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FOOD IN HEALTH AND DISEASE

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ANTIBACTERIAL ACTIVITY OF CHESTNUT HONEY (*Castanea sativa* Mill.) AGAINST *Helicobacter pylori* AND CORRELATION TO ITS ANTIOXIDANT CAPACITY

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original scientific paper

Summary

One of the proven therapeutic properties of honey is its antimicrobial activity. The aim of this study was to examine the antimicrobial activity of chestnut honey against *Helicobacter pylori* and to evaluate a relationship between the content of phenols, antioxidant capacity and antimicrobial activity. The antimicrobial activity of honey was determined by the agar well diffusion method, and the inhibitory effect of different honey concentrations (20%, 50% and 75%) was evaluated. The phenolic content was determined by the Folin-Ciocalteu method while the total antioxidant capacity was determined by the FRAP assay. Water activity and hydrogen peroxide content were also determined. The results showed that the zones of inhibition of *H. pylori* ranged from eight to 21 mm depending on the sample and the concentration of honey, where the concentration of honey of 20% did not have inhibitory effect. The phenolic content ranged from 204.94 to 233.82 mg of GA/kg while FRAP values were between 392.71 and 441.53 μM Fe (II). The honey sample that showed the highest antimicrobial activity against *H. pylori* also had the highest total antioxidant capacity. However, the same correlation was not observed in the other analysed samples. Further research is needed to determine the contribution of individual components of honey to its antimicrobial activity.

Keywords: chestnut honey, *Helicobacter pylori*, antibacterial activity, antioxidant capacity

Introduction

One of the proven therapeutic properties of honey is its antimicrobial effect. Low pH value of honey, high osmotic pressure, hydrogen peroxide, phytochemicals (phenolic components, methylglyoxal), antimicrobial peptides (bee defensin 1 and 2) and lysozyme are considered the main factors responsible for the antimicrobial activity of honey (Manyi-Loh et al., 2010; Samie et al., 2014; Ronsisvalle et al., 2019). Chemical composition and consequently therapeutic properties depend primarily on honeys botanical origin. Scientific studies have shown that darker honeys, like chestnut honey and honeydew honey, have stronger inhibitory effect on microorganisms compared to lighter honey types (Gradvol et al., 2015; Küçük et al., 2007; Güneş et al., 2016). Darker honeys have higher phenolic content and antioxidant capacity as well as higher enzyme activity, especially important is glucose oxidase activity that catalyses production of hydrogen peroxide, a major antibacterial substance in honey (Flanjak et al., 2016a; Flanjak et al., 2016b; Strelec et al., 2018). Chestnut honey (*Castanea sativa* Mill.) is characteristic for the continental area of Croatia and one of the most important unifloral types of honey produced in the Republic of Croatia.

Helicobacter pylori infection is one of the most common human chronic microbial infection worldwide. It is estimated that 50% human population harbors *H. pylori* bacterial strains and to some percent, it causes gastritis and peptic ulcers (Nzeako and Al-Namaani, 2006; Samie et al., 2014). Different treatment regimens for successful eradication of *H. pylori* have been proposed. Generally, a combination of two antibiotics (clarithromycin or amoxicillin and metronidazole) and a proton pump inhibitor or an anti-ulcer agent is most widely used therapy. However, the problem of resistance to antibiotics is growing and the alternative therapies are investigating intensively. Natural products, like plant extracts, honey and probiotics alone or in a combination with antibiotics are evaluated as possible anti-*H. pylori* agents. Studies have shown that honey as *in-vitro* anti-*H. pylori* activity that is mostly related to inactivation of *H. pylori* urease but further studies are needed to prove honeys' antimicrobial activity against *H. pylori in-vivo* (Ayala et al., 2014; Samie et al., 2014; Debraekeleer and Remaut, 2018).

The aim of this study was to examine the antimicrobial activity of chestnut honey (*C. sativa* Mill.) against *H. pylori* and to evaluate a relationship between the content of phenols, antioxidant capacity and antimicrobial activity.

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Materials and methods

Honey samples

Botanical origin of five chestnut honey (*Castanea sativa* Mill.) samples collected in 2019 was confirmed based on the results of pollen analysis (Deutsches Institut für Normung, 2002), electrical conductivity (Bogdanov, 2009) and sensory analysis (International Organization for Standardization, 1987).

Analyses

Water content and hydroxymethylfurfural (HMF) content were determined according to the methods prescribed by International Honey Commission (Bogdanov, 2009). Water activity was determined using HygroLab 3 water activity measuring system which is calibrated in range 0.000 to 1.000 aw range. Semi-quantitive method (Strelec et al., 2018) using MQuant™ peroxide test strips (Merck, Germany) was used for estimation of hydrogen peroxide content. Phenolic content and antioxidant capacity (FRAP assay) were determined according to the methodology described by Flanjak et al. (2016a).

Antibacterial activity

The clinical specimen of *Helicobacter pylori* strain 3639 was isolated from a biopsy of the gastric mucosa of a patient treated under the diagnosis of chronic gastritis. Sample was homogenised and cultured on Columbia brood agar base (OXOID; Basingstoke, UK) with 7% defibrillated sheep blood plus *Helicobacter pylori* Selective Supplement (Dent) (OXOID, Basinstoke, UK). *H. pylori* cultures were incubated under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) with addition of Campy Gen sachet (OXOID, Basinstoke, UK) at 37 °C for 5 days. Identification of grown colonies were Gram strained and observed under microscope. In addition, urease, oxidase and catalase activity tests were performed.
Agar well diffusion method (Dastaouri et al., 2008; Manyi-Loh et al., 2010) was used to test the antibacterial activity. Clinical specimen of *H. pylori* was suspended in sterile saline and adjusted to 4.0 McFarland standard (corresponding to 1.2 x 10^9 CFU/mL). The suspension was smeared with a sterile cotton swab on selective nutrient media. Three wells were made in each Petri dish with sterile tip and in every well 100 µL of honey solution diluted with sterile saline (20%, 50% and 75%; respectively) was added. Amoxicillin (2µg) was used as positive control. Petri dishes were incubated at 37 °C for seven days under the microaerophilic conditions. Antibacterial activity was evaluated by measuring the zone of inhibition against the test microorganism.

Results and discussion

Five honey samples labelled by the beekeepers as unifloral chestnut honey (*Castanea sativa* Mill.) were subjected to botanical origin determination and according to the results presented in Table 1 uniflorality of samples was confirmed. All samples had *C. sativa* pollen share higher than 80% (90 – 98 %) that is a prescribed limit in national regulation (Ministry of Agriculture, Fisheries and Rural Development, 2009) for unifloral chestnut honey. Also, all samples had electrical conductivity higher than 0.8 mS/cm (0.81 – 1.73 mS/cm) that is a minimum for chestnut honey prescribed in national and international regulations (Codex Alimentarius Commission, 2001; Council of the European Union, 2002; Ministry of Agriculture, 2015). Sensory attributes of analysed samples (aroma, taste and colour) was characteristic for chestnut honey (Persano Oddo and Piro, 2004). Besides, the water content (18.0 ± 1.2 %) and HMF content (4.24 ± 2.88 mg/kg) of samples indicate that the collected samples were fresh and properly processed. After botanical origin confirmation and quality assessment, the antibacterial activity of different honey solutions against *H. pylori* was tested. The inhibitory effect of three concentrations of honey solution (20%, 50% and 7 %) on *H. pylori* growth was tested and the results, expressed as zone of inhibition (mm), are presented in Table 2 and Fig. 1. The concentration of 20% of honey solution had no inhibitory effect on *H. pylori* while at concentration of 50% two samples and at 75% three samples had inhibitory potential against *H. pylori*. The inhibitory potential at 50% (zone of inhibition 8 and 18 mm) is in accordance or slightly higher to literature data for the same chestnut honey concentration (Küçük et al., 2007; Kolayli et al., 2008; 2017). Based on the diameter of the inhibition zone (Table 2), the inhibitory potential of chestnut honey samples can be classified as very low (5.5 – 10 mm) or low (11 – 15 mm) and for sample 2 even high inhibitory potential (16 mm or higher) against *H. pylori* (Kolayli et al., 2008; Küçük et al., 2007). At the same time, two samples showed no inhibitory effect to growth of *H. plyori* at any of tested concentrations. Antimicrobial activity of honey is a result synergistic effect of different physical (acidity, osmolarity) and chemical (H_2O_2, phenolic components, lysozyme, bee defensins, methylglyoxal) factors and the contribution of each factor to overall antimicrobial activity is not clear yet (Maddocks and Jenkins, 2013; Gradvol et al., 2015; Samie et al., 2014; Debraekeleer and Remaut,

2018; Quraisiah et al., 2020). One for the reasons is the fact that although in unifloral honey one botanical species prevails (nectar and pollen) and gives a specific melissopalynological, physicochemical and sensory characteristics, there is no 100% unifloral honey. Those botanical species present in lower amounts can contribute to variations in honey properties. Besides, processing and manipulation after extraction of honey by the beekeeper can effect on chemical composition and properties of honey. The difference in antimicrobial potential within the same honey type was also reported by Kolayli et al. (2008) and Gradvol et al. (2015). Antimicrobial activity of honey is mostly attributed to the presence of hydrogen peroxide (H_2O_2) that is a product of the conversion of glucose into gluconic acid catalysed by enzyme glucose oxidase (Strelec et al., 2018). The H_2O_2 content in all analysed honey samples was 147.05 μmol/L h (Table 1) determined by semi-quantitative method. The obtained results in this study are in compliance to previous results for chestnut honey (Strelec et al., 2018). Along with H_2O_2, the high osmolarity and acidity of honey contribute to its antimicrobial activity. Water activity (a_w) of analysed chestnut honey samples was between 0.55 and 0.60 (Table 1). Those a_w values are low enough to create inhospitable environment for most microorganisms (Maddocks and Jenkins, 2013). As mentioned above the non-peroxide substances, like phenolic components, contribute to antimicrobial activity. Many studies available prove the correlation between honey's antioxidant capacity, phenolic content and composition and antibacterial activity (Güneş et al., 2016; Kolayli et al., 2008; 2017; Küçük et al., 2007; Ronsisvalle et al., 2019). Generally, darker honeys (e.g. chestnut honey and honeydew honey) have higher total phenolic content, higher antioxidant capacity and possess higher antimicrobial activity than lighter honey types (e.g. black locust honey, lime honey). Total phenolic content of analysed chestnut honey samples was between 204.94 mg GA/kg and 233.82 mg GA/kg and antioxidant capacity determined by FRAP assay between 392.71 μM (Fe(II)) and 441.53 μM (Fe(II)) (Table 1). The obtained results are in compliance to the literature data (Bertocelj et al., 2007; Beretta et al., 2005; Flanjak et al., 2016a). The honey sample that showed the highest antimicrobial activity against *H. pylori* also had the highest total antioxidant capacity. However, the same correlation was not observed in the other analysed samples. This indicates that phenolic components contribute to the antimicrobial activity of honey but overall antimicrobial activity is a result of synergistic action of different components of honey, only some of which were determined in this study.

Table 1. Specific pollen share, physicochemical characteristics, phenolic content and antioxidant capacity (FRAP assay) of analysed honey samples

Sample	Specific pollen (%)	Water content (%)	Electrical conductivity (mS/cm)	HMF content (mg/kg)	Water activity	Hydrogen peroxide content (μmol/L h)	Phenolic content (mg GA/kg)	FRAP (μM (Fe(II))
1	90	17.2	1.06	8.98	0.58	147.05	219.66	413.50
2	97	17.2	1.09	2.47	0.56	147.05	211.17	441.53
3	88	17.1	1.73	1.50	0.55	147.05	233.82	436.00
4	96	19.2	0.81	3.89	0.59	147.05	204.94	404.82
5	98	19.5	0.81	4.27	0.60	147.05	211.17	392.71
Min-Max	90 - 98	17.1 - 19.5	0.81 - 1.73	1.50 - 8.98	0.55 - 0.60	147.05 - 147.05	204.94 - 233.82	392.71 - 441.53
Mean±SD	94 ± 4	18.0 ± 1.2	1.10 ± 0.38	4.24 ± 2.88	0.58 ± 0.02	147.05 ± 0.00	216.15 ± 11.18	417.71 ± 20.68

Table 2. Antibacterial activity of chestnut honey solutions against *H. pylori*

Honey concentration (%)	Zone of inhibition (mm)				
	sample 1	sample 2	sample 3	sample 4	sample 5
20	0	0	0	0	0
50	8	18	0	0	0
75	15	21	0	12	0

Fig. 1. Zone of inhibition of selected chestnut honey solutions (sample 2) against *H. pylori* on chocolate agar

Conclusion

The obtained data indicate the potential use of chestnut honey in the treatment of *H. pylori* infections but further research is needed to determine the contribution of individual components of chestnut honey to its antimicrobial activity. Also, the efficiency of honey as an alternative ot complementary anti-H. pylori agent sholud be confirmed in in-vivo studies in future research.

References

Ayala, G., Escobedo-Hinojosa, W. I., de la Cruz-Herrera, C. F., Romero, I. (2014): Exploring alternative treatments for *Helicobacter pylori* infection, *World J. Gastroenterol.* 20 (6), 1450-1469.

Beretta, G., Granata, P., Ferrero, M., Orioli, M., Facino, R. M. (2005): Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics, *Anal. Chim. Acta* 533, 185-191. https://doi.org/10.1016/j.aca.2004.11.010

Bertoncelj, J., Doberšek, U., Jamnik, M., Golob, T. (2007): Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey, *Food Chem.* 105 (2), 822-828. https://doi.org/10.1016/j.foodchem.2007.01.060

Bogdanov S. (2009): Harmonised methods of the International Honey Commission, http://www.bee-hexagon.net/files/file/fileE/IHCPapers/IHC-methods_2009.pdf Accessed June 15, 2019.

Codex Alimentarius Commission (2001): Revised Codex Standard for honey, Alinorm 19-26.

Council of the European Union (2002): Council Directive 2001/110/EC of Dec 20, 2001, relating to honey, *Off. J. Eur. Comm.* L10, 47-52.

Dastaouri, M. R., Fakhimzadeh, K., Shayeg, J., Sharaf, J. D., Valilou, M. R., Maheri-Sis, N. (2008): Evaluating antibacterial activity of the Iranian honey through MIC method on some dermal and intestinal pathogenic bacteria, *J. Anim. Vet. Adv.* 7, 409-412.

Debraekeleer, A., Remaut, H. (2018): Future perspective for potential *Helicobacter pylori* eradication therapies, *Future Microbiol.* 13, 671-687. https://doi.org/10.2217/fmb-2017-0115

Deutsches Institut für Normunge (2002): DIN 10760:2002-05 Analysis of honey - Determination of the relative frequency of pollen.

Flanjak, I., Kenjerić, D., Bubalo, D., Primorac, Lj. (2016a): Characterisation of selected Croatian honey types based on the combination of antioxidant capacity, quality parameters, and chemometrics, *Eur. Food Res. Technol.* 242, 467-475. https://doi.org/10.1007/s00217-015-2557-0

Flanjak, I., Strelec, I., Kenjerić, D., Primorac, Lj. (2016b): Croatian produced unifloral honeys characterised according to the protein and proline content and enzyme activities, *J. Apic. Sci.* 60, 39-48. https://doi.org/10.1515/jas-2016-0005

Gradvol, V., Atlaban, N., Lenart, L., Pavlović, H. (2015): Microbilogical quality and inhibitory potential of selected Croatian apiary honeys, *Croat. J. Food Sci. Technol.* 7 (2), 40-46. https://doi.org/10.17508/CJFST.2015.7.2.07

Güneş, M. E., Şahin, S., Demir, C., Borum, E., Tosunoglu, A. (2016): Determination of phenolic compounds profile in chestnut and floral honeys and their antioxidant and antimicrobial activities, *J. Food Biochem.* 41, e12345. https://doi.org/10.1111/jfbc.12345

International Organization for Standardization (1987): ISO 4121 Sensory analysis - Metodology- Evaluation of food products by methods using scales.

Kolayli, S., Aliyazicioğlu, R., Ulusoy, E., Karaoğlu, Ş. (2008): Antioxidant and antimicrobial activities of selected Turkish honeys, *Hacettepe J. Biol. & Chem.* 36 (2), 163-172.

Kolayli, S., Baltas, N., Sahin, H., Karaoglu, S. (2017): Evaluation of anti-*Helicobacter pylori* activity and urease inhibition by some Turkish authentic honeys, *J. Food Eng.* 7, 67-73. https://doi.org/10.17265/2159-5828/2017.02.001

Küçük, M., Kolayli, S., Karaoğlu, Ş., Ulusoy, E., Baltaci, C., Canfan, F. (2007): Biological activities and chemical composition of three honeys of different types of Anatolia, *Food. Chem.* 100, 526-534. https://doi.org/10.1016/j.foodchem.2005.10.010

Maddocks, S. E., Jenkins, R. E. (2013): Honey: a sweet solution to the growing problem of antimicrobial resistance? *Future Microbiol.* 8 (11), 1419-1429. https://doi.org/10.2217/fmb.13.105

Manyi-Loh, C. E., Clarke, A. M., Munzhelele, T., Green, E., Mkwetshana, N. F., Ndip, R. N. (2010): Selected South African honeys and their extracts possess *in vitro* anti-*Helicobacter pylori* activity, *Arch. Med. Res.* 41, 324-331. https://doi.org/10.1016/j.arcmed.2010.08.002

Ministry of Agriculture (2015): Regulation on the quality of honey, *Off. Gazette* 30, 3-5.

Ministry of Agriculture, Fisheries and Rural Development (2009): Regulation on the quality of unifloral honey, *Off. Gazzete* 122, 15-16.

Nzaeko, B. C., Al-Namaani, F. (2006): The antibacterial activity of honey on *Helicobacter pylori*, *Sultan Qaboos Univ. Med. J.* 6 (2), 71-76.

Persano Oddo, L., Piro, R. (2004): Main European unifloral honeys: descriptive sheets, *Apidologie* 35, S38-S81. https://doi.org/10.1051/apido:2004049

Quraisiah, A., Fazalda, A., Alfizah, H., Nur Azlina, M. F. (2020): *In vitro* study of anti-*Helicobacter pylori* activity of honey: A systematic review, *Sains Malays.* 49 (2), 411-420. https://doi.org/10.17576/jsm-2020-4902-20

Ronsisvalle, S., Lissandrello, E., Fuochi, V., Petronio Petronio, G., Straquadanio, C., Crascì, L., Panico, A., Milito, M., Cova, A. M., Tempera, G., Furneri, P. M. (2019): Antioxidant and antimicrobial properties of *Castanea sativa* Miller chestnut honey produced on Mount Etna (Sicily), *Nat. Prod. Res.* 33 (6), 843-850. https://doi.org/10.1080/14786419.2017.1413568

Samie, A., Tanih, S., F., Ndip, R. N. (2014): *Helicobacter pylori* infection-Challenges of antimicrobial chemotherapy and emergence of alternative treatments. In: Trends in *Helicobacter pylori* Infection, Intech Open (ed.), London, United Kingdom, pp. 234-275. https://doi.org/10.5772/57462

Strelec, I., Crevar, B., Kovač, T, Bilić Rajs, B., Primorac, Lj., Flanjak, I. (2018): Glucose oxidase activity and hydrogen peroxide accumulation in Croatian honeys, *Croat. J. Food Sci. Technol.* 10 (1), 33-41. https://doi.org/10.17508/CJFST.2018.10.1.06

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MONITORING OF HONEY CONSUMPTION IN THE AREA OF THE CITY NAŠICE WITH REFERENCE TO THE HEALTH EFFECTS OF HONEY CONSUMPTION

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original scientific paper

Summary

There is a wide range of bee products on the market. The most famous and most accepted by consumers is honey. In Western European countries, the average consumption of honey per capita ranges from three to eight kilograms, while the average consumption of honey in Croatia is very low, 400 grams per capita. The European Union produces only 52% of honey for its own needs. The aim of the research was to study the availability of honey to potential consumers, honey consumption habits, ways of consuming honey, and knowledge of the properties of honey. A survey was conducted online. Respondents were of different genders and different age groups. The survey consisted of 20 questions, and 130 individuals (46% mean and 54% women) from the city of Našice were interviewed. The obtained data were analysed. After conducting research and processing the results, it can be concluded that honey is a product that is accepted by consumers of different age groups. Respondents believe that propolis has better healing properties than honey, but they rarely consume it. Honey is available and affordable to consumers.

Keywords: honey, honey consumption, types of honey

Introduction

The basic raw material for honey production is nectar produced by various plants using nectar glands in their flowers or outside them. Nectar is a sweet and fragrant liquid with a water content of 50 – 75 %, simple sugars content 20 – 25 %, and abundance of minerals, provitamins, essential oils and proteins (Bauer et al., 1999). Honey contains over 180 different compounds and elements that have been identified and it is believed that further studies will find new, yet unknown compounds. A teaspoon of honey contains more than a hundred important ingredients for the body. These are primarily sugars, up to 75%: on average fructose 38%, glucose 30%and the rest are maltose and other disaccharides, water 18%, organic acids 0.3% (mainly gluconic, malic, tartaric, citric), enzymes, minerals 0.2% (iron, copper, manganese, silicon, chlorine, calcium, potassium, sodium, magnesium, etc.), vitamins C and B complex and phytochemicals (flavonoids and phenols) that have oxidizing properties (Laktić and Šekulja, 2008). It has been known for centuries that honey is food and medicine. The nutritional properties of honey have been intensively researched lately. Most research is based on the qualitative and quantitative content of flavanoids in honey. Indirectly, through nectar, pollen and honeydew, honey bees bring polyphenols into their hives. The amount of polyphenols that will be in the honey depends on the quality of bee pasture, honey collection season, geographical area, etc. (Kurtagić, 2017). Lachman et al. (2010) state that the main groups of flavanoids found in honey are flavones, flavonols, and flavones. In addition to flavonoids, other phenols have been found in honey, such as phenolic acids, coumaric, ellagic and ferulic acids, and their esters (Pasupuleti et al., 2016). Their presence in honey depends on the botanical origin, and some flavonoids can be used as markers of the botanical origin of honey (Kaškonienė and Venskutonis, 2010). Andrade et al. (1997) found that ellagic acid identified in honey may be a marker for heather honey (*Erica* sp.). Tomás-Barberán et al. (2001) state that abscisic acid is a possible marker of acacia honey, and that a possible connection with the botanical origin of honey is also shown by folic acid derivatives present in honey. Studies have shown that honeydew honey has a higher phenol content than flower honeys (Meda et al., 2005). The proteins in honey origin from pollen grains (Kochan, 2013). Honey also contains vitamins that have a high pharmacological activity that is stimulated by biogenic substances (enzymes, phytohormones, microelements) present in honey (da Silva et al., 2016). Use of honey in medications for diabetes is mentioned in Ayurveda since ancient times. Honey is normally added to the prepared decoctions. Bee's honey is beneficial for diabetic patients in two ways. One is that honey being sweeter than sugar, one may need a much smaller quantity of honey as a sweetener and honey contain lesser calories than sugar. Further, by providing vitamins B2, B4, B5, B6, B11 and vitamin C, and minerals such as calcium, iron, zinc, potassium, phosphorous, magnesium, selenium, chromium, and manganese. The nutritional values of honey could be altered by feeding the bees with selective food (Arawwawala and Hewageegana, 2017). In the human body, honey is very well digested and is almost completely utilized; its sweetness is approximately the same as

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sucrose. Due to the sugar content, honey is classified as a high-energy food. The energy value of honey ranges from 12 500 to 13 600 kJ/kg, which is less than white sugar (16 000 to 16 500 kJ/kg) (Laktić et al., 2005). Studies have shown that honey has an antibacterial effect based on the absence of resistance, which is also an advantage over antibiotic therapy (Levy and Marshall, 2004). Different degrees of antibacterial action of honey arise from the different characteristics of individual types of honey (Mulu et al., 2004). In general, honey stops the growth of bacteria at the cellular level (Cernak et al., 2012). Today on the market there is a large number of different types of honey with different geographical origin. By morphology of pollen grains, it is possible to determine the pollen composition of honey, and thus determine its botanical origin and classify it into monofloral or polyfloral honeys (Sabo et al., 2011). Mandić et al. (2006) state that people with good sensory abilities who are in frequent contact with different types of honey can well identify botanical origin of honey. Scientific findings, in recent years, have shown that daily consumption of honey in small amounts of at least one teaspoon a day provides a number of beneficial effects on human health (Bauer et al., 1999). Consumption of honey in EU countries is three to eight kilograms per capita per year and in the Republic of Croatia many times less, regardless of all the values contained in this healthy food. The European Union produces only 52% of honey for its own needs, which is a large, but so far untapped export opportunity for Croatian beekeepers (Špoljarić, 2010).

The aim of this paper was to study the consumption of honey in the town of Našice, and the habits of consumers with regard to honey consumption.

Materials and methods

The survey was conducted through Microsoft forms on 130 respondents and was completely anonymous. The survey was posted on a personal Facebook page, and the visitors were asked to access the survey. The survey consisted of 20 questions; the first part of the survey refers to the gender and age of the respondents and whether they consume honey, while the second part refers to specific habits of consuming and knowing the properties of honey. The obtained data were analysed using Microsoft Excell software.

Results and discussion

130 respondents participated in the survey, of which 46% were male and 54% female (Fig. 1). The majority of respondents were at younger age, 38.24% of respondents were aged 18-29. Second largest group were respondents aged between 30-45 (24.32%), followed by age up to 20 years (11.7%). Slightly fewer respondents were present in the population aged 30-35 years (8.6%) and 35-40 years and older (7.8%). This can be explained by the fact that people at a slightly older age are not very eager to participate in online surveys. Honey is available in the diet of a large number of respondents (82.4%) (Fig. 1).

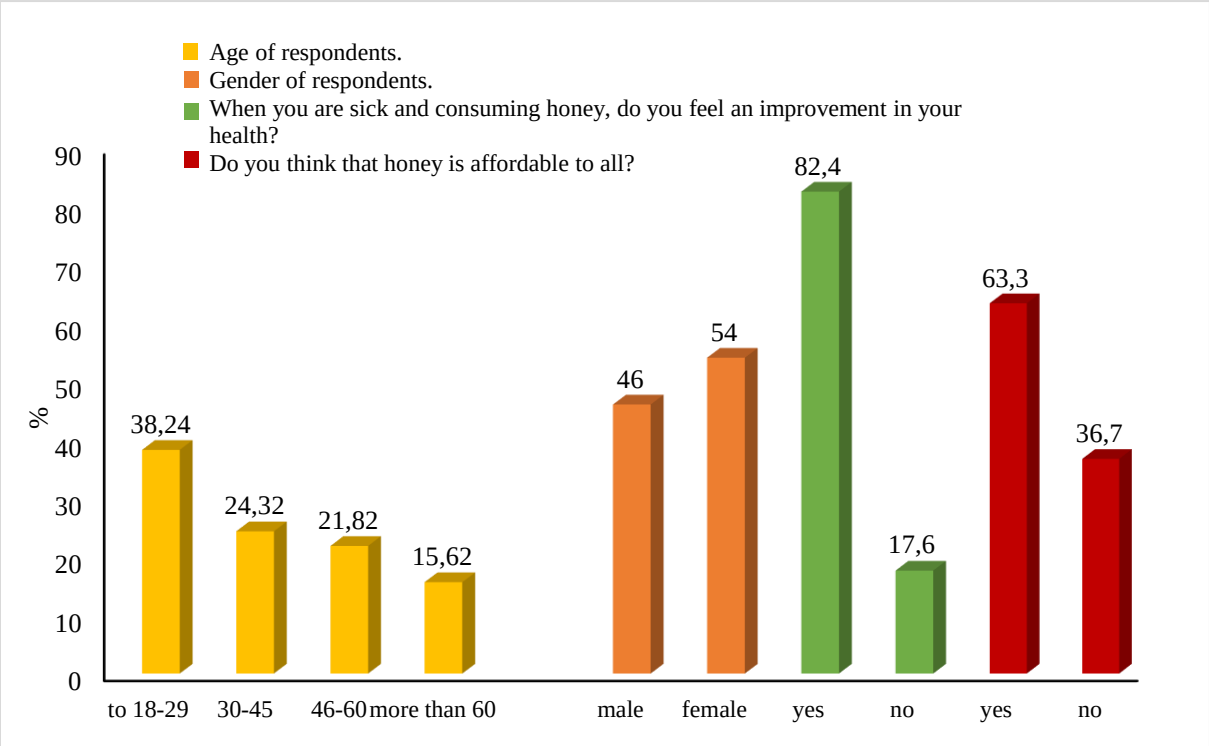

Fig. 1. Answers to questions about age, gender, existence of medicinal properties and availability

Respondents believe that honey contains medicinal properties (99.2%), while only a few of them have the opposite opinion (0.8%) (Fig. 2). Kurtagić (2017) states that the share of polyphenols in honey is relatively small, but they are responsible for the healing properties of honey. By regular consumption of honey, biogenic substances (enzymes, photohormones, mickroelements) from honey will enhance the action of vitamins as well as the healing effect of honey through enzymes, phytohormones and trace elements (Nikolić-Pavljašević and Redžepagić-Dervišević, 2016). Despite the fact that the subjects are aware of the healing properties of honey, they do not consume honey often enough. Only 6.3% of respondents consume honey on daily basis (Fig. 3). Honey is mostly consumed several times a year (41.4%), once a week (25%), and once a month (18%). When buying, the choice of honey is influenced by quality (45.7%), followed by taste (15%), producer (13.4%) and price (11.8%).

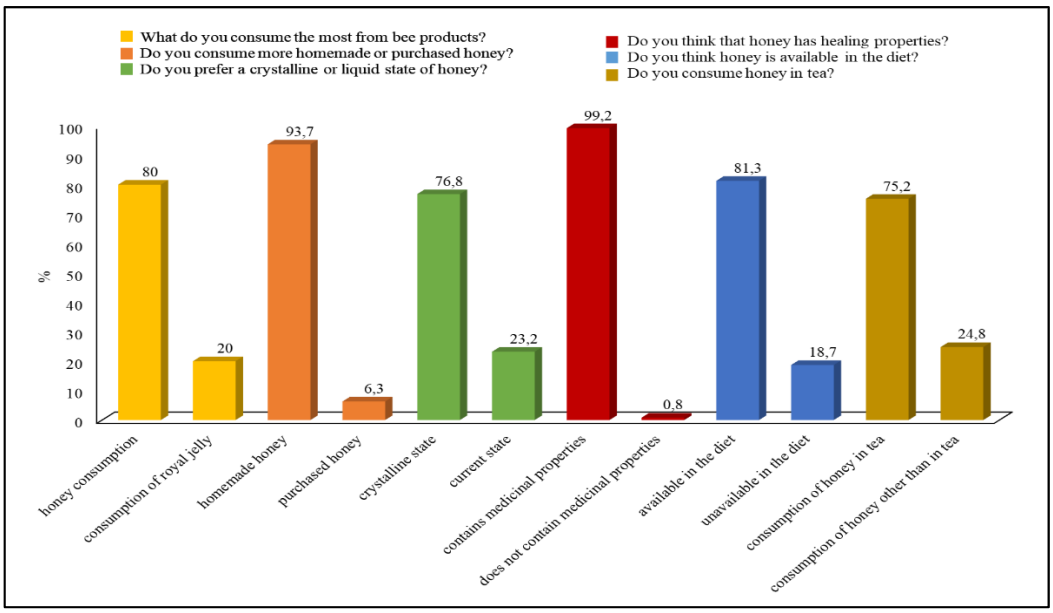

Fig. 2. Answers to questions about the type of bee products consumed, preferences, medicinal properties, availability, method of consumption

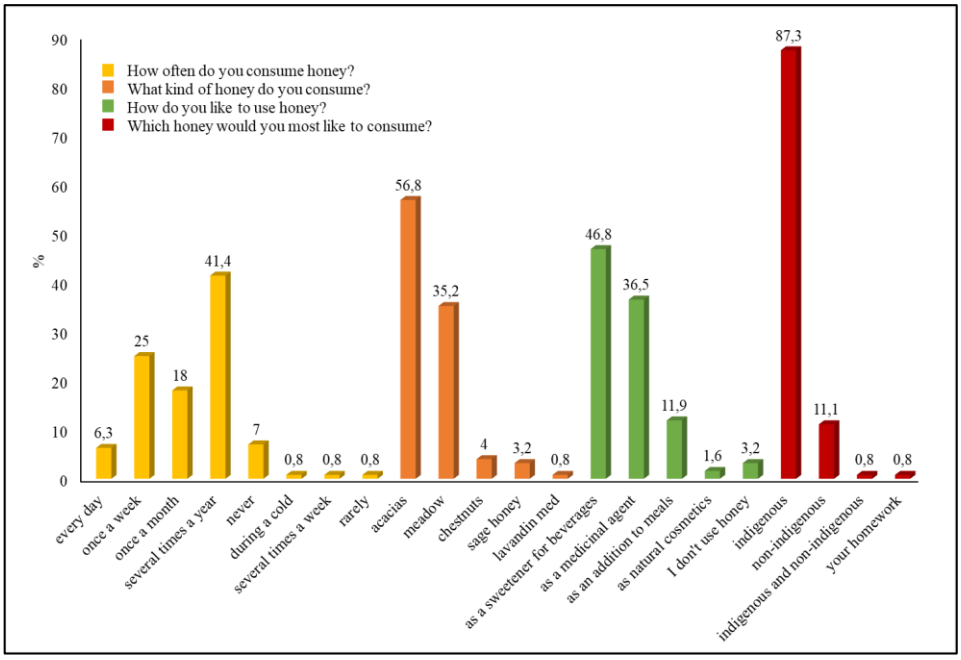

Fig. 3. Answers to questions about the frequency, type, manner and preference of honey consumption

Legislation (Ministry of Agriculture, 2005) sets quite strict quality parameters for honey. However, the question of the credibility of certain types of honey remains. In their research, Kenjerić et al. (2007 and 2008) analysed the characteristic flavonoids in *Robinia* and sage honey, which are evidence of the monofloral origin of honey. In general, the folic acid derivatives present in honey can be linked to the botanical origin of honey. The flavonoids pinocembrin, apigenin, campherol, quercetin, galangin, chrysin, pinobaksin, luteolin, and hesperitin are most commonly present in honey. The presence of these flavanoids in honey depends primarily on the botanical origin of honey. Therefore, individual flavanoids are markers of the botanic origin of honey (Lachman et al., 2010). According to research by Bertoncelj (2008) and Pasupuleti et al. (2016) the proportion of phenolic compounds and flavanoids in honey is correlated with floral and geographical origin on the one hand and antimicrobial activity on the other.

When choosing honey, the country of origin (7.1%), colour (6.3%), and brand (0.8%) have a slightly smaller influence (Fig. 4).

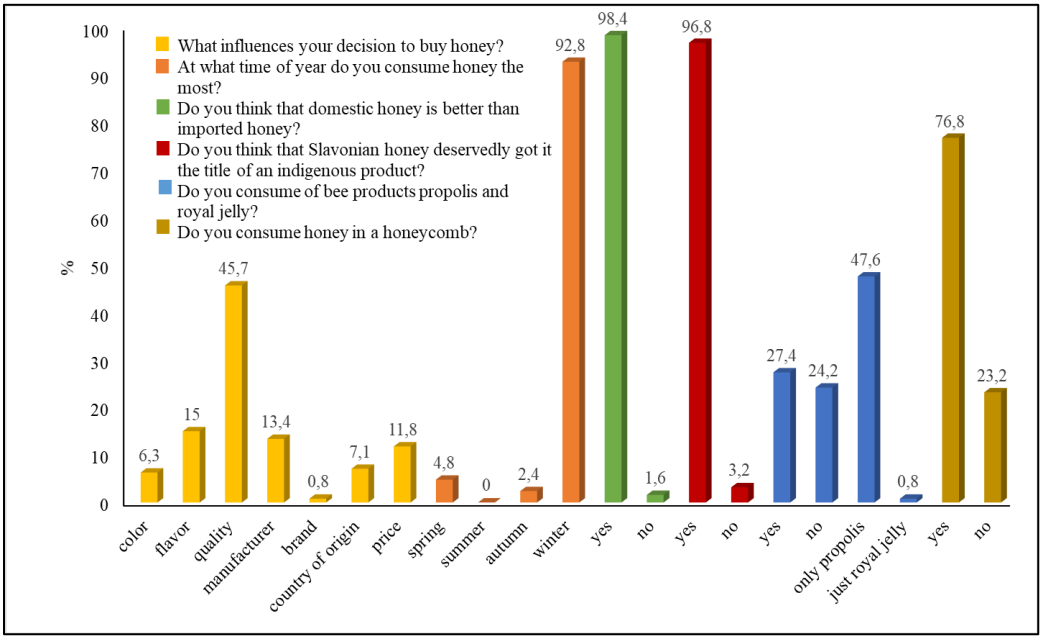

Fig. 4. Answers to questions about buying honey, the season of consumption, the origin of honey, consumption of propolis, royal jelly and honey in the honeycomb

Domestic honey (93.7%) is used more often than purchased (6.3%). In the wider area of the town of Našice, there is large number beekeepers, and consumers have the opportunity to buy honey directly from producers. Respondents prefer honey in the crystallized state (76.8%) to the liquid state (23.2%). Crystallization of honey is a normal property of honey and it is not considered as loss of quality. Many consumers believe that crystallized honey is of poor quality or that it is forged honey (Laktić and Šekulja, 2008), which has not been confirmed in this case. It can be assumed that most of the respondents are familiar with the reasons for the crystallization of honey and that they may associate crystallization with non-adulteration of honey, which would be interesting to further investigate. Fig. 4. shows the consumption of honey according to the seasons. Consumption is most common in winter (92.8%), followed by spring (4.8%), slightly less in autumn (2.4%), and least in summer (0%).

The most consumed is acacia honey (56.8%), followed by meadow (35.2%), chestnut (4%), sage honey (3.2%) and lavander (0.8%). Bauer et al. (1999) recommend the use of acacia honey in insomnia to calm the irritated nervous system. However, perhaps the reason for acacia honey higher consumption is in availability and in organoleptic properties. Namely, acacia honey is extremely bright, yellowish in colour, mild in aroma and taste. Sage honey is an autochthonous type of honey, and in Europe it is produced only in parts of the Croatian coast and islands, and is less known and available on the continent (Kenjerić et al., 2008). So, the consumption of 3.2% is quite satisfactory. Meda et al. (2005) state that the content of phenol in the examined 27 samples of honey ranged from 32.59 to 114.75 mg/100g, and that honeydew honey has a higher content of phenol than flower honey. The value of honey on the market varies based on floral origin. In some northern European countries, honeydew is a favourite type

of honey and more prized than flower honeys (Prodolliet and Hischenhuber, 1998). Consumers in Croatia prefer unifloral honeys. Respondents mostly consume honey in tea (75.2%), but also in some other ways (24.8%). Consumption of honey with chamomile, lemon balm or St. John's wort tea enhances the action of the active substances from tea, as well as those contained in honey itself (Bauer et al., 1999). Honey can be used as a cosmetic preparation and treatment aid. Traditionally, honey is used in the treatment of burns, open wounds, cuts, and skin infections (Kochan, 2013). Recent studies of the therapeutic effect of honey have been associated with the treatment of conjunctivitis, corneal inflammation (Albietz and Lentin, 2006). Honey can be used and diluted with distilled water in a ratio of 1: 1 in the treatment of various infections (Al-Waili, 2004). Respondents believe that domestic honey (98.4%) is much better than imported honey (1.6%), and that Slavonian honey deservedly received the title of indigenous product (87.3%) (Fig. 3), so it is to be assumed that consumers have created certain habits in honey consumption. Geographical and botanical properties are important for the quality of honey, and the taste, smell and colour of honey change according to the nectar from the flower (Kaya et al., 2005). Because of this, respondents would rather consume indigenous honey (87.3%) than others (11.1%), and only a few of them would consume both (0.8%) or their own domestic (0.8%). Sabo et al. (2013) in their research conducted pollen analysis of chestnut, acacia and goldfinch honey in the Našice area, and found that taxonomic variability is greatest in rare groups, followed by a group with a small amount of pollen, a secondary and dominant group. Considering the above, chestnut honey and acacia honey are classified as unifloral honeys, and goldfish honey is polyfloral honey. Identifying the source of honey is a difficult task, still there is no appropriate analytical method for unambiguously determining the botanical origin of honey (Anklam, 1998). Of the other bee products, the respondents consume propolis (47.6%) the most, and a very small number of respondents consume royal jelly (0.8%). The properties of propolis are very well known among the people, but little is known about royal jelly. Beekeepers and nutritionists try to promote royal jelly on their websites, stating that royal jelly has antibacterial and antiviral properties, and that it acts on the human body as a biostimulator, regenerator and development factor, because it contains all important substances necessary for the development and survival of living organisms. New discoveries regarding the active components of royal jelly, their internal mechanisms of action and the possibility of isolation and purification of pure substances represent a starting point for the formulation of new products for therapeutic and pharmacological use as an alternative to conventional antibiotics. From the available literature, royal jelly and its derivative components, such as royalisin, jelleines and 10-hydroxy-2-decenoic acid (10-HDA), have shown high activity against Gram-positive bacteria, while their effectiveness decreases against Gram-negative bacteria (Fratini et al., 2016). Several studies conducted on royal jelly have shown that this product is also effective against many multidrug-resistant bacteria, such as MRSA (methicillin-resistant *Staphylococcus aureus*) (Fratini et al., 2016). Honey is the most common in consumption of all bee products (80%), and many people consume it in honeycomb (76.8%) (Fig. 4). During the manipulation of honey, a large part of vitamin C is lost, while it is preserved in the original honey or honeycomb (Laktić et al., 2005). The data on the high percentage of honey consumption in the honeycomb also speaks of the knowledge of the properties of honey in the honeycomb. Fig. 4. shows that respondents mostly use honey as a sweetener for beverages (46.8%), followed by a medicinal product (35.6%), a food supplement (11.9%) and the least as a natural cosmetic (1.6%). If honey is used as a medicinal agent in combination with antibiotics, honey does not alter the action of antibiotics and no adverse interactions occur (Boateng and Nso Diunase, 2015). It is important to emphasize that the respondents believe that honey is affordable for everyone (63.3%) and that it contains medicinal properties (82.4%).

Conclusions

Based on this research, it can be said that honey is recognized as a product that contributes to health improvement. Consumption is most common in the winter months as a tea sweetener. Considering the answers of the respondents, it can be concluded that honey consumption is related to traditional knowledge about honey. Very few respondents use honey for any purpose other than as a tea sweetener. Many other beneficial effects of honey have been scientifically proven: as a prophylaxis and aid in the treatment of various diseases. So, scientists together with beekeepers, should work intensively and introduce consumers to new knowledge, and contribute to the increase of consumption of honey, especially domestically produced.

References

Albietz, J. M., Lenton, L. M. (2006): Effect of antibacterial honey on the ocular flora in tear deficiency and meibomian gland disease, *Cornea* 25 (9), 1012-1019. https://doi.org/10.1097/01.ico.0000225716.85382.7b

Al-Waili, N. S. (2004): Investigating of antimicrobial activity of natural honey and its effects on pathogenic bacterial infections of surgical wounds and conjunctiva, *J. Med. Food* 7 (2), 210-222. https://doi.org/10.1089/1096620041224139

Andrade, P., Ferreres, F., Gil, M. I., Tomás-Barberán, F. A. (1997): Determination of phenolic compounds in honeys with different floral origin by capillary zone electrophoresis, *Food Chem.* 60 (1), 79-84. https://doi.org/10.1016/s0308-8146(96)00313-5

Anklam, E. (1998): A review of the analytical methods to determine the geographical and botanical origin of honey, *Food Chem.* 63, 549-562. https://doi.org/10.1016/s0308-8146(98)00057-0

Bauer, Lj., Biškupić, I., Brkan, B., Dekanović, I., Dolenec Dravski, M., Domaćinović, V., Kvočić, K., Matijaško, N., Matković Mikulčić, K., Milković, B., Pavlek Moćan, M., Olić, R., Sulimanović, Đ., Zeba, Lj. (1999): Med: pčelarenje i običaji. Zagreb: Pučko otvoreno učilište Zagreb.

Beroncelj, J. (2008): Identifikacija in vsebnost nekaterih antioksidantov v slovenskem medu, Dokt. disertacija, Ljubljana, Univ. v Ljubljana, Biotehniška fakulteta.

Boateng, J., Nso Diunase, K. (2015): Comparing the Antibacterial and Functional Properties of Cameroonian and Manuka Honeys for Potential Wound Healing-Have We Come Full Cycle in Dealing with Antibiotic Resistance? *Molecules* 20 (9), 16068-16084. https://doi.org/10.3390/molecules200916068

Cernak, M., Majtanova, N., Cernak, A., Majtan, A. (2012): Honey Prophylaxis Reduces the Risk of Endophthalmitis During Perioperative Period Eye Surgery, *Phytother. Res* 26 (4), 613-616. https://doi.org/10.1002/ptr.3606

da Silva, P. M., Gauche, C., Gonzaga, L. V., Costa, A. C., Fett. R. (2016): Honey: chemical composition, stability and authenticity, *Food Chem.* 196, 309-323. https://doi.org/10.1016/j.foodchem.2015.09.051

Kaya, Z., Binzet, R., Orcan, N. (2005): Pollen analyses of honeys from some regions in Turkey. *Apiacta* 40, 10-15.

Fratini, F., Cilia, G., Mancini, S., Felicioli, A. (2016): Royal Jelly: An ancient remedy with remarkable antibacterial properties, *Microbiol. Res.* 192, 130-141. https://doi.org/10.1016/j.micres.2016.06.007

Kaškonienė, V., Venskutonis, P. R. (2010): Floral Markers in Honey of Various Botanical and Geographic Origins: A Review, *Compr. Rev. Food Sci. Food Saf.* 9 (69), 620-634. https://doi.org/10.1111/j.1541-4337.2010.00130.x

Kenjerić, D., Mandić, M. L., Primorac, Lj., Bubalo, D., Perl, A. (2007): Flavonoid profile of *Robinia* honeys produced in Croatia, *Food Chem.* 102, 683-690. https://doi.org/10.1016/j.foodchem.2006.05.055

Kenjerić, D., Mandić, M. L., Primorac, Lj., Čačić, F. (2008): Flavonoid pattern of sage (*Salvia officinalis L.*) unifloral honey. *Food Chem.* 110, 187-192. https://doi.org/10.1016/j.foodchem.2008.01.031

Kochan, A. (2013): Honey. Apitherapy, *J. Amer. Apith. Soc.* 20 (1), 68-74.

Kurtagić, H. (2017): Polifenoli i flavonoidi u medu, *Food Health Diseas.* 6 (1), 28-35.

Lachman, J., Orsák, M., Hejtmánková, A., Kovářová E. (2010): Evaluation of antioxidant activity and total phenolic of selected Czech honeys, *LWT-Food Sci. Technol.* 43 (1), 52-58. https://doi.org/10.1016/j.lwt.2009.06.008

Laktić, Z., Bračić. I., Bodakoš, D., Tucak, Z. (2005): Pčelarski priručnik, Osijek: GRAFIKA. Laktić, Z., Šekulja, D. (2008): Suvremeno pčelarstvo, Zagreb: Nakladni zavod Globus.

Levy, S. B., Marshall, B. (2004): Antibacterial resistance worldwide: causes, challenges and responses, *Nat. Med.* 10 (S12), S122-S129. https://doi.org/10.1038/nm1145

Arawwawala, L. D. A. M., Hewageegana H. G. S. P. (2017): Health benefits and traditional uses of honey: A review, *J. Apither.* 2 (1), 9-14. https://doi.org/10.5455/ja.20170208043727

Mulu, A., Tessema, B., Derbie, F. (2004): *In vitro* assesment of the antimicrobial potential of honey on common human pathogenes, *Ethiop. J. Health Dev.* 18 (2), 107-111. https://doi.org/10.4314/ejhd.v18i2.9945

Mandić, M. L., Primorac, Lj., Kenjarić, D., Bubalo, D., Perl, A., Flanjak, I. (2006): Characterization of Oak Mistletoe and Common Thistle Honeys by Physicochemical, Sensory and Melisssopalynology Parameters, *Dtsch. Lebensm.-Rundsch.*. 102. 245-249.

Meda, A., Lamien, C.E., Romito, M., Millogo, L., Nacoulma, O.G. (2005): Determination of total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity, *Food Chem.* 91, 571-577. https://doi.org/10.1016/j.foodchem.2004.10.006

Ministry of Agriculture (2005): Ordinance on honey NN 30/15.

Nikolić-Pavljašević, S., Redžepagić-Dervišević, E. (2016): Bolesti oka i terapijski učinak meda. *Food Health Diseas.* 5 (1) 27-31.

Pasupuleti, V. R., Kumara, T. K., Naguib, S., Siew, H. G. (2016): Biological and therapeutic effects of honey produced by honey bees and stingless bees: a comparative review, *Rev. Bras. Farmacogn.* 26, 657-664. https://doi.org/10.1016/j.bjp.2016.01.012

Prodolliet, J., Hischenhuber, C. (1998): Food authentication by carbohydrate chromatography, *Z Lebensm Unters Forsch.* 207, 1-12. https://doi.org/10.1007/s002170050286

Sabo, M., Potočnjak, M., Banjari, I., Petrović, D. (2011): Pollen analysis of honey from Varaždin Conty, Croatia, *Turk. J. Bot.* 35 (5), 581-587. https://doi.org/10.3906/bot-1009-86

Sabo, M., Glušac, N., Banjari, I., Petrović, D. (2013): Peludna analiza u medu kestena, bagrema i zlatnice s područja Našica, *Glasnik zaštite bilja* 36 (6), 42-49.

Špoljarić J. (2010): Istraživanje o konzumaciji meda za potrebe prodaje u pčelarstvu, EFZG-serija članaka u nastajanju 10-09.

Tomás-Barberán, F. A., Martos, I., Ferreres, F, Radović, B. S., Anklam, E. (2001): HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys, J. Sci. Food Agric. 81 (5), 485-496. https://doi.org/10.1002/jsfa.836

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SAFETY ASPECTS OF BEE POLLEN USE IN NUTRITION

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review paper

Summary

Bee pollen is popular nutraceutical and remedy used in traditional medicine since ancient times. Although it has indisputable beneficial action on human health, in recent years some issues regarding its safety have been raised. Mainly, they are a result of human actions, either indirectly (usage of pesticides, pollution with toxic trace elements) or directly (microbial contamination during handling). This review summarizes findings regarding safety aspects of bee pollen for human consumption.

Keywords: bee pollen, pesticides, toxic elements, bacteria, fungi

Introduction

Bee pollen is term referred to grains that honey bees (*Apis mellifera*) form in pollen baskets by compressing flower pollen mixed with secretion from the mouth (Mekki, 2019). It has long been recognised as a nutraceutical, functional food and remedy in alternative medicine. These attributes pollen owes to its chemical composition, which is providing virtually all essential compounds for human and animal nutrition (Kostić et al., 2020), although contents of specific compounds vary extensively, depending on botanical species, geographical origin and climate (Margaroan et al., 2019).

Major nutritive components in pollen are carbohydrates, proteins and fats. According to Li et al. (2018), carbohydrates take up 40 – 85 % of dry bee pollen, with fructose as a major carbohydrate, followed by glucose, sucrose, oligo- and polysaccharides. Total dietary fibre content ranges between 17.60 and 31.26 %, with cellulose and callose as main components (Thakur and Nanda, 2020).

On average, protein content varies from 10 to 40 g/100 g dry weight (Campos et al., 2008), although range 2.5 – 62 % has been reported (Nicolson, 2011). Although amino acid composition depends on botanical origin of pollen, bee pollen is considered as a valuable source of essential amino acids, among which leucine and lysine are often present in largest quantities (Thakur and Nanda, 2020), along with proline, glutamic and aspartic acid (Mekki, 2019).

Lipid content averages between 1 – 13 % of pollen dry weight, with significant contents of ω-3 fatty acids. Among lipids, Li et al. (2017) reported presence of 41 different phosphatidylcholines, 43 phosphatidylethanolamines, 9 phosphatidylglycerols, 10 phosphatidylserines, 12 lysophosphatidylcholines, 8 ceramides, 27 diglycerides, 137 triglycerides, and 47 fatty acids. Fatty acid profile is highly dependent not only on botanical source, but on geographical origin as well. Most prevalent saturated fatty acids are myristic, stearic and palmitic, and α-linolenic, linoleic and oleic acid are dominant unsaturated acids. Significant levels of arachidonic, behenic, capric, caproic, caprylic, 11-eicosenoic, elaidic, lauric, lignoceric, myristic, oleic and stearic acids have also been reported. On average, saturated fatty acids range from 4.29 – 71.47 %, monounsaturated from 1.29 – 53.24 % and polyunsaturated from 4.33 – 75.1 %. ω-3 fatty acids vary from 8.07 to 44.1 % and ω-6 fatty acids from 1.77 to 38.25 % (Thakur and Nanda, 2020). Kostić et al. (2020) designated pollen as a valuable source of polyunsaturated fatty acids (PUFA), basing their claim upon the fact that different Portuguese pollen samples contained app. 49 – 70 % PUFA, with UFA/SFA ratio 1.9 – 5.9, Serbian pollen samples had app. 22 – 54 % PUFA and Philippine stingless bee pollen samples app. 52% PUFA.

Bee pollen is a significant source of minerals, with 2.5 – 6.5 % of ash content. Over 25 minerals have been reported, among which Ca, Cu, Cr, Fe, K, Mg, Mn, Na, P and Zn are most abundant. However, exact composition and proportions of minerals are affected by soil, climate, geographical origin and botanical species (Thakur and Nanda, 2020). Kostić et al. (2020) accentuate the value of bee pollen as a selenium source, with app. content of 0.02%.

Furthermore, vitamins in pollen comprise up to 0.7% (Kostić et al., 2020), with high contents of B-complex (Thakur and Nanda, 2020) and carotenoids, vitamin A precursors, and polyphenols take up app. 1.6% of pollen (Kostić et al., 2020). Among polyphenols, flavonoids are dominant group (Kostić et al., 2020; Thakur and Nanda, 2020), but different geographical origin of pollen and different plant species result in large diversity of compounds and their contents reported in literature. Often, apigenin, epicatechin, hesperetin, isorhamnetin, catechin,

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kaempferol, luteolin, quercetin, naringenin, etc. and phenolic acids: chlorogenic acid, ferulic acid, caffeic acid, gallic acid, vanillic acid, syringic acid and *p*-coumaric acid are reported (Thakur and Nanda, 2020). The unique nutritional composition of pollen makes it valuable remedy in neurological disorders, from spinal cord injury to Alzheimer's and Parkinson's disease (El-Seedi et al., 2020), anti-inflammatory, anti-tumor and antimicrobial agent (Margaroan et al., 2019). On the other hand, although allergic reactions caused by ingestion of pollen with food are rare, Kostić et al. (2020) do not exclude pollen as a potential allergenic, it may contain pyrrolizidine alkaloids, toxic trace elements (such as arsenic and cadmium), mycotoxins and pathogenic microorganisms. Therefore, safety aspects of bee pollen use should not be disregarded in evaluation of its beneficial effects.

Pesticides

Pesticides used in plant protection have raised great concern among public due to great loss of pollinators, among which are honey bees, and presence of residues in food. As a response, scientists have been focusing on exposure routes and risks of pesticides for bees (reviewed by Zioga et al., 2020), and on different bee products as vectors of further transmission of contaminants to humans.

By definition, "pesticides are toxic chemicals used to kill or repel pests or to interrupt their reproduction, and are some of the most toxic, environmentally stable and mobile substances in the environment" (Andreo-Martinez, 2020). Bees are exposed to them through water, pollen nectar, dust-spray droplets collected on body hairs of bees, guttation drops, and even in the bee hive if beekeepers use them to control parasites. Since they do not have detoxifying enzymes, bees accumulate pesticides in pollen, brood, wax and honey. As a result, acute poisoning may manifest in a number of consequences, from reduced flying ability to increased mortality (Catalayud-Vernich et al., 2018).

Catalayud-Vernich et al. (2018) collected bee pollen from 39 locations in different parts of Spain and screened them for 63 pesticides and their degradation products. They found 14 different pesticides in pollen, 8 of which was for agricultural use and 6 was used in beekeeping. Although some samples were pesticide-free, an average count was 3 pesticides per sample and most commonly found were: coumaphos, fluvalinate and amitraz degradate DMF. Interestingly, there was no difference regarding the number of detected pesticides and their average count per sample between hives located in high- and low agricultural environment. Residues of coumaphos were found in pollen even though they were not applied in hives for months, indicating that, along with environmental contamination, bee pollen may be contaminated with pesticides present in the wax. Also, this proves that pesticides accumulate in hives over a long period of time, posing a risk to bees for prolonged period. Migdal et al. (2018) linked pesticide residues with colony collapse disorder, although clear cause-and-effect relationship is yet to be proven.

Chaimanee et al. (2019) analysed contents of pesticides in bee pollen collected at pollen traps at 16 non-agricultural and 20 agricultural sites in Northern Thailand. They also found no difference in contamination of pollen regarding the site type. They found 8 different pesticides (organophosphate chlorpyrifos, 2,4-dimethylphenyl formamide (DMPF), carbendazim, metalxyl, atrazine, imidacloprid, cypermethryn and fluvalinate) in agricultural sites and 4 (carbendazim, chlopyrifos, fluvalinate and DMPF) in non-agricultural sites. All detected pesticides are among most frequently used in Thailand.

Manning (2018) assessed bee pollen collected in area of canola farms as the only source of nectar. All pollen samples were contaminated with trifluralin. Atrazine was found in 61.5% of samples and chlorpyrifos in 30.8% of samples.

Ostiguy et al. (2018) monitored pesticide residues in bee pollen collected in USA over 4-year period. They reported that 79 pesticides and their metabolites were determined in pollen, with insecticides detected more frequently than other types. The most frequently detected fungicides were carbendazim, azoxystrobin, and propiconazole-1, the most frequent herbicide was atrazine and carbaryl was the most frequent insecticide. Although different pesticides in different concentrations were found depending on season, generally, pollen was more contaminated than wax. Similar conclusion was withdrawn by Raimets et al. (2020), who also found that bee pollen and beebread collected in southeastern Estonia were more frequently contaminated than honey or wax, and that pollen was most frequently contaminated by insecticides and fungicides.

Pesticide residues present in bee pollen and other bee products do not pose risk only to honey bees. Through consummation of bee products, humans are also exposed to these residues. There is evidence that numerous pesticides affect non-target species as well, including humans. For example, atrazine is nowadays banned in the EU due to demasculinizing and defeminizing effect on reptiles, birds, mammals and other species (Vandenberg et al., 2020). However, it is still being used in the USA, China and Australia as a herbicide, particularly in corn production.

Clorpyriphos, widely used insecticide both in agricultural and domestic use, has been linked to neurotoxicity, cytotoxicity, oxidative stress, mutagenicity etc., where humans are esp. sensitive after oral administration (Ubaidurrahman et al., 2020). Carbendazim has been reported to cause embryotoxicity, infertility, hepatocellular dysfunction and other disorders in different mammalian species, including human (Wang et al., 2020).

Toxic trace elements

Toxic trace elements may be found in bee pollen due to man-caused pollution of air, water and soil and uptake of these elements by plants. Uptake of the elements by plants depends on plant species and genotypes, soil type and pH (Radanović and Antić-Mladenović, 2012). Although flower pollen is mixed with nectar, saliva and honey to produce bee pollen and therefore concentration of trace elements is always lower in bee pollen than in flower pollen, bee pollen may contain significant amounts of toxic elements (Silva et al., 2012).

Among them, lead and cadmium are often found, because of industrial pollution and pesticide application. Altunatmaz et al. (2017) reported ranges of 0.006 – 0.181 μg/g for Cd and 0.000 – 0.479 μg/g for Pb in bee pollen collected in different regions of Turkey and Silva et al. (2012) reported 13.98 – 18.19 μg/mL for Pb in bee pollen collected in Teresina region of Brazil. Range of 0.003-0.233 mg/kg was reported for Cd contents in bee pollen collected in south-eastern (Morgano et al., 2010) and 0.0026 – 0.0244 mg/100 g for southern part (Rio Grande do Sul State) of Brazil (Sattler et al., 2016).

Arsenic was also found, mainly due to air and water pollution (Altunatmaz et al. 2017), but through pesticide application as well (Ratnaike, 2003). Altunatmaz et al. (2017) reported 0.006 – 1.035 μg/g of As in Turkish bee pollen and Morgano et al. (2010) reported <0.01-1.38 mg/kg for bee pollen collected in south-eastern Brazil, while Maragou et al. (2017) reported levels below 0.2 μg/g in bee pollen collected in northern and western parts of Greece.

Traces of mercury were also found in bee pollen. Namely, <0.0004-0.0068 mg/kg for Hg was reported for area of south-eastern Brazil (Morgano et al., 2010), 0.0036 – 0.0066 mg/kg for Poland (Roman, 2009) and bee pollen of Greek origin contained < 0.06 μg/g (Maragou et al., 2016). Although very toxic, these levels of mercury are not of concern for safety of bee pollen for human consumption, but they do show that bee pollen may serve as an indicator of environmental pollution with mercury.

However, Cd and Pb levels could pose concern. Long-term exposure to Cd causes damaging of cardiovascular, nervous, respiratory, urinary, skeletal and/or reproductive system and cancer. It has very long biological half-life (10 – 30 years), and can accumulate in body for a very long time (Rahimzadeh et al., 2017) which makes it especially concerning. Lead is non-biodegradable and highly toxic to virtually all organs. The most affected is nervous system and children are especially sensitive. Long-time exposure to Pb may cause behavioural problems, lowered IQ and learning disorders in children, and decreased cognitive performance in adults, and chronic exposure leads to its accumulation in bones and kidneys (Wani et al., 2015).

Arsenic is also highly poisonous. In small amounts it causes gastrointestinal problems, but chronic exposure leads to wide range of symptoms, since it is deposited in liver, kidney, heart, spleen, lungs, nails, hair and skin. Hyperpigmentation, diabetes mellitus, respiratory diseases, malignant changes of all organs are some examples of chronic arsenic poisoning (Ratnaike, 2003).

Microbial contamination

Unlike pesticides and trace elements, microorganisms can contaminate pollen in different stages of collecting and handling. Beev et al. (2018) state that pollen may be contaminated from its natural habitat, bee activities (foraging and transport), human activities (handling during colleting, drying, packaging) and environmental factors (wind, rain-splash, dew or fog drip etc.). Lopez et al. (2020) differentiate primary sources of bacterial contamination of pollen: digestive tracts of honeybees, dust, air, earth and nectar, and post-harvest sources: humans, equipment, containers, pests and water. Viruses, bacteria and fungi have all been detected on pollen, showing that pollen is favourable environment for microbial development. This is due to favourable chemical composition (carbohydrates, proteins and lipids), discussed above.

Beev et al. (2018) analysed 13 fresh and 19 dried pollen samples collected in different areas of Bulgaria. Along with significant difference in water activity (a_w) (0.717 in fresh compared to 0.359 in dried samples) and pH (4.23 in fresh and 5.21 in dried samples), they reported significantly higher total viable count (182153.8 CFU/g compared to 30352.6 CFU/g in dried samples) and fungal load in fresh bee pollen (mean value 10512.3 CFU/g compared to 2418.4 CFU/g). Apparently, difference in pH of samples was not so remarkable to show an effect on microorganisms' growth like water activity. There was no significant difference in *Enterobacteriaceae,*

Staphylococcus spp. and lactic acid bacteria count. Dinkov (2016, 2018a,b) also analysed fresh and dried pollen collected in different regions of Bulgaria, also showing contamination with *Enterobacteriaeae* in fresh and dried pollen over period of years. Belhadj et al. (2014) collected fresh bee pollen samples in the public market in Algeria. Along with different parts of Algeria, Egyptian and Chinese pollen sold at the market were also sampled. Considering total aerobic mesophilic count and total yeast and mold count, all samples were satisfactory, however, *Enterobacteriaceae* were detected in majority of samples, including *Salmonella* and *Listeria* ssp., indicating non-hygienic handling of pollen by bee keepers. Additionally, only one sample was free of *Staphylococcus aureus.*

Among fungi, Beev et. al. (2018) reported that most often *Penicillium* and *Fusarium* species were isolated, indicating that mycotoxin presence could also pose a problem. In research of Belhadj et al. (2014) *Aspergillus*, *Penicillium*, *Alternaria* and *Mucor* were frequently isolated, all of which are mycotoxin producing. Mycotoxin-producing moulds were found in Portuguese (Estevinho et al., 2011), Lithuanian (Sinkevičiene et al., 2019) and Slovakian (Kačaniova et al., 2009) pollen as well, namely *Aspergillus*, *Penicillium* and *Fusarium*. Among mycotoxins, fumonisins, ochratoxins, deoxynivalenol (DON) and zearalenone were found in pollen (Kačaniova et al., 2011; Rodriguez-Carasco et al., 2013).

Dried pollen has better microbial quality, as shown by da Silva et al. (2019) and DeMelo et al. (2015) who did not detect *Salmonella*, *E. coli* and *S. aureus* in dried pollen collected in Brasil. De Arruda et al. (2017) reported presence of *E. coli* in 11% and *S. aureus* in 30% of analysed dehydrated pollen samples collected in Brazil, and Dinkov (2016) found *Enterobacteriacease* in dried pollen collected in Bulgaria, but the incidence and number of present microorganisms are still lower than in the case of above mentioned results for fresh pollen.

Conclusions and Future Remarks

The nutritional and pharmaceutical values of bee pollen are unquestionable, however, safety aspects and quality of pollen must be standardised. There is no standard of bee pollen quality and safety on EU or IHC level, although some countries do have legislation regarding these issues.

Beekeepers should be constantly educated regarding good hygiene practices in apiaries, and during processing and packaging of bee pollen. Namely, high incidence of presence of different *Enterobacteriaceae* species indicates poor hygienic practices. Identification of *Listeria* in some researches is of special concern. This bacterium is capable of reproducing in large temperature range (4 – 45 °C) and is very difficult to eradicate once it establishes in facilities.

In addition, awareness of beekeepers regarding environmental pollution and choice of pasture areas for bees further from industrial and agricultural pollution should be raised. At the same time, crop growers need to be educated regarding integrated pest control, which enables usage of lower quantities of pesticides.

Additional research is needed regarding assessment of bee pollen as a vector of dietary intake of harmful substances.

References

Altunatmaz, S. S., Tarhan, D., aksu, F., Barutcu, U. B., Or, M. E. (2017): Mineral element and heavy metal (cadmium, lead and arsenic) levels of bee pollen in Turkey, *Food Sci. Technol. Campinas* 31 (1), 136-141. https://doi.org/10.1590/1678-457x.36016

Andreo-Martinez, P., Oliva, J., Gimenez-Castillo, J.J., Motas, M., Quesada-Medina, J., Camara, M. (2020): Science production of pesticide residues in honey research: A descriptive bibliometric study, *Environ. Toxicol. and Pharmacol.* 79, 103413. https://doi.org/10.1016/j.etap.2020.103413

Beev, G., Stratev, D., Vashin, I., Pavlov, D., Dinkov, D. (2018): Quality assessment of bee pollen: a cross sectional survey in Bulgaria, *J. Food Qual. Hazards Control.* 5, 11-16. https://doi.org/10.29252/jfqhc.5.1.11

Belhadj, H., Harzallah, D., Dahamna, S., Khennouf, S. (2014): Microbiological quality of marketed pollen, *Der Pharm. Lett.* 6 (2), 37-42.

Campos, M. G. R., Bogdanov, S., de Almeida-Muradin, L. B., Szczesnca, T., Mancebo, Y., Frigeiro, C., Ferreira, F. (2008): Pollen composition and standardisation of analytical methods, *J. Apic. Res.* 47 (2), 156-163.

Catalayud-Vernich, P., Catalayud, F., Simo, E., Pico, Y. (2018): Pesticide residue in honey bees, pollen and beeswax: assessing beehive exposure, *Environ. Pollut.* 241, 106-114. https://doi.org/10.1016/j.envpol.2018.05.062

Chaimanee, V., Chantawannakul, P., Khongphinitbunjong, K., Pettis, J. S. (2019): Comparative pesticide exposure to *Apis mellifera* via honey bee-collected pollen in agricultural and non-agricultural areas of Northern Thailand, *J. Apicult. Res.* 58 (5), 720-729. https://doi.org/10.1080/00218839.2019.1637224

De Arruda, V. A. S., dos Santos A. V., Sampaio, D. F., da Silva Araujo, E., de Castro Peixoto, A. L., Fernandes Estevinho, M. L., de Almeida-Muradian, L. B. (2017): Microbiological quality and physicochemical characterization of Brazilian bee pollen, *J. Apicult. Res.* 56 (3), 231-238. https://doi.org/10.1080/00218839.2017.1307715

Dinkov, D. (2016): Microorganisms in vacuum stored flower bee pollen, *J. Bacteriol. Virol.* 46 (4), 258-268. http://dx.doi.org/10.4167/jbv.2016.46.4.258

Dinkov, D. (2018a): Isolation and identification of bacteria in flower bee pollen, *Walailak J. Sci. Tech.* 15 (3), 225-234.

Dinkov, D. (2018b): Bacterial contamination of vacuum stored flower bee pollen, *J. Microbiol. Biotech. Food Sci.* 7 (4), 387-391. http://dx.doi.org/0.15414/jmbfs.2018.7.4.387-391

El-Seedi, H. R., Khalifa, S. A. M., El-Wahed, A. A., Gao, R., Guo, R., Tahir, H. E., Zhao, C., du, M., Farag, M. A., Musharraf, S. G., Abbas, G. (2020): Honeybee products: an updated review of neurological actions, *Trends Food Sci. Technol.* 101, 17-27. https://doi.org/10.1016/j.tifs.2020.04.026

Estevinho, L. M., Rodrigues, S., Pereira, A. P., Feas, X. (2011): Portuguese bee pollen: palynological study, nutritional and microbiological evaluation, *Int. J. Food Sci. Technol.* 47, 429-435. https://doi.org/10.1111/j.1365-2621.2011.02859.x

Kačaniova, M., Pavličova, S., Haščik, P., Kociubinski, G., Knazovicka, V., Sudzina, M., Sudzinova J., Fikselova, M. (2009): Microbial communities in bees, pollen and honey from Slovakia, *Acta Microbiol. Immunol. Hung.* 56 (3), 285-295. https://doi.org/10.1556/AMicr.56.2009.3.7

Kačaniova, M., Juraček, M., Chlebo, R., Knazovicka, V., Kadasi-Horakova, M., Kunova, S., Lejkova, J., Haščik, P., Mareček, J., Šimko, M. (2011): Mycobiota and mycotoxins in bee pollen collected from different areas of Slovakia, *J. Environ. Sci. Health B* 46, 623-629. http://dx.doi.org/10.1080/03601234.2011.589322

Kostić, A. Ž., Milinčić, D. D., Barać, M. B., Shariatti, M. A. Tešić, Ž. Lj., Pešić, M. B. (2020): The application of pollen as a functional food and feed ingredient – the present and perspectives, *Biomolecules* 10, 84. http://dx.doi.org/10.3390/biom10010084

Li, Q., Liang, X., Zhao, L., Zhang, Z., Xue, X., Wang, K., & Wu, L. (2017): UPLC-Q-Exactiveorbitrap/MS-based lipidomics approach to characterize lipid extracts from bee pollen and their in vitro anti-inflammatory properties, *J. Agricult. Food Chem.* 65 (32), 6848–6860. http://dx.doi.org/10.1021/acs.jafc.7b02285

Li, Q.-Q., Wang, K., Marcucci, M. C., Sawaya, A. C. H. F., Hu, L., Xue, X.-F., Wu, L.-M., Hu, F.-L. (2018): Nutrient-rich bee pollen: a treasure trove of active natural metabolites, *J. Funct. Foods* 49, 472-484. https://doi.org/10.1016/j.jff.2018.09.008

Lopez, A., C., Fernandez, L. A., Alippi, A. M. (2020): Traceability of potential enterotoxigenic *Bacillus cereus* in bee-pollen samples from Argentina throughout the production process, *Int. J. Food Microbiol.* 334, 108816. https://doi.org/10.1016/j.ijfoodmicro.2020.108816

Manning, R. (2018): Chemical residues in beebread, honey, pollen and wax samples collected from bee hives placed on canola crops in Western Autralia, *J. Apicult. Res.* 57 (5), 696-708. https://doi.org/10.1080/00218839.2018.1494889

Maragou, N. C., Pavlidis, G., Karasali, H., Hatjina, F. (2016): Cold vapour atomic absorption and microwave digestion for the determination of mercury in honey, pollen, propolis and bees of Greek origin, *Glob. Nest J.* 18 (4), 690-696.

Maragou, N. C., Pavlidis, G., Karasali, H., Hatjina, F. (2017): Determination of arsenic in honey, propolis, pollen, and honey bees by microwave digestion and hydride generation flame atomic absorption, *Anal. Lett.* 50 (11), 1831-1838. https://doi.org/10.1080/00032719.2016.1244542

Margaroan, R., Strant, M., Varadi, A., Topal, E., Yucel, B., Cornea-Cipcigan, M., Campos, M. G., Vodnar, D. C. (2019): Bee collected pollen and bee bread: bioactive constituents and health benefits, *Antioxidants* 8, 568. https://doi.org/10.3390/antiox8120568

Mekki, I. (2019): Microbial contamination of bee pollen and impact of preservation methods, dissertation, Univeriste Libre de Tunis, Tunisia.

Migdal, P., Roman, A., Popiela-Pleban, E., Kowalska-Goralska, M., Opalinski, S. (2018): The impact of selected pesticides on honey bees, *Pol. J. Environ. Stud.* 27 (2), 787-792.

Morgano, M. A., Teixeira Martins, M. C., Rabonato, M. C., Milani, R. F., Yotsuyanagi, K., Rodriguez-Amaya, D. B. (2010): Inorganic contaminants in bee pollen from southeastern Brazil, *J. Agric. Food Chem.* 58, 5876-6883.

Nicolson, W. S. (2011): Bee food: the chemistry and nutritional value of nectar, pollen and mixture of the two, *Afr. Zool.* 46 (2), 197-204. https://doi.org/10.3377/004.046.0201

Ostiguy, N., Drummond, F. A., Aronstein, K., Eitzer, B., Ellis, J. D., Spivak, M., Sheppard, W. S. (2018): Honey bee exposure to pesticides: a four-year nationwide study, *Insects* 10, 13. https://doi.org/10.3390/insects10010013

Radanović, D., Antić-Mladenović, S. (2012): Uptake, Accumulation and Distribution of Potentially Toxic Trace Elements in Medicinal and Aromatic Plants. *In: Mineral Nutrition of Medicinal and Aromatic Plants* (M. Naeem, M. Masroor A. Khan, Moinuddin, Eds.), Global Science Books.

Rahimzadeh, M. R., Rahimzadeh, M. R., Kazemi, S., Moghadamnia, A. (2017): Cadmium toxicity and treatment: an update, *Caspian J. Intern. Med.* 8 (3), 135-145.

Raimets, R., Bontsutsnaja, A., Bartkevics, V., Pugajeva, I., Kaart, T., Puusepp, L., Pihlik, P., Keres, I., Viinalass, H., Mand, M., Karise, R. (2020): Pesticide residues in beehive matrices are dependent on collection time and matrix type but independent of proportion of foraged oilseed rape and agricultural land in foraging territory, *Chemosphere* 238, 124555. https://doi.org/10.1016/j.chemosphere.2019.124555

Ratnaike, R. N. (2003): Acute and chronic arsenic toxicity, *Postgrad. Med. J.* 79, 391-396. https://doi.org/10.1136/pmj.79.933.391

Rodriguez-Carasco, Y., Font, G., Manes, J., Berrada, H. (2013): Determination of mycotoxins in bee pollen by gas chromatography – tandem mass spectrometry, *J. Agric. Food Chem.* 61, 1999-2005. https://dx.doi.org/10.1021/jf400256f

Roman, A. (2009): Concentration of chosen trace elements of toxic properties in bee pollen loads, *Polish J. of Eniron. Stud.* 18 (2), 265-272.

Sattler, J. A. G., De-Mello, A. A. M., do Nascimento, K. S., de Melo, I. L. P., Mancini-Filho, J., Sattler, A., de Almeida-Muradian, L. B. (2016): Essential minerals and inorganic contaminants (barium, cadmium, lithium, lead and vanadium) in dried bee pollen produced in Rio Grande do Sul State, Brazil, *Food Sci. Technol. Campinas* 36 (3), 505-509. http://dx.doi.org/10.1590/1678-457X.0029

da Silva, E. V. C., Reis, I. F. A., de Almeida, L. N., dos Prazeres, A. R., da Silva, J. B., de Oliveira, A. C. S. (2019): Dehydrated apiculture pollen quality, *Period. Tche Quimica.* 16 (31), 335-340.

Silva, A. S., Araujo, S. B., Souza, D. C., Santos E Silva, F. A. (2012): study of CU, Mn, Pb and Zn dynamics in soil, plants and bee pollen from the region of Teresina (PI), Brazil, *An. Acad. Bras. Cienc.* 84 (4), 881-889.

Sinkevičiene, J., Marcinkevičiene, A., Baliukoniene, V., Jovaišiene, J. (2019): Fungi and mycotoxins in fresh bee pollen. Proceedings of 9th International Scientific Conference Rural Development 2019 69-72. http://doi.org/10.15544/RD.2019.004

Thakur, M., Nanda, V. (2020): Composition and functionality of bee pollen: a review, *Trends Food Sci. Technol.* 98, 82-106. https://doi.org/10.1016/j.tifs.2020.02.001

Ubaidurrahman, H. U., Asghar, W., Nazir, W., Sandhu, M. A., Khalid, N. (2020): A comprehensive review on chlorpyrifos toxicity with special reference to endocrine disruption: evidence of mechanisms, exposures and mitigation strategies, *Sci. Total Environ.* 755 (2), 142649. https://doi.org/10.1016/j.scitotenv.2020.142649

Vandenberg, L. N., Najmi, A., Mogus, P. (2020): Agrochemicals with estrogenic endocrine disrupting properties: lessons learned?, *Mol. Cell. Endocrinol.* 518, 110860. https://doi.org/10.1016/j.mce.2020.110860

Wang, S.-Y. Shi, X.-C., Liu, F.-Q., Laborda, P. (2020): Chromatographic methods for detection and quantification of carbendazim in food, *J. Agric. Food Chem.* 68, 43, 11880-11894. https://doi.org/10.1021/acs.jafc.0c04225

Wani, A. L., Ara, A., Usmani, J. A. (2015): Lead toxicity: A review, *Interdiscip. Toxicol.* 8 (2), 55-64. https://doi.org/10.1515/intox-2015-0009

Zioga, E., Kelly, R., White, B., Stout, J. C. (2020): Plant protection product residues in plant pollen and nectar: a review of current knowledge, *Environment. Res.* 189, 109873. https://doi.org/10.1016/j.envres.2020.109873

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STABILITY OF 10-HDA IN LYOPHILIZED ROYAL JELLY AND IN THE FINISHED PRODUCTS

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professional paper

Summary

This paper aims to examine the stability of lyophilized royal jelly by monitoring the content of its biologically active component, 10-hydroxy-2-decenoic acid (10-HDA). Stability was monitored in the lyophilized royal jelly and in the finished products containing it. Analyses were performed using high performance liquid chromatography (HPLC) with diode array detector. Chromatographic conditions for HPLC with diode array detection were as followed: InterSustain® C18 column, 150 x 40 mm, 5 µm; column temperature, 40 °C; mobile phase was a mixture of methanol: ultrapure water: phosphoric acid (250: 250: 1,25) with a flow rate of 1 mL/min. The detection wavelength was UV 210 nm. The amount of 10-HDA decreased regardless of storage conditions (room temperature and light protection), but the content of 10-HDA in any sample of the lyophilized royal jelly did not decline below the declared 3.5% during the shelf life. During the monitoring period, the analysed finished products also shown a decrease in the amount of 10-HDA, but if stored under recommended conditions, all analysed products contain the amount of 10-HDA in accordance with the declared value.

Keywords: 10-HDA, lyophilized royal jelly, stability

Introduction

Royal jelly has a wide range of positive effects on the human body making it one of the most valuable bee products. Bee glands, such as hypopharyngeal, mandibular, venom and wax, secrete numerous volatile and non-volatile components which are integral part of royal jelly, bee venom, and wax, the only three products that bees synthesize *de novo* (Erler and Moritz, 2016; Sabatini et al., 2009). The largest share in fresh royal jelly takes water, making 62.0 - 68.5% while the rest consists of proteins, lipids, carbohydrates, and to lesser extent vitamins and minerals (ISO, 2016). The main representatives of the proteins are the group of proteins called Major royal jelly proteins (MRJP), which make up the largest part of water-soluble proteins and it is assumed that they are responsible for the growth and development of the queen. A total of 26 amino acids were isolated and identified, of which 8 were essential for human organisms. Lipids are very important components of royal jelly of which more than 80% are free fatty acids while the rest are waxes, steroids, phenols and phospholipids. The most important representative of free fatty acids is 10-hydroxy-2-decenoic acid, which is characteristic of royal jelly, and as such is used as an indicator of quality and authenticity. The carbohydrate composition of royal jelly is similar to that of honey and is mostly composed of glucose and fructose, whose share reaches up to 90% of total carbohydrates, while the remaining share consists of ribose, trehalose, maltose and erlose. The most common vitamins are B vitamins, emphasizing pantothenic acid and niacin, whose shares are 0.095%, while the total share of vitamins A, C, D, and E is about 0.008%. The share of minerals ranges from 0.8% to 3%, and amongst them can be found K, Na, P, S, Ca, Al, Mg, Fe, Cu and Mn (Bogdanov, 2017; Xue et al., 2017; Ramadan and Al-Ghamdi, 2012).

Fresh royal jelly is a yellowish-white, viscous substance with a sour-pungent odour and a sweet-sour taste. This highly beneficial blend has numerous biologically valuable components amongst which 10-HDA stands out as one of the most important (Bogdanov, 2017). 10-HDA has an effect on the activation and modulation of the immune system, acts on the production of collagen and has a mild estrogenic effect, antibacterial, anti-tumour, anti-inflammatory, anti-ulcer and anti-rheumatic effects. Due to the reasons above, royal jelly is often used as a dietary supplement and functional food, as well as an ingredient in cosmetic products (Bogdanov, 2017; Oršolić, 2013; Ramadan and Al-Ghamdi, 2012).

Lyophilized royal jelly has the same biological characteristics as the fresh one due to process of lyophilization where the water is removed from the frozen product in the vacuum, which allows the retention of highly valuable components. Therefore, lyophilization is considered the best way to preserve the quality of royal jelly. During storage, it is extremely important to store lyophilized royal jelly in hermetically sealed containers due to its high hygroscopicity (Bogdanov, 2017; Krell, 1996). The chemical composition of lyophilized royal jelly is not yet legally regulated, but there are recommendations given based on the work (Sabatini et al., 2009; Oršolić, 2013). Although Bogdanov (2017) states storing lyophilized royal jelly for a year at

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refrigerator temperature (3 - 5 °C) or two years at freezer temperature (- 18 °C), manufacturers of lyophilized royal jelly recommend keeping it protected from light and high temperatures, with a shelf life of three years from the date of manufacture.

The aim of this study was to examine the stability of lyophilized royal jelly and finished products containing it when stored at room temperature by monitoring the content of 10-HDA.

Materials and methods

Samples of lyophilized royal jelly

Analyses were performed on 4 samples of lyophilized royal jelly. Stability monitoring of 10-HDA in lyophilized royal jelly samples was performed on samples purchased from two manufacturers in the period from 2011 till 2019. All samples originate from China and are submitted with the appropriate documentation or the certificate of analysis. The samples were stored protected from the light in a constant temperature room (22 ± 1.5 °C) with controlled content of the moisture in the air (47 ± 3 %), in accordance with the manufacturer's instructions. Sample 1, purchased from the first manufacturer, was submitted for analysis in 2011 and several analyses were performed, which were compared with the accompanying manufacturer's analysis certificate. The analyses were performed once a year in the period from 2013 till 2016. Another subsequent analysis was made in 2020 to confirm a further reduction in the share of 10-HDA. The remaining three samples were purchased from another manufacturer. They were analysed twice and the analytical reports were compared with the manufacturer's analysis certificate.

Samples of products with lyophilized royal jelly

Samples of 9 finished products containing lyophilized royal jelly from the same manufacturer, which were stored according to the label, i.e. at the temperature up to 25 °C, protected from light and heat sources, were analysed. The declared value of 10-HDA in the samples ranged from 5 to 20 mg of 10-HDA in 10 ml of the sample. The samples were divided into three groups and analysed at half-life, immediately after the expiration date and one year after.

Chemicals

To prepare the mobile phase methanol HPLC grade (Merck, Germany), 85% phosphoric acid (Sigma-Aldrich, USA) and ultra-pure water obtained by the Letzner water purification system (Hückeswagen, Germany) with an electrical conductivity of up to 0.04 μS/cm and a 10-HDA standard (ChromaDex, USA) purity ≥ 97.4% were used.

HPLC analysis

The analysis of lyophilized royal jelly samples and commercial samples containing it was performed on the Shimadzu HPLC system that includes LabSolution software, two quaternary pumps LC-20ADXR, a column chamber CTO-20AC, a diode array detector SPD-M20A and an autosampler SIL20-ACX. The separation of components was achieved on an InertSustain® C18 column from GL Science, measuring 150 x 40 mm, filled with 5 μm particles.

The mobile phase used for the analysis containing methanol, ultrapure water and phosphoric acid, prepared in a ratio of 250: 250: 1.25, was degassed with a vacuum pump before use. The flow of the mobile phase was 1 ml/min at a column temperature of 40 °C with an injection volume of 5 μl. Spectrum recording was performed in the wavelength range from 190 to 370 nm, while detection was performed at a wavelength of 210 nm. The identification of 10-HDA was performed by comparing the retention times of 10-HDA standard solution and sample solution (Fig. 1) and unquestionable identification was confirmed comparing the specific spectrum of 10-HDA standard solution and sample solution.

The method (Garcia-Amoedo and Almeida-Muradian, 2003) was modified to fit the samples for analysis and the device on which it is applied. For method validation, the latter procedures were performed following good laboratory practice (GLP) and good manufacturing practice (GMP). Linearity, which was tested in the working range from 0.13 to 100 μg/mL which proved to be satisfactory for the expected concentrations of 10-HAD in royal jelly as raw material (1.26 - 2.25 % in fresh and 3.01 - 6.26 % in lyophilized royal jelly) and finished products containing it as an integral component. A limit of detection (LOD) was calculated based on signal-to-noise ratio (3.3:1) and it was 0.048 μg/mL and a limit of quantification (LOQ), calculated based on signal-to-noise ratio (10:1) (ICH, 2020), was 0.145 μg/mL. The precision of the method was tested through repeatability by successive measurements of three different concentrations in one day, giving a relative standard deviation (RSD) <0.46%. The average precision was determined by measuring three different concentrations over three days and RSD values obtained for peak area changes were <1.22%. The accuracy of the method was tested through analytical yield by analysis of three concentrations: 12.5, 50 and 100 μg/mL where analytical yields were obtained: 98.8%, 99.2% and 99.6%, respectively. The robustness of the method was tested by changing two experimental parameters (column

temperature and wavelength of detection), meeting the limits of acceptability for the tested changes (≤15%). Statistical analysis was performed using Microsoft Excel 365 (Microsoft Corp.).

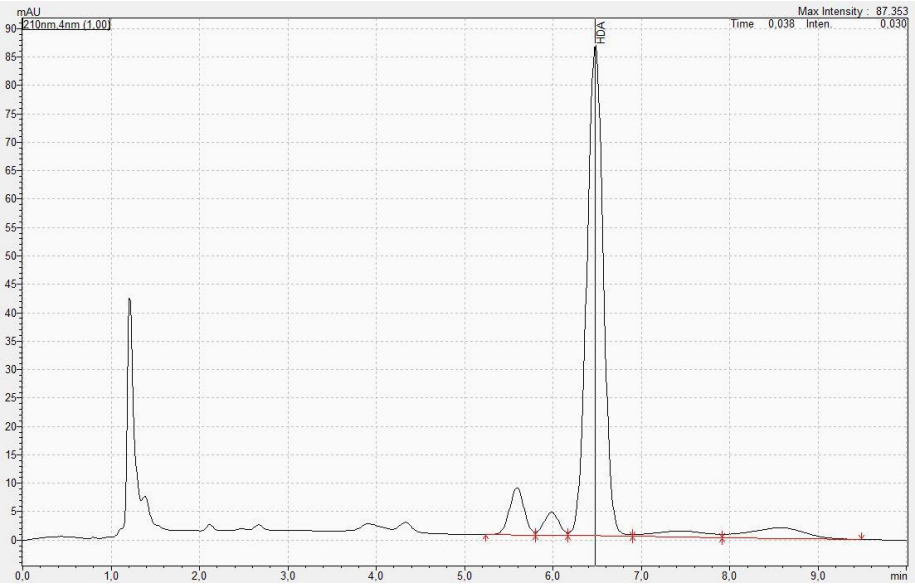

Fig. 1. Chromatogram of the lyophilized royal jelly solution

Results and discussion

Lyophilized royal jelly

The sample of lyophilized royal jelly No. 1 was monitored from the date of production (December 2011) to May 2020 (Fig. 2). This sample was supplied with an analytical certificate stating 4.2% of 10-HDA. During the shelf life, which was declared for 2 years from the date of manufacture, the share of 10-HDA decreased by 2.14%, and in the next year this value falls by 15.95% from the initial value and was at 3.53% of the 10-HDA content, but thus still meets the recommendations for the share of 10-HDA in lyophilized royal jelly. The analysis conducted in the following years showed a further decrease in the share of 10-HDA in the examined sample. Over 10 years, the analyses show a total decrease in the content of 10-HDA in the tested lyophilized royal jelly by 47.62% from the initial value declared on the manufacturer's certificate. Samples 2, 3 and 4 (Fig. 3) were purchased from another manufacturer and supplied with an analysis certificate stating the minimum share of 10-HDA of 5% and the validity period of three years from the date of manufacture. Over 1 year, the share of 10-HDA decreased by <1% and over 2 years to <20%, which is in line with the recommended expiration date.

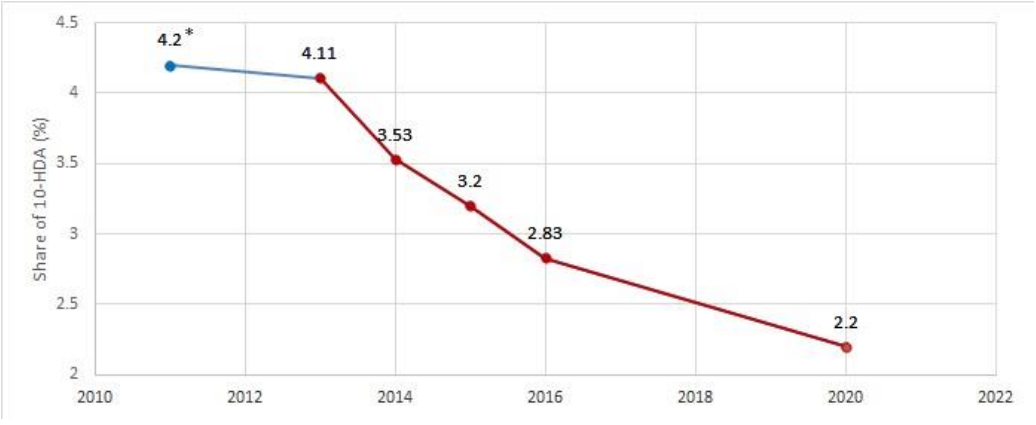

Fig. 2. Monitoring of the share of 10-HDA in sample 1 shown by years (*certified value)

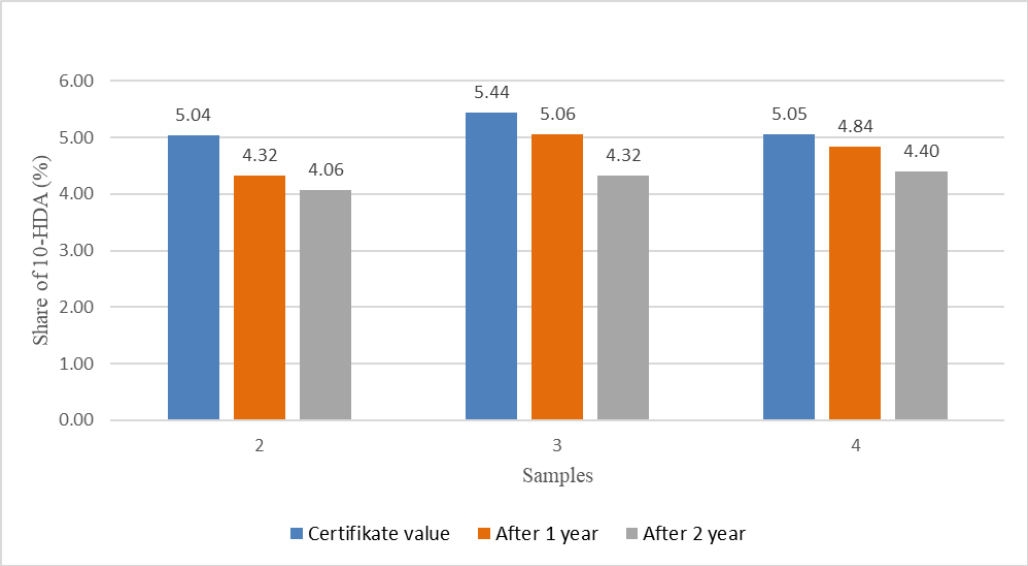

Fig. 3. Demonstration of the reduction of 10-HDA content in lyophilized royal jelly over 2 years

Finished products containing lyophilized royal jelly

Commercially available samples containing lyophilized royal jelly with a declaration stating the value of 10-HDA were analysed. The samples were divided into three groups. The declared value of 10-HDA was compared with the analytically determined share of 10-HDA in the finished product with lyophilized royal jelly one year after production, right after the expiration date and one year after the expiration date. Samples 1, 2 and 3 are the products not yet expired. They show a higher value of 10-HDA than the declared one, on average 33 ± 10.70 %, which is acceptable considering the data indicating a decrease in the value of 10-HDA in the tested samples of lyophilized royal jelly. Products 4, 5 and 6 that are near to the expiration date show the deviation of 11.69 ± 11.94 % from the declared values, which is within legal regulations. Products 7 and 8 expired one year ago show a lower share of 10-HDA than the declared values, 22.89 ± 18.72 %, while product 9 that was also tested one year after the expiration date does not show a significant decrease in the share of 10-HDA (Fig. 4).

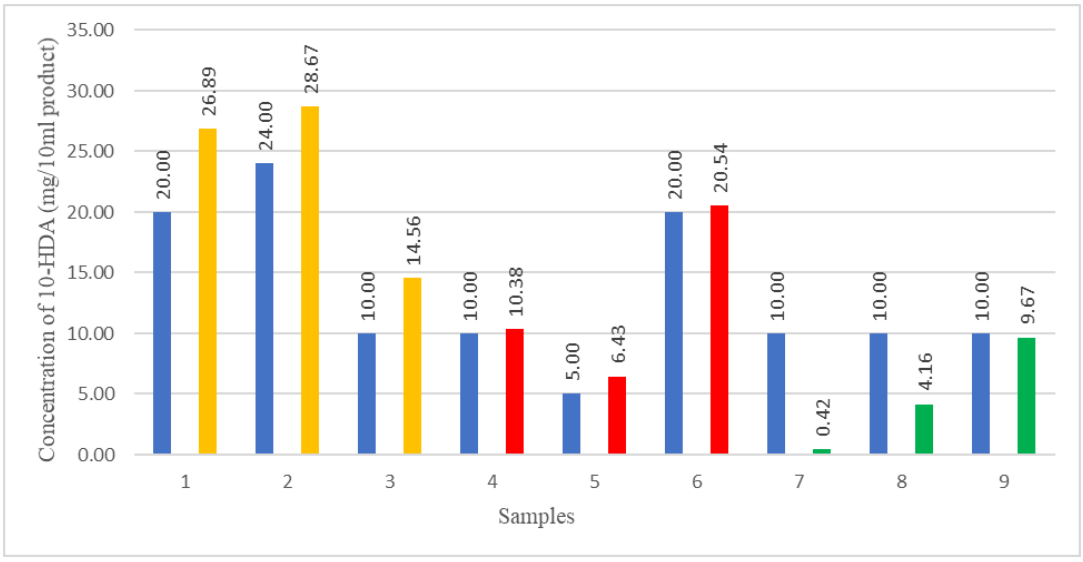

Fig. 4. 10-HDA concentration in commercially available products
(■ declared value, ■ analysis before expiration, ■ analysis after expiration, ■ analysis one year after expiration)

All of the above leads to the question of whether the obtained values of loss of 10-HDA content would be significantly lower if lyophilized royal jelly and finished products containing it were stored in different conditions (in a dark and cool place). Therefore, it would be desirable to continue this pilot study by conducting analyses for the quantitative determination of 10-HDA and to link the results to storage conditions. It would be necessary to store lyophilized royal jelly and finished products in precisely defined conditions of light, temperature (cold chain and room temperature) and humidity, to see if these differences are statistically significant.

Conclusion

The data obtained from the conducted pilot study suggest stability of lyophilized royal jelly under the storage conditions specified by the manufacturer, i.e. at room temperature and protected from a light within the recommended shelf life. The analysis of finished products showed a higher value of 10-HDA than declared in half the shelf life, which is associated with meeting the requirements of the shelf life. According to the legislation, the declared content of 10-HDA must be satisfactory even at the end of the product's shelf life. Therefore, according to the data of reducing the value of 10-HDA in lyophilized royal jelly, it is necessary to increase the share of lyophilized royal jelly as a component of the finished product in order to comply with the regulations, which is (± 10% 10-HDA) at the end of the estimated period.

References

Bogdanov, S.: The Royal Jelly Book. (2017): https://www.bee-hexagon.net/english/bee-products/royal-jelly/. Accessed September 7, 2020.

Erler, S., Moritz, R.F. (2016): Pharmacophagy and pharmacophory: mechanisms of self-medication and disease prevention in the honeybee colony (Apis mellifera). *Apidologie*. 47 (3), 389-411. https://doi.org/10.1007/s13592-015-0400-z

Garcia-Amoedo, L.H., Almeida-Muradian, L.B. (2003): Determination of trans-10-hydroxy-2-decenoic acid (10-HDA) in royal jelly from Sao Paulo State, Brazil. *Cienc. Tecnol. Aliment.* 23, 62-65. http://dx.doi.org/10.1590/S0101-20612003000400012

International Organization for Standardization (ISO). (2016). Royal jelly-specifications, ISO 12824:2016 (E.).

Krell, R: Royal Jelly. In Value-added products from beekeeping. 1996. http://www.fao.org/3/w0076e/w0076e16.htm Accessed September 8, 2020.

Nascimento, A., Moraes, L., Ferreira, N., Moreno, G., Uahib, F., Barizon, E., Berretta, A. (2015): The Lyophilization Process Maintains the Chemical and Biological Characteristics of Royal Jelly. *Evidence-Based Complementary and Alternative Medicine*, 2015, 1-6. https://doi.org/10.1155/2015/825068

Oršolić, N. (2013): Efficacy of biologically active components of royal jelly: analysis and standardization. *Archives of Industrial Hygiene and Toxicology*, 64 (3), 445-460. https://doi.org/10.2478/10004-1254-64-2013-2332

Ramadan, M.F., Al-Ghamdi, A. (2012): Bioactive compounds and health-promoting properties of royal jelly: A review. *Journal of functional foods*, 4 (1), 39-52, 2012. https://doi.org/10.1016/j.jff.2011.12.007

Sabatini, A.G., Marcazzan, G.L., Caboni, M.F., Bogdanov, S., Almeida-Muradian, L. (2009): Quality and standardization of Royal Jelly. *Journal of ApiProduct and ApiMedical Science*, 1 (1), 1-6, https://doi.org/10.3896/ibra.4.01.1.04

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). (2020) https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf. Accessed July 13, 2020.

Xue, X., Wu, L., Wang, K. (2017): Chemical Composition of Royal Jelly. In: Bee Products - Chemical and Biological Properties, J. M. Alvarez-Suarez, Springer, Cham, Switzerland, pp 181-190. https://doi.org/10.1007/978-3-319-59689-1 8

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