Original Article

Effect of ultrasound on the physical, biochemical, antioxidant, and antimicrobial properties of industrial Iranian honey

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ABSTRACT

The aim of this study was to investigate the effects of ultrasound at two different frequencies, namely 30 kHz and 42 kHz, on various aspects of industrial Iranian honey, including its physical, biochemical, antioxidant and antimicrobial properties. Samples were subjected to ultrasound treatment at 30 kHz or 42 kHz for a duration of 1, 5 or 10 minutes at temperatures of 20 °C or 45 °C, respectively. The following parameters were then evaluated on days 1, 30, 90, and 180: HMF content, pH, acidity, proline concentration, total number of aerobic mesophilic bacteria, diastase activity, moisture content, sucrose concentration, fructose concentration, glucose concentration, fructose- glucose ratio, ABTS (antioxidant activity) content, number of osmophiles, phenol concentration, reducing sugar concentration and total sugar concentration. It is noteworthy that both treatment groups exposed to ultrasound showed changes in these parameters. Specifically, the group treated with ultrasound at a frequency of 42 kHz showed a decrease in moisture content, pH, acidity, fructosetoglucose ratio, total sugar concentration, clostridium count, total microbial count, mold count, osmophiles countand HMF content; as well as an increase in diastase activity, proline and phenol concentration. These changes were more pronounced on days 90 and 180. Ultrasound treatment at a frequency of 42 kHz for a duration of 10 minutes at a temperature of 45 °C has the potential to improve the quality and shelf life of industrial honey. Consequently, this technique can be used in the food industry to obtain a better product that is more suitable for export.

Keywords: Industrial Iranian honey; Ultrasound; Antioxidant activity; Antimicrobial activity

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1. Introduction

Honey is a sweet and nutritious product of the bee species Apis mellifera. It contains glucose, fructose, water, minerals, vitamins, organic acids, phenolic compounds and enzymes (1, 2). Honey has various functional properties such as antioxidant, antibacterial, antifungal, prebiotic, and anticancer properties, which are essential for human health. Due to its unique properties, honey is not only known as a nutritious condiment but also as a medicine with healing properties. Honey is a high-density sugar solution that can form coarse crystalswhen conditions change (e.g. glucose content, humidity, temperature, impurities, and ecology) , resulting in a loss of quality (1, 2). The most important factors affecting the crystallization of honey are the ration of fructose to glucose, humidity, storage timeand thermal history. The change in the ratio of glucose and fructose sugar is directly related to the tendency of the honey to crystallize. The higher the glucose content in relation to fructose, the faster the honey crystallizes and vice versa. Heat treatment is often used to decrystallize honey, but this can increase the hydroxymethylfurfural(HMF) content, reduce enzyme activity, increase antioxidant activity and cause browning of honey (3). The HMF content is an important quality indicator for honey. When honey is subjected to heat treatment, it undergoes decrystallization, which leads to an increase in HMF content, browningand increased antioxidant activity. The antioxidant activity of honey is primarily attributed to phenolic compounds, including flavonoids and phenolic acids (4). Recently, ultrasound technology has proven to be an effective and successful technique for liquefying crystallized honey in the food industry. Since ultrasonic technology induces important structural changes associated with cavitation phenomena, it offers an alternative to conventional heating methods. Unlike heat treatment, ultrasound does not affect the integrity of enzymes such as α-amylase and peroxidase, which are essential for the quality of honey. This non-thermal technique, called ultrasonic treatment, can be used to reduce particle size and eliminate crystals (5). Ultrasound treatment can significantly reduce the number of honey crystals without affecting the properties of the honey. The aim of this study was to determine the effect of ultrasound on the textural, physico-chemical, and microbial properties of industrial Iranian honey (6). However, there are few studies on the effects of ultrasound frequency on the quality and shelf life of honey. In most studies, ultrasound was used at a single frequency or a in narrow frequency range, which may not reflect the optimal conditions for treating honey (7) (8). Therefore, the aim of this study was to compare the effect of ultrasound with two different frequencies (30 and 42 kHz) on the textural, physicochemical, and microbial properties of industrial Iranian honey. The hypothesis was that ultrasound at 42 kHz has a more favorable effect than ultrasound at 30 kHz, as it generates more cavitation bubbles and a stronger sound pressure. The results of this study could provide valuable information for the food industry to improve the quality and shelf life of honey using ultrasound technology.

2. Materials and Methods

2.1. Materials

The honey used in this study was purchased from Tehran Industrial Company (Tehran, Iran). The following reagents and chemicals were purchased from Merck (Germany): Folin-Ciocalteu reagent, gallic acid, methylene blue, sodium hydroxide, sulfuric acid, iodine, sodium thiosulfate, zinc acetate and potassium ferrocyanide. Ethanol, Ringer, Fehling A, Fehling B and DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from Sigma Aldrich (Germany). The following culture media were procured from Ibresco Life Science (Iran): Glucose Chloramphenicol Agar (YGC), Sabouraud dextrose agar (SDA), potato dextrose agar (PDA), and sulfite iron agar

2.2. Study Design

The experimental design for the honey samples was as follows. The honey samples were subjected to ultrasound (Branson, USA) at 30 or 42 kHz and 20 or 45 °C for 1, 5or 10 min. The following parameters were measured after 1, 30, 90, and 180 days: HMF, pH, acidity, proline, total aerobic mesophilic bacteria, diastase, moisture, sucrose, fructose, glucose, fructose- glucose ratio, ABTS, osmophiles, phenol, reduced sugars, and total sugars. The changes in the ultrasound-treated samples were compared with the control group (without ultrasound treatment) and with each other.

2.3. Physico-chemical properties

2.3.1. Moisture content

The moisture content of the sample was determined using the White method with a refractometer at 20 ºC according to Horwitz (1975) (9).

2.3.2. pH

A 10 g portion of the honey sample was dissolved in carbon dioxide-free distilled water in a beaker. The pH meter was calibrated with buffers of pH 4 and 7. The pH of the solution was measured at 20 ºC (9).

2.3.3. Acidity

A 10 g honey sample was weighed into a beaker and dissolved in distilled water. The solution was titrated with 0.1 N sodium hydroxide until a pink color was obtained with a phenolphthalein indicator, corresponding to a pH of 8.3. (**Formula 1**) (9).

Formula 1: Acidity (eq/kg) = 1000 × N (V-V1) / W

N= Normality consumable sodium hydroxide

V= Volume= milliliter consumable sodium hydroxide sample

V1= Volume= milliliter consumable sodium hydroxide control sample
W= Weight

2.4. Biochemical Properties
2.4.1. Sugar Content
2.4.1.1. Glucose, fructose, and fructose -glucose ratio
A 25 ml aliquot of honey dissolved in distilled water was transferred to a 250 ml Erlenmeyer flask. Using a pipette, 20 ml of 0.1 N iodine and then 0.5 N sodium hydroxide were added. The flask was kept in the dark for 15 minutes then 5 ml of 2-N-sulfuric acid was added. The excess iodine was titrated with sodium thiosulfate using a starch indicator solution (**Formulas 2 and 3**) (9).

Formula 2: Glucose (%) = 250 × 9.01 × D × 100 / 25 × w × 1000
D=The difference in the titration of sodium thiosulfate consumed by the sample and the control
W= Honey sample weight
9.01 Glucose=1ml iodine 0.1 normal

Formula 3: Fructose (%) = Reducing sugar before hydrolysis – Glucose

2.4.2. Sucrose
It was determined as follows (**Formula 4**) (9):

Formula 4: Sucrose (%) = (S1 – S) × 0.95
S1= Reducing sugar after hydrolysis
S= Reducing sugar before hydrolysis

2.4.3. Total and reducing sugar level
The total and reducing sugar content of the honey sample was determined according to the previously described method (9).

2.5.2. Antioxidant Properties
2.5.2.1. Hydroxymethylfurfural *(HMF)*
A 5 g honey sample was weighed in a beaker and dissolved in distilled water. The solution was transferred to a 50 ml volumetric flask and diluted to the mark. Two drops of ethanol were added to prevent foaming. The first 10 ml of the filtrate was discarded and the remainder was collected. Approximately 5 ml of the filtered honey solution was pipetted into two separate tubes. One tube contained 5 ml distilled water (test tube) and the other 5 ml sodium bisulphite (blank tube). Both tubes are mixed well. The absorbance of the sample tube compared to the blank tube was measured at the wavelengths 284 and 336 nm (**Formula 5**) (9).

Formula 5: HMF (mg/kg) = (A284 – A336) × 149.7 × 5 × D/W
A284= Molar absorption of HMF at 284 nm
A336= Molar absorption of HMF at 336nm
149.7= Absorption to a concentration conversion factor
149.7=126×1000×1000/16830×10×W
16830= Molar absorption coefficient of HMF at 284 nm
126= HMF molecular weight
1000= Convert kilograms to grams and grams to milligrams
D= dilution factor (Volume of final solution divided by 10)

2.5.2.2. Diastase Activity
An aliquot of 10 ml of the honey sample was transferred to a 50 ml volumetric flask and diluted to the mark. A 10 ml portion of the starch solution was transferred to another 50 ml volumetric flask and diluted to the mark. Both flasks were placed in a water bath at 40 ºC for 15 minutes. Then, 5 ml of the starch solution was added to the honey solution and incubated for a further 5 minutes. A 0.5 ml sample of the honey-starch mixture was pipetted and mixed with 5 ml of diluted iodine solution. The volume of the mixture was adjusted with water. The mixture was stirred well. The absorbance of each solution was measured separately at 660 nm. (**Formula 6**) (9).

Formula 6: Diastase activity (DN) = 60 minutes utes/ Time per minute = 0.10 / 0.01 × 1 / 2= 3000 / Time per minute

2.5.2.3. Proline Activity
The following procedure was carried out for the honey sample solution and proline: 0.5 ml of each solution was transferred to two separate tubesand 0.5 ml of water was added to a third tube as a blank. Subsequently, 1 ml of ninhydrin-formic acid was added to each tube, the tubes were capped and vortexed for 15 min. The tubes were then heated in a boiling water bath for 5 min and then cooled in a water bath at 70 ºC. Then 5 ml of 2-propanol was added to each tube, the tubes were released and allowed to stand at room temperature for 45 min to allow color development. Finally, the absorbance of the tubes was measured at a wavelength of 500-520 nm using a spectrophotometer (**Formula 7**) (9).

Formula 7: WP(mg/kg) = EP / ES × M1 / M2 × 80
EP=Absorption of sample solution
ES=Average absorbance of proline standard solution
M1=The original mass of proline in the standard solution
M2=The main mass of the honey sample
80= dilution factor(1g honey)
WP= Weight of proline

2.5.2.4. Phenol content

The phenol content of the honey sample was determined according to the method described above (9).

2.5.2.4. Antioxidant activity

The ABTS radical scavenging test was used to measure the antioxidant activity of the honey samplesaccording to a modified method of Re et al (10).

2.6.2. Antimicrobial Properties

Microbial analysis of the honey samples was performed to determine the numbers of molds, aerobic mesophilic bacteria, osmophilic bacteriaand clostridium, using the method described by Wahab et al (11).

2.7. Statistical Analysis

Data were expressed as mean ± SD, and graphs were created using the GraphPad Prism program (version 9). Data were statistically analyzed using analysis of variance (two-way ANOVA) followed by a post-tukey test, with a *p*-value≤0.05 defined as a significant difference.

3. Results

3.1. Physical Characters

3.1.1. Moisture

Honey samples were treated with ultrasound at 30 and 42 kHz for 10 min at 45 ºC, and their moisture content was measured at days 1, 30, 90, and 180. The results showed that both treatments significantly reduced the moisture conetnt of the honey samples compared to the control (P<0.001), and there was a significant difference between the two treatments (P<0.05) on days 1, 30and 90. (**Figure 1F**).

3.1.2. pH

The pH of honey samples was measured after treatment with ultrasound at 30 or 42 kHz for 1, 5, or 10 min at 20 °C on days 1, 30 and 180. The results showed that the pH of the treated samples increased significantly compared to the control (P<0.001 for 30 kHz and P<0.0001 for 42 kHz) at 1 min, and this trend continued at 5 min (P<0.01 for 30 kHz and P<0.001 for 42 kHz). The pH value of the samples treated with 42 kHz was also higher at both time points than that of the samples treated with 30 kHz (P<0.01). After 10 min, the pH of the treated samples increased further compared to the control (P<0.001 for 30 kHz and P<0.0001 for 42 kHz), and there was a significant difference between the two treatments (P<0.001) (**Figure SF1**). The pH of honey samples was measured after treatment with ultrasound at 30 or 42 kHz for 1, 5, or 10 min at 45 °C on days 1, 30, and 180. The results showed that the pH of the treated samples decreased significantly compared to the control (P<0.0001 for 30 kHz and P<0.0001 for 42 kHz) at 1 min on days 1 and 30, and only for 30 kHz on day 90 (P<0.05). After 10 min, the pH of the treated samples continued to decrease compared to the control and between treatments (P<0.0001) on day 1. On day 30, the pH decrease was more pronounced for both treatments (P<0.001 for 30 kHz and P<0.0001 for 42 kHz) (**Figure SF1**).

3.1.3. Acidity

The acidity of honey samples was measured after treatment with ultrasound at 30 or 42 kHz for 1, 5or 10 min at 20 or 45 °C on days 1, 30, 90 and 180. The results showed that the acidity of the samples treated with 42 kHz at 20 °C for 1 min decreased significantly compared to the control on day 180 (P<0.001) (**Figure 2A**). At 5 min, samples treated with 42 kHz had lower acidity than the control at days 1 and 90(P<0.05), and this effect was also observed for both treatments (P<0.001 for 30 kHz and P<0.0001 for 42 kHz) at day 180 (**Figure 2B**). At 10 min, the acidity of the treated samples decreased compared to the control at day 1 (P<0.05 for both treatments), 30 (P<0.05 for 30 kHz and P<0.01 for 42 kHz) and 90 (P<0.05 for 30 kHz), and there was also a significant difference between the treatments at day 180 (**Figure 2C**).

The acidity of samples treated with ultrasound at 45 °C for 1 min decreased significantly compared to the control (P<0.01 for both treatments) and between treatments (P<0.05) at days 1 and 90, and this trend continued on day 180 (P<0.01 for both treatments), with samples treated at 42 kHz having lower acidity than those treated at 30 kHz (P<0.01) (**Figure 2D**). At 5 min, the acidity of the treated samples decreased compared to the control (P<0.05 and P<0.001 for day 90 and P<0.0001 for both treatments at day 180), and there was also a significant difference between treatments on these days, with lower acidity for the 42 kHz treated samples (P<0.05 for day 90 and P<0.0001 for day 180) (**Figure 2E**). At 10 min, the acidity of treated samples increased compared to control at day 1 (P<0.05 for both treatments) and 30 (P<0.05 for both treatments), but decreased at day 90 (P<0.05 for both treatments), and there was no significant difference between treatments on these days (**Figure 2F**).

3.2 Biochemical Characters

3.2.1 Fructose

The results showed that the only significant increase in the fructose content of the treated samples compared to the control was observed in the samples treated with 30 kHz for 10 min on all days (P<0.05).

3.2.2 Rate of fructose to glucose

The results showed that the only significant decrease in the ratio of fructosetoglucose of the treated samples compared to the control was observed for the samples treated with 42 kHz for 10 min at 45 °C on days 30, 90 and 180 (P<0.05).

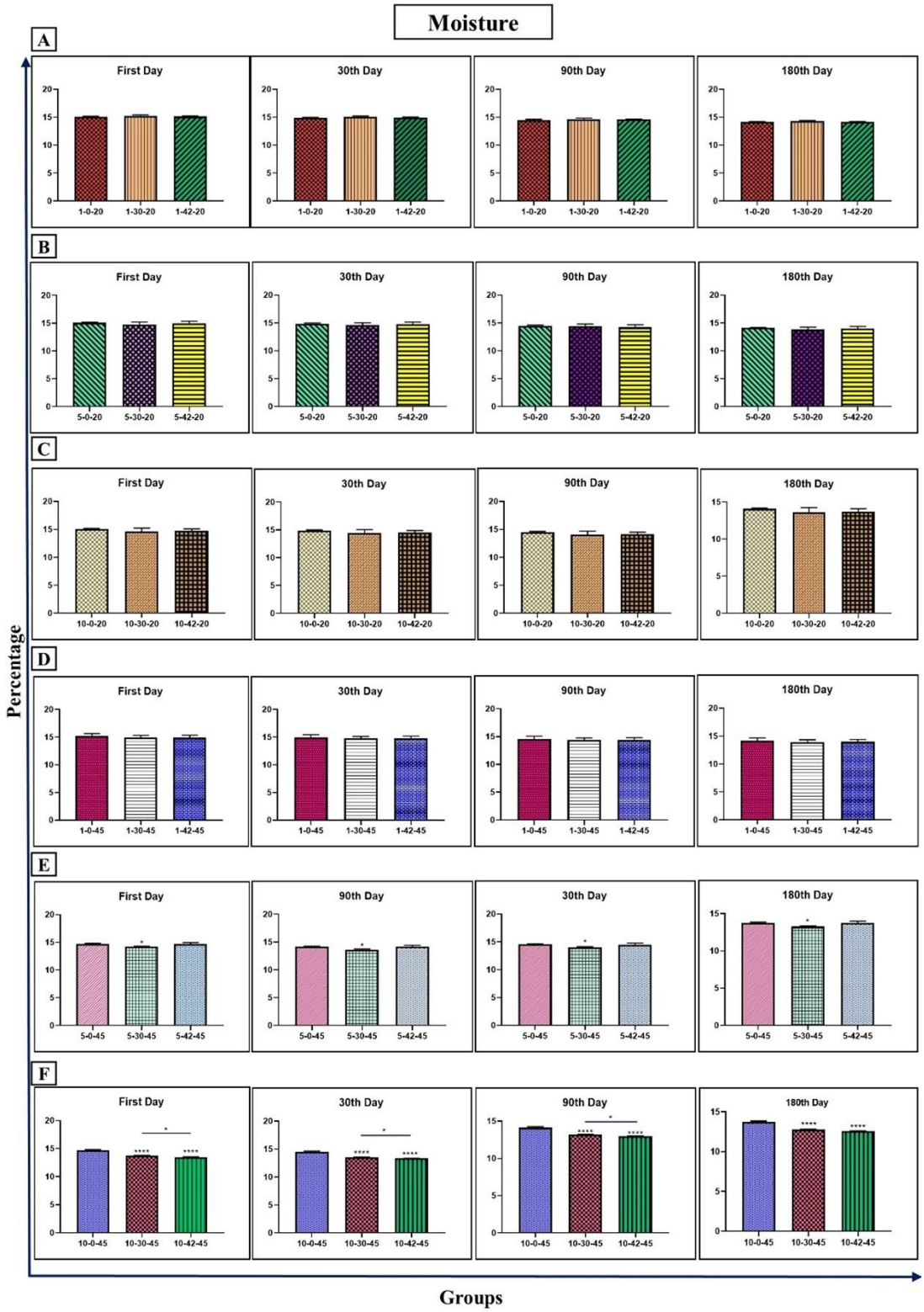

Figure 1. Effect of 0 (as control), 30, and 42 kHz ultrasound on the industrial honey humidity. The samples that treated at 30 kHz for 1 (**A**), 5 (**B**), 10 (**C**) minutes, and 20 °C. The samples that treated at 45 kHz for 1 (**D**), 5 (**E**), 10 (**F**) minutes, and 45 °C. Data are shown as the mean ± SD. The mean values with asterisks are significantly different ($p \leq 0.05$). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

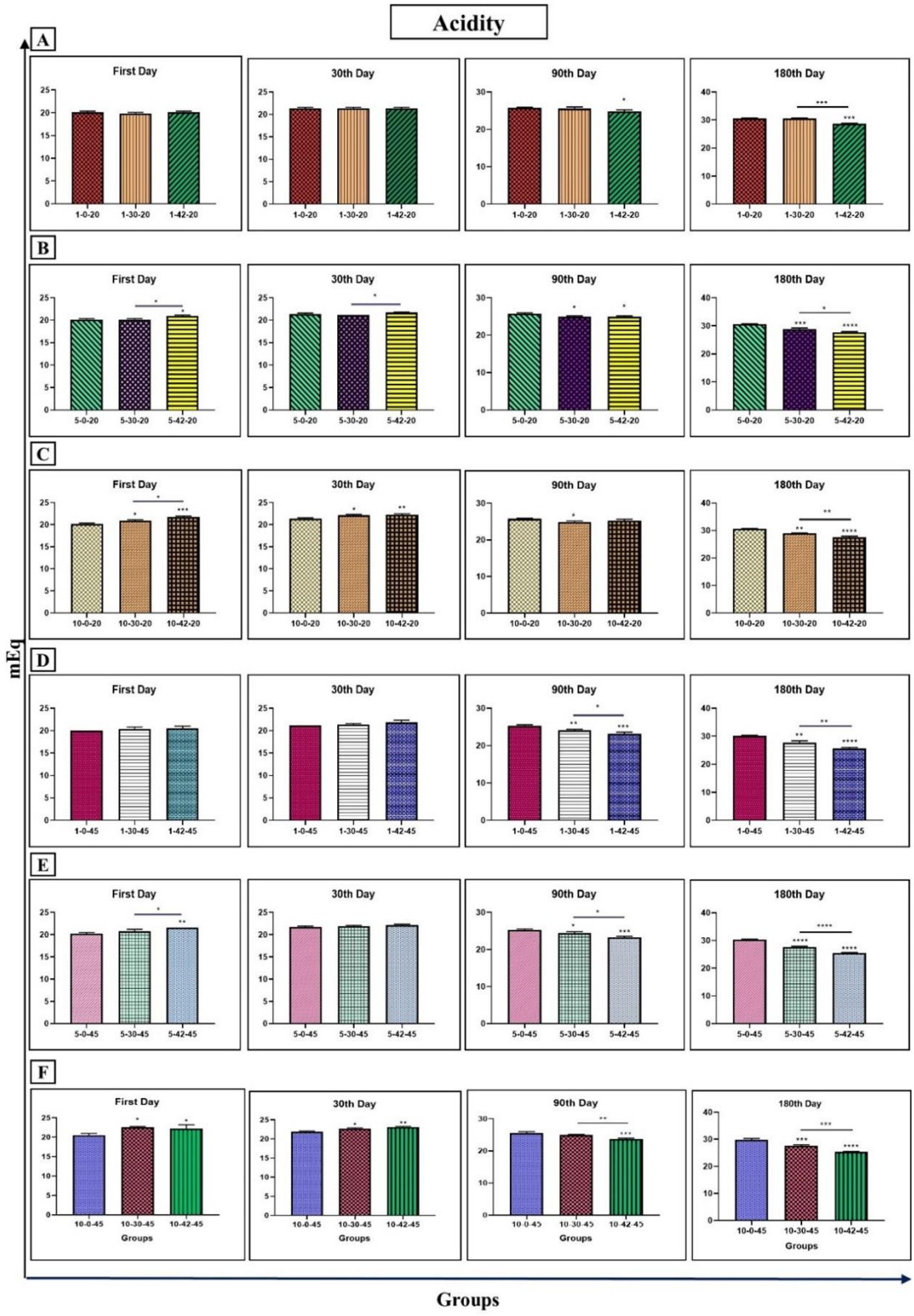

Figure 2. Effect of 0 (as control), 30, and 42 kHz ultrasound on the industrial honey acidity. The samples that treated at 30 kHz for 1 (**A**), 5 (**B**), 10 (**C**) minutes, and 20 °C. The samples that treated at 45 kHz for 1 (**D**), 5 (**E**), 10 (**F**) minutes, and 45 °C. Data are shown as the mean ± SD. The mean values with asterisks are significantly different ($p \leq 0.05$). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

3.2.3. Sucrose

The sucrose content of the honey samples was measured after ultrasonic treatment. The results showed that there was no significant difference in the sucrose content of the treated samples compared to the control (P<0.005), regardless of day, treatment duration or temperature.

3.2.4. Total Sugar

The results showed that the glucose content of the samples treated with 42 kHz for 5 and 10 min at 45 °C decreased significantly compared to the control (P<0.0001) and to the samples treated with 30 kHz (P<0.0001) on all days and treatment durations.

3.2.5. Reducing sugars

The results showed that the glucose content of the samples treated with 42 kHz decreased significantly compared to the control (P<0.0001) and to the samples treated with 30 kHz (P<0.0001) on all days and treatment durations.

3.3. Antioxidative parameters

3.3.1. HMF content

The results showed that the HMF content of the treated samples was lower than that of the control on most days and treatment durations, except for the samples treated with 42 kHz for 1 min at 20 °C on day 180 (P>0.05) and the samples treated with 42 kHz for 10 min at 20 °C on day 180 (P>0.05). The HMF content of the 42 kHz treated samples was also lower than that of the 30 kHz treated samples on some days and at some treatment durations, especially at 45 °C. The details of the statistical analysis are shown in **Figure 3.**

3.3.2. Diastasis

The results showed that the diastase activity of the treated samples varied depending on the treatment duration and frequency. The diastase activity of the samples treated with 42 kHz for 1 min decreased significantly compared to the control and to the samples treated with 30 kHz on day 90 (P<0.001). The diastase activity of the samples treated with 30 or 42 kHz for 5 min increased significantly compared to the control on days 30, 90 and 180 (P<0.01, P<0.05, P<0.0001, or P<0.0001) and compared to each other on all days (P<0.0001). The diastase activity of the samples treated with 30 or 42 kHz for 10 min increased significantly compared to the control on days 90 and 180 (P<0.0001) (**Figure SF2**).

3.3.3. Proline

Proline levels increased significantly in the samples treated with 30 and 42 kHz ultrasound (20 °C for 1 minute) on days 90 and 180 compared to the control sample (P<0.05 for 30 kHz and P<0.0001 for 42 kHz on day 90). The 42 kHz group also had higher proline levels than the 30 kHz group on day 180 (P<0.00001) (**Figure 4A**). Similarly, honey treatment (20 °C for 5 minutes) increased proline levels on day 90 and 180compared to the control sample (P<0.0001 for both frequencies on day 90). The 42 kHz group also had higher proline levels on day 180 than the 30 kHz group (P<0.05) (**Figure 4B**). However, extending the treatment time from 1 to 10 minutes led to a reduction in proline levels in all study groups on days 90 and 180 compared to the control group and the other groups (P<0.001) (**Figure 4C**). At 45 °C, ultrasound treatment with 30 or 42 kHz for 1, 5 or 10 minutes on days 1, 90 and 180 significantly increased the proline content of the honey samples compared to the control group (P<0.001). The proline content was higher in the samples treated with 42 kHz ultrasound than in the samples treated with 30 kHz ultrasound for the same duration and at the same time (P<0.05). The proline content also increased with increasing ultrasound duration from 1 to 10 minutes at both frequencies and all time points (P<0.001). Figure 4D-F shows the changes in proline content **(Figure 4D-F)**.

3.3.4. Phenol

The effect of ultrasound treatment at 42 kHz and 20 °C on the phenol content of the honey samples was significant after 1 and 30 days compared to the control group (P<0.05 and P<0.001, respectively) (Figure 5A). Phenol content continued to increase after 5 min of treatment on days 1, 30, 90 and 180 (P<0.01 and P<0.001 for 42 kHz; P<0.01 and P<0.0001 for 30 kHz) (**Figure 5B**). The 42 kHz treatment resulted in higher phenol levels than the 30 kHz treatment on all days (P<0.05, P<0.001, P<0.001, and P<0.0001 for days 1, 30, 90 and 180, respectively) (**Figure 5B**). After 10 min of treatment, both ultrasound frequencies significantly increased the phenol content compared to the control group on all days (P<0.0001 for both frequencies) (**Figure 5C**). The 42 kHz treatment also showed higher phenol levels than the 30 kHz treatment on all days (P<0.0001, P<0.001, P<0.0001 and P<0.001 for days 1, 30, 90and 180, respectively) (**Figure 5C**). The effect of ultrasound treatment at 30 and 42 kHz and 45 °C on the phenol content of the honey samples was also significant on all days compared to the control group (P<0.05 and P<0.01 for both frequencies) (**Figure 5D**). Phenol levels continued to increase after 5 minutes of treatment on all days (P<0.01 and P<0.01 for both frequencies on day 1; P<0.01 and P<0.001 for both frequencies on day 30; P<0.01 for both frequencies on day 90; P<0.001 and P<0.001 for both frequencies on day 180) (Figure 5E). After 10-min of treatment, both ultrasound frequencies significantly

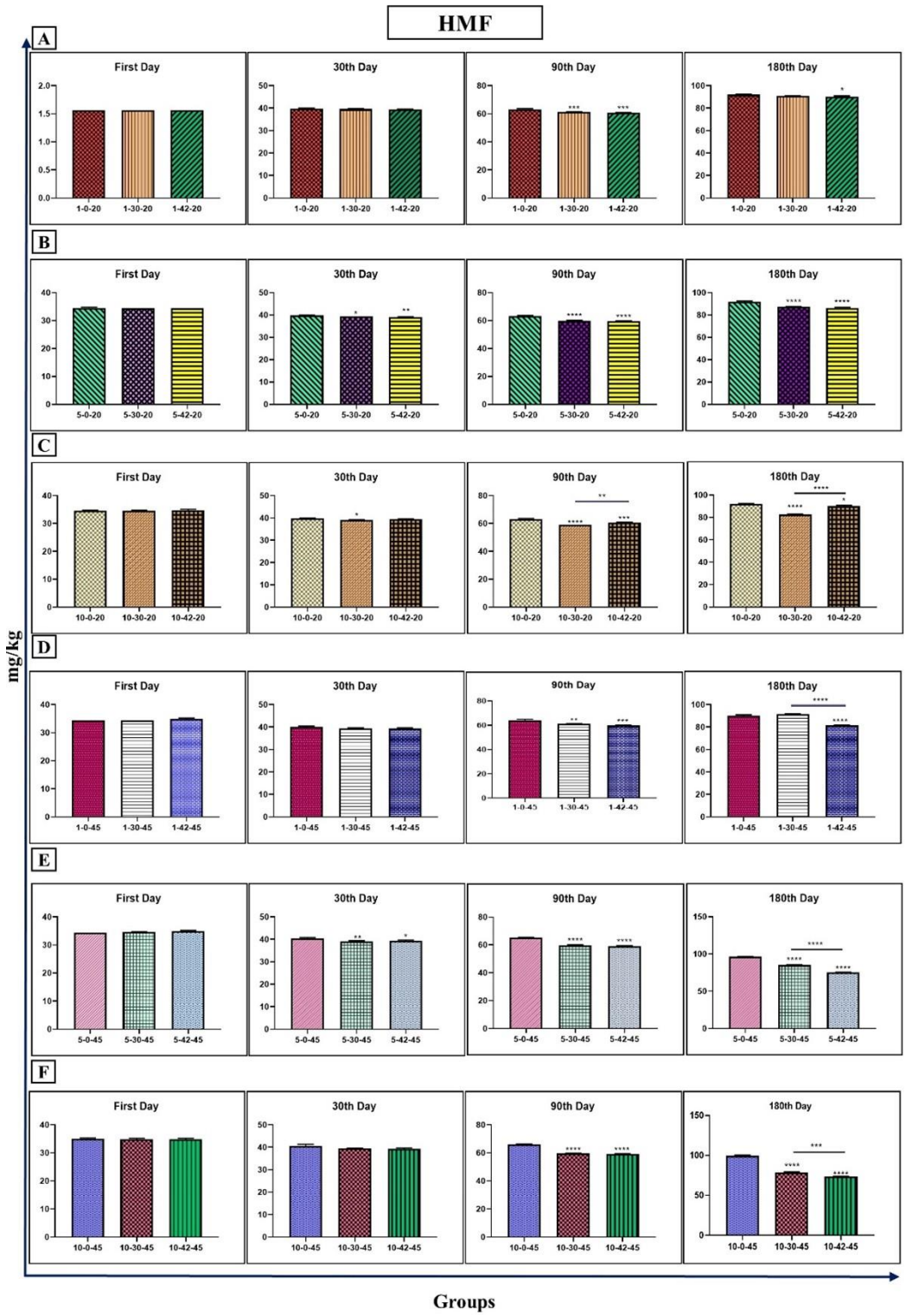

Figure 3. Effect of 0 (as control), 30, and 42 kHz ultrasound on the industrial honey HMF. The samples that treated at 30 kHz for 1 (**A**), 5 (**B**), 10 (**C**) minutes, and 20 °C. The samples that treated at 45 kHz for 1 (**D**), 5 (**E**), 10 (**F**) minutes, and 45 °C. Data are shown as the mean ± SD. The mean values with asterisks are significantly different ($p \leq 0.05$). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

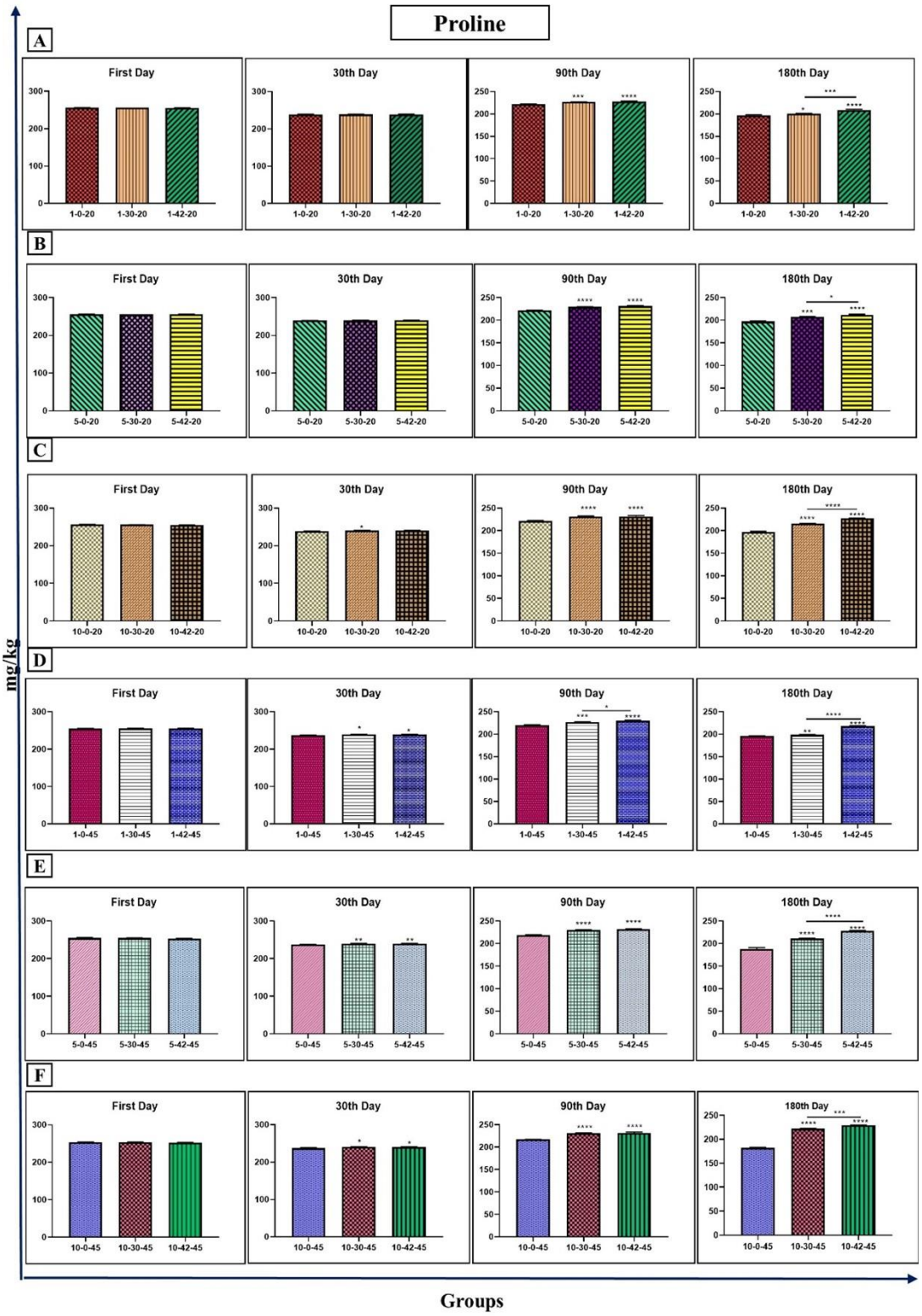

Figure 4. Effect of 0 (as control), 30, and 42 kHz ultrasound on the industrial honey proline. The samples that treated at 30 kHz for 1 (**A**), 5 (**B**), 10 (**C**) minutes, and 20 °C. The samples that treated at 45 kHz for 1 (**D**), 5 (**E**), 10 (**F**) minutes, and 45 °C. Data are shown as the mean ± SD. The mean values with asterisks are

increased phenol content compared to the control group on all days (P<0.0001 and P<0.0001 for both frequencies on day 1; P<0.001 and P<0.0001 for both frequencies on day 30; P<0.0001 and P<0.0001 for both frequencies on day 90; P<0.001 and P<0.0001 for both frequencies on day 180) (**Figure 5F**). The 42 kHz treatment resulted in higher phenol levels than the 30 kHz treatment after one and 90 days (P<0.01 and P<0.001, respectively) (**Figure 5F**).

3.3.5. ABTS content

The ABTS content of the samples treated with 30 and 42 kHz ultrasound at 20 °C for 1 minute decreased significantly after one day compared to the control group (P>0.05 and P<0.001, respectively) (Figure 6A). ABTS levels continued to decrease at 30, 90 and 180 days (P<0.01 and P<0.05 for both frequencies at day 30; P<0.05 for both frequencies at day 90; P<0.001 and P<0.01 for both frequencies at day 180) (Figure 6A). After 5-min treatment at 20 °C, both ultrasound frequencies significantly reduced ABTS content compared to the control group on all days (P<0.01 and P<0.0001 for both frequencies on day 1; P<0.05 and P<0.01 for both frequencies on day 30; P>0.05 and P<0.01 for both frequencies on day 90; P<0.05 and P<0.01 for both frequencies on day 180) (Figure 6B). After 10-min treatment at 20 °C, both ultrasound frequencies significantly reduced the ABTS content compared to the control group on all days (P<0.01 and P<0.001 for both frequencies on day 1; P<0.01 and P<0.001 for both frequencies on day 30; P<0.05 and P<0.01 for both frequencies on day 90; P<0.01 and P<0.001 for both frequencies on day 180) (Figure 6C). The ABTS content of the samples treated with 42 kHz ultrasound at 45 °C for 1 minute decreased after 1, 30 and 180 days compared to the control group (P<0.05, P<0.05, and P<0.01, respectively) (Figure 6D). The ABTS content was also lower after 30, 90 and 180 days than that of the samples treated with 30 kHz ultrasound (P<0.01, P<0.01and P<0.001, respectively) (Figure 6D). After a 5-min treatment at 45 °C, both ultrasound frequencies significantly reduced the ABTS content compared to the control group on all days (P<0.01 and P<0.001 for both frequencies on day 1; P<0.001 for both frequencies on day 30; P<0.01 and P<0.001 for both frequencies on day 90; P<0.001 for both frequencies on day 180) (Figure 6E). After 10 min treatment at 45 °C, both ultrasound frequencies significantly reduced the ABTS content compared to the control group on all days (P<0.001 for both frequencies on day 1; P<0.01 and P<0.001 for both frequencies on day 30; P<0.01 for both frequencies on day 90; P<0.001 for both frequencies on day 180) (Figure 6F).

3.4 Microbiological Parameters

The honey samples were examined for microbiological parameters (clostridium, total microbial count, moldand osmophiles). None of the treatment and control groups showed statistically significant changes in clostridium.

3.4.1 Total Count of Aerobic Mesophilic Bacteria

The effect of ultrasound (20 °C) on the total number of aerobic mesophilic bacteria (AMB) in the samples was significant. Ultrasound at 42 kHz reduced AMB more than at 30 kHz, and a longer exposure (5 minutes) reduced AMB more than a shorter exposure (1 minute). The reduction was observed at all time points (1, 30, 90and 180 days) compared to the control sample, except for 30 kHz ultrasound for 1 minute at day 180. Ultrasound (45 °C) significantly reduced the total number of AMB in the samples. The reduction was more pronounced with 42 kHz than with 30 kHz ultrasound and with longer exposure (10 minutes) than with shorter exposure (1 or 5 minutes). The reduction was observed at all time points (1, 30, 90 and 180 days) compared to the control sample, except for 30 kHz ultrasound for 1 minute on day 1. The statistical significance of the differences is shown in **Figure SF3**.

3.4.2 Mold

The results showed that ultrasound reduced the levels of mold in all experimental groups compared to the control group, except for the group treated with 30 kHz ultrasound at 20 °C on the first day. The reduction was more significant with higher frequency and higher temperature, suggesting that these factors enhanced the antimicrobial effect of ultrasound. The reduction was also the same at all time points (1, 90and 180 days), suggesting that ultrasound has a long-lasting effect on inhibiting mold growth. The statistical significance of the differences between the groups is shown in Figure 7-I.

3.4.3. Osmophiles

Ultrasound (20 °C for 1 minute) reduced the osmophil content in the honey samples after 90 and 180 days compared to the control sample (P<0.0001). There was no difference between 30 and 42 kHz ultrasound at 90 days, but 42 kHz ultrasound was more effective than 30 kHz ultrasound at 180 days (P<0.001) (Figure 7-II-A and B). Ultrasound at 45 °C for 1 and 5 minutes also reduced the osmophil content in the honey samples after 180 days compared to the control (P<0.001) (Figure 7-II-C and D).

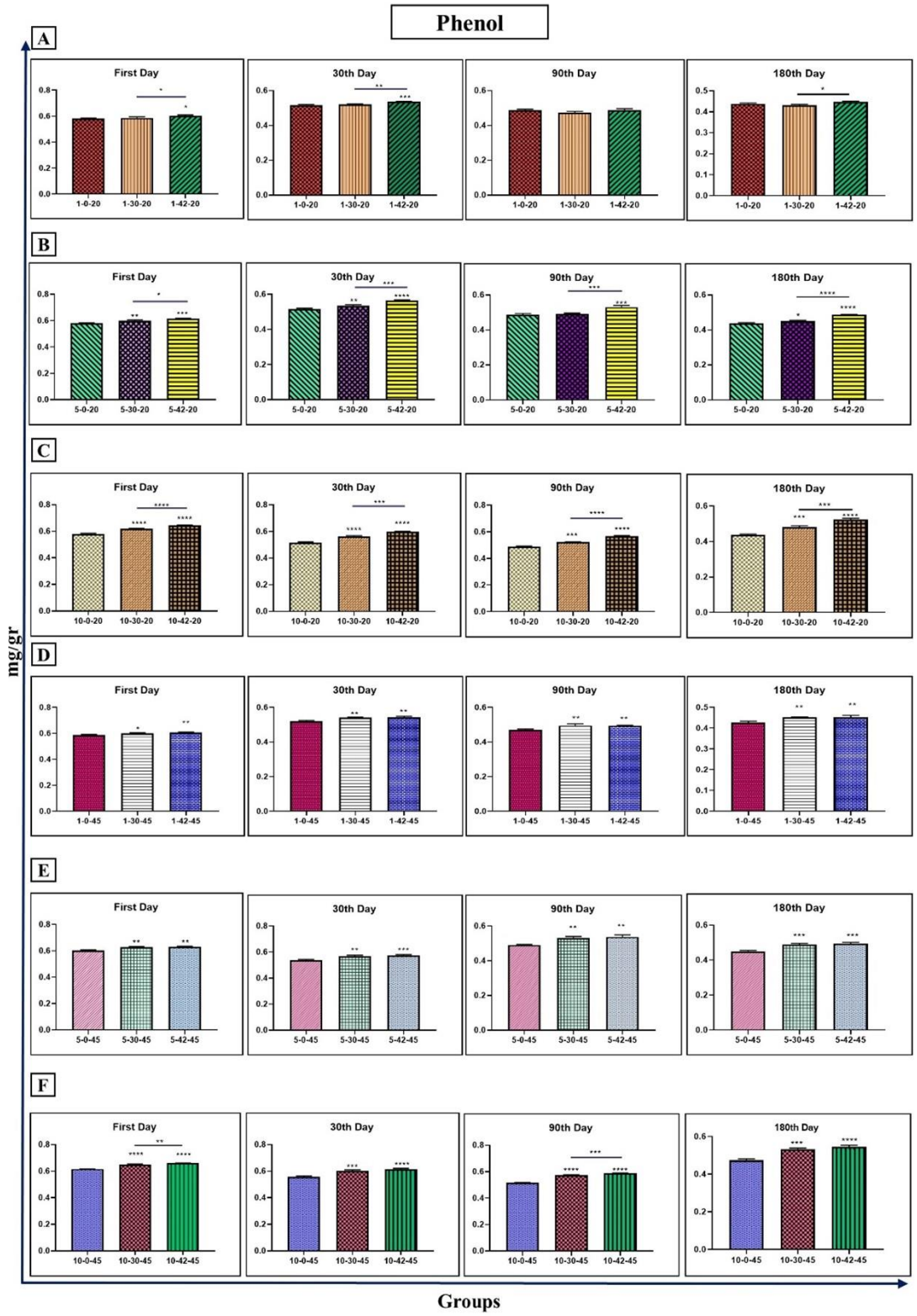

Figure 5. Effect of 0 (as control), 30, and 42 kHz ultrasound on the Industrial honey phenol. The samples that treated at 30 kHz for 1 (**A**), 5 (**B**), 10 (**C**) minutes, and 20 °C. The samples that treated at 45 kHz for 1 (**D**), 5 (**E**), 10 (**F**) minutes, and 45 °C. Data are shown as the mean ± SD. The mean values with asterisks are significantly different ($p \leq 0.05$). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

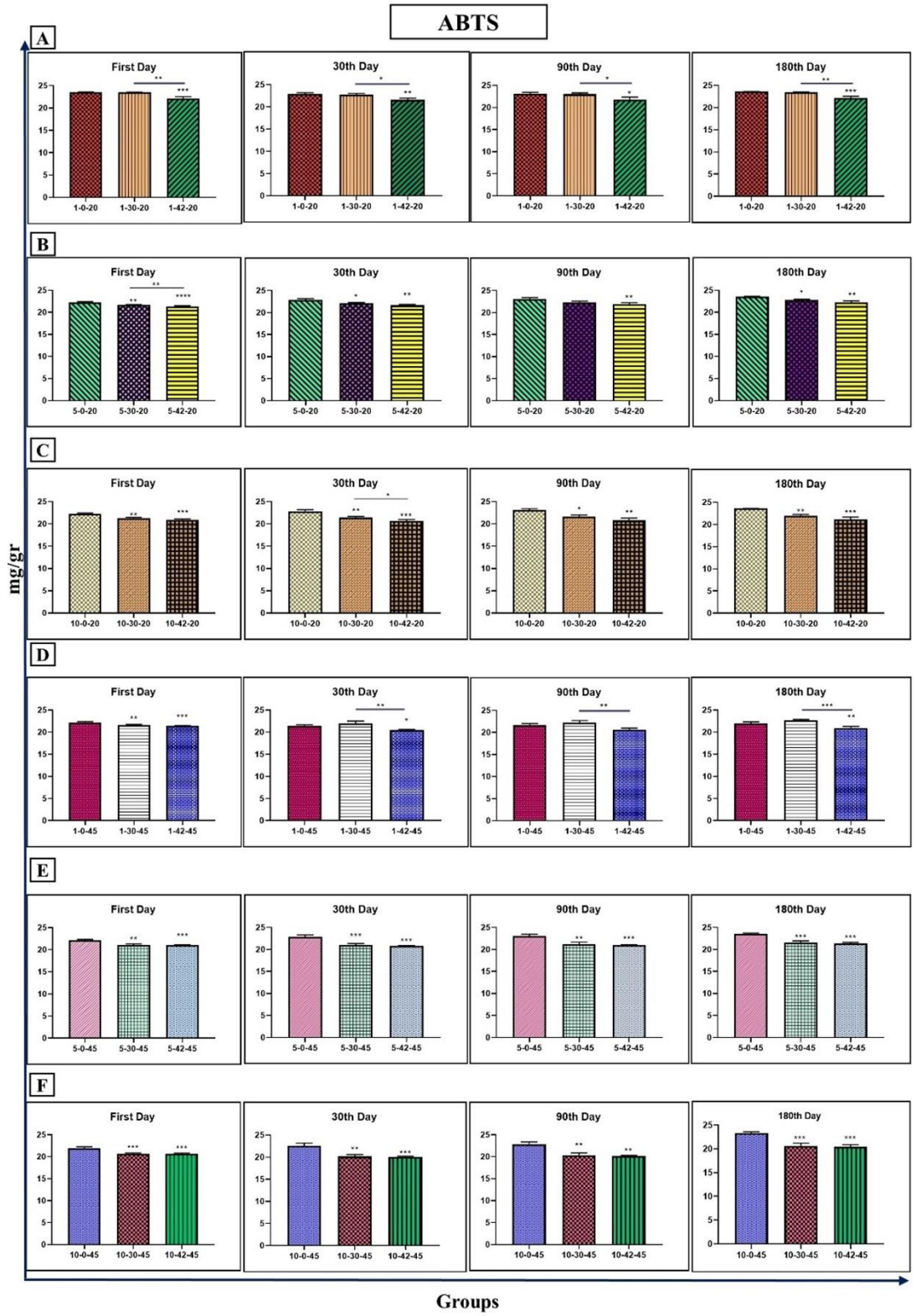

Figure 6. Effect of 0 (as control), 30, and 42 kHz ultrasound on the Industrial honey ABTS. The samples that treated at 30 kHz for 1 (**A**), 5 (**B**), 10 (**C**) minutes, and 20 °C. The samples that treated at 45 kHz for 1 (**D**), 5 (**E**), 10 (**F**) minutes, and 45 °C. Data are shown as the mean ± SD. The mean values with asterisks are significantly different ($p \leq 0.05$). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

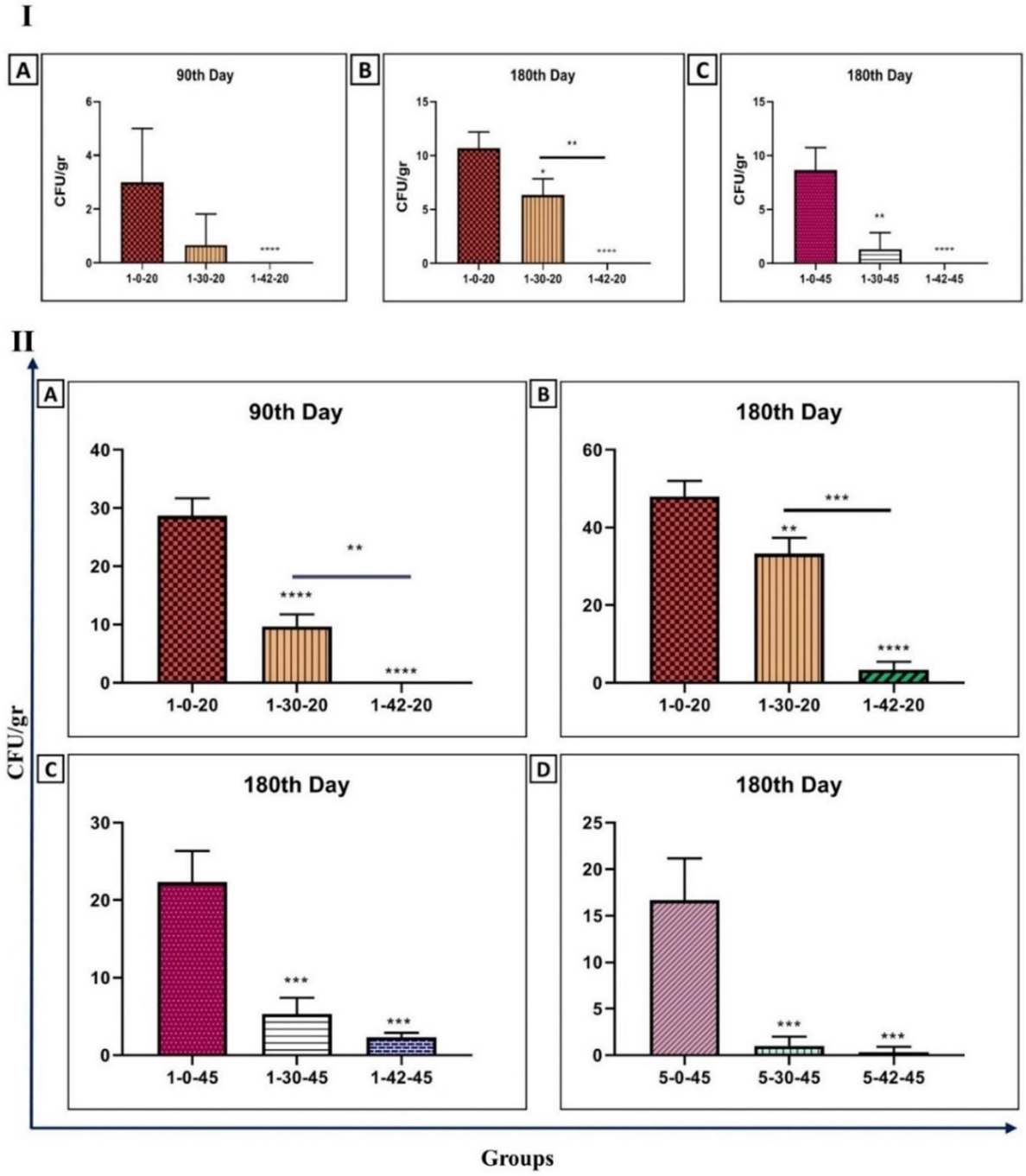

Figure 7. Effect of 0 (as control), 30, and 42 kHz ultrasound on the Industrial honey mold (**I**) and osmophiles (**II**). The samples were treated at 30 kHz and 42 kHz ultrasound for (20 °C for 1 minute) after 90 days (**I-A**), 180 days (**I-B**), and for (1 minute, and 45 °C) after 180 days (**I-C**). The samples that were treated at 30 kHz and 42 kHz ultrasound for (20 °C for 1 minute) after 90 days (**II-A**), 180 days (**II-B**), and for (1 minute, and 45 °C) after 180 days (**II-C**), and for (5 minutes, and 45 °C) after 180 days (**II-D**). Data are shown as the mean ± SD. The mean values with asterisks are significantly different ($p \leq 0.05$). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

4. Discussion

This study investigated how ultrasound affects the textural, physicochemical and microbial properties of industrial Iranian honey. Honey samples were treated with ultrasound of 30 or 42 kHz at 20 and 45 °C for 1, 5 and 10 minutes and the changes in the different parameters were monitored on the following days. Water and moisture are important factors for the quality, stability, shelf life, and crystallization of honey. In previous studies, the moisture content of single-flower eucalyptus honey from Portugal was reported as 16.65% (12), monofloral honeys from Turkey as 17.1-20% (13) and of honey samples from Argentina as 15-21% (14). In this study, our results showed that the moisture content of the groups treated with 30 and 42 kHz ultrasound (at 45 °Cfor 10 minutes) after 180 days was (12.76±0.94% and 12.57±1.13%) respectively, and was lower than that of the control group. The acidity of honey can vary according to its geographical origin and affect its texture (15). The pH range for single-flower eucalyptus honey from Portugal was reported as 3.83. Our results are consistent with previous studies, and the pH of the groups treated with 30 and 42 kHz ultrasound (at 45 °C for 10 minutes) after 180 days was (27.6±2.26 and 25.33±4.53, respectively). The acidity of honey is an important indicator of its fermentation. In previous studies, the acidity of Nigerian honey samples was reported to be 6.15-41.2 (eq/kg) (16) and Turkish samples 18.2-47.5 (eq/kg) (13). Our results are consistent with these findings. In this study, the acidity of the groups treated with 30 and 42 kHz ultrasound (at 45 °C for 10 minutes) after 180 days was (27.6±2.26 mEq and 25.33±4.53 mEq, respectively) after 180 days. The sugar content of the honey can help distinguish nectar honey from honeydew. The higher the ratio of fructosetoglucose, the more resistant the honey is to crystallization (17). The glucose (28.83±0.01% and 28.83±0.01%), fructose (45.13% and 44.83±0.12%), fructose-to-glucose (1.56±0.01% and 1.54±0.01%) and sucrose (4.69±0.43% and 4.66±0.2%) contents of the groups treated with 30 and 42 kHz ultrasound (at 45 °C, for 10 minutes) wer measured after 180 days. The breakdown product of fructose and glucose is HMF, and its production process depends on many factors, such as low pH, high water contentand high temperature (18). Since the pH of honey produced during food processing is higher than that of flower honey, HMF formation is also higher in flower honey than in factory-produced honey. The HMF content in other studies was 9.41mg/kg in eucalyptus honey in Portugal, 3.91 (mg/kg) in the study by Hasan (2013) in Iraqand 31.28 mg/kg in the study by Ozcan and Olmez (2014) in Turkey (17). The HMF rate in the groups treated with 30 and 42 kHz ultrasound (at 45 °C for 10 minutes) was (78.96±0.5 mg/kg and 74.35±0.5 mg/kg) after 180 days. Solis-Silva et al. (2017) investigated the effect of ultrasound on bioactive compounds and antioxidant activity in 5 samples of single-flower honey from Mexico during the storage period (19). The ultrasonic treatments were performed at 42 kHz for 5, 10and 15 minutes. The honey treated with ultrasound showed higher levels of phenolic acids, flavonoids, and antioxidant activity than the control sample, with the highest content observed in the 15-minute ultrasound treatment. Ultrasound was evaluated as a suitable alternative to the thermal method of honey without altering HMF (19). The HMF content in our samples was higher than in these studies due to the differences in plant species, climate and soil productivity. HMF is a degradation product of fructose and glucose, and its formation depends on several factors, such as low pH, high water content and high temperature. Flower honey has a higher pH than processed honey, so it also contains more HMF. In earlier studies, the HMF content of eucalyptus honey from Portugal was reported as 9.41mg/kg, of honey samples from Iraq as 3.91 (mg/kg) and of honey samples from Turkey as 31.28 mg/kg (17). The HMF content of the groups treated with 30 and 42 kHz ultrasound (at 45 °C for 10 minutes) was (78.96±0.5 mg/kg and 74.35±0.5 mg/kg) after 180 days. Solis-Silva et al. (2017) investigated the effect of ultrasound on bioactive compounds and antioxidant activity in 5 single-flower honey samples from Mexico during storage (19). The ultrasonic treatments were performed at 42 kHz for 5, 10 and 15 minutes. The treated honey showed higher levels of phenolic acids, flavonoids, and antioxidant activity than the control sample, with the highest levels observed in the 15 min ultrasonic treatment. Ultrasound was a suitable alternative to the thermal method of honey without altering the HMF (19). Our samples had a higher HMF content than these studies, which could be due to differences in plant species, climateand soil productivity. In this study, the diastase enzyme activity, proline contentand total phenol content of the ultrasonically treated honey were measured. These parameters provide information about the freshness, qualityand antioxidant activity of the honey. The group treated with 42 kHz ultrasound (at 45 °C for 10 minutes) shwoed the highest diastase enzyme activity (13.71±0.5 DN) and proline content (229.78±10.12mg/kg) after 180 days, which was comparable to previous studies on Iranian honey (17). The total phenol content of our samples was 0.55±0.001 (mg/gr), which was lower than other studies on monofloral honey from different countries (17) (20). Honey contains important antioxidant compounds that can protect cells from free radicals, and its phenolic content correlates with its antioxidant activity (20). In this study, the effect of ultrasound on the crystallization and quality

of honey was investigated. Previous studies reported that honey samples treated with ultrasound had higher microbiological quality than untreated control samples, and no yeasts and molds were detected in some of them. However, ultrasound also altered the hydroxymethylfurfural and diastase content of the honey, with values varying depending on the origin of the flowers (21). The antioxidant activity of honey was measured by two methods (DPPH and ABTS), and the results showed that the darkest and multifloral honey samples had the highest activity (21) (22). Janghu et al. (2017) evaluated the microbiological quality of honey treated with ultrasound and heat. Ultrasonic treatment (42 kHz, 45 °C, 10 minutes) reduced the total microbial count and preserved the clostridium, mold and osmophile levels after 180 days. Ultrasonic treatment also affected the physicochemical properties of honey, such as moisture, pH, diastase activity, HMF content and color (19). In the current study, the ultrasonically treated honey showed lower microbial load and better quality than the control honey. Honey is rich in antioxidants but can deteriorate over time due to crystallization and microbial growth. Ultrasonication is an alternative technology that can improve the shelf life and quality of honey by influencing its physical, biochemical, antioxidantand antimicrobial properties. This study showed that 42 kHz ultrasound at 45 ºC for 10 minutes was the most effective treatment to reduce the moisture, pH, acidity, sugar, microbial countand HMFand to increase the diastase, proline, and phenol of industrial honey. Ultrasound can be used in the food industry to produce and export higherquality honey.

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Authors' Contribution

Concept and design of the study: S.R
Collection of data: S.A.A
Analysis and interpretation of data: B.J
Drafting of the manuscript: S.A.A
Critical revision of the manuscript for important intellectual content: H.A
Statistical analysis: H.A
Administrative, technical, and material support: S.A

Ethics

Not applicable.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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