

**CLASSICAL AND CORRELATIVE ANALYTICAL METHODS FOR ORIGIN
IDENTIFICATION OF HUNGARIAN HONEYS**

Short running title: Detection of authenticity of honey

ZS. BODOR^{a,b}, CS. BENEDEK^{b*}, T. KASZAB^a, JL. ZINIA ZAUUU^a, I. KERTÉSZ^a, and Z. KOVACS^a

^aDepartment of Physics and Control, Faculty of Food Science, Szent István University, Somlói street 14-16, Hungary

^bDepartment of Dietetics and Nutrition, Faculty of Health Sciences, Semmelweis University, Vas Street 17, 1088 Budapest, Hungary

Honey is produced by honeybees from nectar, sap of plant parts or the juicy material secreted by sucking insects living on trees. It is rich in nutritionally useful components, the occurrence of which highly depends on the botanical and geographical origin of honey. Our goal is to develop a new, rapid and accurate combination of analytical methods for identification of botanical and geographical origin.

Physicochemical parameters such as pH, electrical conductivity, moisture and ash content, colour (L*a*b*) and antioxidant properties were determined besides correlative electronic tongue and near infrared spectroscopy techniques. For the statistical evaluation ANOVA, principal component analysis, and linear discriminant analysis were applied.

Results showed significant differences ($p < 0.05$) for the physicochemical, colour and antioxidant capacity according to the botanical origin of honeys. Electronic tongue (ET) and near infrared spectroscopy (NIR) techniques were useful for the identification the botanical and geographical origin with generally good accuracy.

The physicochemical parameters are important and can serve as reference methods, while NIR and ET are promising, but need further improvement for the determination of origin of honey.

Keywords: honey, antioxidant, electronic tongue, NIR, authenticity

*To whom correspondence should be addressed
Phone: +36 1 486 4822; e-mail: benedek.csilla@se-etk.hu

According to EU legislation, honey is produced by honeybees (*Apis mellifera*) from nectar, sap of plants or honeydew (E.C., 2001). It is rich in nutrients like vitamins, antioxidants, organic acids, minerals and enzymes, although the occurrence of these components depends on the botanical and the geographical origin of the product (ANKLAM, 1998). The valuable nutritional content and high market price of honey makes it a target of various forms of adulteration globally. These forms can be the with sugar syrups, mixing honeys from different geographical origins without labelling or even feeding bees with sugars during the collecting period (ZÁBRODSKÁ & VORLOVÁ, 2014). Current analytical methods such as HPLC, NMR, IRMS or ICP-OES are useful for tracking some of these fraudulent activities (ARIES *et al.*, 2016; SPITERI *et al.*, 2016) nevertheless, they remain relatively expensive, time consuming and require high qualification and practice. Furthermore, they are not well standardized for the continuously developing trends of honey adulteration.

Origin identification of honey has often been regarded as a complex task, as, legislations for compositional limits or ranges of physicochemical parameters (like electrical conductivity, pH, sugar composition, etc.) are missing for the different unifloral honeys in the European Union. Only national directives are available in some countries like Germany, Italy (THRASYVOULOU *et al.*, 2018) or in Hungary (for acacia and linden honey) (HUNGARIAN FOOD BOOK, 2002). The physicochemical properties might be also used for the determination of botanical origin of honeys when it is applied as a multivariable, combined data set for analysis. The individual parameters cannot give satisfactory differentiation (ANKLAM, 1998). Moreover, the determination of these parameters also time-consuming quite expensive as a result of they require high quantity of chemical reagents. Therefore, there is an urgent demand for an accurate, control system and a well-defined, rapid universal method for monitoring authenticity of honey. Near infrared spectroscopy (NIR) and electronic tongue (ET) (DI ROSA *et al.*, 2018; WEI &

WANG, 2011; WEI *et al.*, 2009) are rapid and easy-to-use techniques with a potential to fulfil, these requirements.

NIR was successfully used for discrimination of Chinese honeys based on their botanical origin: discriminant analysis showed 87.4 and 85.3% accuracy for the recognition (training) and prediction (validation) abilities for five botanical groups (CHEN *et al.*, 2012; GAN *et al.*, 2016) used NIR spectroscopy and electronic tongue for the classification of botanical origin and detection of adulteration of honeys. Their results showed that recognition and prediction abilities were 100% and >80%, based on the results of both NIR and electronic tongue.

The aim of our study was to build up a database based on the physicochemical, colour and antioxidant properties of different unifloral honeys to enlarge the data available for Hungarian honeys. On the other hand as a second goal to test the application possibilities of NIR and ET rapid, correlative methods in the determination of botanical and geographical origin of honey.

1. Materials and methods

1.1 Honey samples

In our studies more than 100 honeys from different botanical and geographical origin were collected to build up a robust database. In this work four different type of honey, such as eleven acacia (*Robinia Pseudoacacia*), four rape (*Brassica napus*), five chestnut (*Castanea sativa*) and six sunflower (*Helianthus annuus*) honeys were analysed, which honey types are commonly consumed in Hungary.

Acacia samples originated from Hajdú-Bihar, Heves, Jász-Nagykun-Szolnok, Nógrád, Pest and Szabolcs-Szatmár-Bereg counties, rape samples from Békés, Heves and Jász-Nagykun-Szolnok counties, chestnut samples from Zala, Vas, Nothern-mountains and Győr-Moson-Sopron counties, and sunflower samples from Békés, Heves and Nógrád counties of Hungary.

1.2 Physicochemical, colour and antioxidant parameters of honey

Physicochemical parameters (ash content, electrical conductivity, pH, total soluble dry matter) were determined according to the International Honey Commission guidelines (BOGDANOV, 2002), as quality indicators. Measurements were performed in three replicates per sample, resulting in a total of 78 observations for the whole sample set.

Antioxidant properties were characterized by the measurement of total polyphenol content (TPC), cupric ion reducing antioxidant capacity (CUPRAC) and, ferric reduction antioxidant power (FRAP) using a Thermo Helios- α spectrophotometer (APAK *et al.*, 2008; BENZIE & STRAIN, 1996; PRIOR *et al.*, 2005; SINGLETON & ROSSI, 1965). Analyses were performed in five replicates per sample, resulting in 130 observations for the whole sample set.

Colorimetric measurements were determined in CIE L*a*b* tristimuli coordinate system with a Konica Minolta CR410 colorimeter. For each sample in five replicates were obtained, this resulting in 130 observations for the whole sample set.

1.1 Rapid techniques

As rapid measurement techniques electronic tongue (ET) and near infrared spectroscopy (NIR) were applied for the analysis of the honey samples. Transflectance spectra (1000-2500 nm) were recorded with the spectral step of 2 nm with a PMC Spectralyzer 10-25 infrared spectrophotometer. The acquisition of spectra was performed with Spectralyzer Operating Software, version 1.44. (METRIREP, Hungary) Approximately 5 g of honey sample was put in ring cup cuvette and scanned after standard per each sample. The sample set was scanned in two runs, on two different consecutive days. In both repeated series samples were recorded in randomized order. Three consecutive spectra were recorded for each sample at each time. Therefore one sample was represented by six spectra in total, from the two measurement days, this resulting in 156 spectra for the whole sample set.

ET measurements were performed with an Alpha ASTREE (ALPHAM.O.S., Toulouse, France) electronic tongue composed of seven ISFET sensors developed for food applications (ZZ, CA, BB, GA, HA, JB, JE). The honey samples were diluted with distilled water prior to the ET tests: 10 g of honey was weighted and filled up to volume in a 100 ml volumetric flask. Honeys were tested three times on three independent days with nine consecutive measurements every day resulting in 18 values for each sample and a total of 468 observations.

1.3 Statistical analysis

The mean and standard deviation were determined for each botanical group for the physicochemical, antioxidant and colour parameters. Significant differences between different botanical groups were determined by ANOVA method, followed by Tukey post hoc test at $p < 0.05$ significance level.

Multivariate statistical methods were used for the evaluation of the results of NIR and ET measurements after the relevant pre-treatment of the data: for both measurements. Outliers were identified based on the exploratory data evaluation (principal component analysis - PCA) and omitted before the further data evaluation, resulting in 124 spectra for the NIR measurements and 288 observations in total for the electronic tongue data set. As raw data pretreatment, drift correction was applied in the case of ET to decrease the effect of the sensor drift during the different days (SZÖLLÖSI, 2015). In the case of NIR, spectra in the range 1100-1800 nm were evaluated by chemometrics. Savitsky-Golay smoothing (2nd order polynomial and 21 points) and multiplicative scatter correction (MSC) were applied on raw spectra to reduce noise and baseline variation. PCA was also used for the pattern recognition of results of ET and NIR, with special attention to the separation of botanical and geographical origin of honeys. Linear discriminant analysis (LDA) was applied to build models for the classification of botanical and geographical groups based on the results of the ET (threefold cross validation) and NIR (five-

fold cross validation). For the statistical evaluation of the data Microsoft Excel 365 and R-project 3.5.2 software were used.

2. Results and discussion

2.1 Results of physicochemical, colour and antioxidant measurements of honey

Table 1 shows the results of the physicochemical and colour parameters for each botanical type. The lowest antioxidant capacity, ash content, electrical conductivity was obtained in acacia honeys – the results of these parameters were significantly lower than in the case of chestnut and sunflower honeys. Acacia honeys were also the lightest (L^*), less yellow (b^*) and had the lowest a^* value in the green range. These results are in accordance with the literature, where acacia honey had the lowest antioxidant capacity and highest L^* , while chestnut honeys had higher antioxidant capacity and lower L^* (BERTONCELJ *et al.*, 2007; GÜL & PEHLIVAN, 2018).

Table 1

Rape honeys showed the highest pH value and had significantly lower antioxidant properties, electrical conductivity and ash content than sunflower and chestnut honeys. Sunflower honeys had significantly higher yellow colour than the other honey types, while they had significantly lower FRAP and TPC values, electrical conductivity and ash content than chestnut honeys. These results are also in a good agreement with the data acquired by the IHC group (ODDO, L. P., & PIRO, R. 2004).

2.2 Identification of botanical and geographical origin of honey by near infrared spectroscopy

The PCA score plots of evaluated spectra of honey showed the best separation of points of sunflower and rape honeys from the groups of chestnut and acacia honeys (Figure 1).

Figure 1.

This separation can be seen through the first principal component (PC1), which describes 92.31% of the total variance. The PCA loadings (Figure 2) showed that 1584 nm, 1444 nm and 1368 nm had the highest role in the formation of PC1. These peaks belong to the 1st overtone: peak at 1444 can be assigned to the free water content of the samples, while peak at 1584 could show the difference in the sugar composition (BÁZÁR *et al.*, 2015.)

Figure 2.

These results are in accordance with our results obtained in the water content measurement: rape and sunflower honeys had higher water content, and the sugar structure was different from the chestnut and acacia honeys, which can be due to their crystallized structure. It is well known that the main determining factor in crystallization of honeys is their crystal structure (BOGDANOV, 1993).

LDA classification models built for the classification of the different botanical origin of the honey samples are presented in Figure 3.

Figure 3.

Similarly to PCA, root 1, shows the separation of sunflower and rape honeys from chestnut and acacia, nonetheless, based on root 2 a tendency of separation also can be detected for chestnut and acacia honeys. The LDA model presented average recognition and prediction abilities of 88.12% and 62.52%, respectively for the four groups. The best results were obtained for acacia honeys: LDA model recognises 97.9% and predicts correctly 92.4% of the acacia honeys, misclassifying 7.6% as belonging to chestnut honeys. This minimal overlapping with the chestnut honeys is caused by the crystal structure: these two honey types were not crystallized like sunflower and rape samples, which data points separated well enough from the chestnut and acacia honeys. The model recognises 96.4% and predicts 72.3% of sunflower samples correctly, misclassifying 3.6% and 27.7% to rape honeys. On the other hand, misclassification between the rape and sunflower honeys can be explained by their similar

crystallized structure. The results show that NIR instrument was sensitive to the stage of crystallization patterns. Our results lag behind those mentioned in the literature. For instance CHEN *et al.* (2012) reached to better classification results of 250 spectra of five different type of unifloral honey samples (acacia, jujube, vitex, rape and linden) resulting in 85.3 % correct prediction using FT-NIR spectrometer (Antaris, Thermo Nicolet, USA) in the 10000 cm^{-1} to 4200 cm^{-1} (1000 nm - 2380 nm) spectral range. The weaker classification results obtained in our research can be attributed to the less precise instrument and the used narrower wavelength range.

PCA score plot including all botanical groups showed no clear separation based on the geographical origin, this pointing on the higher discriminative power of botanical origin over the geographical one. Therefore, LDA models were built for the individual botanical groups of honey separately, resulting in better discrimination of the groups of honey samples obtained from different parts of Hungary. Origin of rape honeys ($n=11$) was classified correctly. For the origin of sunflower ($n=22$) samples LDA model provided average recognition and prediction abilities of 100% and 82.3% respectively (mutual misclassification was found only for honeys from Békés and Heves). For chestnut ($n=15$) honeys, recognition and prediction abilities of the LDA models were 98.4% and 75.0% (mutual misclassification was found only for honeys from Zala and the region of the Northern mountains). In the case of acacia ($n=49$) honeys classification accuracy was not as good as for the previous botanical types, average recognition and prediction abilities were significantly lower (68.6% and 46.7%, respectively) and mutual misclassification was found between each of the groups from Hajdú-Bihar, Heves, Jász-Nagykun-Szolnok, Nógrád, Pest and Szabolcs-Szatmár-Bereg counties. This can be explained on one hand by the fact that acacia honeys are poor in nutritionally active components (BERTONCELJ *et al.*, 2007), on the other hand the samples originated mostly from eastern and

middle part of Hungary, where the climate and the topographic conditions are not substantially different.

2.3 Results of identification of botanical and geographical origin of honey by electronic tongue

PCA results of the electronic tongue measurements of the tested honeys showed some separation tendency according to both their botanical and geographical origins, but the dominance of botanical origin over the geographical one was observed (Figure 4).

Figure 4.

LDA results of electronic tongue for the classification of the honey samples from different botanical origin (Figure 5) presented average recognition and prediction abilities of 92.1 % and 91.8%, respectively. The model correctly classified the chestnut honeys, reflecting thus the rich aroma of these honeys. Second best results were obtained for sunflower honeys; LDA model recognised 99.1% of sunflower honeys misclassifying 0.9 % to rape honeys. It predicts correctly 98.2%, misclassifying only 1.8% as belonging to rape samples. For acacia honeys recognition and prediction abilities were 92.0% and 91.5% respectively, some samples were misclassified as being rape honeys. Rape samples were classified correctly in 77.5 % (misclassifying 22.5% belonging to acacia honeys) during both the model building and validation. The overlapping between rape and acacia honeys can be explained by their relatively weak aroma and similar nutrient content.

Figure 5.

The classification based on geographical origin provided higher accuracy when the geographical locations of the tested honey samples were far from each other and thus climatic conditions were different. The geographical origin identification of chestnut honeys ($n=66$) presented 100% correct classification for the four areas. LDA model of rape honeys ($n=49$) also resulted in 100% correct classification for the three geographical groups. Classification

model for sunflower ($n=55$) honeys resulted again in high prediction and recognition abilities of 100 and 97.25 % (mutual misclassification was found for honeys from Békés and Nógrád county, while honeys from Heves were classified correctly). Similarly to NIR results, the lowest classification accuracy was obtained in the case of acacia honeys ($n=118$), where the prediction and recognition abilities were 72.7% and 63.4 %, respectively and misclassifications were found for each geographical origin.

3. Conclusion

Physico-chemical and colour attributes of honeys showed significant differences according to the botanical type. These results are promising in identification of the botanical origin of the honey types investigated. Even though these are cheap and affordable measurements, they are time-consuming and can only be applied in authentication as a complex, multivariable dataset, requiring a high number of samples. On the other hand, these attributes can be used as valuable descriptive reference parameters for different unifloral honeys, supporting thus the creation of calibration models for NIR and ET.

Near infrared spectroscopy was able to classify the different botanical groups: average recognition and prediction abilities were 88.12% and 62.52 %, respectively. Our results suggest that geographical origin of honey can be identified reliably only if models are built for the individual floral types. When botanical types were evaluated separately, high accuracy was obtained for identification of geographical origin of sunflower, rape and chestnut honeys. Electronic tongue provided better results for the classification of botanical groups than NIR, average recognition and prediction abilities were above 90%. It was also applicable for determination of geographical origin: models of rape, sunflower and chestnut honeys provided accuracies higher than 90%.

Further enlargement of the honey database is envisaged to improve the performance of differentiation according to both floral and geographical origin of honeys, exploring the potential of NIR and ET as rapid and affordable analytical tools.

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Table 1 Results of physicochemical and colour parameters of the 26 honey samples

	TPC mg GAE/100g sample	CUPRAC $\mu\text{mol TEQ/g}$ sample	FRAP mg AAE/100 g sample	L*	a*	b*	Ash content %	Total dry soluble matter %	pH	Electrical conductivity ($\mu\text{S/cm}$)
Acacia	5.0 \pm 0.5 ^a	10.8 \pm 1.8 ^a	6.6 \pm 1.4 ^a	58.2 \pm 0.2 ^c	-1.7 \pm 0.2 ^a	14.5 \pm 1.0 ^a	0.04 \pm 0.00 ^a	81.8 \pm 0 ^b	3.9 \pm 0 ^b	155.9 \pm 0.3 ^a
Chestnut	12.2 \pm 0.6 ^c	45.0 \pm 3.0 ^b	44.9 \pm 1.4 ^c	48.2 \pm 0.2 ^a	5.6 \pm 0.1 ^b	27.6 \pm 0.2 ^c	0.32 \pm 0.01 ^c	83.2 \pm 0 ^c	4.3 \pm 0 ^c	629.2 \pm 0.8 ^d
Rape	5.8 \pm 0.6 ^a	17.8 \pm 2.5 ^a	18.0 \pm 2.6 ^b	55.0 \pm 0.5 ^b	-1.5 \pm 0.0 ^a	23.2 \pm 0.1 ^b	0.07 \pm 0.01 ^a	80.6 \pm 0 ^a	4.0 \pm 0 ^b	222.5 \pm 0.3 ^b
Sunflower	8.9 \pm 0.8 ^b	56.2 \pm 7.0 ^b	37.9 \pm 5.3 ^c	53.4 \pm 0.2 ^b	0.1 \pm 0.1 ^a	38.6 \pm 0.2 ^a	0.16 \pm 0.01 ^b	80.8 \pm 0 ^{ab}	3.8 \pm 0 ^a	479.9 \pm 0.8 ^c

Mean \pm Standard deviation, The letters are showing the significant differences based on the results of Tukey post hoc method ($p < 0.05$)

Figure 1 PCA score plots of results of NIR spectra by botanical origin of honey after outlier detection (n= 124)

● Rape ■ Chestnut ▲ Acacia ◆ Sunflower

Figure 2 PCA loadings (PC1-PC2) of results of NIR spectra by botanical origin of honey

Figure 3 LDA score plots of results of NIR spectra for the classification of unifloral honey types after outlier detection(n=124)

● Rape ■ Chestnut ▲ Acacia ◆ Sunflower ○ Rape validation □ Chestnut Validation △ Acacia validation ◇ Sunflower Validation

Figure 5 PCA score plots of results of electronic tongue by botanical origin of honey after outlier detection (n=288)

● Rape ■ Chestnut ▲ Acacia ◆ Sunflower

Figure 5. LDA score plots of results of electronic tongue for the classification of unifloral honey types after outlier detection (n=288).

● Rape ■ Chestnut ▲ Acacia ◆ Sunflower ○ Rape validation □ Chestnut Validation △ Acacia validation ◇ Sunflower Validation





