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THE EFFECT OF STORAGE TIME ON THE ANTIOXIDANT ACTIVITY AND POLYPHENOLIC PROFILE OF FROZEN AND LYOPHILIZED DRONE BROOD FIXED IN HONEY

S u m m a r y

Background. The purpose of the study was to evaluate the effect of drone brood fixation in rape honey and to analyze changes in its antioxidant activity during storage. The fixation was carried out for frozen and lyophilized drone brood, which were mixed with honey in various proportions (1, 2, and 4 % w/w). The antioxidant activity (DPPH and FRAP methods) and the total content of polyphenolic compounds (TPC) after 3, 6, and 9 months of storage were analyzed. After 9 months of storage, the polyphenolic profile (HPTLC method) and physicochemical parameters of samples were assessed.

Results and conclusion. The addition of drone brood to honey increased the antioxidant activity of the final product considerably (by 33 to 110 %), while only slightly affected the physicochemical parameters (conductivity and a diastase number) compared to control honey. Moreover, honey with the addition of drone brood still continued to meet the requirements for honey standard. The polyphenol profile obtained by HPTLC method for honey with the addition of drone brood was enriched mainly with ellagic and ferulic acids compared to control honey. It was found that fixing the drone brood in honey allows to maintain its antioxidant properties for 6 months, whereas a significant decrease in reducing power (FRAP) and polyphenolic content (TPC) during prolonged storage were observed (from 8 to 26 %). Due to the fact that lower losses were observed for the addition of frozen than for lyophilized drone brood after 9 months of storage, preserving the frozen brood in honey (up to 5 % w/w) can be recommended as an effective and inexpensive method available in apiary conditions.

Key words: drone brood, treatment, honey, antioxidant activity, polyphenolic profile

Introduction

Drone brood homogenate is a bee product obtained from drone larvae collected from drone cells in a honeycomb at various stages of development. In the form of a

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homogenate, because of its similar chemical properties, it is considered to be the male equivalent of royal jelly. It is commonly used as Apilarnil, which is obtained from drone brood subjected to homogenization, then frozen at -65 °C and fixed by lyophilization to preserve its biological properties [4].

Drone brood, as a nutrient-rich little-known beekeeping product has many beneficial healing properties. It is a valuable source of protein (13.28 %) and minerals (e.g. phosphorus, sodium, sulfur, magnesium, calcium), but also contains phenols (ellagic acid, chlorogenic acid) and steroid hormones (testosterone, progesterone, estradiol) [16]. Due to its low acquisition cost and effectiveness, it was used as a remedy for various diseases. Some of the biological and therapeutic effects of drone brood have been confirmed in *in vitro* laboratory studies [13, 16].

It is known that drone brood loses its biological activity in a very short time, so it is important to fix it as quickly as possible. The simplest and cheapest method is freezing (up to 6 days at -2 °C or up to 10 months at -18 °C). Drone brood can be frozen in liquid nitrogen (-196 °C) or lyophilized (shelf life up to 2 years). However, drone brood can be deposited on a stable absorbent such as honey. For this purpose, a previously prepared homogenate of drone brood is added to honey in the amount of up to 1 ÷ 5 % of the final volume. The storage of the final product at room temperature allows its properties to be retained for 6 months [5].

The effect of honey variety as a fixing agent has not been studied in detail. However, rape honey is best suited for this purpose. It is one of the best-known varieties in Poland due to the earliest harvesting time, a low price, and easy availability. In a liquid form, it has a light color and crystallizes the fastest among all honey varieties found in Poland (3 ÷ 7 days). After crystallization, it turns white or changes its color to creamy. Rape honey contains essential oils, tannins, bitter compounds, flavonoids (mainly quercetin, kaempferol, apigenin) [23]. This honey type nourishes and regenerates an exhausted organism [12]. Recently, it has been promoted to replace unhealthy sugar with honey, which cannot be overdosed because of its sweet taste and quick satiation with sweet taste.

The aim of the study was to fix drone brood (frozen or lyophilized) in rape honey and to analyze changes in the antioxidant activity of obtained mixtures during storage.

Materials and methods

Chemicals

Chemicals [2,2-diphenyl-1-picrylhydrazyl; 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); 2,4,6-Tris(2-pyridyl)-s-triazine], reagents (Folin–Ciocalteu reagent), were obtained from Sigma Aldrich (St. Louis, MO, USA). Solvents and acids (ethyl alcohol, ethyl acetate, formic acid, acetic acid) were purchased from Chempur (Piekary

Śląskie, Poland) and reagents for HPTLC visualization (natural product reagent, PEG 400) were obtained from Carl Roth GmbH (Karlsruhe, Germany).

Material Collection

The drone brood was collected from an apiary in the south-eastern part of Poland in the June 2020 season. The drone brood of the *Apis mellifera carnica* breed families were selected by hand from a drone frame, immediately sealed in sterile containers, and transferred to the laboratory. The samples were homogenized using a tissue homogenizer (TH 02, Omni International, Kennesaw, GA, USA) with 7 mm Omni Tips ™ plastic tips. The material was then frozen at -18°C or lyophilized (using Alpha 1–2, LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Rape honey was purchased from a local apiary located in the Podkarpackie Province in the 2020 beekeeping season.

As a method of drone brood fixation, both the frozen (F) or lyophilized (L) drone brood were introduced to honey during creaming. Liquefied rape honey (100 g) was weighted in a glass jar (200 cm^3), inoculated with crystallized honey (99:1) and mixed with a kitchen mixer for 60 s, four times a day to start the crystallization process. Six honey samples were creamed. Subsequently, frozen drone brood was added to honey in increasing amounts of 1, 2, and 4 % (w/w*)*. For lyophilized drone brood, the addition was recalculated taking into account a loss of water during lyophilization as 0.3, 0.6 and 1.2 % w/w, but for the better understanding, it was marked as the same as the dose of frozen brood dose (corresponded value 1, 2 and 4 % w/w). The whole mixture was mixed again with the kitchen mixer for 60 s. Samples prepared in such a manner were stored in a refrigerator at 4 °C for three days and mixed two times a day to obtain homogeneous consistency. After complete crystallization, the honeys were stored at 21 ± 2 °C without exposure to sunlight for 3, 6, 9 months until analyses.

DPPH Test

The inhibition of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was measured by following the assay procedure described by Dżugan et al. [2], with minor modifications. The reduction of DPPH radicals was calculated using the following t AA % = $[(A_0 - A_s)/A_0] \times 100$, where A0 is the absorbance of the control and As is the absorbance of the tested samples and expressed as μmol of Trolox (TE) equivalents per 100 g of honey (μmol/100 g) based on a calibration curve ($y = 15.553x$, $r^2 = 0.9970$), prepared for Trolox in the range of 25-300 nmol/cm^3.

FRAP Assay

The FRAP (ferric ion reducing antioxidant parameter) assay was carried out according to Dżugan et al. [2]. The results were expressed as μmol of Trolox (TE) equiv-

alents per 100 g of honey (μmol/100 g) based on a calibration curve (y = 0.152*x*, r^2 = 0.9998), prepared for 0.1 mmol Trolox in the range of 25-300 nmol/cm^3.

Total Phenolic Content Determination (TPC)

The total phenolic content was determined using a Folin-Ciocalteu reagent, according to Dżugan et al. [2], with minor modifications. The phenolic content expressed as mg of gallic acid (GAE) equivalents per 100 g of honey (mg GAE/100 g) was calculated based on a calibration curve prepared for gallic acid in the range of 0 ÷ 150 μg/mL (y = 0.336*x*, r^2 = 0.9914).

Polyphenolic profile using HPTLC chromatography

The polyphenolic profiles for honeys were performed on HPTLC Silica Gel 60 F_{254} plate (20 × 10 cm) purchased from Merck (Darmstadt, Germany). The samples for an analysis were prepared by making a 20 % solution of honeys in water acidified with HCl (pH = 2). Subsequently, the solutions were passed through the C-18 Sep-Pak cartridges (Waters, Milford, USA) retaining phenolic compounds. Ballast compounds were rinsed with 10 mL of acidified water and finally polyphenols were rinsed with 2.5 mL of methanol. Five μL of such prepared honey samples were applied to the HPTLC Silica Gel 60 F_{254} plate as 7 mm bands from the lower edge of a plate, at the rate of 100 nL/s, using a semi-automated HPTLC application device (Linomat 5, CAMAG, Muttenz, Switzerland). Chromatographic separation was performed in a chromatographic tank saturated for 20 min with the mobile phase (ethyl acetate: water: formic acid: acetic acid; 15:2:1:1) and developed to a distance of 70 mm. The results obtained were documented using an HPTLC imaging device (TLC Visualizer, CAMAG) at 366 nm. Additionally, the plate was derivatized using an automated TLC plate derivatizer (CAMAG Derivatizer) with Natural Product Reagent (diphenylboric acid β-aminoethyl ester complex) and in the second step with an alcoholic PEG 400 solution. The chromatographic image obtained was analyzed using HPTLC software (Vision CATS, CAMAG). The profile of polyphenolic compounds of honey with the addition of drone brood was analyzed in comparison to control honey and drone brood fingerprints in terms of the pattern of bands, their color and intensity. Single phenolic compounds was identified based on Rf values determined for standard substances (ellagic acid, ferulic acid, chlorogenic acid, quercetin, apigenin) separated under the same conditions (not shown on the chromatogram).

Physicochemical parameters determination

The water content, active acidity and free acidity, conductivity and diastase number were determined for all tested samples strictly as described in our earlier paper [15], in accordance with the legal requirements for honey [11].

Statistical analysis

All calculations were made in triplicate. Three-way analysis of variance followed by HSD Tukey's test ($p < 0.05$) was applied to find significant differences in antioxidant activity regarding the used drone brood addition value, the time of storage and the form of added drone brood. The Student's t-test was used to check whether the differences between the control sample and other means are statistically significant. Correlation coefficients (r Pearson) were calculated. All calculations were performed using Statistica 13.3 software (StatSoft, Tulsa, USA).

Results and discussion

Changes in antioxidant activity and total phenolic content of drone brood fixed in rape honey during storage

Rape honey (control) showed antioxidant activity measured using a DPPH method at the level of 3.1 μmol TE/100 g, which gave almost 10 % of the ability to scavenge free radicals (Fig. 1a). The addition of 1 % of frozen drone brood to honey significantly increased the antiradical capacity by 64.5 %. However, the addition of 2 % of drone brood did not have a direct effect on a double increase in the antiradical activity. The highest increase in the analyzed parameter, compared to control honey, was demonstrated for the highest addition of frozen drone brood (an increase of 77.4 %).

The storage time did not influence the activity of the tested honeys significantly. However, after 9 months of the storage of honey, a significant decrease in antioxidant activity was found in all the analyzed samples. The ability of rape honey (control) to scavenge free radicals increased by 6.45 %, which is characteristic of stored honey (Maillard reaction) [21]. On the other hand, as regards the honeys to which drone brood was added, a decrease in antioxidant activity was found (9.3 ÷ 36.95 %). The greatest decrease was found for honey with a 4 % addition of frozen drone brood. Despite a decrease in activity after 9 months of storage, the honey retained a higher activity compared to the control honey.

A similar relationship was found for the results of ferric ion reducing antioxidant power (FRAP) (Fig. 1b), which was confirmed by a correlation among the results after 3 months of storage ($r = 0.532$). However, the addition of lyophilized drone brood enriched honey with bioactive compounds to a lower extent, but the highest increase compared to the control was found for the 4 % addition of frozen brood (60.53 %). The storage of drone brood fixed in honey for 9 months decreased the ability to reduce ferric ions in all samples significantly. Compared to the honeys stored for 6 months, the activity of rape honey decreased by 17.31 %, while in the case of honeys with brood addition, it ranged from 2.84 to 32.95 %.

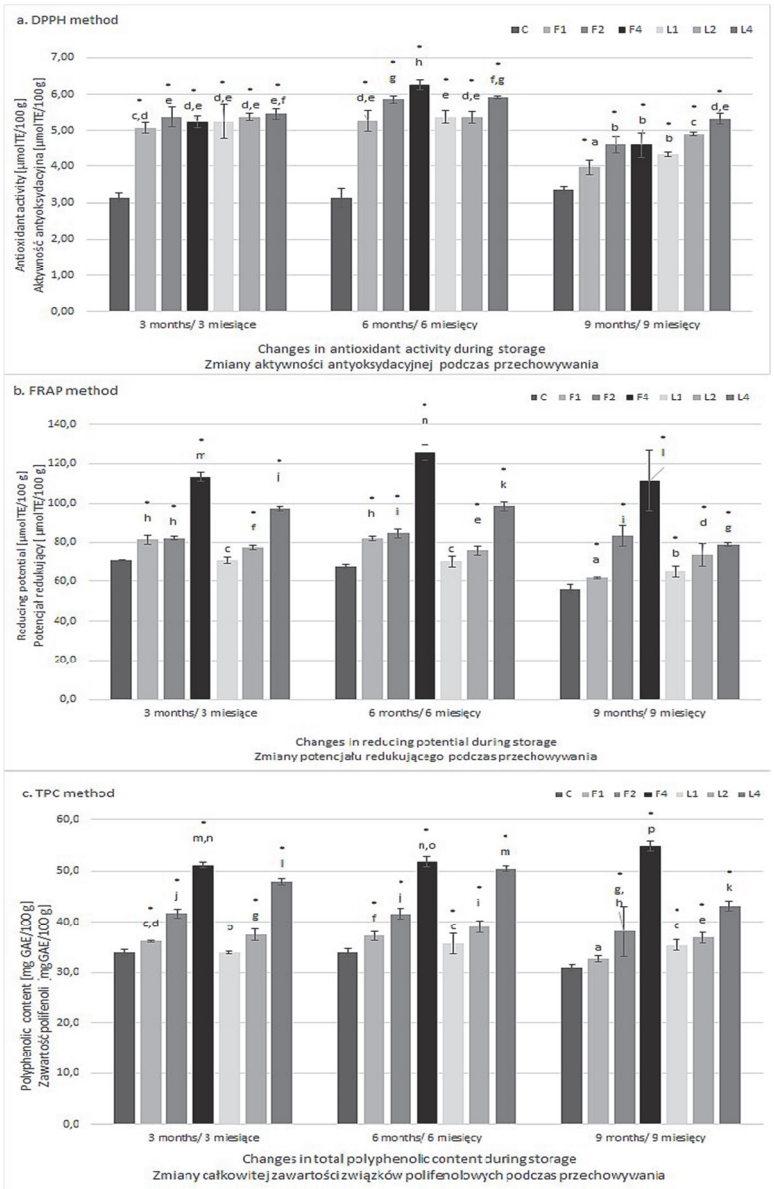

Fig. 1. Changes in the antioxidant properties of drone brood fixed in honey compared to control rape honey measured using the DPPH method (a), the FRAP method (b), and as total phenolic content (c); (n=3)

Ryc. 1. Zmiany właściwości przeciwutleniających czerwia trutowego utrwalonego w miodzie w porównaniu z kontrolnym miodem rzepakowym mierzone metodą DPPH (a), metodą FRAP (b) oraz jako całkowita zawartość polifenoli (c); (n=3)

Explanatory notes / Objaśnienia: $^{a, b, c, d, e, f, g}$ - means marked with different letters differ significantly / średnie oznaczone różnymi literami różnią się istotnie ($p < 0.05$); * significant differences compared to control honey / istotnie różne w porównaniu do próby kontrolnej ($p < 0.05$)

The synergistic effect of the mixture of various bee products with honey has been already reported. Habryka et al. [3] enriched honey with 25 % bee pollen and achieved eight-and-a-half times higher DPPH activity of the finished product compared to control honey. However, the 5 % addition contributed to a two-and-a-half-fold increase in activity. The authors [3] obtained a similar effect by enriching honey with propolis at various concentrations (0.1 ÷ 1 %). Socha et al. [17] analyzed the effect of adding bee bread to honey. A FRAP analysis showed that after the addition 20 % of bee bread to honey, the reducing ability increased 10 times, which was statistically significant.

The total polyphenol content in the rape honey was 33.9 mg GAE/100 g (Fig. 1c). The highest increase was recorded for the greatest addition of frozen and lyophilized drone brood by 51.03 and 41.29 %, respectively. Furthermore, the addition of drone brood increased the content of phenolic compounds significantly, except for the addition of 1 % brood lyophilisate compared to control honey. After 6 months of storage, the content of polyphenolic compounds in the enriched honey increased on average by 3-5 %, which was statistically significant and strongly correlated with the FRAP results ($r = 0.925$). Moreover, the influence of the form of the additive, the amount of the additive and the storage time on changes in the antioxidant potential and the content of polyphenols was found. All interactions were statistically significant ($p < 0.05$). The 9-month storage of drone brood fixed in honey resulted in a decrease in the content of polyphenolic compounds by 14.29 % for honey with a 4 % addition of lyophilized drone brood. Habryka et al. [3] enriched honey with 5 % bee pollen and showed a significant increase in the total content of polyphenolic compounds. Majewska and Trzanek [8] obtained similar results.

The comparison of the HPTLC polyphenolic profiles of drone brood fixed in rape honey after 9 months of storage

The comparison of polyphenolic profiles of drone brood fixed in rape honey was provided in Fig. 2.

The visualization of drone brood extract (DB) at 366 nm shows a phenolic profile (Rf = 0.12, 0.20, 0.25, 0.31, 0.45, 0.84, 0.91), which can be considered unique because the bands are visually different in color (light blue, blue). Additionally, chlorogenic acid (Rf = 0.03) was identified at the start line as the light blue band. Rape honey showed a different phenolic profile than drone brood. For honey, the most intense bands were found at Rf (0.12, 0.21, 0.31). This fingerprint was dominated by brown (Rf = 0.05 ÷ 0.25), and light yellow bands (Rf above 0.90). In addition, the presence of light yellow and light blue bands was found. Some bands that were barely visible in the control honey showed higher intensity in honey with the addition of drone brood at Rf = 0.31, 0.12 (blue bands) and 0.95 (yellow band) identified as apigenin. Increasing the

intensity and visibility of the bands, as well as increasing the addition share, proves that the content of phenolic compounds in honey becomes higher with the addition of drone brood. The results obtained were in line with the Stanek and Jasicka-Misiak's results [18], who compared the phenolic profile of nectar honeys. They showed dominant bands of yellow, light blue, blue and black, among which they identified the presence of p-coumaric acid, chlorogenic acid and myricetin in nectar honeys. However, Tomczyk et al. [19] analyzed the phenolic profile of Polish honeys, showing orange, blue, yellow, and green bands in rape honey. The composition of polyphenols in drone brood was similar to that in the previous studies [14, 15]. Blue bands dominated in the drone brood homogenate chromatogram, which proves the presence of ferulic acid (Rf

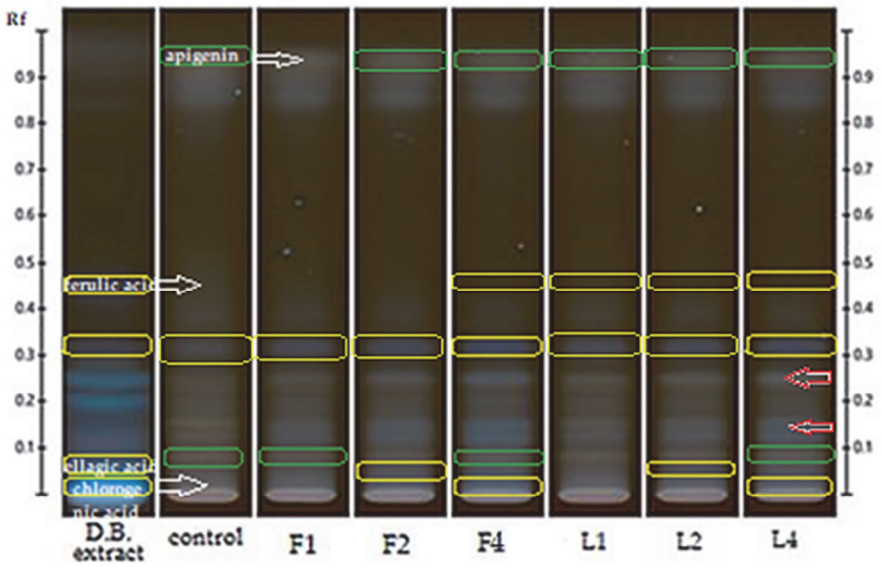

Fig. 2. Polyphenolic profiles (using the HPTLC method) after 9 months of storage of frozen (F1, F2, and F4) and lyophilized (L1, L 2 and L 4) drone brood fixed in honey. DB - drone brood extract and control - rape honey were used for the comparison.

Rys. 2. Profile polifenolowe (metodą HPTLC) po 9 miesiącach przechowywania zamrożonego (F1, F2 i F4) i liofilizowanego (L1, L 2 i L 4) czerwiu trutowego utrwalonego w miodzie. Dla porównania użyto DB - ekstrakt z czerwia trutowego i kontrolny miód rzepakowy.

Explanatory notes / Objaśnienia:

Selected compounds which migrated from the drone brood extract to honey are marked in yellow, compounds originated from rape honey are marked in green. The red arrow indicates the small amount of polyphenols present in honey, which the content of which was increased by drone brood addition (bands which were intensified).

Na żółto zaznaczono wybrane związki migrujące z ekstraktu czerwiu trutowego do miodu, na zielono zaznaczono związki pochodzące z miodu rzepakowego. Czerwona strzałka wskazuje polifenole obecne w miodzie w niewielkiej ilości, których zawartość została zwiększona przez dodanie czerwiu trutowego (prążki, które zostały zintensyfikowane).

= 0.48) and ellagic acid (Rf = 0.08) in the tested material. Moreover, apigenin as yellow band was identified [15]. Based on the HPTLC results, the beneficial effect of drone brood additive on polyphenolic profile of rape honey was shown, the higher share of drone brood, the more polyphenols were observed.

Physicochemical parameters and organoleptic evaluation of enriched honeys after 9 months of storage under legal regulations for honey

After 9 months of storage, the organoleptic evaluation of the honeys was carried out. During the evaluation of honeys with frozen drone brood, it was found that their taste and smell corresponded to the typical characteristics of rape honey and no foreign ones were found. Only the color of the honey with 4 % addition was slightly darker and the consistency more fluid, however, these observations did not reduce the overall quality of the honey. The honeys enriched with lyophilized drone brood behaved similarly. In this case, their taste and smell did not differ from that of the control honey. The color of the honey was very similar to that of the control honey, also with the 4 % additive. The honey enriched with lyophilized drone brood retained a more stable consistency compared to the addition of frozen brood due to low water content. In addition, it was observed that the highest addition stabilized the consistency and was the most stable among all of the honeys being evaluated.

Bee honey is obtained according to generally accepted beekeeping practice and is always subjected to quality control before being placed on the market. The physicochemical parameters, thanks to which the quality of honey is controlled based on a laboratory analysis, include, among other things, water content, electrical conductivity, acidity, pH, and diastase number. A good quality of the rape honey was confirmed, which was in line with the findings of other authors for Polish [7, 10, 19] and European [1, 6, 22] rape honey. The analysis of physicochemical parameters after 9 months of the storage of the honeys did not show deviations from the norm for nectar honeys [11]. The addition of 4 % of frozen drone brood did not exceed allowed water content. However, the value was significantly different from the control honey. Meanwhile, a beneficial increase in acidity, conductivity and diastase number was observed for the enriched honey, resulting from native acidity, high bioelement content, and diastatic activity of raw drone brood [15]. However, the values observed were still within the legal limits for honey quality standard.

Developing new products and introducing them to the market is extremely difficult and should take into account market requirements and consumer preferences. Most importantly, a new product and its ingredients should neither be hazardous to nor mislead the consumer [9]. Due to the hormonal activity of drone brood, detailed studies should be performed to confirm safe consumption and the maximum daily dose should be established before introducing it as a dietary product.

Table 1. Physicochemical properties of honey enriched with drone brood after 9 months of storage (n=3).

Tabela 1. Właściwości fizykochemiczne miodu wzbogaconego czerwiem trutowym po 9 miesiącach przechowywania (n=3).

Sample / Próbka	Water content Zawartość wody [%]	pH	Acidity Kwasowość [mval/kg]	Conductivity Przewodność właściwa [mS/cm]	Diastase number Liczba diastazowa
Control	17.00	4.70	13.15	0.215	11.07
F 1	17.20	4.52	16.10*	0.225	13.69*
F 2	17.30*	4.59	19.60*	0.249*	17.16*
F 4	19.70*	4.45	24.80*	0.256*	17.50*
L 1	17.15	4.63	15.90*	0.216	13.53*
L 2	17.00	4.59	17.20*	0.225	14.00*
L 4	17.00	4.56	22.70*	0.244*	14.96*
Standard requirements PN-88/A-77626	<20	-	<50	0.2-0.8	>8

Explanatory notes / Objaśnienia:
*significant differences compared to control honey / istotnie różne w porównaniu do miodem kontrolnym ($p < 0.05$)

Conclusions

1. The addition of drone brood to rape honey significantly increased its antioxidant activity and enriched polyphenolic profile in a dose-dependent manner.
2. The use of frozen drone brood in the highest dose was more beneficial than the lyophilized additive, for this product less changes in antioxidant activity were observed during prolonged storage.
3. The use of honey to fix the drone brood did not change significantly its physicochemical parameters, which still meet the requirements of honey quality standards.
4. Fixing frozen drone brood in honey seems to be the most applicable method under apiary conditions.

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WPŁYW CZASU PRZECHOWYWANIA NA WŁAŚCIWOŚCI ANTYOKSYDACYJNE I PROFIL POLIFENOLOWY MROŻONEGO I LIOFILIZOWANEGO CZERWIU TRUTOWEGO UTRWALONEGO W MIODZIE

S t r e s z c z e n i e

Wprowadzenie. Celem pracy była ocena wpływu utrwalenia czerwiu trutowego w miodzie rzepakowym oraz analiza zmian jego aktywności antyoksydacyjnej podczas przechowywania. Utrwalanie przeprowadzono na mrożonym i liofilizowanym czerwiu trutowym, który zmieszano z miodem w różnych proporcjach (1, 2 i 4 % w/w). Analizowano aktywność przeciwutleniającą (metodami DPPH i FRAP) oraz całkowitą zawartość związków polifenolowych (TPC) po 3, 6 i 9 miesiącach przechowywania. Po 9 miesiącach przechowywania oceniono profil polifenolowy (metoda HPTLC) oraz parametry fizykochemiczne.

Wyniki i wnioski. Dodatek czerwiu trutowego do miodu silnie zwiększył aktywność przeciwutleniającą produktu końcowego (o 33 do 110 %), a jedynie nieznacznie wpłynął na parametry fizykochemiczne (przewodność i liczbę diastazową) w porównaniu z miodem kontrolnym. Ponadto miód z dodatkiem czerwiu trutowego nadal spełnia wymagania normy dla miodu. Profil polifenolowy uzyskany metodą HPTLC dla miodu z dodatkiem czerwiu trutowego został wzbogacony głównie o kwas elagowy i ferulowy w porównaniu z miodem kontrolnym. Stwierdzono, że utrwalanie czerwiu trutowego w miodzie pozwala na zachowanie jego właściwości antyoksydacyjnych przez 6 miesięcy, natomiast zaobserwowano znaczne spadki mocy redukującej (FRAP) i zawartości polifenoli (TPC) podczas dłuższego przechowywania (od 8 do 26 %). W przypadku dodatku czerwiu mrożonego, po 9 miesiącach przechowywania obserwowano mniejsze straty niż w przypadku liofilizatu, dlatego przechowywanie mrożonego czerwiu w miodzie (do 5 % m/m) można polecić jako skuteczną i niedrogą metodę dostępną w warunkach pasiecznych.

Słowa kluczowe: czerw trutowy, utrwalanie, miód, aktywność antyoksydacyjna, profil polifenolowy