30th International Conference Ecological Truth & Environmental Research 2023

Proceedings

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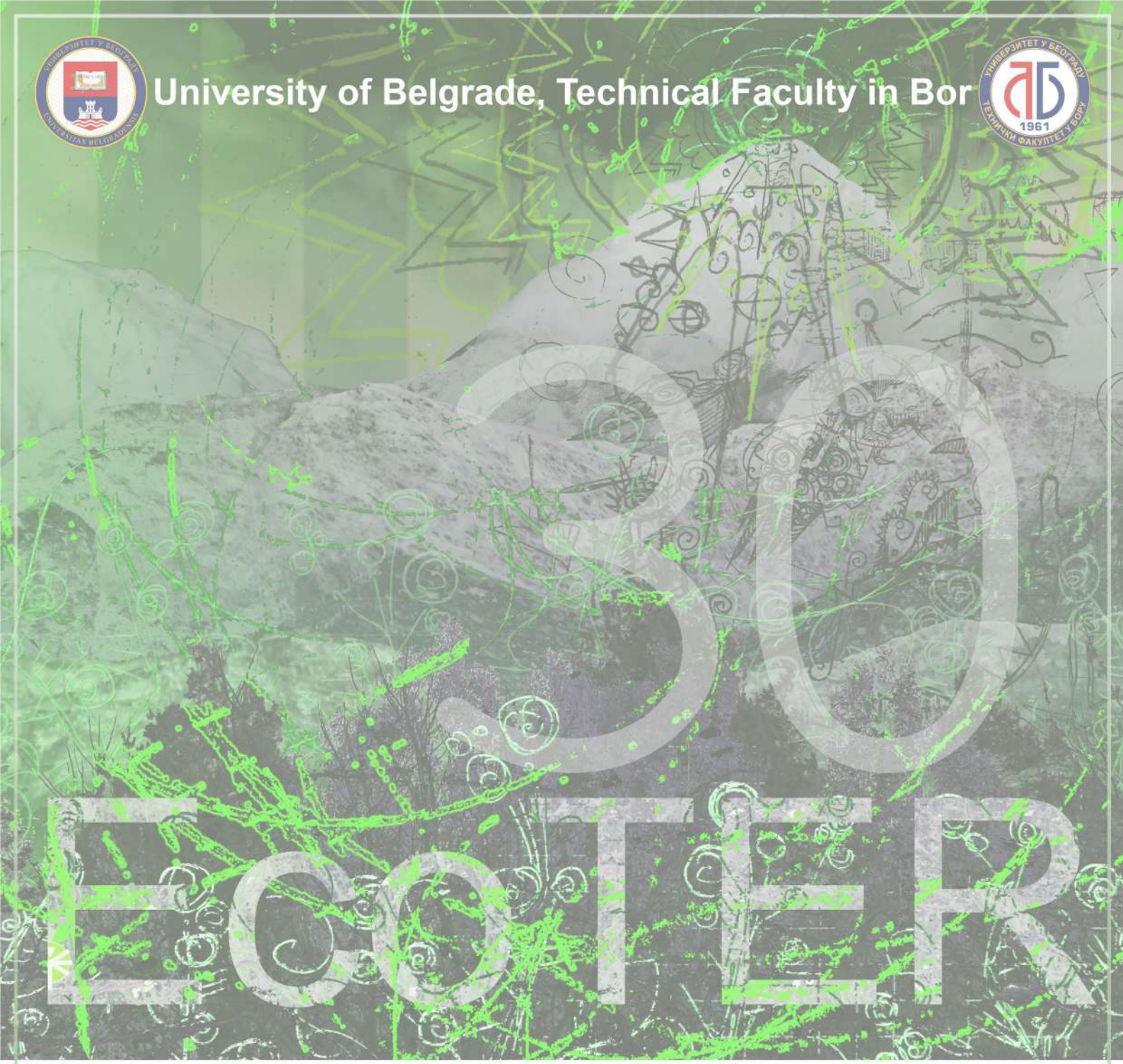

University of Belgrade, Technical Faculty in Bor

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PROCEEDINGS

30th INTERNATIONAL CONFERENCE

ECOLOGICAL TRUTH AND ENVIRONMENTAL RESEARCH – EcoTER'23

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PREFACE

The 30th international conference Ecological Truth & Environmental Research – EcoTER'23 kept three areas in focus: ecology, environmental protection and sustainable development. The conference will be held on Mt Stara Planina in hotel Stara Planina, Serbia, 20–23 June 2023. The monograph is published on the occasion of the 30th anniversary of the conference. On behalf of the scientific and organizing committee, it is a great honor and pleasure to wish all the participants a warm welcome to the conference.

The monograph is published on the occasion of the 30th anniversary of the conference.

We hope to convey the message of the conference, which is that a transformation of attitudes and behavior would bring the necessary changes. This is also an opportunity for the participants who are experts in this field to exchange their experiences, expertise and ideas, and also to consider the possibilities for their collaborative research.

The 30th international conference Ecological Truth & Environmental Research – EcoTER'23 is organized by the University of Belgrade, Technical Faculty in Bor, and co-organized by the University of Banja Luka, Faculty of Technology, the University of Montenegro, Faculty of Metallurgy and Technology – Podgorica, the University of Zagreb, Faculty of Metallurgy – Sisak, the University of Pristina, Faculty of Technical Sciences – Kosovska Mitrovica and the Association of Young Researchers, Bor.

These Proceedings 103 papers from the authors coming from the universities, research institutes and industries in 11 countries: Australia, USA, Brazil, Spain, Portugal, Libya, Italy, Bulgaria, Bosnia and Herzegovina, North Macedonia, and Serbia.

As a part of this year's conference, the 5th Student Session – EcoTERS'23 is being held. We appreciate the contribution of the students and their mentors who have also participated in the conference.

The support of the Gold donor and their willingness and ability to cooperate has been of great importance for the success of the EcoTER'23. The organizing committee would like to extend their appreciation and gratitude to the Gold donor of the conference for their donation and support.

We appreciate the effort of all the authors who have contributed to these Proceedings. We would also like to express our gratitude to the members of the scientific and organizing committees, reviewers, speakers, chairpersons and all the conference participants for their support to the EcoTER'23. Sincere thanks go to all the people who have contributed to the successful organization of the EcoTER'23.

Prof. Snežana Šerbula,

President of the scientific and organizing committee

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ADVANCED OPTICAL TOOLS APPLIED ON HONEY SAMPLES FOR BEE HEALTH STATUS MONITORING

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Abstract

Honey bees have a very important role in pollinating plants, in addition to honey production. Bee diseases are one of the reasons of a significant decrease in bee colonies in the world in recent years. Honey samples originating from hives with different infestations with N. ceranae were analyzed. Total phenolic content and total protein content were determined by the Folin-Ciocalteu assay and the Bradford assay, respectively. Fluorescence spectroscopy combined with PARAFAC was used to determine the spectral components originating from proteins (PFSC1) and phenolics (PFSC2) in honey samples, and their ratios were calculated. Phenols and proteins content in the honey samples, obtained by spectrophotometric quantification, decreases with increasing infestation levels in the respective hives infected with N. ceranae. A negative correlation was obtained for the ratio of PARAFAC components PFSC2/PFSC1 and the level of infestation with N. ceranae in honey samples. These results indicate that fluorescence spectroscopy combined with PARAFAC could be used as an optical tool for non-invasive and rapid screening of honey to estimate bee health status.

Keywords: *Apis mellifera*, *Nosema ceranae*, fluorescence spectroscopy, proteins, TPC.

INTRODUCTION

Honey bees (*Apis mellifera*) are source of unique, natural products of honey, wax, propolis and royal jelly. However, bees have an important ecological role related to the pollination of numerous agricultural and wild plant species, thus the high annual losses of bees in recent years have attracted a lot of public and scientific attention. Consequently, scientists have devoted much of their recent work to uncovering the stresses that affect bees [1,3]. Several studies have focused on assessing the relationship between bee colony health and the effects of multiple biotic (parasites and pathogens) and abiotic (pesticide exposure, poor nutrition, and low temperature) stressors [4,5]. Monitoring the health status of bee colonies can help us identify potential threats to their survival, and take action to protect them.

Special attention of the scientific public is focused on the influence of the parasite microsporidia *Nosema ceranae*, on bee colonies. Research has shown that the infestation with microsporidia *N. ceranae* leads to disturbances in the protein metabolism of bees, a decrease in the amount of protein in the hypopharyngeal glands of bees, then leads to a decrease in the growth of bees and the loss of bee colonies [6,7]. The negative influence of *N. ceranae* on the yield of honey originating from infected bee colonies has been shown in several studies [8,9].

MATERIALS AND METHODS

Samples

Five honey samples were collected during 2018 from the apiary in the yard of the Faculty of Veterinary Medicine in Belgrade. At the moment when the honey was taken for further analysis, the bees were also taken to assess nosema infestation.

Microscopic detection of *N. ceranae* spores and quantification of the infestation level

A microscopic examination of bee samples was performed to detect nosema spores in accordance with the recommendations of the World Organization for Animal Health (World Organization for Animal Health, OIE, 2017). From each hive, 30 adult foraging bees were collected from the hive, and then the bees were frozen. Each bee was macerated with 2–3 ml of water, and then the obtained suspension was examined under a microscope (magnification x400). Figure 1 shows a microscopic examination where the presence of spores of the genus *N. ceranae* was observed. At the moment when the bees were taken for the assessment of nosema infestation, the honey samples were also taken for further analysis. All samples were collected during one season from one apiary.

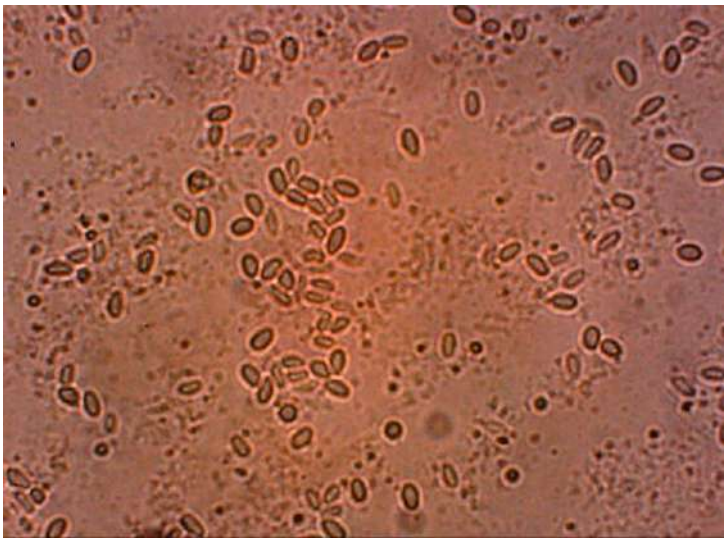

Figure 1 *Microscopic examinations of the macerated bees with the presence of N. ceranae spores*

Determination of Total Phenolic Content (TPC)

Each honey sample (5 g) was diluted with ultrapure water at 50 mL volumetric flask and filled to the mark. The total phenolic content was determined spectrophotometrically by the Folin–Ciocalteu method [10]. The sample solution (0.3 mL) and deionized water (6 mL) were

mixed with Folin-Ciocalteu reagent (0.5 mL) and incubated for 6 min at room temperature. About 3 mL sodium carbonate solution (20 %) was added in the sample solution. Afterwards, the sample was kept at 40 °C for 30 min and the absorbance was measured at 765 nm using a Shimadzu UV-160 spectrophotometer (Kyoto, Japan). Gallic acid was used as the standard, and the calibration curve of gallic acid was prepared in the concentration range between 0 and 250 mg L^{-1}. A mixture of water and Folin–Ciocalteu reagent was used as the blank. The results are expressed as the gallic acid equivalent (GAE) per kg of honey.

Determination of Total Protein Content

The total protein content was determined using the Bradford procedure [11]. Each honey sample (5 g) was mixed with ultrapure water (10 mL). Coomassie Brilliant Blue (200 μL) was added in the honey solution (5 μL). After 5 min of incubation, the absorbance was measured at 595 nm against a standard solution of bovine serum albumin (10–100 μg/0.1 mL). The total protein content was quantified and expressed as g/kg of honey.

Fluorescence measurements

Fluorescence spectra of honey samples were recorded using a Fluorolog FL3-221 spectrofluorimeter (Jobin Yvon Horiba, Paris, France), with the FluorEssence 3.5 software package (Horiba Scientific, Kyoto, Japan); the light source was a xenon lamp 450 W. All measurements were made in the front face configuration at an angle of 35°, with an integration time of 0.1 s, while the width of the opening for passing the excitation and emitted light (slits) was 2 nm. Emission spectra of honey samples were recorded in the range from 280 to 500 nm with excitation wavelengths of 260–380 nm. The wavelength step in the excitation measurements was 5 nm and the emission step was 1 nm.

PARAFAC

The EEMs of honey samples were packed in three-way data arrays for PARAFAC analysis. PARAFAC is able to decompose a three-way data into trilinear components, which number depends on the number of fluorophores in the samples. PARAFAC Components should correspond to the fluorophores in the sample [12].

Correlation analysis

Correlation analysis was performed using basic Data analysis add in the Microsoft office Excel 365, by calculating Pearson's correlation coefficient between PARAFAC scores and the rest of the studied variables. Statistical significance of correlations was estimated by Student's *t*-test.

RESULTS AND DISCUSSION

The results of spectrophotometric quantification of proteins and phenols in honey samples are presented in Figure 2 and Figure 3. It can be observed that the values of total proteins and phenols content in the honey samples decrease with the increase in the infestation *N. ceranae* of the corresponding bee colonies. A high linear dependence is confirmed with R^2 values for proteins and phenols 0.8174 and 0.7423, respectively.

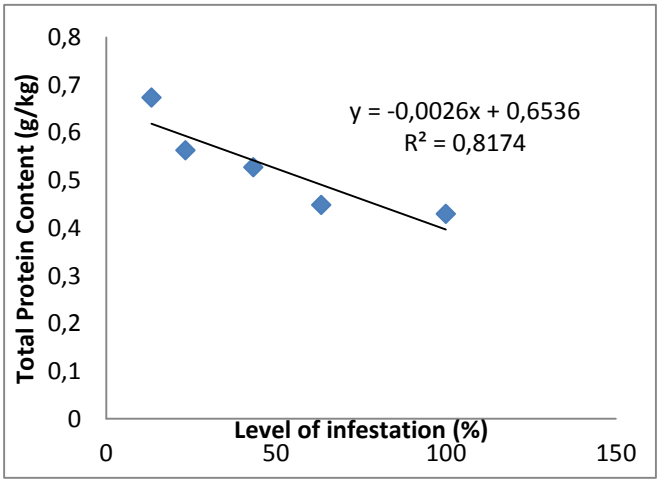

Figure 2 *Dependence of total protein on the level of hives infestation with N. ceranae*

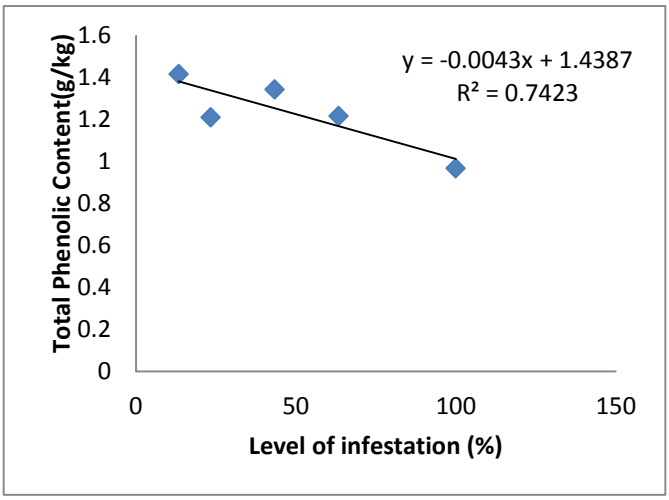

Figure 3 *Dependence of phenolic content on the level of hives infestation with N. ceranae*

Figure 4 shows, as an example, the fluorescence EEM contour map for the honey sample obtained from the beehive with 10% *N. cerenae* infestation. In all honey emission spectra, there were two broad characteristic maxima, the first maximum emission about 340–360 nm assigned to proteins and the second about 400–450 nm assigned to phenolic compounds.

The PARAFAC models were built by successive increase in number of components, from 1 to 5. Investigation of residuals and changes in the core consistency suggested that the spectral data are best described by 2 components. Considering low number of honey samples validation of PARAFAC models by split-half analysis was not possible. The loading vectors of the two PARAFAC components obtained by decomposition of EEMs are given in the Figure 5. These two should represent the inherent pure emission (a) and excitation (b) spectra of the characteristic honey fluorophores.

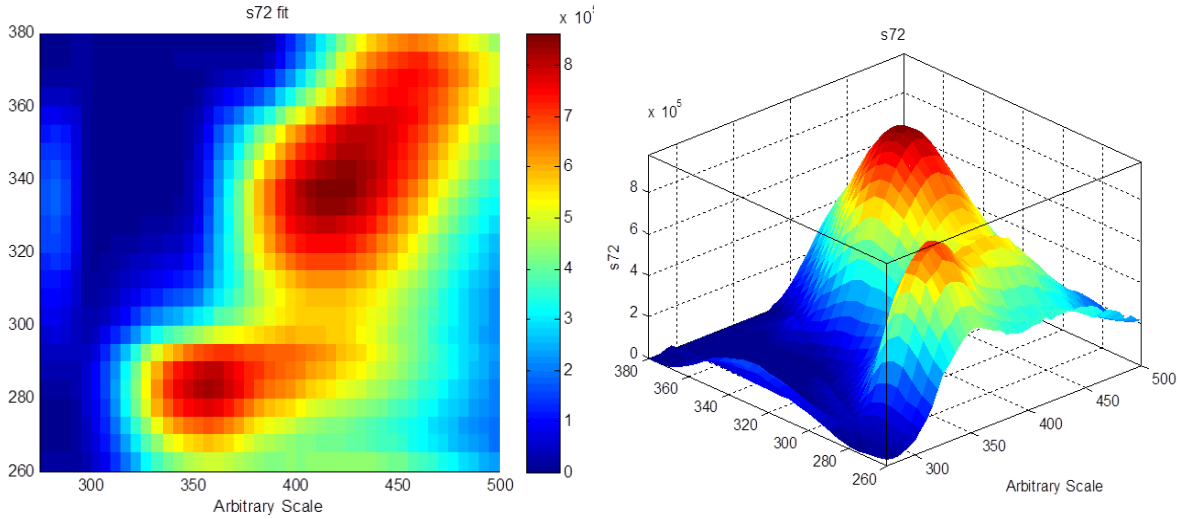

Figure 4 *The Excitation Emission Matrix (EEM) contour map for the raw spectra of the honey sample obtained from the beehive with 10% N. cerenae infestation*

The emission loading vector of the first PARAFAC component (PFC1) has the maximum at 325 nm, while its corresponding excitation loading vector reaches the maximum above 360 nm. According to findings this component can be attributed to the protein fluorophore in honey [13]. In the case of the second component (PFC2) one small emission maximum (at 325 nm) and two prominent emission maxima (in the range of 370–400 nm) are observed. Excitation loading vector of PFC2 shows significant intensity from 260–345 nm. This can be attributed to the phenolic compounds present in honey samples [14].

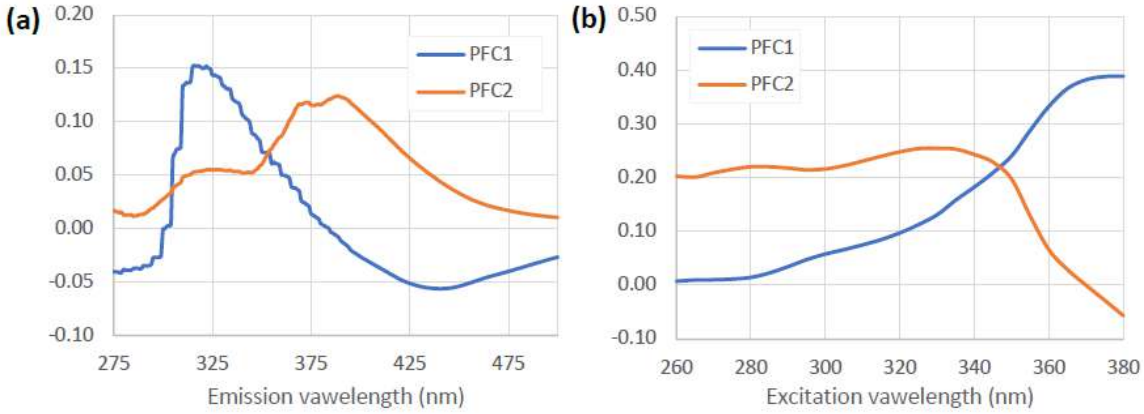

Figure 5 *Emission (a) and excitation (b) loading vectors for the two components (PFC1, and PFC2) PARAFAC model*

In order to explore possible connections of the spectral features with biochemical properties of honey samples, especially the level of infestation (%), a simple correlation matrix, based on Pearson's correlation coefficient was calculated between PARAFAC scores of both components and their ratios (PFSC2/PFSC1). The total proteins and TPC are significantly negatively correlated with the infestation level. Also, a statistically significant

negative correlation (-0. 71, R= -0. 71, p=0.05) between the ratio PFSC2/PFSC1 and the level of infestation was found.

CONCLUSION

The results presented in this research show the relationship between different parameters in honey samples and the infestation of colonies with *Nosema ceranae* as a type of stress of bee colonies. Corresponding protein and phenolic components in the emission spectrum of honey were obtained using the PARAFAC method which was applied to the excitation-emission matrices of honey samples. The ratio of spectral PFSC2/PFSC1 PARAFAC components is a negatively correlated with the level of infestation. It was shown that the contents of total proteins and total phenolics decrease with the level of *N. ceranae* infestation of the corresponding bee colonies, and they are significantly negatively correlated with the infestation level. Observed changes in honey may indicate changes in the state of the bee colony that is exposed to biotic stress, specifically the infestation of colonies with *Nosema ceranae.* Spectrofluorimetric assessment of the relative content of phenols and proteins may have an advantage over the existing methods used for the quantification of proteins and phenols in honey, because it is done without a sample preparation procedure. This method enables the simultaneous determination of proteins and phenols, because their maxima in the emission spectra are clearly separated. The results obtained in this research can be the basis for developing new optical tools applied on honey samples for bee health status monitoring.

ACKNOWLEDGEMENT

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