that the pH value of the honey samples decreased due to the increase in

Table 2. Some physicochemical properties of honey samples (on a dry basis).

Type of honey	Lemon juice concentrate (%)	Brix (%)	pH	Free acidity (meq kg^{-1})	Reducing sugar (%)	Total sugar (%)	Sucrose (%)
Pine	0	82.00 ± 0.00^{a}	4.46 ± 0.01^{a}	17.34 ± 0.22^{k}	90.46 ± 0.27^{f}	96.44 ± 0.31^{f}	5.68 ± 0.04^{e}
	1	81.17 ± 0.41^{d}	3.97 ± 0.01^{c}	32.06 ± 0.37^{h}	90.33 ± 0.23^{f}	95.71 ± 0.26^{g}	5.11 ± 0.03^{g}
	2	80.00 ± 0.00^{e}	3.69 ± 0.01^{d}	47.76 ± 0.81^{e}	91.53 ± 0.38^{e}	96.45 ± 0.42^{f}	4.68 ± 0.04^{i}
	3	80.00 ± 0.00^{e}	3.50 ± 0.01^{f}	65.44 ± 0.50^{b}	90.37 ± 0.27^{f}	94.66 ± 0.30^{h}	4.08 ± 0.02^{j}
Flower	0	80.00 ± 0.00^{e}	3.99 ± 0.01^{b}	8.48 ± 0.19^{l}	88.96 ± 0.22^{g}	97.05 ± 0.27^{e}	7.68 ± 0.04^{a}
	1	80.00 ± 0.00^{e}	3.35 ± 0.04^{g}	25.79 ± 0.58^{i}	88.93 ± 0.16^{g}	96.65 ± 0.19^{ef}	7.33 ± 0.03^{b}
	2	79.00 ± 0.00^{f}	3.12 ± 0.02^{j}	43.14 ± 0.79^{f}	88.24 ± 0.26^{h}	95.50 ± 0.30^{g}	6.90 ± 0.04^{c}
	3	78.83 ± 0.41^{f}	3.02 ± 0.01^{k}	59.51 ± 0.49^{c}	87.47 ± 0.14^{i}	93.72 ± 0.16^{i}	5.93 ± 0.02^{d}
Chestnut	0	81.85 ± 0.00^{ab}	3.61 ± 0.00^{e}	22.17 ± 0.37^{j}	105.08 ± 0.18^{a}	111.10 ± 0.40^{a}	5.72 ± 0.21^{e}
	1	81.50 ± 0.55^{bcd}	3.33 ± 0.01^{h}	38.39 ± 0.49^{g}	103.40 ± 0.35^{b}	109.05 ± 0.39^{b}	5.37 ± 0.04^{f}
	2	81.33 ± 0.52^{cd}	3.14 ± 0.01^{i}	56.87 ± 0.50^{d}	102.27 ± 0.27^{c}	107.73 ± 0.30^{c}	5.19 ± 0.03^{g}
	3	81.67 ± 0.52^{abc}	3.00 ± 0.01^{k}	72.26 ± 0.59^{a}	100.86 ± 0.22^{d}	106.00 ± 0.31^{d}	4.88 ± 0.17^{h}

Means in similar column with different lowercase superscripts are significantly different ($p < 0.05$).

the proportion of LJC, which had higher acidity than honey. A positive correlations of 0.93 and 0.76 were observed between pH level and Brix values in pine and flower honey, respectively.

The difference in pH values of different/same types of honey could be explained by the fact that the nectars collected by the bees may vary depending on the environment, organic acid amount, and mineral content of the nectars (Majewska *et al*., 2019). In a study conducted on some honey in Turkey, it was reported that the pH values of the honey samples were in the range of 3.19 - 4.39 (Yucel and Sultanoglu, 2013). They reported that the lowest pH value belonged to parsley flower honey, and the highest value belonged to Calluna honey. They reported the pH value as 3.58 in flower honey. In a study examining monofloral honey in Sicily, the pH value of chestnut honey was found to be 5.90 (Fallico *et al*., 2004).

The free acidity values of honey samples were found in the range of 8.48 - 72.26 meq kg^{-1} (Table 2). When control (without the addition of LJC) honey samples were examined, it was seen that the lowest free acidity level belonged to flower honey, and the highest free acidity level belonged to chestnut honey ($p < 0.05$). As the amount of LJC in honey samples increased, the free acidity values also increased. Strong negative correlations were found between free acidity level and pH values in pine, flower, and chestnut honey at the levels of -0.97, -0.94, and -0.99, respectively. The acids in honey originate from the nature of the nectar collected by honeybees, and are produced by honeybees from sugars. This situation is used to determine the geographical region where honeybees collect nectar.

The acids in honey are directly related to the chemical properties such as the colour, aroma, and pH of the honey (Silva *et al*., 2016). In different studies, the free acidity values of honey samples were determined between 21.50 - 57.07 meq kg^{-1} (Yucel and Sultanoglu, 2013; Qamer *et al*., 2013; Silva *et al*., 2016). According to the Turkish Food Codex Honey Communiqué and Codex Alimentarius, the amount of free acid in flower and secretion honey should not exceed 50 meq kg^{-1} (TGK, 2020; Guerzou *et al*., 2021). In the present work, the free acidity values of honey samples in the control groups were found in the range of 8.48 - 22.17 meq kg^{-1}.

The sugar composition in honey is generally composed of monosaccharides, namely glucose and fructose. Honey is mostly composed of a supersaturated solution of glucose, and can therefore be converted to glucose monohydrate form at room temperature (crystallization). The amount of sugar in honey depends on the type of honey and the geographical region where it was created by honeybees (Cavia *et al*., 2002; Haroun, 2006; Silva *et al*., 2016). In the present work, the reducing sugar values of honey samples were determined in the range of 87.47 - 105.08% (on a dry basis) (Table 2). Among the honey varieties, the highest reducing sugar value belonged to chestnut honey, and the lowest reducing sugar value belonged to flower honey ($p < 0.05$). In addition, as the amount of added LJC increased, the reducing sugar content of the samples decreased.

The total sugar values of honey samples were observed in the range of 93.72 - 111.10% (on a dry basis) (Table 2). While the control chestnut honey had the highest total sugar value, the flower honey with 3% LJC added had the lowest total sugar value. Very high negative correlations were obtained between the reducing sugar and free acidity in flower (-0.91) and chestnut (-0.98) honey. Positive correlations were observed between reducing sugar and Brix (0.85), sucrose (0.95), and total sugar (0.99) in flower honey. There were positive correlations between reducing sugar and pH values (0.98), sucrose (0.94), and total sugar (0.99) in chestnut honey. Positive correlations were also observed between total sugar and pH (0.97) and sucrose (0.96).

The sucrose content of the honey samples was found in the range of 4.08 - 7.68 g/100 g (on a dry basis) (Table 2). The sucrose contents of the control groups (without the addition of LJC) of pine, chestnut, and flower honey samples were 5.68, 5.72, and 7.68 g/100 g, respectively. As the LJC in the samples increased, the amount of sucrose generally decreased. According to Turkish Food Codex Honey Communiqué and Codex Alimentarius, the amount of sucrose in flower and secretion honey should be at most 5 g/100 g (on a wet basis). In the present work, the sucrose content of control flower honey was determined slightly above the limit value. Many similar cases can be found in the literature (Anupama *et al*., 2003; TGK, 2020). This may occur when cheap sweeteners such as sugar syrups, refined beet sugar, or cane sugar are added directly into honey, or some sugary products are collected by honeybees instead of nectar, and turned into honey. The amount of sucrose in honey is an important quality criterion that indicates the maturity level of honey. Harvesting sucrose before it is completely converted to glucose

and fructose may also result in high sucrose content (Silva *et al*., 2016).

Total phenolic content

The TPC of honey samples were measured in the range of 4.14 - 218.7 mg GAE/100 g (on a dry basis) (Table 3). When the control honey samples were examined, it was seen that the honey with the lowest TPC value was flower honey with 4.14 mg GAE/100 g, followed by chestnut honey with 53.90 mg GAE/100 g and pine honey with 56.10 mg GAE/100 g, respectively. It was also observed that the TPC increased as the LJC in the sample increased, and during the storage period. Although the TPC in pine honey showed a positive high correlation with free acidity (0.95), it exhibited significant negative correlations with sucrose content (-0.94), pH values (-0.93), and Brix values (-0.94). In flower honey, the TPC had significant negative correlations with the sucrose content (-0.80) and pH values (-0.92), and a positive correlation with the free acidity values (0.88). In chestnut honey, the TPC had significant negative correlations with the reducing sugar amount (-0.89), the total sugar level (-0.88), the sucrose level (-0.82), and pH values (-0.92), a positive correlation with the free acidity values (0.88).

In some studies on flower and secretion honey, the total phenolic content of honey samples was reported as between 32.53 - 85.34 mg GAE/100 g (Çakır *et al*., 2017; Malkoç *et al*., 2019). In literature, the ascorbic acid and phenolic content of LJC were reported as 31 mg/100 g and 1371 mg GAE/L, respectively (Al-Zubaidy and Khalil, 2007; Ucan *et al*., 2016). In the present work, it was found that increasing LJC increased the TPC of honey samples. In a study on the determination of phenolics by the Folin-Ciocalteu method, it was shown that glucose, HMF, furfural, and vitamin B_{12} did not interfere with the phenolic estimation, but ascorbic acid did strongly (Bastola *et al*., 2017). Stevanato *et al*. (2004) found that TPC analyses using the Folin-Ciocalteu method were significantly affected by ascorbate, citrate, and sulphites. The present work suggests that ascorbic and citric acid from LJC and their degradation products/derivatives were effective in determining the TPC contents of honey samples. The increase in TPC as storage time and temperature increased can be explained by this mechanism. Phenolic compounds in honey directly affect some of its characteristic features. These compounds generally occur as glycosides in the nectars collected by honeybees, and are included in the content of honey in hydrolysed form as flavonoids. The geographical region where honeybees collect nectar primarily determines the content of honey. The variety and amount of phenolic components in honey also vary according to the geographical region where honey is produced (Silici *et al*., 2010).

Antioxidant activity

Many studies have shown that honey is a source of natural antioxidants that have a positive effect on diseases such as heart disease, cancers, immune system, cataracts, and inflammation. It is also used in foods due to its functions such as inhibiting oxidation, pathogenic/degrading bacteria, and enzymatic browning (Bertoncelj *et al*., 2007). The antioxidant activity (AA) by DPPH method values of honey samples were found in the range of 1.01 - 18.15 mg GAE/100 g (on a dry basis) (Table 4). In control samples, pine honey had the highest AA value, while flower honey had the lowest AA value ($p < 0.05$).

The flavonoid groups of phenolic compounds in honey are effective in determining the antioxidant activity of honey. The hydroxyl number and position of flavonoid compounds, and the glycosylation of flavonoid compounds, have an important function in determining antioxidant activity (Guzel and Bahceci, 2019). As stated in many studies, the relationship between AA and TPC contents of honey samples was observed to be generally significant (Bertoncelj *et al*., 2007; Silva *et al*., 2016). Therefore, in parallel with TPC, it was measured that AA increased with increasing amounts of LJC and during storage. It can be said that phenolic compounds, ascorbic acid, and Maillard reaction products (especially HMF) from LJC are effective in the increase in AA value. Many studies have shown that HMF has antioxidant activity (Bertoncelj *et al*., 2007). In this context, the HMF formed during storage may also have contributed positively to the AA value.

HMF content

HMF is formed by a non-enzymatic browning reaction (Maillard), dehydration of sugars in an acidic environment (caramelisation), and decomposition of ascorbic acid. The existence of elevated HMF levels in honey products poses considerable issues about consumer safety and regulatory compliance. HMF is a compound that forms during the heating and storage of honey, and its concentration serves as an important

Table 3. Total phenolic content of honey samples in mg GAE/100 g (on a dry basis).

Type of honey	Lemon juice concentrate (%)	Temperature (°C)	Storage (mth)		
			0	3	6
Pine	0	25	56.10 ± 1.18^{e}	79.96 ± 0.92^{p}	76.19 ± 0.77^{x}
		35	56.10 ± 1.18^{e}	87.71 ± 0.51^{l}	106.05 ± 1.43^{n}
		45	56.10 ± 1.18^{e}	129.56 ± 0.35^{e}	195.16 ± 0.93^{d}
	1	25	60.56 ± 1.66^{d}	86.22 ± 0.86^{m}	84.82 ± 0.56^{t}
		35	60.56 ± 1.66^{d}	90.52 ± 1.28^{k}	112.60 ± 0.27^{k}
		45	60.56 ± 1.66^{d}	133.39 ± 0.88^{d}	200.69 ± 0.81^{c}
	2	25	74.84 ± 0.95^{b}	68.91 ± 0.97^{s}	87.15 ± 0.24^{s}
		35	74.84 ± 0.95^{b}	94.11 ± 1.40^{i}	120.44 ± 0.58^{j}
		45	74.84 ± 0.95^{b}	137.46 ± 0.71^{c}	206.54 ± 0.35^{b}
	3	25	78.47 ± 2.03^{a}	74.78 ± 1.26^{r}	91.62 ± 0.39^{r}
		35	78.47 ± 2.03^{a}	97.30 ± 0.34^{h}	123.54 ± 0.51^{i}
		45	78.47 ± 2.03^{a}	148.35 ± 1.03^{a}	218.70 ± 0.95^{a}
Flower	0	25	4.14 ± 0.10^{g}	24.67 ± 0.14^{B}	36.68 ± 0.39^{F}
		35	4.14 ± 0.10^{g}	39.54 ± 0.39^{y}	48.49 ± 0.80^{C}
		45	4.14 ± 0.10^{g}	53.76 ± 0.64^{v}	82.42 ± 0.89^{u}
	1	25	5.22 ± 0.20^{g}	29.27 ± 0.49^{A}	40.05 ± 0.43^{E}
		35	5.22 ± 0.20^{g}	44.04 ± 0.60^{x}	54.97 ± 0.35^{A}
		45	5.22 ± 0.20^{g}	64.82 ± 0.89^{t}	96.57 ± 0.21^{p}
	2	25	5.36 ± 0.34^{g}	31.81 ± 0.47^{z}	44.72 ± 0.49^{D}
		35	5.36 ± 0.34^{g}	50.37 ± 0.34^{w}	63.98 ± 0.89^{z}
		45	5.36 ± 0.34^{g}	88.29 ± 0.53^{l}	109.25 ± 0.35^{m}
	3	25	5.78 ± 0.22^{g}	39.21 ± 0.66^{y}	49.68 ± 0.30^{B}
		35	5.78 ± 0.22^{g}	56.04 ± 1.55^{u}	70.99 ± 0.45^{y}
		45	5.78 ± 0.22^{g}	92.66 ± 0.66^{j}	131.32 ± 0.37^{h}
Chestnut	0	25	53.90 ± 1.74^{f}	78.38 ± 1.22^{q}	77.33 ± 1.02^{w}
		35	53.90 ± 1.74^{f}	82.56 ± 0.57^{o}	93.76 ± 0.27^{q}
		45	53.90 ± 1.74^{f}	113.33 ± 0.84^{g}	132.23 ± 0.80^{h}
	1	25	56.66 ± 0.97^{e}	78.10 ± 0.70^{q}	81.17 ± 0.64^{v}
		35	56.66 ± 0.97^{e}	83.96 ± 0.92^{n}	101.50 ± 0.43^{o}
		45	56.66 ± 0.97^{e}	128.23 ± 1.13^{f}	154.05 ± 1.20^{g}
	2	25	72.73 ± 2.21^{c}	80.10 ± 1.96^{p}	84.22 ± 0.47^{t}
		35	72.73 ± 2.21^{c}	90.00 ± 0.64^{k}	111.01 ± 0.27^{l}
		45	72.73 ± 2.21^{c}	136.28 ± 0.94^{c}	168.45 ± 0.58^{f}
	3	25	74.52 ± 1.22^{bc}	85.66 ± 0.41^{m}	85.12 ± 0.22^{t}
		35	74.52 ± 1.22^{bc}	93.26 ± 1.10^{ij}	111.87 ± 0.27^{kl}
		45	74.52 ± 1.22^{bc}	139.03 ± 0.68^{b}	179.71 ± 1.33^{e}

Means in similar column with different lowercase superscripts are significantly different ($p < 0.05$).

Table 4. Antioxidant activities of honey samples in mg GAE/100 g (on a dry basis).

Type of honey	Lemon juice concentrate (%)	Temperature (°C)	Storage (mth)		
			0	**3**	**6**
Pine	0	25	8.35 ± 0.21^{a}	7.59 ± 0.07^{ij}	7.79 ± 0.33^{k}
		35	8.35 ± 0.21^{a}	9.07 ± 0.23^{ef}	14.40 ± 0.06^{c}
		45	8.35 ± 0.21^{a}	14.00 ± 0.43^{a}	18.15 ± 0.12^{a}
	1	25	8.06 ± 0.22^{b}	7.37 ± 0.14^{jk}	7.63 ± 0.13^{klm}
		35	8.06 ± 0.22^{b}	8.25 ± 0.68^{gh}	13.81 ± 0.17^{d}
		45	8.06 ± 0.22^{b}	12.86 ± 0.26^{b}	16.01 ± 0.05^{b}
	2	25	8.48 ± 0.18^{a}	4.41 ± 0.38^{op}	7.74 ± 0.48^{kl}
		35	8.48 ± 0.18^{a}	8.49 ± 0.14^{fgh}	13.34 ± 0.52^{e}
		45	8.48 ± 0.18^{a}	10.04 ± 0.12^{d}	16.09 ± 0.12^{b}
	3	25	7.58 ± 0.10^{c}	4.85 ± 0.49^{o}	7.88 ± 0.17^{k}
		35	7.58 ± 0.10^{c}	8.81 ± 0.08^{efg}	11.64 ± 0.09^{fg}
		45	7.58 ± 0.10^{c}	10.21 ± 0.30^{d}	16.09 ± 0.24^{b}
Flower	0	25	1.01 ± 0.03^{j}	2.42 ± 0.13^{r}	3.01 ± 0.23^{u}
		35	1.01 ± 0.03^{j}	3.35 ± 0.18^{q}	4.08 ± 0.34^{s}
		45	1.01 ± 0.03^{j}	4.65 ± 0.30^{o}	7.95 ± 0.22^{k}
	1	25	1.27 ± 0.03^{i}	2.75 ± 0.22^{r}	3.60 ± 0.31^{t}
		35	1.27 ± 0.03^{i}	4.04 ± 0.37^{p}	4.65 ± 0.18^{r}
		45	1.27 ± 0.03^{i}	4.93 ± 0.39^{o}	7.88 ± 0.37^{k}
	2	25	1.53 ± 0.09^{h}	3.98 ± 0.26^{p}	4.03 ± 0.32^{st}
		35	1.53 ± 0.09^{h}	5.81 ± 0.80^{mn}	5.24 ± 0.28^{q}
		45	1.53 ± 0.09^{h}	7.55 ± 0.10^{ij}	8.97 ± 0.19^{j}
	3	25	1.89 ± 0.03^{g}	4.38 ± 0.12^{op}	4.68 ± 0.17^{r}
		35	1.89 ± 0.03^{g}	7.53 ± 0.65^{ij}	5.69 ± 0.18^{p}
		45	1.89 ± 0.03^{g}	8.06 ± 0.29^{hi}	10.06 ± 0.12^{i}
Chestnut	0	25	6.07 ± 0.07^{f}	5.82 ± 0.39^{mn}	5.90 ± 0.46^{p}
		35	6.07 ± 0.07^{f}	5.95 ± 0.30^{mn}	6.63 ± 0.26^{o}
		45	6.07 ± 0.07^{f}	11.03 ± 0.32^{c}	10.07 ± 0.14^{i}
	1	25	6.27 ± 0.08^{e}	5.47 ± 0.72^{n}	6.40 ± 0.09^{o}
		35	6.27 ± 0.08^{e}	7.68 ± 0.95^{ij}	7.20 ± 0.42^{mn}
		45	6.27 ± 0.08^{e}	8.80 ± 0.04^{efg}	10.74 ± 0.15^{h}
	2	25	7.07 ± 0.16^{d}	6.35 ± 0.14^{lm}	7.08 ± 0.22^{n}
		35	7.07 ± 0.16^{d}	7.41 ± 0.35^{jk}	7.29 ± 0.70^{lmn}
		45	7.07 ± 0.16^{d}	9.35 ± 0.44^{e}	11.34 ± 0.10^{g}
	3	25	7.89 ± 0.12^{b}	6.83 ± 0.09^{kl}	7.52 ± 0.82^{klmn}
		35	7.89 ± 0.12^{b}	7.14 ± 0.28^{jk}	7.67 ± 0.53^{klm}
		45	7.89 ± 0.12^{b}	11.18 ± 0.13^{c}	11.91 ± 0.15^{f}

Means in similar column with different lowercase superscripts are significantly different ($p < 0.05$).

indicator of honey quality. Increased HMF levels may signify inadequate storage conditions, excessive heat exposure, and possible adulteration, which can jeopardise the safety and quality of honey products. When the prescribed dietary limit is exceeded, HMF consumption may cause carcinogenic, genotoxic, and organotoxic effects (Burdurlu *et al*., 2006; Shapla *et al*., 2018). HMF content in honey has been set as a maximum of 40 mg kg^{-1} (80 mg kg^{-1} in tropical honey) by the Codex Alimentarius Commission (Shapla *et al*., 2018).

In the present work, the HMF values of the honey samples were measured in the range of 12.41 - 7646.5 mg kg^{-1} (Table 5). While the HMF values of the control flower and pine honey were below the limit value, the HMF value of chestnut honey was above the limit value with 144.8 mg kg^{-1}. The increase in the amount of HMF generally occurs as a result of the storage conditions, heating of the honey, and the Maillard reaction. However, the amount of sugar in the honey, the presence of organic acids, pH, Brix value, and the geographical region where honeybees collect nectar can also be effective on HMF formation (Silva *et al*., 2016). In the present work, it was observed that the increase in the amount of added LJC, storage temperature, and time increased the HMF level in the samples. Considering the formation mechanisms of HMF, it is thought that LJC added to honey samples increased this formation due to the ascorbic acid (decomposition) and citric acid (sugar-acid interaction) it contained. In addition, considering that honey can contain low levels of amino acids, the pH decreased with the addition of LJC may have accelerated the amine group-reducing sugar interaction (Ajandouz *et al*., 2001). Various strategies can be used to reduce HMF formation in honey and other food products. Adjusting the pH balance (Wu *et al*., 2023), managing the ionic composition (Kocadağlı and Gökmen, 2016), utilising activated carbon (Altıok *et al*., 2021), fine-tuning storage temperatures (Karadeniz *et al*., 2024), and implementing UV-C treatment (Gök, 2021) are among the strategies suggested to lower HMF levels in final products.

In the present work, high positive correlations were found between HMF level and TPC, free acidity, and AA (0.81 - 0.99). On the other hand, high negative correlations were observed between sucrose, reducing sugar, total sugar, pH, and Brix value ((-0.80) - (-0.99)).

HMF formation kinetics

It was observed that HMF formation kinetics were compatible with zero-order reaction (Eq. 1). Using HMF formation kinetics, HMF levels of the samples at the end of 24 months (average shelf life determined by commercial companies) were estimated (Table 5). At the end of 24 months, it was estimated that the HMF level of honey without LJC stored at room conditions would be ND - 724.1 mg kg^{-1}. The HMF contents of honey containing 1% LJC and stored at 25, 35, and 45°C were estimated to be 259.8 - 912.7, 849.8 - 5656, and 11331 - 21595 mg kg^{-1}, respectively. The HMF contents of honey containing 2% LJC and stored at 25, 35, and 45°C were estimated to be 260.0 - 1157, 2975 - 8041, and 16903 - 25875 mg kg^{-1}, respectively. The HMF contents of honey containing 2% LJC and stored at 25, 35, and 45°C were estimated to be 471.8 - 1404, 4364 - 9134, and 22691 - 30039 mg kg^{-1}, respectively.

Table 6 shows the zero-order rate constants (k) and Arrhenius equation coefficients of the HMF formation reactions of the honey sample. It was determined that the reaction rate constant increased as the temperature and LJC ratios increased. The highest rate constant was observed in chestnut honey, followed by pine and flower honey. Similar findings were reported in the previous studies. Grainger *et al*. (2017) studied the formation of HMF in New Zealand mānuka honey. They reported the zero-order rate constants of honey supplemented with different amino acids and 2000 mg/kg DHA in the ranges of 1 - 3 × 10^{-4}, 3 - 7 × 10^{-4}, and 2.3 - 9.6 × 10^{-3} mmol kg^{-1} day^{-1} for honey samples stored at 20, 27, and 37°C, respectively. Turkut *et al*. (2018) investigated the kinetics of HMF formation in heat-treated (50, 70, and 80°C for 0 - 48 h) honey from different floral sources. The zero-order rate constants of multifloral, honeydew, and chestnut honey samples heated at 50°C were found to be 0.0307, 0.0712, and 0.2173 mg kg^{-1} h^{-1}, respectively. In another study by Yap and Chin (2020), the zero-order rate constant of *kelulut* honey was reported as 0.0831 mg kg^{-1} h^{-1}. The results obtained in the present work and literature studies show that honey type and applied temperature value significantly affect HMF formation kinetics.

The activation energy (E_a) values of the reactions ranged from 126.2 - 219.7 kJ mol^{-1} (Table 6). There are many studies in the literature on the formation kinetics of HMF in both food and model systems. Studies show that the kinetics of HMF

Table 5. HMF levels of honey samples in mg kg^{-1} (on a dry basis).

Type of honey	Lemon juice concentrate (%)	Temperature (°C)	HMF level during storage (mth)			
			0	3	6	24 (predicted)
Pine	0	25	25.44 ± 0.75^{def}	27.91 ± 0.08^{stu}	31.50 ± 0.39^{tu}	49.68
		35	25.44 ± 0.75^{def}	85.07 ± 5.24^{rstu}	246.2 ± 9.65^{pqrs}	908.6
		45	25.44 ± 0.75^{def}	806.0 ± 6.78^{j}	1383 ± 23.35^{l}	5458
	1	25	26.39 ± 4.69^{de}	79.60 ± 3.04^{rstu}	92.38 ± 1.34^{stu}	290.3
		35	26.39 ± 4.69^{de}	272.0 ± 8.89^{op}	729.9 ± 24.63^{n}	2840
		45	26.39 ± 4.69^{de}	2060 ± 22.39^{f}	3535 ± 92.66^{h}	14061
	2	25	32.35 ± 0.52^{d}	103.0 ± 11.74^{rst}	186.9 ± 7.01^{qrst}	650.4
		35	32.35 ± 0.52^{d}	418.3 ± 13.28^{m}	1088 ± 34.76^{m}	4256
		45	32.35 ± 0.52^{d}	2935 ± 21.39^{d}	5018 ± 147.78^{e}	19975
	3	25	33.67 ± 0.50^{d}	145.4 ± 6.69^{qr}	242.5 ± 5.99^{pqrs}	868.8
		35	33.67 ± 0.50^{d}	540.8 ± 11.38^{l}	1428 ± 35.38^{l}	5612
		45	33.67 ± 0.50^{d}	3998 ± 41.75^{b}	6628 ± 270.57^{b}	26412
Flower	0	25	ND	ND	ND	ND
		35	ND	15.85 ± 0.75^{tu}	31.78 ± 6.27^{tu}	113.9
		45	ND	415.4 ± 64.09^{m}	1506 ± 55.02^{l}	6011
	1	25	12.41 ± 0.36^{g}	25.75 ± 3.40^{stu}	74.25 ± 26.21^{stu}	259.8
		35	12.41 ± 0.36^{g}	115.8 ± 7.61^{rs}	221.7 ± 20.14^{qrs}	849.8
		45	12.41 ± 0.36^{g}	1282 ± 55.29^{h}	2842.01 ± 182.61^{i}	11331
	2	25	17.97 ± 0.36^{fg}	46.39 ± 0.83^{stu}	78.48 ± 52.56^{stu}	260.0
		35	17.97 ± 0.36^{fg}	215.5 ± 5.66^{pq}	757.1 ± 159.66^{n}	2975
		45	17.97 ± 0.36^{fg}	2037 ± 31.41^{f}	4239 ± 89.13^{f}	16903
	3	25	23.06 ± 1.18^{ef}	70.42 ± 5.49^{rstu}	135.2 ± 4.80^{rstu}	471.8
		35	23.06 ± 1.18^{ef}	320.6 ± 14.40^{no}	1108 ± 73.30^{m}	4364
		45	23.06 ± 1.18^{ef}	2656 ± 99.34^{e}	5690 ± 58.81^{c}	22691
Chestnut	0	25	144.8 ± 4.35^{b}	249.4 ± 6.07^{op}	289.6 ± 3.76^{pqr}	724.1
		35	144.8 ± 4.35^{b}	473.5 ± 18.37^{lm}	1041 ± 53.87^{m}	3731
		45	144.8 ± 4.35^{b}	1954 ± 65.16^{g}	3836 ± 13.76^{g}	14910
	1	25	134.1 ± 10.93^{c}	292.5 ± 3.36^{op}	328.8 ± 18.76^{pq}	912.7
		35	134.1 ± 10.93^{c}	673.5 ± 36.16^{k}	1515 ± 64.69^{l}	5656
		45	134.1 ± 10.93^{c}	2864 ± 147.71^{d}	5499 ± 273.98^{d}	21595
	2	25	149.1 ± 10.80^{b}	297.8 ± 3.82^{op}	401.2 ± 7.33^{op}	1157
		35	149.1 ± 10.80^{b}	845.6 ± 22.10^{j}	2122 ± 37.19^{k}	8041
		45	149.1 ± 10.80^{b}	3653 ± 35.13^{c}	6581 ± 228.70^{b}	25875
	3	25	182.7 ± 4.86^{a}	390.4 ± 5.38^{mn}	488.0 ± 20.84^{o}	1404
		35	182.7 ± 4.86^{a}	950.5 ± 62.30^{i}	2420 ± 124.17^{j}	9134
		45	182.7 ± 4.86^{a}	4115 ± 253.28^{a}	7646 ± 245.60^{a}	30039

Means in similar column with different lowercase superscripts are significantly different ($p < 0.05$).

Table 6. Rate constants (*k*) (1/mth) at different temperatures and activation energy (E_a) (kJ/mol) values for HMF formations in different honey samples.

Type of honey	LJC (%)	25°C		35°C		45°C		Arrhenius equation parameter		
		k (mg kg^{-1} mth^{-1})	R^2	k (mg kg^{-1} mth^{-1})	R^2	k (mg kg^{-1} mth^{-1})	R^2	A (mg kg^{-1} mth^{-1})	E_a (kJ mol^{-1})	R^2
Pine	0	1.01	0.9887	36.80	0.9342	226.34	0.9926	4.49E+38	219.7	0.9684
	1	11.00	0.8888	117.25	0.9705	584.79	0.9916	4.61E+28	156.9	0.9917
	2	25.75	0.9976	176.00	0.9764	830.94	0.9911	4.20E+25	137.0	0.9982
	3	34.80	0.9984	232.45	0.9758	1099.10	0.9866	4.20E+25	136.1	0.9985
Flower	0	ND	ND	4.75	0.9953	250.46	0.9364	1.86E+31	176.7	0.9313
	1	10.31	0.9027	34.89	0.9999	471.60	0.9965	2.29E+27	150.0	0.9503
	2	10.09	0.9988	123.20	0.9326	703.53	0.9994	2.52E+30	167.5	0.9929
	3	18.70	0.9920	180.87	0.9363	944.49	0.9983	1.69E+28	154.7	0.9949
Chestnut	0	24.14	0.9383	149.42	0.9768	615.22	0.9999	7.69E+23	127.7	0.9971
	1	32.44	0.8840	230.08	0.9843	894.21	0.9999	2.09E+24	130.9	0.9927
	2	42.02	0.9894	328.84	0.9720	1071.90	0.9973	7.69E+23	127.9	0.9816
	3	50.89	0.9584	372.97	0.9682	1244.00	0.9990	7.69E+23	126.2	0.9851

formation conform to zero, first, and second-order kinetics. Turkut *et al*. (2018) reported E_a of heat-treated (50, 70, 80°C for 0 - 48 h) multifloral, honeydew, and chestnut honey samples as 204.6, 174.2, and 138.3 kJ mol^{-1}, respectively. Yap and Chin (2020) stated that the zero-order E_a of *Kelulut* honey was 104.1 kJ mol^{-1}. A high E_a value is an indicator of temperature sensitivity (Turhan *et al*., 2008). In the present work, pine honey was the most sensitive to temperature in terms of HMF formation.

Conclusion

In the present work, some physicochemical properties of the honey samples (Brix, pH, free acidity, reducing sugar, total sugar, and sucrose content) were determined by adding LJC at different ratios to three different types of honey. The HMF levels, AA, and TPC of the samples stored at three different temperatures (25, 35, and 45°C) were monitored for six months. The degree of reaction, rate constants, and Arrhenius coefficients were calculated using kinetic data in HMF formation. Based on the recommended expiration date, the estimated HMF values at the end of two years were calculated using the kinetic model. It was revealed that the increase in the amount of added LJC, storage time, and storage temperature dramatically increased the formation of HMF. It can be said that the addition of LJC to honey and honey-based mixtures will provide a positive effect in terms of health and nutrition, but may lead to undesirable effects on HMF formation. It has been observed that storing these products at high temperatures may drastically accelerate the formation of HMF. It is recommended that this and similar products be stored at lower temperatures and/or LJC be neutralised before addition.

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