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Analysis of the amino acid composition of pollen and honey

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SUMMARY

Nowadays, an important direction in food analytical research is the development of methods for detecting honey counterfeiting. The amino acids in honey come mainly from pollen, raising the possibility of origin determination based on the examination of amino acid composition [1]. In our research, the free amino acid composition of single flower honeys and the pollen from their original plants were compared. According to our results, there is no correlation between the amino acid profiles of the pollen and the honeys, which is mainly due to the fact that it is rare that the pollen composition of single flower honeys strongly reflects the flower character. Our results support the hypothesis that proline comes from the bees, and the proline content of the pollen contributes to the high proline content of honey only marginally. Pollen pellet samples also contain large amounts of nectar and glandular secretions in addition to the pollen, and thus have a significantly lower free amino acid content than pollen samples collected directly from the flowers. In the course of the research, the total amino acid content of the pollen pellets was in the 6 to 16% range. Compared to other amino acids, proline was present in a significantly higher proportion in the free form than in the protein-bound form. There are contradictory data in the literature regarding the ability of bees to select among different pollens according to their needs [2, 3]. According to our results, the amino acid composition of pellets selected by the bees reflects their amino acid requirements better than unifloral samples. The rapeseed pellet, which is particularly preferred by the bees, has an amino acid composition that is different from the optimal one, but it has an outstanding essential amino acid content, so it is likely that a quantitative, and not qualitative protein intake regulation is carried out by the bees.

Introduction and literature review

Pollen is a microscopic, granular material that provides the male genetic material of flowering plants. When nectar is collected, it easily adheres to the body of the bees and eventually appears in the honey **[4]**. Pollen analysis can thus be used to determine the botanical origin of honeys, but with several limitations. Individual identification of the pollen grains requires a long time and special expertise, and is therefore relatively expensive. The reliability of the analysis is influenced by the experience and current judgment of the expert carrying out the investigation. In order to eliminate these factors, characterization of honeys based on chemical parameters has been preferred **[5]**. According to our knowledge, with the exception of proline, amino acids found in honey come from the pollen, so examination of the amino acid profile, together with other parameters, may be potentially useful in the determination of the botanical origin of honeys **[1]**.

The beekeeping sector, in addition to producing widely sought-after export products, is of vital ecological and economic importance: by pollinating flowers, bees ensure the proliferation of wild and cultivated plants, thus contributing to maintaining biodiversity and increasing agricultural productivity

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[6]. Pollen production has a tradition in only a few countries, but there has been a significant increase in demand on the international level over the last decade. Worldwide, more than 10,000 tons of pollen is consumed annually. Because of its natural conditions, in addition to Brazil, Argentina, Spain and China, Hungary also produces outstanding amounts of pollen **[7]**. Due to the natural and landscape conditions, as well as centuries-old cultural traditions, favorable conditions for the production of beekeeping products have developed in Hungary. In addition to their nutritional benefits, pollen pellets may pose a number of food safety risks, the most important of which are pesticides, toxic metals, pyrrolizidine alkaloids and mycotoxins **[8, 9, 10, 11]**. Pollen and the pellets made from it are extremely valuable in terms of nutrient content: they contain carbohydrates (13-55%), proteins (10-40%), fats (1-13%), dietary fiber (0.3-20%) and minerals (2-6%) **[12]**. In addition, some studies emphasize their antioxidant, antibacterial, anti-inflammatory, anticarcinogenic and antiallergic properties **[13]**. The importance of the topic is demonstrated by the fact that in the last 10-15 years the number of research projects concerning the nutrient content of pollen, as well as its vitamin, mineral and polyphenol composition has increased significantly. Based on this, pollen is considered to be a very valuable nutrient source in the human diet: pollen pellets can be used as functional dietary supplements due to their antioxidant activity, mineral content, fatty acid composition and their applicability in the prevention of certain diseases **[7, 14]**.

In our experience, research on nutritional ingredients and food safety risks is generally not accompanied by a full examination of the organoleptic characteristics. We found only a few scattered research results regarding the organoleptic properties of pollen. The quality of sensory data is determined by the sensory judges, and it is therefore necessary to continuously monitor the performance of trained and expert judges, for which several methods have been developed **[15, 16, 17, 18]**. Linking of the preference values of sensory consumers and the intensity values of trained judges is solved both from a method, statistical and software point of view **[19, 20, 21]**. One of the solutions that provide the most information is the integration of the organoleptic and instrumental results of smell, taste and texture **[22, 23, 24, 25, 26]**.

Due to the enormous number of bee deaths worldwide, research projects have been focusing on the protection of the health of bees, according to which the nutritional status of bees greatly influences their resistance to parasites, infections and various stress factors, and thus their mortality **[27, 28]**. In general, it can be stated that pollens have a variety of nutrient profiles and a wide variety of bioactive components are found in them. They contain large amounts of proteins, amino acids, usable carbohydrates and lipids, as well as phenolic compounds, enzymes, coenzymes, vitamins and minerals **[29]**. These compounds are essential for the proper physiological development of bees, so a smaller part of the family specializes in pollen collection. Pollen collecting bees accumulate pollen adhering to their bodies in the baskets on their hind legs, shape and compact it with their glandular secretions and nectar, so they can easily transport it to the hive. Under optimal conditions, strong families collect much more pollen than they need, and therefore, beekeepers can collect pellets from them using a special tool, and the pellets are used as dietary supplements and for apitherapeutic purposes **[30, 31]**. It is a known fact that bees have different preferences for different types of pollen. For example, pollen from rapeseed is preferred to other pollen available at the same time, while pollen from other plants is only collected only periodically or under extreme weather conditions **[30, 32, 33]**. Many researchers have found that able to select among different pollen species according to their amino acid requirements, but there are studies that refute this assumption **[2, 3]**.

Nowadays, an important direction in food analytical research is the development of methods for detecting honey counterfeiting. The amino acids in honey come mainly from pollen, raising the possibility of origin determination based on the examination of amino acid composition **[1]**. At present, little information is available on the relationship between the amino acid composition of pollens and that of single flower honeys.

The aim of our work was to draw a parallel between the free amino acid profiles of some single flower honeys, pollen pellets from their main source plants collected by the bees, and pollen collected directly from the plants, with special emphasis on the proline content. We aimed to clarify the hypothesis that bees are able to select among different pollens according to their needs. To this end, we aimed to examine whether there was a correlation between the amino acid composition of rapeseed pollen, particularly preferred by the bees, and the amino acid requirement of the bees.

Materials and methods

Our analytical samples included rapeseed, linden, Japanese pagoda tree, goldenrod and sunflower honey, as well as pollen collected directly from their source plants. When selecting the pollens, our goal was to examine the amino acid composition of plants from which nectar is collected by the bees for the production of single flower honey, of flowers that produce no nectar but are used by the bees as pollen sources, as well as of flowers from which pollen is collected only under exceptional circumstances. Data for the samples used are summarized in ***Table 1***.

The free amino acid composition of pollen from common hibiscus and aster, which are nutrient sources for the bees, and of pollen from ragweed and pelargonium, rarely visited by the bees, have also been determined **[31, 32]**. The free and protein-bound amino acid composition of pollen pellets from rapeseed and sunflower, as well as summer pollen pellets selected by the bees were also examined.

The pollen composition of the honeys was determined by a traditional, microscopic method by the expert of the Feed Testing National Reference Laboratory of the National Food Chain Safety Office, according to standard specifications **[34]**. The source plant can be identified on the basis of the microscopic properties of the pollen grains.

For the determination of the free amino acid composition, sample preparation was performed as follows:

In the case of pollens collected from flowers 0.2 g, in the case of honeys 3.0 g and in the case of pollen pellets 1.0 g of homogenized sample was weighed into a 50 ml Erlenmeyer flask with analytical accuracy. Pellet samples were crushed in a mortar before weighing in order to maximize the dissolution of amino acids. To the weighed samples, 5 ml of trichloroacetic acid was added, and extraction was carried out at 100 rpm for 1 hour on a shaker. The extract was then first filtered through filter paper into a test tube, then through 0.22 µm pore size syringe filters into Eppendorf tubes. The samples thus prepared were stored frozen until the analysis.

For the determination of total amino acid composition, sample preparation was performed as follows:

Pollen pellets were crushed in a mortar and 0.15 g was weighed into hydrolysis tubes with analytical accuracy. 10 ml of 6 M hydrochloric acid was added to the samples, and nitrogen was bubbled through the solutions. Hydrolysis of the sealed solutions was carried out at 110 °C for 24 hours. After cooling, the hydrolyzate was washed with 20 ml of 4 M NaOH solution into a 25 ml round-bottom flask, and it was filled to mark with distilled water. The solution obtained after neutralization was first filtered through filter paper into a test tube, then through 0.22 µm pore size syringe filters into Eppendorf tubes. The samples thus prepared were stored frozen until the analysis.

Free amino acid composition and total amino acid composition were determined using an INGOS AAA 400 amino acid analyzer at the Department of Food Chemistry and Nutrition of Szent István University, using an ion exchange chromatography method. The parameters of INGOS AAA 400:

- Cation exchange column: Ionex Ostion LCP 5020, column size: 200 x 3.7 mm,
- Column temperature: 50 °C-60 °C
- Reaction temperature:120 °C
- Analysis time: 200 min
- Eluent: Li citrate buffers (Li citrate, LiCl and citric acid)
- Sample volume:100 µl
- Detection: 440 nm, 570 nm
- Eluent flow rate: 0.30 ml/min
- Ninhydrin flow rate: 0.25 ml/min
- Detection limit: 0.5 µmol/l

The assay is carried out in a strongly acidic medium, with a series of eluents of gradually weakening acidity, with step gradient elution (*buffer 1:* 0.18 M Li citrate, pH 2.80; *buffer 2:* 0.20 M Li citrate, pH 3.05; *buffer 3:* 0.36 M Li citrate, pH 3.35; *buffer 4:* 0.33 M Li citrate, pH 4.05; *buffer 5:* 1.20 M Li citrate, pH 4.65). Amino acids are detected spectrophotometrically on the basis of their color reaction with ninhydrin. The color reaction of proline is different from that of the other amino acids, therefore it is detected at 440 nm, while the other amino acids can be detected at 570 nm. The color reaction takes place by post-column derivatization at 120 °C. Chromatograms were evaluated using the CHROMuLAN082 program, by comparison with standard amino acid mixtures. Of the chromatograms obtained in our studies, ***Figure 1*** shows the chromatogram of linden pollen, while ***Figure 2*** shows the chromatogram of summer pellet.

Results and conclusions

Pollen analysis results showed that rapeseed honey alone has a main pollen ratio above 90%. Sunflower and Japanese pagoda tree honey contained pollen from the plant indicated in 66 and 60%, respectively. Linden and goldenrod honey contained mainly pollen not from the indicated source plant, and also contained large amounts of pollen from plants that do not bloom at the same time, suggesting that the finished products were not properly treated. Pollen compositions of the honeys are shown in ***Table 2***.

The concentration of free amino acids was 0.40-0.77 mg/g in honeys, 15.06-47.53 mg/g in pollen collected directly from the plants, and 10.40-22.41 mg/g in the pellets collected by the bees. Proline was predominant in all three product types, with its ratio to the total free amino acids being 56-76% in the case of honeys, 29-81% in the case of pollen, and 69-82% in the case of pollen pellets. The concentration of free proline was approximately two orders of magnitude higher in pollen than in honey. However, honey ha a low pollen content, so it is estimated that only 1.8% of the free proline content of honey comes from pollen, which means that our results support the hypothesis that the amino acid composition of the product is altered by the bees during the transformation of the nectar through their metabolic processes.

Table 3 compares the free amino acid content of single flower honeys and of the pollen of the plants indicated as the sources of the nectar. According to the data, there is a difference of two or three orders of magnitude between the amino acid profiles of the honeys and the pollens. This is mainly due to the fact that the pollen composition of the honeys is very varied, and the proportion of the main pollen rarely reaches 90%. Our results can also be traced back to the fact that the amino acid composition of pollen is influenced by extreme environmental conditions, and that the free amino acid composition of honeys is influenced by the amino acid content of the nectar, the transformation activity of the bees, as well as storage time **[35]**.

The total amino acid content of the pellets was 157.91 mg/g in the case of rapeseed, 61.84 mg/g in the case of sunflower and 65.96 mg/g in the case of the mixed product collected in the summer, 24% of which was proline on average. Our results confirm literature data, according to which the majority of proline is present in the free form in pollen collected by the bees **[36]**. ***Figure 3*** shows pellet composition with respect to amino acids essential for the bees, indicating that the rapeseed product contains approximately two to three times the amount of these components compared to the other two samples.

Measurement results of the essential amino acid content of pollen pellets were compared to literature data that discuss the ratios of amino acids essential to the bees **[37]**. ***Figure 4*** shows the relative amino acid requirements of honey bees and the proportion of the amino acids found in the samples. When optimizing bee nutrition, it is worth keeping in mind that isoleucine was the limiting amino acid in the products, as bees require this amino acid in the highest amount, but it was present in the pellet samples only in small to medium proportion. The second limiting amino acid in the case of rapeseed and the summer mixed product was methionine, while in the case of sunflower it was threonine.

Based on the results, we reject the hypothesis that bees select among pollen types according to their needs and choose the pollen to be collected to serve as the most suitable nutrition for them, since the rapeseed pollen most preferred by them does not have the optimal amino acid composition. It is most likely that the amount of pollen on the plant, its protein content, easy availability (suitable flower structure) and the color, smell and distance of the given plant from the hive play a greater role in the preference.

Summary

In our experimental work, the amino acid composition of the honeys tested, of pollen collected from the flowers by us and of commercially available pollen pellets collected by the bees were compared. Some of the experiments were aimed at finding a correlation between the amino acid content of pollen and the collection preferences of the bees, while others sought to determine whether the amino acid composition of the pollen of the source plants is reflected in the amino acid content of the honeys. Pollen serves as an almost exclusive source of amino acids and proteins for bees. Many researchers have concluded that bees are capable of selecting among different pollen species according to their needs, however, our results refute this claim, because the rapeseed pollen most preferred by them does not have the optimal amino acid composition. It is more likely that the amount of pollen on the plant, its protein content, easy availability (suitable flower structure) and the distance of the given plant from the hive all play a role in the preference. The pollen content of honey comes from the flowers that bees visit for either nectar or pollen, and so in certain cases it can serve as evidence of the origin of the honey. Our studies included relatively high single flower pollen content honeys and honeys with lower pollen content.

It is a known fact that proline is the predominant free amino acid in both honeys and pollens. Proline acts as a kind of “stress hormone” in plants, i.e., it serves as a precursor to other amino acids when needed, therefore, it is produced in large quantities in different parts of the plants. According to our test results, the free proline content of pollen is orders of magnitude higher than the amount of other free amino acids, but according to our calculations, pollen contributes only marginally to the proline content of honey. Consequently, the theory that bees transform the collected material during honey production can be confirmed. Contact with the bee’s body changes the proportion of amino acids in the product. Since proline is not essential to bees, its proportion in the product increases, while essential amino acids are utilized by their body, thus their proportion decreases in the honey. It is advisable to carry out further experiments to investigate the pollen preference of bees under experimental conditions where the plant species are available in approximately the same amounts and availability, thus eliminating the distorting effect of easy accessibility, among other things. Some studies also suggest that bees also prefer proline-rich nectar sources. Since proline is not essential to bees, this study can be refined by comparing nectar sources (sugar solutions) with exactly the same sugar composition and smell in proline-enriched and non-proline variants.

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