Bulgarian Journal of Agricultural Science, 31 (No 4) 2025, 829–840

Changes in physicochemical parameters and antimicrobial properties of some monofloral honeys at different temperatures

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Abstract

Gruznova, O. A., Gruznov, D. V., Sokhlikov, A. B., Lobanov, A. V. & Chesnokova, I. P. (2025). Changes in physicochemical parameters and antimicrobial properties of some monofloral honeys at different temperatures. *Bulg. J. Agric. Sci., 31*(4), 829–840

Natural bee honey can change its physicochemical and biological properties during storage. According to the requirements of Interstate Standard 19792–2017, honey should be stored at a temperature not exceeding 20°C. There is literature data on long-term storage of honey at low temperatures (from 0 to -20°C), which ensures the stability of some physicochemical parameters, in particular the content of hydroxymethylfurfural (HMF). However, despite the prospects for the practical use of such temperature regimes, it is necessary to take into account their possible negative impact on other physicochemical parameters of honey. The aim of this work was to study the influence of different temperature conditions of various botanical origins of honey storage on its physicochemical and biological parameters for a long time.

The samples of freshly pumped linden (n = 31), buckwheat (n = 34) and sunflower honey (n = 36) were used in studies. Generally accepted, standard and proprietary methods were used in this work.

For the first time, a wide range of physicochemical parameters of different botanical origins honey was analyzed before and during storage for 12 months at temperatures of 18, 10, 5, 0, -5, -10 and -18 (±2)°C. The evaluation of the physicochemical parameters of the samples carried out before storage showed their full compliance with the requirements of the Interstate Standards. The obtained initial values were taken as control. During the entire storage period, HMF level remained stable at -18°C, while a significant increase was observed at higher temperatures: after 12 months, at 18°C an increase of 472.5–488.1% was recorded (depending on botanical origin). However, MPC (25 mg/kg) was not exceeded. The decrease in the activity of the enzymes diastase, D-glucose-1-oxidase and catalase was revealed at all temperature conditions already in the 1st month of storage. Minimal changes were observed at 0 and 5°C. In this temperature range, hydrogen peroxide (H_2O_2) was stable: its concentration decreased by no more than 12.2% at the 12th month. Moisture content, total mass fraction of reducing sugars and acidity of all samples did not change significantly: the difference from the control was less than 5%. The antimicrobial test using cultures of *Escherichia coli* (strain 1257), *Staphylococcus aureus* (strain 209-P) and *Bacillus cereus* (strain 96), showed that honey samples stored at 5 and 0°C had the greatest inhibitory effect.

The data obtained demonstrates that the optimal temperature range for long-term storage of honey is between 5 and 0°C. These findings can be used as supplementary guidance when making revisions to regulatory documentation governing storage requirements for this product.

Keywords: bacteria; different temperature; honey; physicochemical parameters

Introduction

For many centuries, natural honey has been considered not only a valuable food, but also a medicinal product with pronounced antimicrobial activity, which is mainly due to its constituent monosaccharides, enzymes, organic and inorganic acids and phenolic compounds (Bucekova et al., 2019; Almasaudi, 2021; Proaño et al., 2021; Seraglio et al., 2021; Ayoub et al., 2023). According to some literature data, the antimicrobial activity of honey depends on the content of hydrogen peroxide in it (Mohammed et al., 2019; Brudzynski, 2020; Alygizou et al., 2021).

The beneficial properties of honey are preserved for a long time, but only if certain storage conditions are followed. According to the requirements of the Interstate Standard "Natural honey. Technical conditions" (2017), this product should be stored in places inaccessible to direct sunlight, at a temperature not exceeding 20°C in tightly sealed containers for 12 months from the date of examination, in hermetically sealed containers for 24 months from the date of packaging, and 12 months after opening hermetically sealed packaging.

There is data on storing honey at low temperatures (Kędzierska-Matysek et al., 2016; Ribeiro et al., 2018; Braghini et al., 2020; Pasias et al., 2022). Most often, this approach is used to suppress fermentation that can occur in honey with a moisture content of more than 20%, as well as to prevent crystallization (Zaikina, 2012; Kędzierska-Matysek et al., 2016; Villacrés-Granda et al., 2021; Ji et al., 2023). It is known that exposure to low temperatures (from 0 to -20°C) for a long time leads to a slowdown in chemical processes and, as a result, stabilization of such physical and chemical indicators of honey as electrical conductivity, humidity, acidity, content of phenolic compounds (Kędzierska-Matysek et al., 2016). It is also important to note that storing honey at these temperatures slows down the formation and accumulation of hydroxymethylfurfural (HMF), a product of monosaccharide dehydration that has neurotoxic, cytotoxic, genotoxic and mutagenic effects (Salhi et al., 2020; Besir et al., 2021; Laolue and Lerdsri, 2023).

Thus, the storage of honey at low temperatures might appear promising for practical application. However, it is essential to bear in mind that, while attempting to prevent the formation of HMF (hydroxymethylfurfural), we must should also take into account the potential adverse effects of low temperatures on other physico-chemical parameters of honey, particularly those that determine its antimicrobial activity.

In Russia, CIS countries and Europe, some of the most common and important honey plants are buckwheat (buckwheat, *Fagopyrum esculentum* Moench.), linden (lime linden, *Tilia cordata* Mill.) and sunflower (annual sunflower, *Helianthus annuus* L.). For example, in Russia (mainly in the forest-steppe zone of the European part of the country, Altai Territory, Transbaikalia and the Primorye-Amur region), as well as in Kazakhstan, buckwheat provides over 50% of marketable honey (Esenkina, 2022a). The honey productivity of buckwheat can reach 60–362 kg/ha (Naumkin, 2002). Another valuable honey plant, small-leaved linden, occupies a large area in the broad-leaved forest zone of the European part of Russia, spreading to the Urals, and is also found in the Crimea and the Caucasus. Its honey productivity averages 700–1000 kg/ha (Yakimov et al., 2022). Annual sunflower is a honey-bearing and pollen-bearing crop that provides honey production in Russia, Kazakhstan, Ukraine, as well as in the countries of southern and south-eastern Europe. Honey productivity is at the level of 40–50 kg/ha (Mazalov and Naumkin, 2021).

In connection with the above, the purpose of this work was to study the influence of different temperature storage conditions of honey of different botanical origins on its physicochemical and biological parameters over a long period of time.

Material and Methods

Object of study: samples of sunflower *Helianthus annuus* L. (n = 36), linden *Tilia cordata* Mill. (n = 31) and buckwheat honey *Fagopyrum esculentum* Moench. (n = 34), admitted to the laboratory of veterinary sanitation and environmental safety in beekeeping All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology, from the following regions of Russia: Rostov, Volgograd, Kursk, Voronezh and

Saratov regions, as well as from the Krasnodar Territory in the period 2021 – 2022. The samples were stored in climatic test chambers M-60/100-500 KTVH (JSC LOIP, Russia).

Confirmation of sample monoflorality

The monoflorality of honey samples was confirmed using microscopic analysis of palynological composition in accordance with Interstate Standard "Honey. Determination of the relative frequency of pollen" (2019). Light microscopy of honey samples was carried out using an AmScope T390C trinocular microscope (AmScope, China) at a magnification of ×400.

The frequency of pollen grains occurrence (X_p) was calculated using the formula:

$$X_p\,(\%) = G \cdot 100n^{-1}, \quad (1)$$

where $G = \Sigma G_i$ is the number of heather pollen grains in all counting fields; $n = \Sigma n_i$ – total number of counted pollen grains in all counting fields; 100 – coefficient for converting relative shares into percentages.

The photographic image of pollen grains was obtained using a Levenhuk M1000 PLUS digital camera (Levenhuk, USA).

Physicochemical studies

Physicochemical studies of honey were carried out in accordance with current Interstate Standards, the requirements of which are no less stringent than those of the Codex Alimentarius (1981) and Council Directive 2001/110/EU (2002). Also, it should be noted that the methods of some analyzes in the above standards are for the most part similar to the methods of Harmonized methods of the international honey commission (2009).

Determination of the mass fraction of water

The mass fraction of water (moisture) of the studied samples was determined by the refractometric method according to Interstate Standard "Honey. Refractometric method for determination of water" (2018) using an IRF-454 B2M refractometer (JSC Kazan Optical-Mechanical Plant (KOMZ), Russia).

Determination of the HMF

HMF was detected by RP-HPLC in accordance with Interstate Standard "Natural honey. Methods for determination of hydroxymethylfurfural" (2019). The analysis was carried out using a Shimadzu LC-20 Prominence chromatograph, an Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm), with a diode array detector (simultaneously detected wavelength range 210–400 nm, working wavelength 283 nm), in gradient mode at a flow rate of 1.0 mL/min. Peak retention time – 3.83 min. Commercial HMF was used with a content of the main substance not <99% (Sigma-Aldrich, USA), from which standard solutions were prepared with concentrations: 150.0; 100.0; 50.0; 30.0; 25.0; 20.0; 15.0; 10.0; 5.0; 1.0 μg/mL to create a calibration curve. Carrez solutions I and II for precipitation of proteins and stabilization of HMF in aqueous solution were also prepared according to the specified Interstate Standard.

The 5-HMF amount in honey samples (mg/kg) was calculated based on a previously constructed calibration curve using the formula:

$$M_{5\text{-}HMF} = C_{5\text{-}HMF} \cdot V_{sample} / m_{money}, \quad (2)$$

where $C_{5\text{-}HMF}$ – the 5-HMF concentration, determined from the calibration graph (μg/ml); V_{sample} – volume of analyzed sample (mL); m_{money} – mass of the analyzed honey sample (g).

Determination of the total mass fraction of reducing sugars

Determination of the glucose and fructose was carried out according to Interstate Standard "Honey. Method for determination of sugars" (2018) using the colorimetric method. The essence of this method is to determine the optical density of a solution of potassium iron sulfide – $K_3[Fe(CN)_6]$ (≥ 99.5%, LenReaktiv, Russia) after its interaction with reducing sugars of honey. The analysis was carried out on a KFK-2 photocolorimeter (JSC Zagorsk Optical-Mechanical Plant, Russia) at a wavelength λ = 440 nm.

Determination of the free acidity

In accordance with Interstate Standard "Honey. Method determination of pH and free acidity" (2019), to determine free acidity, aqueous solutions of honey samples were titrated with a 0.1 M solution of sodium hydroxide – NaOH (99.2%, LenReaktiv, Russia) to pH 8.30. The pH value was monitored using a potentiometric analyzer (pH meter) – Hanna edge with pH electrode HI11310 (Hanna Instruments, USA). The results were expressed in milliequivalents of HCl in 1 kg of honey – meq/kg.

Free acidity (K) in milliequivalents of HCl in 1 kg of honey was calculated using the formula:

$$K = V \cdot 10, \quad (3)$$

where V – the volume of sodium hydroxide solution of concentration c(NaOH) = 0.1 mol/dm^3, consumed for titration, cm^3; 10 – conversion factor for the mass of honey 1 kg.

Determination of the diastase activity

Diastase activity was determined based on Interstate Standard "Honey. Methods for determination of sucrose ac-

tivity, diastase activity, insoluble matters" (2017) by the colorimetric method and expressed by the amount of cm^3 of 1% (wt.) starch solution, digested in 1 h by amylolytic enzymes contained in 1 g of anhydrous honey.

The value of diastase activity (Gothe units) was calculated using the formula:

$$X = 100 \cdot 80(D_C - D_{test}) \cdot D_C^{-1} \cdot (100 - W)^{-1}, \quad (4)$$

where 80 – the conversion factor; D_C – optical density of the control solution; D_{test} – optical density of the test solution; W – mass fraction of water in honey (%).

Determination of the catalase activity

Catalase activity was determined in accordance with the method developed by Aganin (1985), according to which the activity was expressed in mm^3 of oxygen released, when catalase contained in 1 g of honey is exposed to 10 ml of 1% (wt.) H_2O_2 solution for 24 h.

Determination of the D-glucose-1-oxidase activity

D-glucose-1-oxidase activity was measured according to the method described in the work of Flanjak et al. (2015). The essence of this method is the reduction of H_2O_2 to water by peroxidase using 3,3'-dimethoxybenzidine as a substrate. The resulting colored product was detected spectrophotometrically and had a maximum absorption at a wavelength of $\lambda = 400$ nm. The reaction mixture consisted of 0.7 ml of 2.14 mM glucose (≥ 99.5%, Merck, Germany), dissolved in 100 mM sodium phosphate buffer (pH 6.1), 0.1 mL of ethanol solution 3, 3'-dimethoxybenzidine (1 mg/mL) (Merck, Germany), 0.1 mL horseradish peroxidase (Serva, Germany), prepared in 100 mM sodium phosphate buffer, pH 6.1 and 0.1 mL honey solution (0.2 g/mL), prepared in 100 mM sodium phosphate buffer (pH 6.1). After adding the honey solution, the reaction mixture was incubated for 30 min at 37°C, and stopped by adding 0.1 mL of 1 M hydrochloric acid – HCl (≥ 37%, LenReaktiv, Russia). The absorption of the mixture was measured at $OD_{400\ nm}$ (spectrophotometer PE5400UF "Ekroskhim", Russia), against a control sample consisting of all reaction components except honey. The measured absorbance was then corrected to the absorbance of the zero minute reaction mixture. The zero-minute reaction mixture consisted of all components, but the honey solution was added after the addition of hydrochloric acid. The results were expressed in μg H_2O_2/h g honey.

Determination of the Hydrogen Peroxide content

The H_2O_2 content in honey was determined using a previously adapted and modified by us spectral-iodometric method, the essence of which is that when potassium iodide interacts with H_2O_2 in an acidic medium, molecular iodine is released, forming a complex anion with an excess of iodide anion, which is recorded by the electron absorption method spectroscopy (Lobanov et al., 2008). Analytical solutions of honey samples were obtained by weighing 1.0±0.01 g of each sample, after which 2.0 mL of HPLC water ("Khimiya XXI Vek", Russia), was added to them and mixed for 3-5 min on Vortex mixer VM-300S (Joan Lab Equipment, China), or ultrasonic homogenizer Bandelin Sonopuls HD 2200 (Bandelin, Germany) until completely dissolved. From the resulting analytical solutions, 1.0 mL was taken and 1.0 mL of 0.2 M sulfuric acid (H_2SO_4, ≥ 1.0 m LenReaktiv, Russia) was added, after which carbon dioxide was passed through. At the last stage of sample preparation, a 5% solution of potassium iodide (KI, 99.5%, Rushim, Russia) was added to the analytical solutions, through which carbon dioxide was previously passed. The prepared analytical solutions of honey samples were incubated at room temperature for 24 h in a dark place. Electronic absorption spectra were recorded using a PE5400UF spectrophotometer (Ekroskhim, Russia). The absorption maximum was observed at $\lambda = 351$ nm.

The H_2O_2 concentration was calculated using the formula:

$$C = R \cdot (D - D_0) / \varepsilon, \quad (5)$$

where C – the concentration of H_2O_2 in the analytical solution (μmol/L); D – optical density of the analytical solution ($\lambda = 351$ nm); D_0 – optical density of the control solution (distilled water); R – coefficient taking into account dilution; ε – molar extinction coefficient ($\varepsilon_{351} = 26400\ L \cdot mol^{-1} \cdot cm^{-1}$).

Determination of the antimicrobial activity

The antimicrobial activity of the studied honey samples before and on the 12th month of storage, at the indicated temperatures was determined using the method of diffusion into agar with microorganisms from daily test cultures. *Escherichia coli* (strain 1257), *Staphylococcus aureus* (strain 209-P) and *Bacillus cereus* (strain 96), were taken as test microorganisms (collection of All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology). From the washout of each culture, a suspension was prepared with the number of microbial cells in 1 mL equal to 10^4 (the number was set according to the turbidity standard), and sown in pre-prepared sterile Petri dishes with MPA with a well in the center (6 dishes for each microorganism-honey sample pair), in which contained honey samples weighing 0.1±0.01 g. The cultures were incubated for 24 h at a temperature of 37°C The results were recorded based on the diameter of the growth inhibition zone around the honey sample. As a control, Petri dishes with a test culture were used, into which no honey sample was added.

Statistical Analysis

Statistical processing of the results was carried out using MS Excel 2010 software. All measurements were performed in triplicate. The significance of differences in mean values was established using Student's *t*-test at a significance level of $p < 0.05$.

Results and Discussion

According to Interstate Standard "Monofloral honeys. Technical conditions" (2022), natural linden and buckwheat honey should contain at least 30% of dominant pollen grains, sunflower honey – at least 45%. Moreover, the total number of pollen grains during palynological analysis cannot be less than 500. Table 1 shows data on the average content of pollen grains (in %) in the studied honey samples.

As can be seen, the studied samples fully complied with the specified requirements: the total number of pollen grains of linden honey exceeded 600, of which 42.2% were identified as pollen grains of linden (*Tilia*). The results of palynological analysis of buckwheat and sunflower honey samples were also positive: the total number of pollen grains was 620 and 680, respectively. Of these, the dominant grains were 45.3% (*Fagopyrum*) and 63.2% (*Helianthus*). The morphological characteristics of all pollen grains completely matched with the data given in literature sources (Burmistrov and Nikitina, 1990; Karpovich et al., 2015).

Before storing samples of freshly pumped linden, buckwheat and sunflower honey, their physicochemical parameters were assessed (Table 2).

As can be observed from the table, all parameters governed by Interstate standards were within established limits. The determination of the activity of catalase and D-glucose-1-oxidase, as well as the H_2O_2 content is currently not regulated by the legislation of Russia. However, there are literature sources that provide methodologies for their determination, as well as data demonstrating the effectiveness of their application when analyzing the chemical composition of honey (Aganin, 1985; Flanjak et al., 2015; Gruznova et al., 2022).

In order to study changes in physicochemical and biological properties of honey during prolonged storage under different temperature conditions, initial values obtained were used as a control.

The equal weight of honey samples (100±0.1 g) were placed in plastic containers with hermetically screwed lids, and stored in climatic chambers at temperatures of 18, 10, 5, 0, -5, -10 and -18 (± 2)°C, humidity 55 (±3)% for a period of 12 months. The choice of temperatures was due to the fact that 18 ± 2°C is the maximum temperature limit for honey

Table 1. Average content of pollen grains in honey samples

Botanical name of honey plants	Pollen grains number, %
Linden honey	
Linden (*Tilia cordata* Mill.)	42.2
Hybrid clover (*Trifolium hibridum* L.)	5.3
Clover (*Trifolium pratense* L.)	4.2
White clover (*Trifolium repens* L.)	4.1
Umbelliferae (*Apiaceae* Lindl.)	11.1
Bedstraw (*Galium* L.)	4.1
Willow (*Salix* L.)	6.2
Mint (*Mentha* L.)	1.6
Meadowsweet (*Filipendula ulmaria* (L.) Maxim.)	6.4
Common Vetch (*Vicia* L.)	2.6
Greater Knapweed (*Centaurea scabiosa* L.)	1.5
Cornflower meadow (*Centaurea jacea* L.)	3.4
Raspberry (*Rubus idaeus* L.)	3.3
Honeydew elements	1.9
Undefined	2.1
Buckwheat honey	
Buckwheat (*Fagopyrum esculrntum* M.)	45.3
Crucíferae (*Cruciferae* Juss.)	14.8
Sweet clover (*Melilotus officinalis* (L.) Lam.)	7.5
Fireweed (*Chamaenerion angustifolium* L.)	1.2
Cornflower (*Centaurea cyanus* L.)	2.3
Umbelliferae (*Apiaceae* Lindl.)	8.6
Raspberry (*Rubus idaeus* L.)	3.5
Clover (*Trifolium pratense* L.)	2.7
White clover (*Trifolium repens* L.)	3.8
Loosestrife (*Lythrum salicaria* L.)	1.4
Meadowsweet (*Filipendula ulmaria* (L.) Maxim.)	4.7
Honeydew elements	1.4
Undefined	2.8
Sunflower honey	
Sunflower (*Helianthus annuus* L.)	63.2
Crucíferae (*Cruciferae* Juss.)	4.3
Cornflower (*Centaurea cyanus* L.)	3.6
Sweet clover (*Melilotus officinalis* (L.) Lam.)	5.6
White clover (*Trifolium repens* L.)	2.3
Clover (*Trifolium pratense* L.)	3.1
Hybrid clover (*Trifolium hibridum* L.)	2.6
Umbelliferae (*Apiaceae* Lindl.)	3.8
Scrophulariáceae (*Scrophulariaceae* Juss.)	1.4
Field thistle (*Cirsium arvense* (L.) Scop.)	6.6
Bird's-foot trefoil (*Lotus corniculatus* L.)	1.2
Honeydew elements	1.1
Undefined	1.2

Table 2. Analysis of the chemical composition of linden, buckwheat and sunflower honey samples

Analyzed parameter, units	Regulated norm by Interstate Standards	Samples		
		Linden honey	Buckwheat honey	Sunflower honey
Interstate standard 31766-2022				
Moisture content, %	not more than 20.0*	18.2±0.5	17.9±0.7	16.8±0.8
	not more than 19.0**			
	not more than 18.0***			
Reducing sugars content, %	at least 66.0*	86.6±3.9	86.8±3.6	89.9±1.6
	at least 68.0**			
	at least 71.0***			
Free acidity, meq/kg	10.0–25.0*	24.6±0.8	26.3±1.1	25.1±0.9
	10.0–40.0**			
	10.0–30.0***			
Diastase, Gothe units	at least 8.0*	17.5±0.8	40.2±0.8	16.5±1.4
	at least 18.0**			
	at least 15.0***			
Interstate standard 19792-2017				
HMF content, mg/kg	not more than 25.0	2.36±0.11	3.25±0.15	2.44±0.1
Non-regulated parameters				
Catalase, mm^3 O_2	–	330±14	485±22	850±22
D-glucose-1-oxidase, μg H_2O_2/h g	–	432.1±10.5	385.9±12.3	187.8±9.1
H_2O_2 concentration, × 10^{-4} M	–	1.71±0.05	1.48±0.07	0.79±0.03

Note: Interstate Standards requirements for: *linden honey, **buckwheat honey, ***sunflower honey.

storage according to Interstate Standard requirements (no more than 20°C), storage at 10, 5, 0, -5, -10 and -18 (±2)°C – modeling the effect of low temperatures, at which changes in the chemical composition of honey were observed. The resulting dynamics are presented in Figure 1.

The mass fraction of HMF in all analyzed samples gradually increased at all temperatures, with the exception of -18°C. This process began to occur most intensively after the 3rd month of storage. The dependence of the increase in HMF on storage temperature is monitored in Figure 1. The maximum HMF content was observed in samples stored at 18°C. At the 12th month, its increase relative to the initial value was found to be 472.5% in samples of linden honey, and by 484.0 and 488.1% for buckwheat and sunflower honey. However, the maximum permissible concentration (MPC), which is 25 mg/kg, was not exceeded. Throughout the entire study period, the HMF content was stable at -18°C. In samples of linden and sunflower honey during storage, a slight decrease was noted: in the 12th month – by 5.9 and 2.5%, respectively. The data obtained are consistent with the results of research

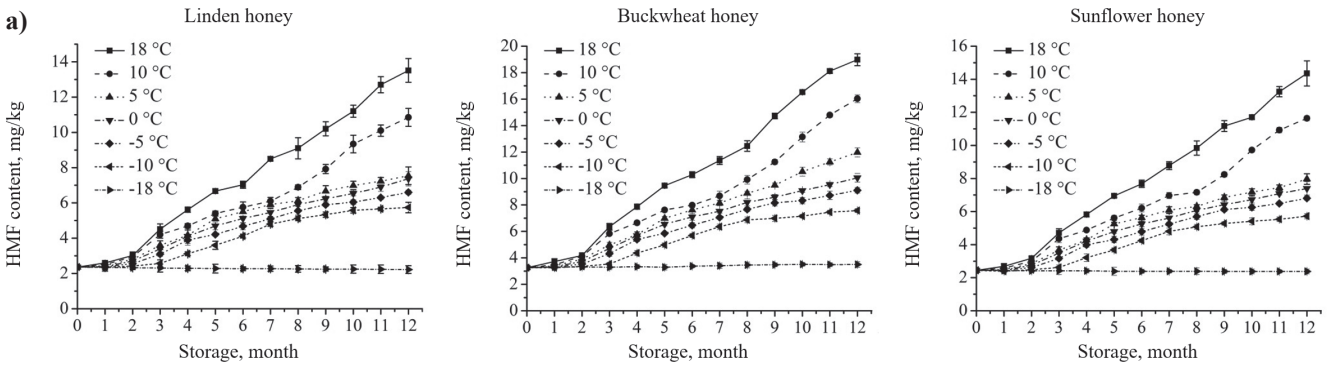

Fig. 1. Changes in the physicochemical parameters: HMF content (a), diastase activity (b), D-glucose-1-oxidase activity (c), catalase activity (d), H_2O_2 concentration (e) of linden, buckwheat and sunflower honey samples during storage for 12 months at different temperatures. 0 month – initial data (control) obtained before storing samples

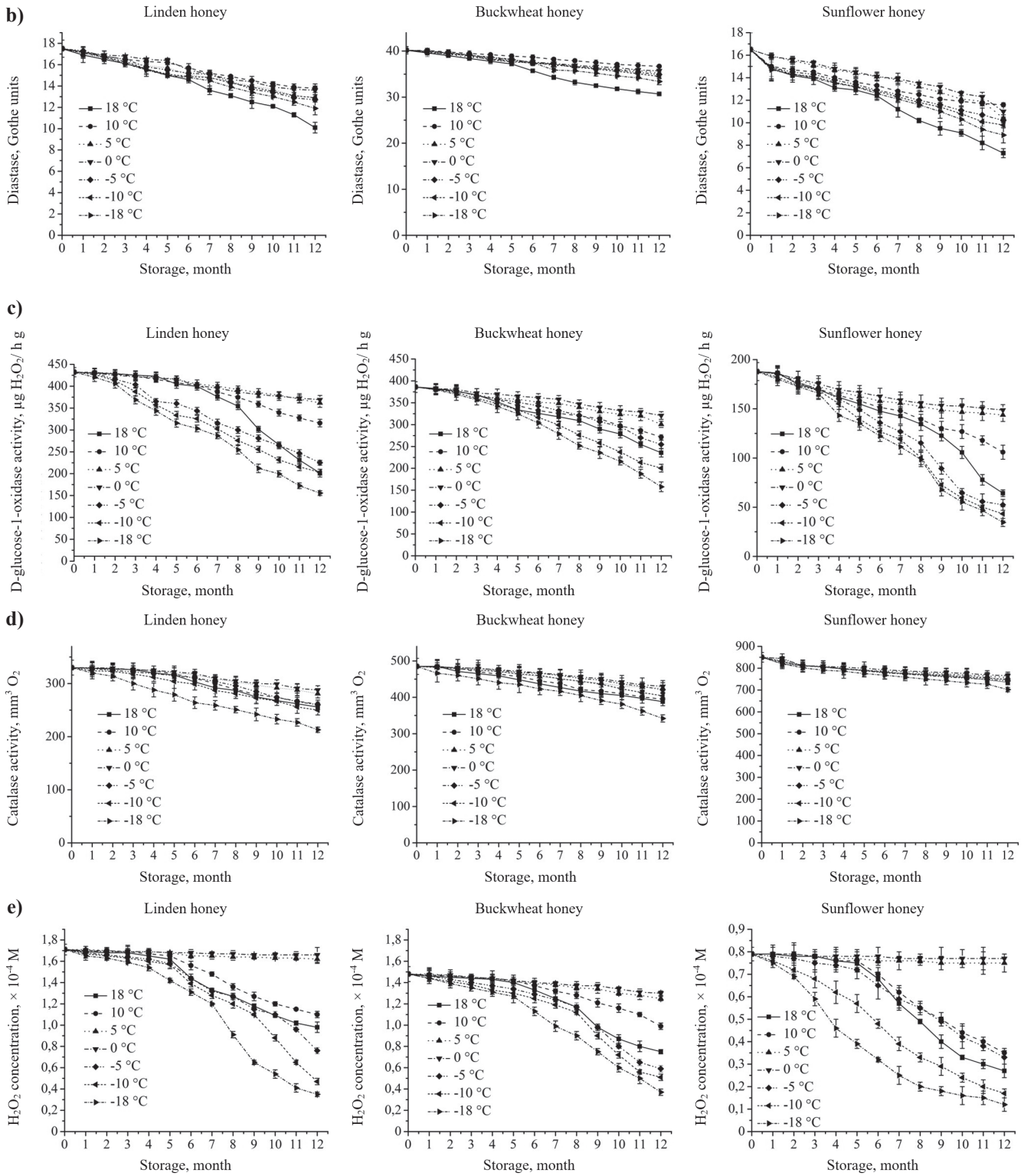

Fig. 1. Continued

by Pasias et al. (2022) and Kędzierska-Matysek et al. (2016), which demonstrated the stability of HMF in honey samples of various botanical origins stored at subzero temperatures, and an increase in the level of this indicator in samples stored at room temperature.

A decrease in diastase activity of honey samples was observed already in the 1st month of storage at all temperature conditions (Figure 1). However, for buckwheat honey samples stored at all specified temperatures for the entire study period, this parameter was within the limits established by Interstate Standard. The same trend was observed for linden honey, with the exception of samples stored for 12 months at 18°C (average deviation from the standard – 0.9 Gothe units). The diastase of sunflower honey samples was below the permissible norm in all experimental variants, except for storage at 0 and 5°C for 3 months.

It should be noted that as a result of storage at 18°C, the diastase activity of all samples decreased significantly by the end of the study. Thus, in the 12th month of the experiment, in samples of linden honey it decreased by 42.3%, in samples of buckwheat honey – by 66.4%, in sunflower honey – by 55.8%. Temperature -18°C also had an unfavorable effect: at the 12th month in linden honey, the diastase number was 11.9 ± 0.96 Gothe units, which is 32.0% less than before storage; in buckwheat – 33.4±0.7 Gothe units (difference from the initial value of 16.9%), and in sunflower – 8.9±0.7 Gothe units (the difference was 46.1%). We noted that minimal changes in this parameter occurred at 0, 5 and 10°C. Pasias et al. (2022) when studying samples of pine, eucalyptus, cotton and thyme honey (Lamia, Greece), showed a decrease in diastase by 33.0-44.4% as a result of storage at room temperature for 12 months and by 5.6-9 .1% at 0°C, as well as no significant changes in this indicator at -18°C. Due to the fact that the researchers recorded the results once every three months, it is quite difficult to track at what stage the decrease in diastase began to occur. Kędzierska-Matysek et al. (2016) reported a decrease in diastase activity of rapeseed honey (*Brassica napus* L.), stored for a year at -20°C, by more than 7% relative to the control. Thus, there is no data on this parameter that completely coincides with each other. It can probably be explained by differences in the resistance of this enzyme to low temperatures depending on the botanical origin of the honey.

The D-glucose-1-oxidase activity began to fall in the first month of storage at all temperature conditions just like the diastase number (Figure 1). The intensity of the decrease in enzyme activity depended on the storage temperature: the maximum was observed in the 12th month at -18°C: by 64.0, 59.1 and 81.6% (compared to the control) for samples of linden, buckwheat and sunflower honey, respectively. Also, significant changes (by 34.0-76.9% depending on the botanical origin of honey) occurred as a result of storage at -10, -5 and 18°C. The most suitable storage condition was found to be at temperatures between 5 and 0°C, as the enzyme activity in the 12-month study period decreased by only 14.6–23.5%.

Analysis of catalase enzyme content in stored honey samples demonstrated its greater resistance to both low and room temperatures, compared to the enzymes described above. Despite the fact that, as in the case of diastase and D-glucose-1-oxidase, a gradual decrease in its activity was noted throughout the entire period of research, this process did not occur so intensely at all temperature conditions (Figure 1). It should be noted that in samples of sunflower honey, characterized by an initially high content of catalase (850±22 mm^3 O_2), minimal changes in this indicator were noted: in the 12th month, catalase decreased by 10.0-17.4%, depending on the storage temperature. At the same time, for samples of linden and buckwheat honey, in which the initial level of catalase activity was significantly lower (330 ± 14 and 485 ± 19 mm^3 O_2, respectively), the activity decreased by 12.7-35.5% and 11.3-29.5%, respectively, by the end of the experiment. In general, it can be noted that, regardless of botanical origin, minimal changes in enzyme activity were observed at temperatures of 5 and 0°C.

The content of H_2O_2 in honey samples was quite stable at temperatures of 5 and 0°C: in the 12th month, its concentration in linden honey samples decreased by 5.3 and 2.9%, respectively, in buckwheat samples – by 15.5 and 12.2%, respectively, sunflower – by 5.1 and 2.5%, respectively. In samples stored at 18°C, a significant decrease in H_2O_2 began at the 6th month, which coincides with our previously obtained data. The same trend was observed for samples stored at 10 and -5°C. At the same time, storage at -10 and -18°C led to a decrease in this indicator already in the first month (Figure 1). Changes in H_2O_2 that occurred during storage, may be due to the following factors: instability of the H_2O_2 molecule itself at temperatures outside the range of 0-5°C; a decrease at 18°C, as well as at low temperatures, in the activity of D-glucose-1-oxidase, under the influence of which H_2O_2 is formed in honey as a result of a two-stage redox reaction; relative stability of the enzyme catalase, which hydrolyzes H_2O_2 to water and oxygen (Brudzynski, 2020).

It was found that as a result of 12-month storage of honey samples at the indicated temperatures, moisture content, total mass fraction of reducing sugars and free acidity, did not change significantly (the difference from the control is less than 5%), and were within the limits regulated by Interstate Standards. In this regard, Table 3 shows the data obtained at the end of the experiment – at the 12th month of storage, as well as the initial values of these indicators (control).

Table 3. Content of moisture, reducing sugars and free acidity in linden, buckwheat and sunflower honey samples stored at different temperatures for 12 months

Analyzed parameter	Control	Storage temperature, °C						
		18	10	5	0	-5	-10	-18
Linden honey								
Moisture content, %	18.2±0.5	17.3±0.2	17.4±0.2	17.4±0.2	17.4±0.3	17.5±0.3	17.6±0.3	17.9±0.6
Reducing sugars content, %	86.6±3.9	83.8±3.7	84.5±2.9	84.6±1.7	84.8±3.5	85.1±3.8	85.2±3.5	85.7±2.3
Free acidity, meq/kg	24.6±0.8	25.7±1.2	25.6±0.3	25.5±1.2	25.5±0.9	25.4±0.7	24.8±0.6	24.7±0.2
Buckwheat honey								
Moisture content, %	17.9±0.7	17.1±0.5	17.2±0.6	17.3±0.5	17.4±0.4	17.5±0.7	17.8±0.3	17.8±0.5
Reducing sugars content, %	86.8±3.6	83.9±1.3	84.3±2.6	84.9±2.4	85.3±2.8	85.6±2.8	86.2±1.9	86.7±3.1
Free acidity, meq/kg	26.3±1.1	27.3±0.7	27.1±0.9	27.0±0.5	26.9±1.3	26.8±1.1	26.8±1.2	26.6±0.6
Sunflower honey								
Moisture content, %	16.8±0.8	16.0±0.2	16.1±0.3	16.2±0.1	16.2±0.1	16.2±0.2	16.3±0.3	16.4±0.2
Reducing sugars content, %	89.9±1.6	88.4±1.4	89.4±2.7	89.5±2.9	89.6±3.1	89.7±2.8	89.8±2.5	89.8±3.8
Free acidity, meq/kg	25.1±0.9	25.8±1.3	25.6±1.2	25.6±0.8	25.5±1.2	25.4±0.7	25.3±0.9	25.2±1.1

The maximum decrease in the moisture content of linden honey samples was 4.9% at a temperature of 18°C (relative to the control), buckwheat and sunflower honey – 4.5% and 4.8%, respectively, at the same temperature. Minimal deviations from the initial values were observed at 0°C and negative temperatures. The results obtained are consistent with the data obtained by Kędzierska-Matysek et al. (2016). The stability of the moisture of samples stored at low temperatures can be explained by the faster transition of water molecules into a bound form (Bakier, 2006).

The same trend was observed in relation to reducing sugars: the smallest amount of them was noted in the analyzed samples stored at 18°C. Thus, for samples of linden and buckwheat honey the deviation was 3.3%, and for sunflower honey – 1.7%. According to Ribeiro et al. (2018), the content of reducing sugars in honey samples stored at -18°C, for 180 days changed by 4.9%. The results presented in the work of Esenkina (2022b), demonstrated a deviation of this indicator by 4.2% after 3 months of storage at a similar temperature.

Free acidity for 12 months at all temperatures, on the contrary, it increased slightly. The following pattern was identified: the higher the storage temperature, the higher the acidity value. At 18°C, this parameter increased by 4.5% (for linden honey), 3.8% for buckwheat honey, and 2.7% for sunflower honey. Braghini et al. (2020) noted the same trend, when studying honey stored at 22°C.

The changes that occurred in the content of reducing sugars and free acidity, are due to the speed of the dehydration process, as a result of which simpler compounds are formed from monosaccharides, including organic acids, as well as HMF (which explains its stabilization at low temperatures). This is consistent with the findings of Kędzierska-Matysek (2016) who, in her studies of rapeseed honey showed that when the storage temperature of this product was lowered, chemical reactions in it proceeded much more slowly.

In order to study the effect of temperature conditions during long-term storage of honey on its antimicrobial effect, a test was carried out on *Escherichia coli* (strain 1257), *Staphylococcus aureus* (strain 209-P) and *Bacillus cereus* (strain 96, vegetative form). These microorganisms are the most common test cultures when conducting microbiological studies, as well as studying various biochemical mechanisms. In addition, *E. coli* and *S. aureus* are known as contaminants of the mucous and skin epithelium of humans and animals, therefore, under certain conditions, they can cause inflammatory processes and food poisoning. *B. cereus*, although a representative of soil bacteria, can also be a cause of toxic infections (Chhawchharia et al., 2022; Bucekova et al., 2023; Krupyanskii et al., 2023; Rahnama et al., 2023).

The studied samples of freshly pumped honey of various botanical origins had a clear inhibitory effect on the growth of all test cultures (Figure 2).

The antimicrobial activity of linden honey against *S. aureus* (the average diameter of the growth inhibition zone is 38 mm), was superior to the effect on *E. coli* (32 mm) and on *B. cereus* (23 mm). Buckwheat honey also had a greater inhibitory effect on *S. aureus* (31 mm) than on *E. coli* (26 mm) and *B. cereus* (19 mm). Sunflower honey similarly suppressed the growth of *Staphylococcus aureus* (20 mm) to a greater extent than *E. coli* (18 mm) and *B. cereus* (12 mm).

Staphylococcus aureus showed the least resistance to honey, regardless of its botanical origin, which is explained by the different resistance of microorganisms to the influence of external factors.

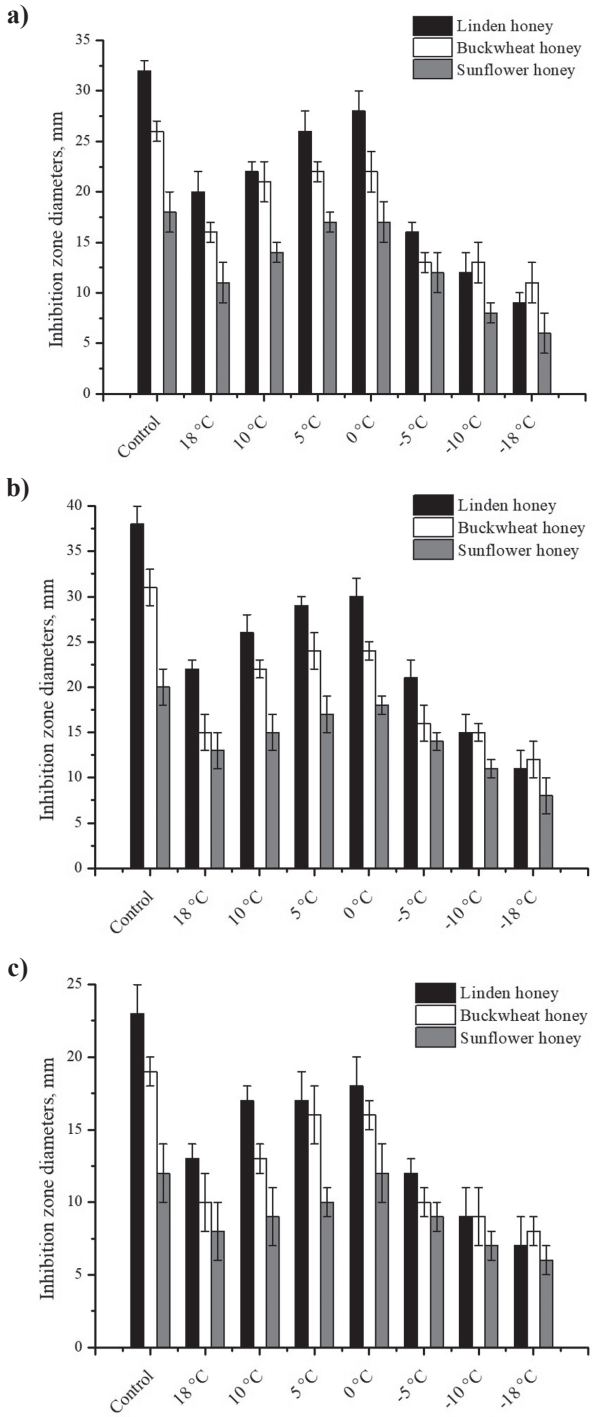

Fig. 2. Inhibition of the growth of *E. coli* (a), *S. aureus* (b) and *B. cereus* (c) by samples of linden, buckwheat and sunflower honey: before storage (control), as well as on the 12th month of storage at temperatures: 18°C, 10°C, 5°C, 0°C, -5°C, -10°C and -18°C

In all honey samples, depending on the storage temperature for 12 months, a decrease in antimicrobial activity was observed. The level of its decrease from greater to less corresponded to the following temperatures: -18, -10, -5, 18, 10, 5, 0°C. This dependence was observed for all types of honey examined.

Thus, the average degree of decrease in the antimicrobial activity of linden honey (compared to the control) against *S. aureus* at -18 and 0°C was 71% and 21%, respectively, for *E. coli* – 72% and 12.5%; *B. cereus* – 69.5% and 21.7%. The inhibitory effect of buckwheat honey against *S. aureus* decreased by 61.3% and 22.6% at -18 and 0°C, respectively; for *E. coli* – by 57.7% and 15.4%; *B. cereus* – by 57.9% and 15.8%. The same trend was noted for sunflower honey: 60.0% at -18°C and 10.0% at 0°C for *S. aureus*; 66.7% and 5.5% for *E. coli*; 50.0% and 0.0% (not including deviation from the mean) for *B. cereus*.

To a large extent, these changes were associated with the level of H_2O_2 in honey samples, which is known to be a stable reactive oxygen species that causes oxidative stress to the microbial cell (Karbyshev and Abdullayev, 2018; Faúndez et al., 2023). However, the dependence was not direct, which is explained by the influence of other factors on it as well.

Conclusions

Based on the data obtained, we can conclude that carried out for 12 months physicochemical and biological analysis of samples of linden, buckwheat and sunflower honey stored at different temperatures, made it possible to identify certain changes in the chemical composition and biological activity of the studied samples.

A positive result of using negative temperatures (-5, -10 and, in particular, -18°C) for long-term storage of honey was a slowdown in the rate of chemical processes, which ultimately led to minimal changes in the content of the toxic compound – HMF. However, such temperature storage conditions had a negative impact on the enzymatic activity and H_2O_2 concentration, which, along with the content of sugars and acids, are factors in the antimicrobial activity of honey. Similar changes occurred with samples stored at 18 and 10°C, in which, in addition, a higher content of HMF was noted. Chemical processes occurred less intensely at 5 and 0°C. The HMF content in samples stored at these temperatures for 12 months ranged from 7.37 to 11.97 mg/kg, depending on the botanical origin of honey, which is significantly below the MPC.

At the 12th month of storage, all honey samples met the requirements of Interstate Standards for all indicators. The exception was diastase activity in samples of sunflower

honey. However, a test conducted to study the antimicrobial activity of honey against *E. coli, S. aureus* and *B. cereus* demonstrated that samples stored at 5 and 0°C had the greatest inhibitory effect. Thus, these temperatures seem to be optimal for storing honey for a long time. The results obtained can be used as additional recommendations, when making changes to the regulatory documentation governing the storage requirements for this product.

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Received: **April, 22, 2024**; *Approved*: **July, 20, 2024;** *Published:* **August, 2025**