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# THE CHEMICAL COMPOSITION OF POLLEN, STAMINATE CATKINS, AND HONEY OF *CASTANEA SATIVA* MILL.

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## ABSTRACT

The chemical composition of pollen and honey primarily depends on the botanical and geographical origin of the species, as well as other factors – climatic conditions, soil type, plant species, etc. The present study was to knowledge the biochemical profile of pollen, staminate catkins, and honey samples of *Castanea sativa* Mill. which were examined under conditions of Ukraine. Proteins are the major components of pollen and staminate catkins (169.0 ±1.60 g.kg$^{-1}$ and 69.8 ±1.67 g.kg$^{-1}$, respectively), while saccharides are predominant in honey samples (38.0 ±1.32 g.kg$^{-1}$ fructose, 32.5 ±0.68 glucose g.kg$^{-1}$ and 6.1 ±0.06 g.kg$^{-1}$ sucrose). Glutamic acid (13.30 g.kg$^{-1}$), aspartic acid (13.05 g.kg$^{-1}$), and proline (12.45 g.kg$^{-1}$) were predominant nonessential amino acids in the chestnut pollen. The content of macro and microelements was found in the pollen and staminate catkins much higher than in the honey. All *Castanea sativa* samples are a very valuable source of potassium as the main mineral element contained in pollen (7400 mg.kg$^{-1}$), staminate catkins (7760 mg.kg$^{-1}$), and honey (981 mg.kg$^{-1}$). Microelements such as manganese and iron prevailed in pollen (478 mg.kg$^{-1}$ Mn and 461 mg.kg$^{-1}$ Fe), and staminate catkins (247 mg.kg$^{-1}$ Mn and 109 mg.kg$^{-1}$ Fe), and heavy metals (Hg, Sr, Sn, Sb, Li) are present only in the pollen samples with the most abundant Sr (12.8 mg.kg$^{-1}$) and Sn (1.9 mg.kg$^{-1}$) content and can be used as indicator suggesting the environmental pollution status in the region. Regarding the vitamin content, vitamin C was the most represented in all samples. Obtained results indicate that chestnut is species with important constituents such as amino acids and vitamins, with low content of heavy metals and high content of biogenic elements that may be used in phytotherapy and phytopharmacology.

**Keywords:** *Castanea sativa*; staminate catkins; pollen; honey; amino acid

## INTRODUCTION

*Castanea* Mill. (Chestnut), belonging to the family *Fagaceae* is distributed in over ten species in temperate climate regions, including Asia, Europe, and North America. Among chestnut trees, *C. sativa, C. crenata, C. dentata,* and *C. mollissima* are important species consumed as a crop. Chestnut trees are valuable not only as food but also as a honey source plant, are already classified as major honey plants in many countries. The inflorescence is composed either of male or female flowers, or staminate and pistillate catkins occur together. Only the male flowers produce nectar that is secreted on the surface of the dislocated at the base of filaments in staminate catkins, from which is possible to produce 27.2 kg of honey per 100 trees **(Farkas and Zajácz, 2007)**. Chestnut pollen, bee pollen, and bee bread were also identified to contain functional and specific substances **(Ivanišová et al., 2015; Hudz et al., 2017a; Hudz et al., 2017b; Nikolaieva et al., 2017)**, mineral

elements **(Motyleva et al., 2018b)** which show anti-inflammatory, antimicrobial **(Avşar et al., 2016)**, antioxidant activity **(Feas et al., 2012; Alves et al., 2013; Avşar et al., 2016; Kızılpınar Temizer et al., 2018; Nikolaieva et al., 2019)**, and hepatoprotective effect **(Yıldız et al., 2013)** was high in *C. sativa* honey **(Turski et al., 2016)**.

Major honey components are carbohydrates (mainly monosaccharides, fructose, glucose, and sucrose) and water. In small amounts, honey also contains minerals, proteins, organic acids, volatile components, phenolics, vitamins, and pigments. Although present in small amounts, these components are very important for honey characterization and nutritive properties **(White, 1975)**. A much better parameter for botanical and geographical origin identification was the analysis of free amino acids in honey, which was reported by several authors **(Hermosín, Chicón and Cabezudo, 2003; Cotte et al., 2004; Nozal et al., 2004; González Paramás et al., 2006)**. Pollen

contains an average of 1500 mg.100g$^{-1}$ of amino acids, which means approximately ten times more than the average concentration in honey (**Davies, 1975**). The major amino acid in honey is proline (80 – 90% of the total amino acids) except lavender, thyme, and rosemary kinds of honey where the major amino acid is phenylalanine (**Hermosín, Chicón and Cabezudo, 2003; Cotte et al., 2004**). The presence of proline is of animal origin and the quantity of proline depends on the time spent by the nectar in the bee's crop (**Petrov, 1974**). The proline content was proposed by the International Honey Commission as a honey ripeness and possible sugar adulteration indicator, with a minimum value of 180 mg.kg$^{-1}$ for fresh, genuine honey (**Bogdanov et al., 1999**). Besides proline, 26 amino acids (both essential and nonessential) were also identified in honey (**Hermosín, Chicón and Cabezudo, 2003**).

Due to ecological-geographical conditions very important are not only differences in some compounds (biogenic elements, heavy metals), which can increase/decrease the quality of pollen or honey but also evaluation of pollen sizes (**Grygorieva et al., 2013; Grygorieva et al., 2017; Motyleva, Gruner and Semenova, 2018a, Horčinová Sedláčková et al., 2018, Horčinová Sedláčková et al., 2020**).

The chemical composition of honey primarily depends on the botanical origin of species or cultivars. However, climatic conditions and/or geographical origin can also affect the chemical composition even within the same honey type (**Anklam, 1998**).

The aim of the present study was to knowledge the biochemical profile of pollen, staminate catkins, and honey samples of *Castanea sativa* cultivar introduced under conditions of Ukraine, such as determination of saccharides, carotenoids, vitamins, amino acids, minerals (macro- and microelements) and heavy metals were examined. Comparative data of the mineral and amino acid composition of pollen and staminate catkins from different ecological-geographical zones little reflected in the literature.

## Scientific hypothesis
*Castanea sativa* is a major honey plant in many countries is a potential source of phytochemical compounds used in food and health products against less-researched and less-used staminate catkins and chestnut pollen. The scientific hypothesis of this study was to examine the pollen, staminate catkins, and honey samples of *Castanea sativa* species and compared them due to their phytochemical, amino acid, and mineral profiles as appropriate substances of phytotherapeutic and phytopharmacology preparations.

## MATERIAL AND METHODOLOGY
### Biological Material
We examined the biological material of *C. sativa* cultivar (staminate catkins, chestnut dried pollen, and monofloral chestnut honey) introduced in M. M. Gryshko National Botanical Garden of Ukraine in Kyiv (Figure 1 and Figure 2). Staminate catkins and pollen were harvested in June 2020 in dry conditions and with no precipitation. Samples were obtained from one cultivar of three trees.

### Chemicals
Ethanol (Centralchem s.r.o., Bratislava, Slovakia, p.a.).

Acetonitrile (Fisher Chemical, Loughborough, UK, HPLC grade).

Petroleum ether (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, Sigma Grade, ≥99%).

Ninhydrin (Ingos, Czech Republic), nitric acid (Analytika Praha Ltd, Czech Republic).

Hydrochloric acid (Analytika Praha Ltd, Czech Republic).

Methyl cellosolve (Ingos, Czech Republic).

Filter with 0.45 µm pore size (Labicom, Czech Republic).

Tin chloride ($SnCl_2$) (Centralchem s.r.o., Bratislava, Slovakia, p.a.).

### Instruments
HPLC system with an ELSD detector (Agilent Technologies 1260 Infinity, Santa Clara, CA, USA).

HPLC system with ninhydrin and a VIS detector (Model AAA-400 amino acid analyzer, Ingos, Czech Republic).

UV-VIS spectrophotometer (UV Jenway Model 6405, UV/VIS, England).

ICP-OES system (Ultima 2, Horiba Scientific, France).

ES column (Zorbax SB-C18, 4.6x25.0 mm, 5 µm particle size, Agilent, Santa Clara, CA, USA).

Microwave oven (Milestone 1200, Milestone, Italy).

Vertical shake table (GFL, Germany).

Centrifuge (EBA 21, Hettich, Germany). Cation exchanger (LG ANB sodium cycle, Laboratory of Spolchemie).

### Laboratory Methods
### Phytochemical analyses
#### *Determination of dry matter, ash, and protein content*
Total dry matter, ash, and protein content were determined according to the EN method (**ČSN EN 12145, 1997**). Total lipid content was determined according to methods specified in the ISO method (**ISO 659, 1998**).

#### *Determination of saccharides*
For the determination of saccharides, 1 g of sample was extracted with 10 mL of extraction solution (ultrapure water and ethanol mixed in ration 4:1) in a 50 mL centrifugation tube placed on a vertical shake table (GFL, Germany). After 1 h of extraction, samples were centrifuged for 4 min at 6000 rpm in a centrifuge (EBA 21, Hettich, Germany); the supernatant was filtered using a filter with 0.45 µm pore size (Labicom, Czech Republic) and filled up to 50 mL in a volumetric flask with ultrapure water. An Agilent Infinity 1260 liquid chromatography (Agilent Technologies, USA) equipped with an ELSD detector was used for the determination of saccharides. A Prevail Carbohydrates ES column (250/4.6 mm) was used as a stationary phase and acetonitrile (VWR) mixed with water in a 75:25 volume ratio was used as the mobile phase.

#### *Determination of carotenoid*
Total carotenoid content expressed as beta-carotene was analyzed at a wavelength of 445 nm spectrophotometrically (VIS spectrophotometer UV Jenway Model 6405 UV/VIS). Sample (1 g) was disrupted with sea sand and extracted with acetone until complete discoloration. Petroleum-ether was added and then water, in purpose to the separation of phases. After the separation, the petroleum ether-carotenoid phase was obtained and the absorbance was measured (**ČSN 560053, 1986**).

**Figure 1** Middle phase of blooming *Castanea sativa* Mill. cultivar.



**Figure 2** Inflorescences of staminate catkins *Castanea sativa* Mill.

### *Determination of mineral contents*

Sample for elemental analysis was prepared using the wet ashing method in a microwave oven (Milestone 1200, Milestone, Italy). A total of 0.25 g sample matrix was decomposed in a mixture of nitric acid (6 mL) (Analytika Praha Ltd, Czech Republic) and hydrochloric acid (2 mL) (Analytika Praha Ltd, Czech Republic).

After the decomposition sample was filtered using a filter with 0.45 μm pore size and filled up to 25 mL in a volumetric flask with ultrapure water. Elemental analysis was performed using ICP-OES (Ultima 2, Horiba

Scientific, France) according to the procedure described by **Divis et al. (2015)**.

### *Determination of amino acids*

Amino acids were determined by ion-exchange liquid chromatography (Model AAA-400 amino acid analyzer, Ingos, Czech Republic) using post-column derivatization with ninhydrin and a VIS detector. A glass column (inner diameter 3.7 mm, length 350 mm) was filled manually with a strong cation exchanger in the LG ANB sodium cycle (Laboratory of Spolchemie) with average particles size 12 μM and 8% porosity. The column was tempered within the range of 35 to 95 °C. The elution of the studied

amino acids took place at a column temperature set to 74 °C. A double-channel VIS detector with the inner cell volume of 5 μL was set to two wavelengths: 440 and 570 nm. A solution of ninhydrin (Ingos, Czech Republic) was prepared in 75% v/v methyl cellosolve (Ingos, Czech Republic) and in 2% v/v 4 M acetic buffer (pH 5.5). Tin chloride ($SnCl_2$) was used as a reducing agent. The prepared solution of ninhydrin was stored in an inert atmosphere ($N_2$) in darkness at 4 °C. The flow rate was 0.25 (mL.min$^{-1}$) and the reactor temperature was 120 °C.

**Determination of vitamins**

*Determination of Vitamin C concentration*

Vitamin C concentration was determined according to the method described by **Vikram, Ramesh and Prapulla (2005)**. Precisely 1 g of the sample was mixed with meta-phosphoric acid and centrifuged at 10,000 rpm for 10 min. at 4 °C in a refrigerated centrifuge (Model H-2000C). The supernatant was sieved through Whatman No 4 filter paper. The filtrate was diluted with 1 mL of 0.8% meta-phosphoric acid and filtered with a 0.450 Millipore filter and 20 μL of the sample injected into the HPLC (Shimadzu 20 A-Series, with column -NH2 -LUNA-100A (250X4.6mm), Diameter – 5uLand Refractive Index Detector.

*Determination of Vitamin A content*

The method described by **Okwu (2004)** was used to determine vitamin A. Sample (1 g) was weighed into a beaker and macerated with a 10 mL mixture of acetone and n-hexane (1:1) and filtered. Thereafter, 10 mL of 50% $(NH_4)_2SO_4$ solution was added, vigorously shaken, and allowed to settle. The upper layer was collected, and the absorbance read in Spectrophotometer (Spectro21D, Pec Medicals, USA) at 450 nm against hexane as blank beta-carotene served as standard. Retinol equivalent was obtained by the conversion factor: 1 mg retinol equal to 12 mg of dietary beta-carotene.

*Determination of Vitamin A content*

Analysis Tocopherols and tocotrienols were analyzed by normal-phase HPLC using a Merck-Hitachi system (Merck, Darmstadt, Germany) with fluorescence detection (excitation: 292 nm, emission: 330 nm). Chromatographic separation was achieved within 45 min at 35 ±10 °C using a Eurospher-100 DIOL column (250 × 4.0 mm, 7 μm), preceded by a Eurospher-100 DIOL guard column (5×4.0 mm, 7 μm) (both from Knauer, Berlin, Germany). An isocratic mobile phase of n-hexane/MTBE (98:2, v/m) was used at a flow rate of 1.5 mL.min$^{-1}$. Individual tocochromanols were identified by comparing their retention times with those of external standards and quantified by 5-point calibration curve of externalstandards.

*Determination of Vitamin B1, B2, and B6*

**Reagents**

All reagents and standards were of analytical grade. Vitamins standards were from Sigma-Aldrich with a purity ≥99%. Potassium hydroxide (KOH), concentrated sulfuric acid ($H_2SO_4$), and acetic anhydride was purchased from Mikrochem (Pezinok, Slovakia). Ethyl acetate, n-hexane, and sodium sulphate anhydrous were purchased from Centralchem s.r.o. (Bratislava, Slovakia). Methanol, HPLC grade was purchased from Fisher Chemical (Loughborough, UK).

**Standard**

Preparation 1 mg.mL$^{-1}$ of working standards i. e. thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, and folic acid were dissolved in methanol. Solutions were further serially diluted to the concentration of 5 – 10 μg.mL$^{-1}$ for thiamine chloride hydrochloride, 1.5 – 2 μg.mL$^{-1}$ for riboflavin, 5 – 10 μg.mL$^{-1}$ for pyridoxine hydrochloride, and 1 – 2 μg.mL$^{-1}$ for folic acid. 50 μl of each solution was used for analysis.

**Sample**

Preparation. Each sample was weighed and triturated into fine powder. The powdered sample was suspended in 50 mL of methanol, sonicated for 15 min, and dilute to 100 mL with methanol. 50 μL of each was for HPLC analysis.

Chromatographic Conditions Shimadzu LC system (LC 20A pump) equipped with UV/Visible detector was used for analysis. A single wavelength of 254 nm was used for simultaneous detection. A Promosil HPLC column C18 was used at 35 ºC. Methanol – 0.05 M sodium acid phosphate (80 + 20, v/v) at pH 7.0 was used as mobile phase. The flow rate was adjusted to 0.8 mL.min$^{-1}$. 50 μL of sample and standard solutions were injected in a single injection.

**Description of experiment**

*Sample preparation*

Freshly flowers (male catkins) were collected randomly from the three genotypes at the ballon stage (June 2020). Pollen samples released from male flowers (catkins) were further dried under laboratory conditions at 20 – 25 °C and prepared for analysis.

*Honey samples*

A total of 5 honey samples were collected from Ukrainian beekeepers. The samples were stored at room temperature in the dark and analyzed within the month after extraction. To confirm the beekeeper's denomination, the samples were subjected to preliminary analyses. Uniflorality of samples was confirmed according to electrical conductivity.

**Number of samples analyzed:** 18
**Number of repeated analyses:** 3
**Number of experiment replication:** 1

**Statistical Analysis**

Basic statistical analyses were performed using PAST 2.17. Data were presented as mean ±standard error of the mean (SEM) and differences between means were considered at $p$ <0.05. The variability of all these parameters was evaluated using descriptive statistics.

**RESULTS AND DISCUSSION**

Bee pollen contains high amounts of carbohydrates, amino acids, fatty acids, minerals, and vitamins. The contents of these ingredients depend on the botanical origin of the pollen. The presence of these components shows that bee pollen can be used for human nutrition **(Campos et al., 2008)**.

In honey, the content of proteins, including the enzymes, is relatively low and has a minor nutritive significance. On the other hand, the proteins, including the enzymes, are usually used as honey quality evaluation parameters. This is because protein content and enzyme activities vary regarding the botanical and geographical origin of the honey **(Rodriguez-Otero et al., 1994; Flanjak et al.,**

**2016; Anklam, 1998; Won et al., 2008). Roulston, Cane, and Buchmann (2000)** present that pollen contains from 2.5 to 61% of protein.

The phytochemical compounds in samples of pollen, staminate catkins, and honey of *Castanea sativa* are shown in Table 1. Our results show that the pollen and staminate catkins have the highest content of cellulose (954.6 g.kg$^{-1}$ and 190.6 g.kg$^{-1}$, respectively) and proteins (169.0 g.kg$^{-1}$ and 911.3 g.kg$^{-1}$). In the dried pollen from different plant species, the protein content varied from 142.1 – 314.0 g.kg$^{-1}$ **(Human and Nicolson, 2006)**, 150.4 – 276.9 g.kg$^{-1}$ DW **(Carpes et al., 2009)**, 242.3 – 341.8 g.kg$^{-1}$ DW **(Estevinho et al., 2012)**, 191.0 – 271.0 g.kg$^{-1}$ DW **(Feas et al., 2012)**, 125.0 – 251.5 g.kg$^{-1}$ DW **(Nogueira et al., 2012)**, 142.4 – 289.5 g.kg$^{-1}$ DW **(Yang et al., 2013)**, 160.3 – 323.4 g.kg$^{-1}$ **(Lilek et al., 2015)**. **Flanjak et al. (2016)** compared the protein content of various species and their study presented that chestnut honey had the highest protein content (the mean was 94.8 mg.100g$^{-1}$) followed by sage (the mean was 79.2 mg.100g$^{-1}$), honeydew honey (the mean was 59.4 mg.100g$^{-1}$). Black locust honey had the lowest protein content (the mean was 30.4 mg.100g$^{-1}$). These differences are probably due to the different plant species and different environmental conditions. The protein content may be a bioactive indicator that contributes to the pharmacological activities of honey **(Tonks et al., 2003; Yusof et al., 2007; Chua, Lee and Chan, 2015)**.

Three free sugars, namely, fructose, glucose, and sucrose, were detected in all samples, while the honey samples have the highest content of carbohydrates (fructose 38 g.kg$^{-1}$, glucose 32.5 g.kg$^{-1}$, and sucrose 6.1 g.kg$^{-1}$). Saccharides represent the main components of honey, and many papers have been published for using sugars as an indication of adulteration but cannot be used to identify the floral or geographical origins of honey **(Sivakesava and Irudayaraj, 2002)**.

**Kim et al. (2020)** presented free sugar content and sugar content per catkin in floral nectar of various *Castanea* spp. Fructose was present in the interval from 61.5 (*C. mollissima*) to 74.8% (*C. crenata*), glucose in the range of 33.7 (*C. dentata*) to 20.5% (*C. crenata*), sucrose ranged from 3.2 (*C. sativa*) to 13.4% (*C. dentata*), free sugar content varied from 22.6 (*C. crenata*) to 82.5 µg.µL$^{-1}$ (*C. mollissima*), sugar content per catkin was determined in the range from 0.31 (*C. sativa*) to 1.35 mg.catkin$^{-1}$ (*C. crenata*) and nectar volume per catkin in the interval from 8.7 (*C. dentata*) – 42.7 µL.catkin$^{-1}$ (*C. crenata*) **(Kim et al., 2020)**.

**Carocho et al. (2014)** presented results of saccharides of *C. sativa* staminate catkins: fructose – decoctions (152.08 –160.41 mg.g$^{-1}$), infusions (123.58 – 148.94 mg.g$^{-1}$); glucose – decoctions (149.09 – 191.91 mg.g$^{-1}$), infusions (145.71 – 164.07 mg.g$^{-1}$); sucrose – decoctions (25.69 – 27.01 mg.g$^{-1}$), infusions (26.67 – 35.68 mg.g$^{-1}$).

Regarding the vitamin content, vitamin C was the most represented in all samples in the following amounts in pollen 95 mg.kg$^{-1}$, staminate catkins 71 mg.kg$^{-1}$, and honey 55 mg.kg$^{-1}$.

The fat content predominates in pollen and staminate catkins against chestnut honey in amounts 55.4, 30.0, and 0.3 g.kg$^{-1}$, respectively.

The fat content of the polyfloral bee pollen from different plant species varied in the intervals 10.0 to 130.0 g·kg$^{-1}$ DW **(Campos et al., 2008)**, 23.3 – 33.2 g.kg$^{-1}$ **(Estevinho et al., 2012)**, 43.0 – 63.0 g.kg$^{-1}$ DW **(Feas et al., 2012)**, 23.5 – 33.3 g.kg$^{-1}$ DW **(Nogueira et al., 2012)**, 37.0 – 65.0 g.kg$^{-1}$ DW **(Soares de Arruda et al., 2013)**, 6.6 – 107.9 g.kg$^{-1}$ DW **(Yang et al., 2013)**, 60.7 g.kg$^{-1}$ – 157.9 g.kg$^{-1}$ **(Lilek et al., 2015)**.

**Table 1** Contents of some phytochemical compounds in pollen, staminate catkins and honey of *Castanea sativa* Mill.

| Components | Pollen (*mean ±SD*) | Staminate catkins (*mean ±SD*) | Honey (*mean ±SD*) |
|---|---|---|---|
| **Cellulose (g.kg$^{-1}$)** | 954.6 ±29.32 | 190.6 ±3.10 | <1 |
| **Dry matter (g.kg$^{-1}$)** | 138.2 ±2.86 | 911.3 ±19.66 | – |
| **Water (%)** | – | – | 14.43 ±0.11 |
| **Proteins (g.kg$^{-1}$)** | 169.0 ±1.60 | 69.8 ±1.67 | 6.5 ±0.16 |
| **Fats (g.kg$^{-1}$)** | 55.4 ±0.15 | 30.0 ±0.11 | 0.3 ±0.01 |
| **Beta Carotene (mg.kg$^{-1}$)** | 3.0 ±0.04 | 2.4 ±0.009 | 2.0 ±0.01 |
| **Vitamin A (mg.kg$^{-1}$)** | <0.1 | <0.1 | <0.1 |
| **Vitamin E (mg.kg$^{-1}$)** | <0.1 | 24.0 ±0.13 | <0.1 |
| **Vitamin B1 (mg.kg$^{-1}$)** | 1.5 ±0.04 | 5.8 ±0.03 | 2.1 ±0.04 |
| **Vitamin B2 (mg.kg$^{-1}$)** | <0.5 | 1.3 ±0.01 | 0.5 ±0.03 |
| **Vitamin B6 (mg.kg$^{-1}$)** | <0.5 | 3.2 ±0.01 | 1.1 ±0.009 |
| **Vitamin C (mg.kg$^{-1}$)** | 95.0 ±2.10 | 71.0 ±2.17 | 55.0 ±0.96 |
| **Folic acid (mg.kg$^{-1}$)** | 0.7 ±0.02 | 1.8 ±0.02 | 0.6 ±0.01 |
| **Nicotinamide (mg.kg$^{-1}$)** | <0.5 | 5.8 ±0.05 | 10.9 ±1.21 |
| **Fructose (g.kg$^{-1}$)** | 3.0 ±0.01 | 4.1 ±0.05 | 38.0 ±1.32 |
| **Glucose (g.kg$^{-1}$)** | 1.8 ±0.04 | 2.2 ±0.02 | 32.5 ±0.68 |
| **Sucrose (g.kg$^{-1}$)** | 4.6 ±0.05 | 2.1 ±0.04 | 6.1 ±0.06 |

**Note:** *mean* – arithmetic mean; *SD* – standard error of the mean.
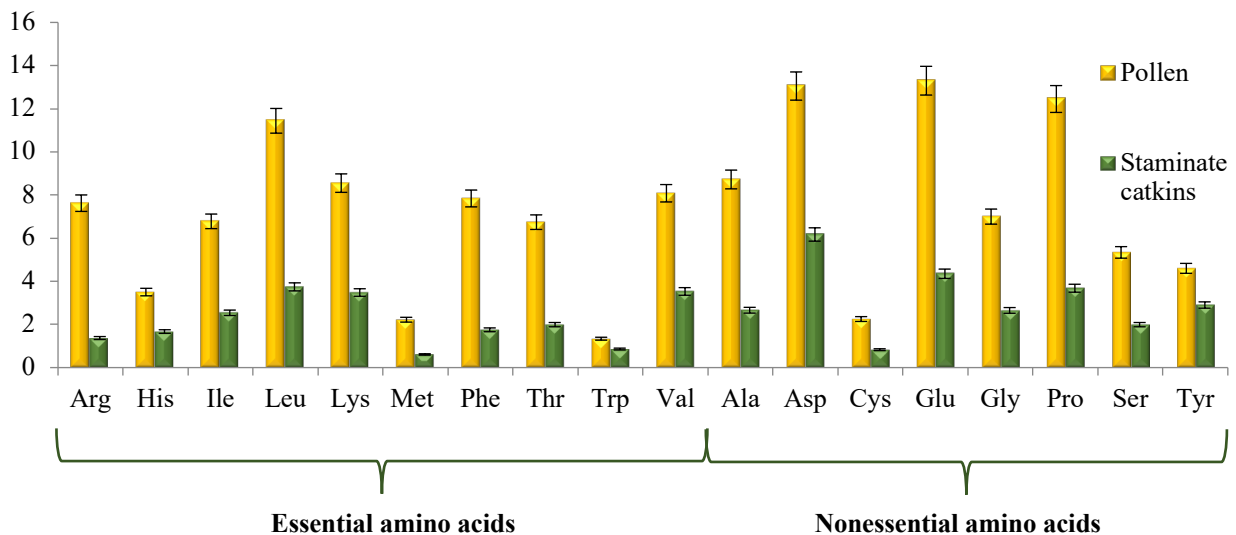
**Figure 3** Amino acid composition from pollen and staminate catkins of *Castanea sativa* Mill. (g.kg⁻¹).
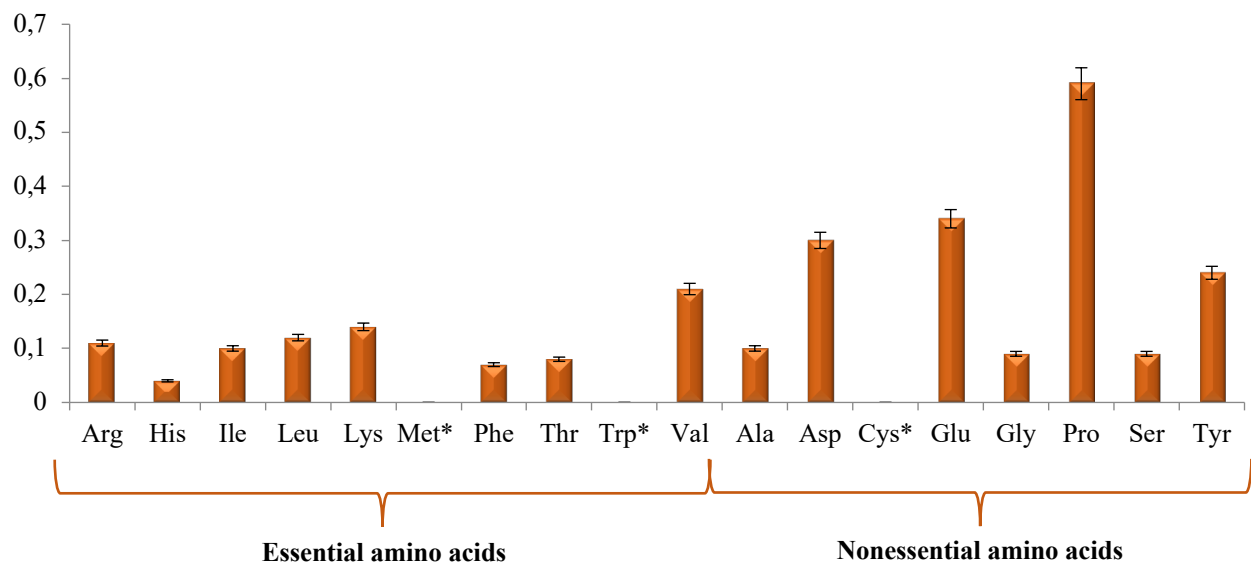


**Figure 4** Amino acid composition from the honey of *Castanea sativa* Mill. (g.kg⁻¹).
Note: * – methionine, tryptophan and cysteine determined at the trace level (<0.01).

Data presented in Figure 3 showed that eighteen (10 essential and 8 non-essential) amino acids were found in pollen and staminate catkins samples. Pollen had the greatest content of amino acids in comparison with staminate catkins and honey. Glutamic acid (13.30 g.kg⁻¹), aspartic acid (13.05 g.kg⁻¹), and proline (12.45 g.kg⁻¹) were the predominant nonessential amino acids in pollen of *C. sativa*. Their content represents 38.41% of total amino acids. High amounts of leucine (11.44 g.kg⁻¹) are also found in the pollen of *C. sativa* where its content is almost comparable to proline one.

According to many authors in bee pollen predominate aspartic acid, glutamic acid, glycine, and leucine (**Lilek et al., 2015; Taha, Al-Kahtani and Taha, 2019**). **De Simone et al. (1980)** in their studies noted that proline almost always appears as the dominant amino acid in pollens of all examined species. According to them in *Pinus pinea* and *Pinus nigra*, proline represents more than 81% of the total amino acids. **Lilek et al. (2015)** demonstrate that in fresh pollen *C. sativa* predominates proline (2.96 g.100g⁻¹), slightly less glutamic acid (1.91 g.100g⁻¹) and aspartic acid (1.77 g.100g⁻¹).

The most abundant amino acid in staminate catkins was found to be aspartic acid (6.16 g.kg⁻¹), slightly below glutamic acid (4.35 g.kg⁻¹), which is 13.19 and 9.30% of the sum of total amino acid.

**Kim et al. (2020)** studied the nectar of *Castanea* spp. male flowers and results showed that the predominant amino acids as asparagine, proline, glutamic acid were detected in amounts of 12.9% (*C. crenata*) – 41.0% (*C. sativa*) of asparagine, 7.3% (*C. mollissima*) – 29.6% (*C. crenata*) of proline and 7.8% (*C. sativa*) – 13.9% (*C. crenata*) of glutamic acid of the total amino acids.

**Table 2** The concentration of the determined macro- and microelements and heavy metals in the pollen, staminate catkins and honey samples of *Castanea sativa* Mill.

| Components (mg.kg⁻¹) | Pollen (mean ±SD) | Staminate catkins (mean ±SD) | Honey (mean ±SD) |
|---|---|---|---|
| К | 7400 ±117 | 7760 ±219 | 981 ±77 |
| P | 3845 ±87 | 1819 ±109 | 96 ±1.6 |
| Ca | 0.27 ±0.010 | 5940 ±227 | 170 ±1.7 |
| Mg | 1730 ±66 | 2040 ±169 | 69 ±1.4 |
| Na | 98 ±1.8 | 31.7 ±0.07 | 25.1 ±0.5 |
| Fe | 461 ±56 | 109 ±13 | 13.6 ±0.6 |
| Mn | 478 ±43 | 247 ±34 | 3.7 ±0.3 |
| Zn | 126 ±19 | 30.1 ±0.8 | 2.1 ±0.05 |
| Cu | 16.7 ±0.9 | 10.4 ±0.3 | <1 |
| Ni | 41.5 ±1.1 | –* | –* |
| Sr | 12.8 ±0.5 | –* | –* |
| Sn | 1.9 ±0.03 | –* | –* |
| Hg | 0.027 ±0.001 | –* | –* |
| Se | <0.05 | –* | –* |
| Sb | <0.25 | –* | –* |
| Li | <0.5 | –* | –* |

Note: mean – arithmetic mean; *SD* – standard error of the mean; * – non-sampled.
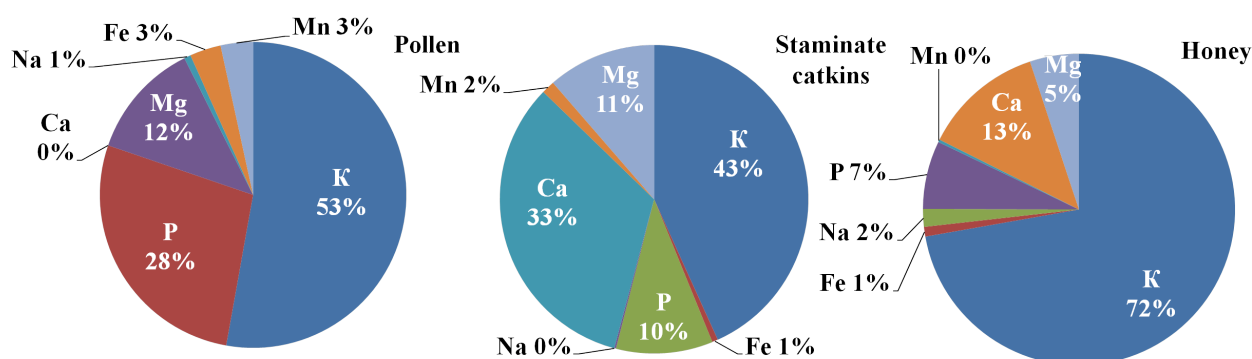


**Figure 5** Macro- and microelement percentage composition in the pollen, staminate catkins and honey samples of *Castanea sativa* Mill.

The main amino acids found for honey were proline (0.59 g.kg⁻¹), which is 22.52% of the sum of total amino acids. Lower but also important amounts of glutamic acid (0.34 g.kg⁻¹), aspartic acid (0.30 g.kg⁻¹), tyrosine (0.24 g.kg⁻¹) and valine (0.21 g.kg⁻¹) were present (Figure 4). Among the component methionine, tryptophan and cysteine are found to be present at the trace level (<0.01).

Honey contains approximately 0 – 1.5% amino acids. The proline is the main amino acid in pollen **(Ball, 2007; Wang and Li, 2011)** accounting for 50 – 85% of total amino acids **(Anklam, 1998; Czipa, Borbély and Győri, 2012)**. Lower values indicated immaturity or falsification of honey with sugar **(Von der Ohe, Dustmann and Von der Ohe, 1991; Bogdanov et al., 1999; Hermosín, Chicón and Cabezudo, 2003)**. Furthermore, amino acid content can serve as a useful indicator for estimating the floral origin of honey **(Iglesias et al., 2004; Senyuva et al., 2009)**. Proline originates from the salivary secretions of honey bees during nectar conversion **(da Silva et al., 2016)**. The amount of proline is important not only as a criterion in determining the quality value and maturity of the honey but also in determining the detection of its

adulteration with sugar. **Bayram and Demir (2018)** reported that they found proline in *C. sativa* honey amount 503.46 to 692.88 mg.kg⁻¹. **Yılmaz and Küfrevioğlu (2001)** found proline values in the range of 300 – 860 mg.kg⁻¹ in their studies on honey (from different plant species) collected from the Eastern and South-eastern Anatolia Regions. **Oddo and Piro (2004)** reported that they found an average proline amount of 468 in 721 mg.kg⁻¹ honeydew honey samples. **Manzanares et al. (2011)** found proline values in the range of 664 – 1689 mg.kg⁻¹ in pine honey and 310 – 1057 mg.kg⁻¹ in flower honey. **Flanjak et al. (2016)** in a comparative study presented that the lowest proline content had the black locust honey with a mean of 157.0 mg.1000g⁻¹. Chestnut honey had the highest proline content with a mean of 699.0 mg.1000g⁻¹ **(Flanjak et al., 2016)**. **Czipa, Borbély and Győri (2012)** found that the proline value of 143 honey samples of different plant origin obtained from countries such as Hungary, Tasmania, New Zealand, Malaysia, Thailand, South Africa, Finland from 242 to 2283 mg.kg⁻¹. The honey proline content was in the range 39 – 632 mg.g⁻¹ (acacia honey), 99 – 617 mg.g⁻¹ (lime honey), and 84 – 450 µg.g⁻¹ (rape honey) **(Kečkeš et al.,**

**2013)**. The average proline content in the analyzed honey varied from 140.14 to 389.66 µg.g$^{-1}$ **(Kowalski et al., 2017)**. **Chen et al. (2017)** discovered higher proline content (an average of about 317 µg.g$^{-1}$ and a maximum above 600 µg.g$^{-1}$) in rape honey. It was determined that the proline content of honey samples from different plant species in the North-Eastern Anatolia Region of Turkey (Erzurum, Narman, Oltu, Tortum) was between 443 – 715 mg.kg$^{-1}$ **(Cengiz, Tosun and Topal, 2018)**.

The present study, bioaccumulation, and biosorption of minerals and heavy metal concentration (K, P, Ca, Mg, Na, Fe, Mn, Zn, Cu, Ni, Sr, Sn, Hg, Se, Sb, Li) were observed in pollen, staminate catkins and chestnut honey (Table 2 and Figure 5).

Macro-elements (Na, K, Ca, Mg, P) are the most represented group, dominated by potassium contained in pollen (7400 mg.kg$^{-1}$), staminate catkins (7760 mg.kg$^{-1}$) and honey (981 mg.kg$^{-1}$). Potassium is the main mineral element with an average of one-third of the total. Some research has suggested that the trace element content in honey is mainly based on the botanical origin of honey, for instance, light blossom honey having a lower content than dark honey such as honeydew, chestnut, and heather **(Sevlimli, Bayulgen and Varinlioglu, 1992; Gonzalez-Miret et al., 2005)**. The content of mineral substances in honey samples ranges from 0.2 to 10.3 g.kg$^{-1}$ **(White, 1975)**. **Rodriguez-Otero et al. (1994)** determined the macro- and microelements content of 91 original honey samples. Potassium was the most abundant of the elements with an average content of 1500 mg.kg$^{-1}$.

Calcium is the 2$^{nd}$ most abundant element in the staminate catkins (5940 mg.kg$^{-1}$) and honey (170 mg.kg$^{-1}$), while in pollen it is only in a small amount (0.27 mg.kg$^{-1}$). Calcium was the prominent element in the honey sample in the amount of 170 mg.kg$^{-1}$. According to the data, the Ca contents of the honey sample in the present study was higher than the ones established in Serbia (20.1 mg.kg$^{-1}$) **(Jevtić et al., 2012)**, Portugal (40.3 mg.kg$^{-1}$) **(Alves et al., 2013)**, Hungary (47.9 mg.kg$^{-1}$) **(Czipa, Andrási and Kovács, 2015)**, but lower than in Italy (356 mg.kg$^{-1}$) **(Pisani, Protano and Riccobono, 2008)**.

We can compare the percentages of individual components, which show that potassium was the most represented element in the honey (72%), pollen (53%), and staminate catkins (43%). Phosphorus was the second most abundant element in pollen (28%) of the total minerals, while calcium represented in staminate catkins 33% and the honey samples 13% of the total minerals.

Microelements (Mn, Zn, Cu, Fe, Se, and Ni) are the second represented group of biogenic elements, where the content of manganese and iron prevailed in all samples. The content of manganese and iron predominate in pollen (478 mg.kg$^{-1}$ Mn and 461 mg.kg$^{-1}$ Fe) and staminate catkins (247 mg.kg$^{-1}$ Mn and 109 mg.kg$^{-1}$ Fe) than in the honey samples (13.6 mg.kg$^{-1}$ Fe and 3.7 mg.kg$^{-1}$ Mn).

**Kızılpınar Temizer et al. (2018)** determined heavy metal content in three unifloral honey samples of *Castanea sativa* from the Black Sea region in Turkey. Fe contents in honey samples were found in amounts from 6.280 mg.kg$^{-1}$ to 20.515 mg.kg$^{-1}$ and showed similarity to the other study in Turkey **(Yücel and Sultanoğlu, 2013)**, and higher than performed in Portugal (0.70 – 7.06 mg.kg$^{-1}$) **(Alves et al., 2013)**, in Serbia (0.58 – 4.21 mg.kg$^{-1}$) **(Jevtić et al., 2012)**,

and New Zealand (0.67 – 3.39 mg.kg$^{-1}$) **(Vanhanen, Emmertz and Savage, 2011)**. **Kızılpınar Temizer et al. (2018)** determined Mn contents in amounts from 21.58 to 26.52 mg.kg$^{-1}$, which are higher compared to our honey sample. The mineral content in honey is also related to the geographical origin, therefore it is possible to be used to differentiate between honey of different geographical origins **(Davies, 1975; Wang and Li, 2011)**. **Fernandez-Torres et al. (2005)** reported that the concentrations of Zn, Mn, Mg, and Na in eucalyptus, heather, orange blossom, and rosemary honey from Spain were strongly dependent on their botanical origin.

Heavy metals (Hg, Sr, Sn, Sb, Li) are present only in the pollen samples with the most abundant of Sr (12.8 mg.kg$^{-1}$), Sn (1.9 mg.kg$^{-1}$), Hg (0.0027 mg.kg$^{-1}$) content and other in the trace level (<0.05 mg.kg$^{-1}$).

Heavy metals such as Cd, Pb, Cr, and Ni in honey can be used as indicators suggesting the environmental pollution status in the region **(Porrini et al., 2003; Wang and Li, 2011)**. It has been reported that Pb contamination of honey in polluted and nonpolluted areas was not significantly different because of the high variability of the data, whereas the highest values were often found in polluted areas **(Bogdanov, 2006)**.

**CONCLUSION**

The chemical composition, amino acid content, and minerals determined in pollen, staminate catkins, and honey obtained from the *Castanea sativa* cultivar were analyzed due to evaluate the representation of individual components in selected chestnut parts and the chestnut product. Total amino acids predominate in the pollen than in the staminate catkins and honey. Glutamic acid, aspartic acid, and proline were the most abundant. The fat content predominates in pollen and staminate catkins. The content of macro and microelements was found in the pollen and staminate catkins much higher than in the honey. All *Castanea sativa* samples are a very valuable source of potassium, which is necessary for the water and electrolyte balance. Vitamin analyses showed the presence of vitamin C in predominant quantity. Heavy metals present only in the pollen samples can be used as indicators suggesting the environmental pollution status in the region. These results showed significant amounts of biogenic substances in dried pollen, staminate catkins, and honey samples, which indicated that various plant parts are appropriate for phytotherapy and phytopharmacology.

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