Short thesis for the degree of doctor of philosophy (PhD)

Comparative analysis of honeys and their application to estimate environmental changes

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I. Introduction and objectives

Honey is an import-export product of great commercial value, with numerous health benefits and medicinal importance due to the mineral, enzyme, protein and vitamin content. In addition to the enjoyment value of honey and its role in folk medicine, it is also useful for estimating environmental changes: as the product of a bioaccumulation process, it is suitable for preserving information regarding the environment of the hives. If the amount of a component changes in the soil, water or air in the collection area, it will be reflected in the composition of the plants, including the nectar and pollen collected by the bees. In this way, honey from a given area becomes an excellent indicator of the bees' environment. The composition and quality of honey depend greatly on the botanical and geographical origin, as well as the conditions of handling and storage. The agricultural and industrial emission of pollutants influences the composition of the pollen grains and nectar, therefore the final bee products. Thus, honeys can be considered as important biomarkers of specific environmental assessments.

Analysis of honey is important regarding food safety and environmental protection. The mineral and trace element content of honey samples and bee products could indicate local and extended environmental pollutions. However, compared to other food matrices, the analysis of honeys can be more challenging from an analytical point of view due to their varied composition and physical properties. One bottleneck of environmental analysis to asses and estimate environmental condition is a sufficiently large number of samples, which requires the optimization of cost-effective measurement methods suitable for routine analysis.

It is a common perception of honeys that they can be stored and consumed for relatively long. However, with the passage of time, the amount of volatile compounds and enzymes decrease, their taste, color and fragrance may also change, but the mineral components do not suffer a quality change even during extended storage. This characteristic of honeys raises the question whether beekeeping products can indicate the environmental changes of the

present, and honeys from the past can be used for environmental reconstruction purposes.

In this study, our first aim was to develop a cost-effective multielemental analytical method for microwave plasma atomic emission (MP-AES) technique to quantitatively determine the inorganic components of honey samples. Then, to test the optimized method by a comparative analysis on more than 250 Hungarian honey samples collected from different areas and plants. Statistical analysis was carried out to explore the relationship between honeys and their botanical and geographical origin, the year of collection and the amount of inorganic components (minerals, trace elements and toxic metals). We intended to investigate the applicability of honeys for the indication of environmental changes over a longer period of time by a unique sample series containing old honeys. We aimed to study the time-dependent effect of handling, storage and processing habits on the composition and quality of honeys. The development of a method for the analysis of metal ions in honey by capillary electrophoresis was also planned using universal internal standard (IUS) and indirect UV detection.

II. Methods

Work was started by contacting various beekeepers and beekeeping associations. Received honeys were divided into two main groups: *„modern"* and *„old"* samples which we treated separately. For the optimization of the cost-effective MP-AES method, we used commercially available honey samples (acacia and multifloral) and it was tested by the elemental analysis of 187 honey samples from 2016.

In this study, we had the opportunity to work with a unique Hungarian sample series, which contains honeys from 1959 to the present, covering several nectar producing plant species and areas. The special feature of this collection is that most of the samples come from one beekeeper, from one geographical area, with known botanical origin. During the elemental analysis of these *"old"*

honeys, we analyzed 65 samples of acacia, sunflower, rape and forest honeys collected between 1959 and 2020. For the determination of *5-(Hydroxymethyl)-furfural* (HMF) content, a total number of 69 samples were measured.

For the elemental analysis, honey samples were digested under atmospheric conditions using nitric acid and hydrogen-peroxide. We used Agilent Technologies 4200 MP-AES and 5100 ICP-OES (inductively coupled plasma atomic emission spectrometer) to determine the elemental composition of the obtained sample solutions. Statistical tests on the elemental analytical results were performed using IBM SPSS and Canoco for Windows programs.

The determination of the HMF content of the honey samples was carried out on an Agilent Technologies Cary 60 type photometer based on the scaled down and optimized method of White's procedure developed in 1989.

We performed the capillary electrophoresis measurements on Agilent Technologies HP 3 DCE instrument.

III. New scientific results

1. A sample preparation and measurement method was developed on MP-AES technique for the cost-effective analysis of honey samples. The applicability of the method was verified for the quantitative determination of the most important inorganic macro and micro components of honey.

In the absence of a certified honey reference material (CRM), the effectiveness of the developed sample preparation and measurement process was checked using an internal standard addition method. We carried out the elemental analysis of the samples with the internal standards for the most important macro and micro elements, and also determined the limit of detection (LOD) and quantification (LOQ) of the MP-AES method. Results were compared to the concentration values obtained by inductively coupled plasma atomic emission spectrometer used in routine analysis and a good agreement was found.

2. It was proved that based on the measured elements honey species can be separated using statistical tools and the botanical aspects have a higher effect on the elemental composition of the studied Hungarian honeys. In a geographical context, the varieties are characterized by a greater degree of homogeneity based on the concentration of the studied elements.

From different nectar producing plants and regions, 19 elements (Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn Na, Ni, Pb, Sr and Zn) of the collected 187 „*modern*" honey samples were determined by the optimized MP-AES method. Performing canonical discriminant analysis (CDA) on the elemental analytical results to investigate the relationship between the botanical origin and the concentration of the measure elements, the separation of five species was observed (*Fig. 1*).

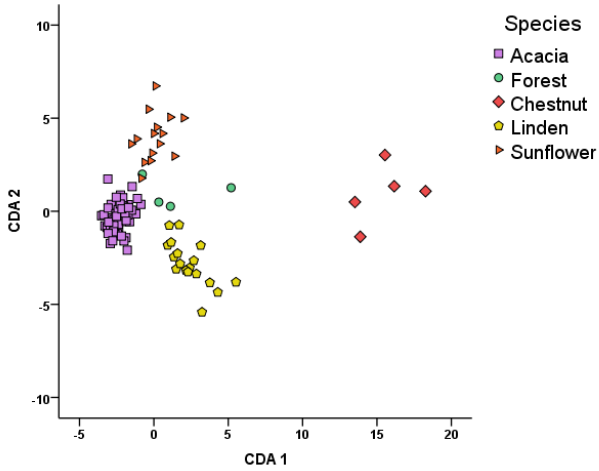

Figure 1 „Modern" honey samples grouped by their botanical origin. The first function accounts for 67% discriminating ability, 16% is the value accounting for the second function. The canonical correlation values are: 0.973 and 0.900, respectively. The cumulative percentages are 67% (CDA1) and 83% (CDA2).

3. For the first time, we studied the time-dependent composition changes of trace elements, minerals and toxic metals through a series of *„old”* honey samples (1959-2020) to gain environmental related information of a longer time scale.

3.1 It was proved that honeys can be considered not only as indicators of the present but can also be used for long-term environmental monitoring purposes.

The used sample series originates from the same location, species and beekeeper excluding heterogeneity from botanical, geochemical, handling and storage conditions of the elemental analytical data. Thus, any change observed in composition is considered to be time-dependent. Samples were grouped based on their decades of origin, then canonical discriminant analysis was performed on the results of the elemental analytical measurements, which is illustrated in *Fig. 2* in the example of acacia honeys.

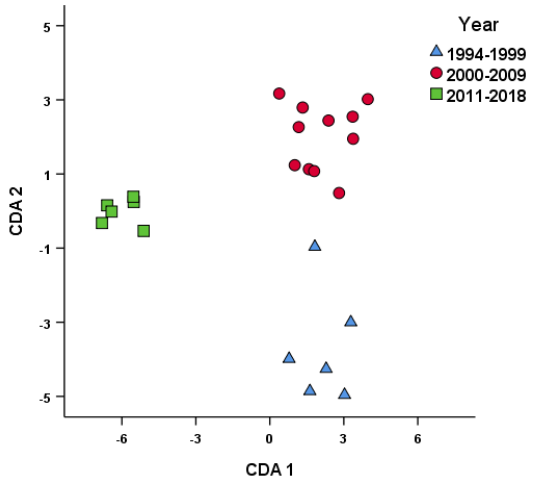

Figure 2 Acacia honey samples from one botanical and geographical origin and the same beekeeper grouped by their year of origin. The first function accounts for 70% discriminating ability, 30% is the value accounting for the second function. The canonical correlation values are: 0.967 and 0.929. The cumulative percentages are 70% (CDA1) and 100% (CDA2).

The separation of decades proves that the composition of the analyzed honey samples is not just affected by the geographical and botanical origin but also by the collection time.

By observing the decreasing tendency in the amount of certain metals, we proved that natural and anthropogenic environmental factors are preserved in honey. Old samples thus indicate the recent and past soil composition, airborne pollution and the effects of beekeeping processes. Sunflower honeys from Mesterháza illustrate the change in the amount of iron and lead over time in *Figure 3.*

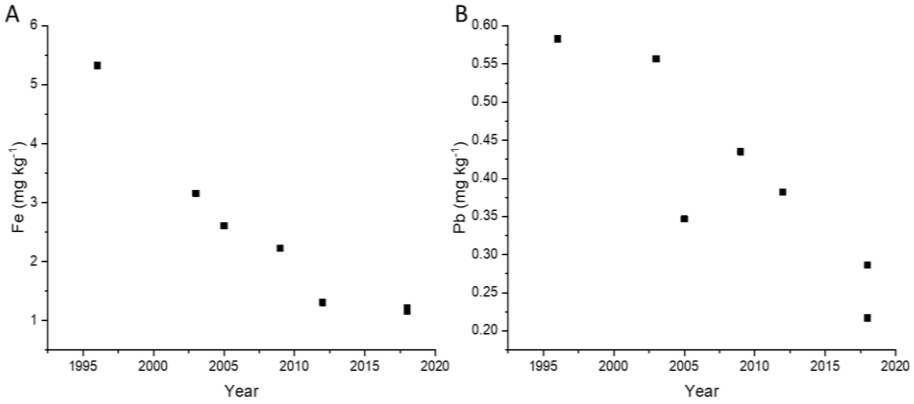

*Figure 3 The Fe (**A**) and Pb (**B**) concentration of honeys plotted against the year of origin through the example of sunflower samples from the sampling location of Mesterháza.*

3.2. We proved that the amount of inorganic components present in honey depends not only on soil properties and the type of nectar-producing plant species, but is also influenced by climatic and weather conditions.

A correlation (p=0.016) was found between the amount of the measured elements in acacia honeys and the amount of precipitation in May (*Fig.4-a*). The redundancy graph (*Fig. 4-b*) also indicates a relationship between precipitation and elemental composition. The observed relationship between the mineral content and rainfall may be related to the vegetation period of acacia since its blooming time in Hungary is at the end of May and early June. Some of the measured elements may be mobilized from the soil and rock by the increased amount of spring precipitation and transported to the pollen producing parts of the trees.

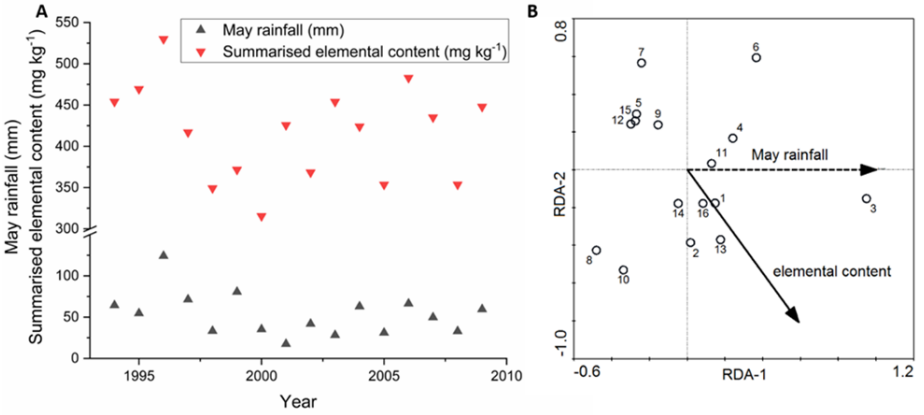

Figure 4 ***A****: Interaction between the May rainfall (mm) and the elemental content of acacia honeys originating from the same pollen producing region (Gór, Hungary).* ***B****: RDA triplot showing correlation between the May rainfall data (dashed arrow) and elemental content (solid arrow) of acacia honeys originating from the same pollen producing region (Gór, Hungary). Numbers indicate the samples from 1994 to 2009.*

4. We implemented a scaled down and optimized White's method for the quantitative determination of the HMF content of honey samples. For the first time, we examined the long-term effect of the processing, handling and storage habits on the consumptionability of honey through changes in the HMF content. We found that the HMF content of acacia honeys increases with the storage time.

The HMF content of acacia honeys increased almost linearly with storage time, the health limit value (40 mg kg^{-1}) was reached in about 5 years (*Fig. 5*).

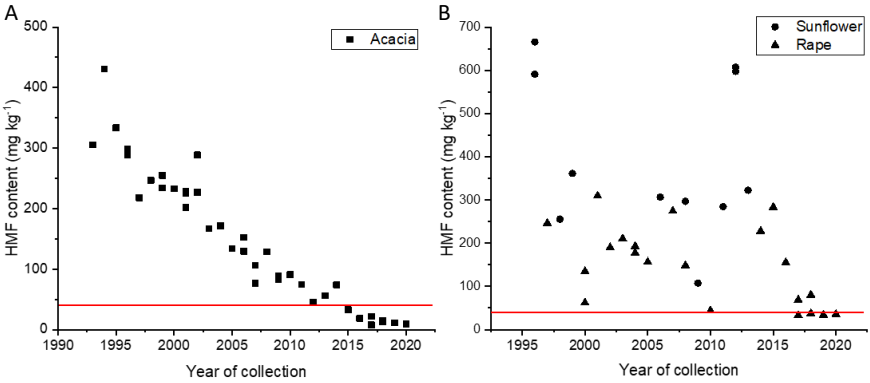

*Figure 5. Changes of HMF content in acacia, - (**A**), rape, - and sunflower honeys (**B**) with the storage time (origin: Gór, Mesterháza). The red line represents the health limit (40 mg kg^{-1}).*

However, the correlation between storage time and HMF content of rape and sunflower honeys shows less of the trend seen in acacia honeys, which may even be due to the nature of the botanical variety. In addition, these agricultural field-origin honeys reached the health limit value (40 mg kg^{-1}) faster, after 1-2 years of storage. Thus, based on our own results, the shelf life of honey samples are approximately 5 years for acacia honeys and 1 year for rape and sunflower honeys.

5. We proved that capillary electrophoresis can be used to determine the metal ion content of undiluted honey samples using electrokinetic injection and universal internal standard method.

Honey, as a highly viscous solution, is an excellent sample type to utilize the special features of capillary electrophoresis like electrokinetic injection and indirect UV detection. With this type of sample introduction, the components enter the capillary via electrophoretic migration, in which case external calibration cannot be applied. For the quantitative determination of metal cations, we used the IUS method, previously developed by the research group, using Ba as internal standard.

Figure 6 shows the result of the electrokinetic injection from an undiluted honey sample, which we were able to identify nine metal ions. Using Ba as an internal universal standard, four metal ions with similar electrophoretic mobility (Ca, Mg, K, Na) were quantified.

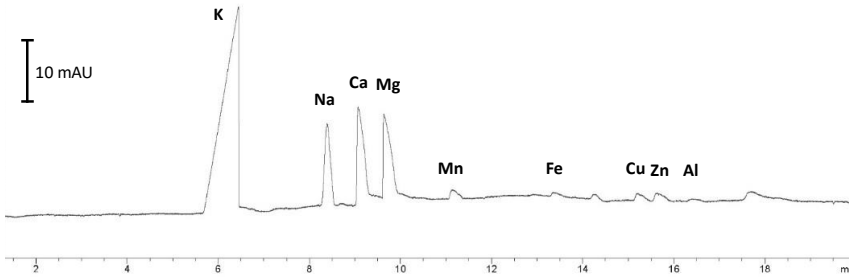

Figure 6 Electropherogram of undiluted honey. Injecting parameters: 5 kV × 5 s. BGE: 0.3 M acetic acid, 0.15 M lactic acid, 0.03 M imidazole, pH=3.0. Indirect UV detection: λ=214 nm (ref.) and λ =580 nm (det.).

IV. Possible utilization of the results

The determination of honey origin is of particular importance in identifying the adulteration of beekeeping products with high export potential. Atomic spectrometric data is a useful statistical variable in the origin determination of honey; however, the amount of available measurement results creates a bottleneck for the practical applicability partly due to the analysis costs.

The optimized method for the MP-AES instrument enables a cost-effective multielemental analysis of honey samples, which can easily be adapted for research and routine analytical laboratories. Our results can also contribute to the creation of statistical database to ensure the authenticity of honey being vital to protect this high market potential agricultural product from adulteration. Elemental composition can also reflect local pollution since toxic elements can accumulate in bee products, which is important from environmental point of view and for food safety. We pointed out that honeys can also be used for environmental assessment and reconstruction over a longer period of time, which can open a new chapter in research regarding beekeeping products as long-term biological indicators. With the comprehensive analysis of the samples and the dating of the old samples, the geological and botanical conditions of the nectar-giving areas can be characterized, the environmental conditions of the past decades can be reconstructed, and the changes in the nutrient content of the soils can be studied.

The HMF determination method, optimized for smaller sample weight and reagent quantity, is a more cost-effective alternative to chromatographic techniques. Our conclusions regarding the HMF content of *„old”* honeys are important for food analysis and food safety.

The developed capillary electrophoretic method can be applied for the direct determination of metal ions from a small amount (~30 μL) of undiluted honey sample, which can be a supplementary method for atomic spectrometry. The method enables a relatively simple, simultaneous qualitative and quantitative analysis of high-viscosity samples.

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Candidate: Zsófi Sajtos
Doctoral School: Doctoral School of Chemistry
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List of publications related to the dissertation

Foreign language scientific articles in international journals (4)

1. **Sajtos, Z.**, Varga, T., Gajdos, Z., Burik, P., Csontos, M., Lisztes-Szabó, Z., Jull, A. J. T., Molnár, M., Baranyai, E.: Rape, sunflower and forest honeys for long-term environmental monitoring: Presence of indicator elements and non-photosynthetic carbon in old Hungarian samples.
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14. Vallejo-Cuzco, G., Varga, K., Varga, D., Soltész, A., **Sajtos, Z.**, Berta, C.: A Vissi-holt-Bodrog neolimnológiai vizsgálata Cladocera közösségek által.
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17. Szvák, E., Sklánitz, A., Szabó, L., Kiss Mészáros, Z., Béni, Á., János, I., Dobróné Tóth, M., Szikossy, I., Szikszai, Z., Kertész, Z., Molnár, M., Major, I., Győry, H., Biacsi, K., Molnár, E., Hajdu, T., Szeniczey, T., Baranyai, E., **Sajtos, Z.**, László, O., Scheffer, K., Kovács, J., Hölzl, R., Tucom-Novak, V., Szirmai, L., Szőke, A., Zsíros, A., Erdődi, M., Rosendahl, W., Zesch, S., Zink, A. R., Maixner, F., Jäger, H. Y., Piombino-Mascali, D., Pálfi, G., Pap, I.: Two ancient Egyptian mummified heads of unknown origin from the Aurél Török Collection: Preliminary results.
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Total IF of journals (publications related to the dissertation): 25,515

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