Fungal Diversity and Over-Represented Non-Nectariferous Plants Pollen in Honey. Case Study on Acacia Honey Authenticity, Analyzed in APHIS Laboratory

Otilia BOBIȘ¹, Daniel Severus DEZMIREAN², Victorița BONTA¹, Adriana Cristina URCAN³, Adela Ramona MOISE¹, Rodica MĂRGĂOAN^{4*}

¹*Life Science Institute, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.*

²*Department of Apiculture and Sericulture, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.*

³*Department of Microbiology, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.*

⁴*Advanced Horticultural Research Institute of Transylvania, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania.*

**corresponding author, e-mail: rodica.margaoan@usamvcluj.ro*

Bulletin UASVM Animal Science and Biotechnologies 77(2)/2020

ISSN-L 1843-5262; Print ISSN 1843-5262; Electronic ISSN 1843-536X

DOI:10.15835/buasvmcn-asb: 2020.0009

Abstract

Pollen analysis is the basic method for the determination of the botanical and geographical origin of honey. However, the presence of over-represented pollen in honeys may lead to false results of the analysis. This can be more severe if this pollen is present in unifloral under-represented honeys of commercial importance (e.g. thyme or acacia honey). In the present study, we investigated the abundance of nectarless pollen grains on several quality characteristics in honey samples. In particular, the physico-chemical (diastase activity, electrical conductivity, sugars and HMF content) analysis were carried out in order to confirm the declared botanical origin. Spectrophotometric method was used for diastase activity determination, electrical conductivity was determined by potentiometry and chromatographic determinations for HMF content (photodiode array detection) and sugars (HPLC refractive index detection). The present study confirms that, in the case of non-nectariferous pollen presence in honeys, a second count must be made, excluding this pollen type and pollen analysis alone cannot give reliable results for the determination of the botanical origin. Consequently, pollen analysis should be combined with other analyses, especially in honeys with under-represented pollens, to give precise results for the botanical characterization and labeling of honeys.

Keywords: honey, melissopalynology, non-nectariferous plant pollen, over-represented pollen, physico-chemical analysis.

Introduction

Honey is a natural sweet substance produced by the *Apis mellifera* bees, from nectar and honeydew, which they collect, combine with own substances and let for maturation in the comb (Council Directive 2001/110/EC). A melliferous plant produces substances that may be collected by

the bees and transformed into honey. Melliferous plants are divided into: nectariferous plants, polleniferous plants and mixed plants. Logically, nectariferous plants are the ones with under-represented pollen, and polleniferous plants are plants with over-represented pollen.

The plants listed to be with under-represented pollen are: *Citrus* (10-20%); *Lavandula* sp. (10-20%); *Rosmarinus* (10-20%); *Salvia* (10-20%); *Robinia* (20-30%); *Tilia* (20-30%) and *Medicago* (20-30%). Also *Epilobium* and some *Cucurbitaceae* pollens are considered to be under-represented (Louvreaux *et al.*, 1978). As expected, honeys coming from these nectars, will have low number of absolute pollen grains number. Accordingly, monofloral honeys from the above mentioned plants with a high absolute number of pollen grains, must be treated with suspicion, identifying all pollen species and additional chemical analysis are required. On the other hand, different plants are considered to have over-represented pollen. Among these, *Myosotis* sp., *Castanea sativa* and *Cynoglossum officinale*, being considered highly melliferous (Louvreaux *et al.*, 1978). Consequently, honeys from these nectars are expected to have high number of absolute pollen grains number.

A third situation in melissopalynological determination is the presence in high number of non-nectariferous pollens in honeys, which can't be denominated accordingly, thus physico-chemical and/or sensory analysis are required. Identification of other pollen types beside those belonging to non-nectariferous plants, leads to the classification of that honey as multifloral, even if it is declared as monofloral by the producer.

According to the principal source of carbohydrates used as raw material, honeys are classified in monofloral (where a certain nectar and pollen type is predominant towards the others present), multifloral (several nectar and pollen source are present in honey in similar amounts) and honeydew (when the carbohydrate source comes from secretion of other parts of plants than nectariferous glands, or excretion of aphides from the leafs and branches of different trees). Authenticity in respect of origin, implies both geographical and botanical origin (Soares *et al.*, 2017; Mădaş *et al.* 2019). The quality of honey is generally determined by sensory, physico-chemical and microbiological analysis (Khalil *et al.*, 2012; Oroian *et al.*, 2016; Bayran, 2019; Tanleque-Alberto *et al.*, 2019; Geana and Ciucure, 2020). Palynology is the basic method for the differentiation of nectar and honeydew honey and monofloral or multifloral honey (Louvreaux *et al.*, 1978; Pita-Calvo and Vázquez, 2016; Rodopoulou *et al.*, 2018). Additional to melissopalynology, the

chemical determinations help in the denomination of certain monofloral honeys (Devillers *et al.*, 2004; Karabagias *et al.*, 2018; Machado *et al.*, 2020) and separation from honeydew honeys.

The aim of the present study was to identify the influence of different fungus and non-nectariferous pollen in under-represented pollen honeys, as well as the influence of beekeeping practices in establishing of honey quality.

Materials and methods

This case study was realized on two samples of black locust honey samples, analyzed in Laboratory for Quality Control of Bee Products and Bee Diseases from University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. The samples were atypical for denominated black locust honey, through the presence of high number of non-nectariferous plants pollen and different types of hyphae and fungus in these monofloral honeys.

Pollen analysis was performed after the method of Louvreaux *et al.* (1978), using a non-acetolytic method with some modifications in the time and speed of centrifugation for pollen residue preparation. Ten grams of honey were dissolved in 15 ml of 5% sulfuric acid in distilled water and centrifuged at 4000 rpm for 30 minutes. The supernatant was removed, leaving about 2 ml with the pellet. Distilled water was added up to 10 ml, shaken and centrifuged for 20 min at 3500 rpm. The operation was performed twice. The final pollen residue from each honey sample was left on a microscope slide in two distinctive drops, dried, stained and fixed with fuchsin in gelatine-glycerine mixture. Light microscopy (400×) was used to examine the samples. The pollen spectrum of honey sample was determined by counting 1000 pollen grains using the whole slide if necessary. Relative frequency classes were determined according to the international melissopalynological nomenclature: D – “dominant pollen” (more than 45% of pollen grains counted), A – “secondary pollen” (representing 15-45% of the pollen spectrum), I – “important minor pollen” (3-15%), R – “minor pollen” (less than 3%). National legislations in different European countries have established minimal limits for specific pollen, in order to declare the respective honeys as monofloral (Table 1).

Physic-chemical analysis. Physic-chemical analysis of investigated honey implies water content determination, electrical conductivity, sugar spectrum and HMF content. All these parameters are stated in national standards and international regulations (SR-784/2009; Honey Directive 110/2001).

Water content was measured refractometrically, using the Abbe Refractometer, according to International Honey Commission Analysis Methods (Bogdanov, 2009), by reading the refractive index, making the temperature corrections when necessary. Electrical conductivity was determined potentiometrically, using International Honey

Commission analysis method. Diastase activity was determined using Amylazime as described in International Honey Commission methods. Sugar content determination in the laboratory is made according to International Honey Commission Method, using high performance liquid chromatography and refractive index detection for principal sugars present in honey (Bonta *et al.*, 2008). The HPLC analysis of the carbohydrates is carried out using a modified Alltima Amino 100Å stainless steel column (4.6 mm diameter, 250 mm length, particle size 5 µm). The SHIMADZU instrument equipped with degasser, two pumps, autosampler, thermostat oven, controller and refractive

Table 1. Minimum percentage (%) of pollen required for monofloral honeys in different European countries, according to national legislations (standards, decisions, guidelines) (SR 784-3/2001; Thrasyvoulou *et al.*, 2018)

Honey type	Romania	Croatia	Greece	Germany	Italy	Serbia
<i>Arbutus unedo</i>	-	10	-	-	-	-
<i>Brassica napus</i>	-	60	-	80	-	-
<i>Calluna vulgaris</i>	-	20	-	-	-	20
<i>Castanea sativa</i>	-	85	87	90	-	85
<i>Citrus spp.</i>	-	10(5*)	3	20	10	-
<i>Gossypium</i>	-	-	3		-	-
<i>Erica spp.</i>	-	-	45	45	-	-
<i>Eucalyptus spp.</i>	-	-	-	85	-	-
<i>Helianthus annuus</i>	40	-	20	50	-	40
<i>Medicago sativa</i>	-	-	-	-	-	>30
<i>Mentha spp.</i>	20	-	-	-	-	-
<i>Lavandula spp.</i>	-	10(5*)	-	-	-	-
<i>Phacelia tanacetifolia</i>	-	60	-	-	-	-
<i>Robinia pseudoacacia</i>	30(25**)	20	-	-	-	20
<i>Rosmarinus officinalis</i>	-	-	-	-	-	20
<i>Rubus idaeus</i>	25	-	-	-	-	-
<i>Salvia officinalis</i>	-	15(10*)	-	-	-	-
<i>Satureja montana</i>	-	20	-	-	-	-
<i>Taraxacum officinalis</i>	-	-	-	-	-	20
<i>Thymus spp.</i>	-	-	18	-	15	-
<i>Tilia spp.</i>	30	25(10*)	-	20	-	25
<i>Trifolium spp., Melilotus spp.</i>	-	-	-	70	-	-

*With characteristic organoleptic properties of honey for particular plant species (smell, taste, color);

**Differences between superior quality and first class quality.

index detector. The mobile phase is a mixture of acetonitrile and ultrapure water (80/20 v/v). The injection volume was 10 µl and the flow rate was 1.3 ml/min. Calibration curves of pure standards were plotted using the same method, to quantify the sugars separated from honey samples and expressed as g/100g honey.

HMF analysis was made following the method described in the International Honey Commission (2009) and validated in APHIS laboratory. The determination uses a Discovery HS C18 (5µm; 4.6 x250 mm) column, equipped with a C18 pre-column, mobile phases: ultrapure water (solvent A) and HPLC grade methanol (solvent B), injection volume 20µl and 0.8 ml/min flow rate. Different HMF standard concentrations were used for constructing the calibration curve, in order to quantify HMF from honey samples.

All determinations were made in triplicate, the presented values representing the average ±SD.

Results and discussions

Honey samples subjected to this case study were analyzed in APHIS Laboratory to determine their authenticity. Palynological analysis of the studied honey samples, reveal that the botanical origin declared by the beekeeper do not correspond with the national regulations for monofloral denomination (Table 1 and Figures 1, 2 and 3).

It is known that *Robinia pseudoacacia* have a flower with under-represented pollen, because is a nectariferous flower. After palynological analysis of these two samples, chemical analysis was made (Table 2), to elucidate the results obtained in pollen analysis.

Analyzing the first acacia honey sample, a very high amount of pollen (absolute number) was identified (more than 25.000 pollen grains). According to von der Ohe *et al.*, (2004) and Louvreaux *et al.* (1977) honeys are classified according to the total number of plant elements (N) into 5 classes: Class I: $N \leq 20 \times 10^3$ (unifloral honeys with under-represented pollen; Class II: $21 \times 10^3 \leq N \leq 100 \times 10^3$, multifloral honeys, honeydew honeys and mixtures of flower and honeydew honeys; Class III: $101 \times 10^3 \leq N \leq 500 \times 10^3$, unifloral honeys with over-represented pollen and honeydew honey; Class IV: $501 \times 10^3 \leq N \leq 10^6$ unifloral honeys with strongly over-represented pollen and some pressed honeys; Class V: $N > 10^6$ only pressed honey. At first appearance and from palynological point of view, the sample is multifloral, even if the producer denominates it as black locust, as also the F/G ratio show this (1.42). The sample have also high content of diastase activity (it is known that acacia honey has, generally, low amount of invertase – diastase activity). The predominant pollen present in the sample belong to *Rosaceae* family, *Filipendula* spp. (Figure 1 and 2).

Table 2. Chemical composition (mean ± S.D.) of the analyzed honey samples

Parameter	Acacia declared Sample 1	Acacia declared Sample 2
Fructose (g/100g)	43.60±2.42	43.11±3.21
Glucose (g/100g)	30.68±4.21	32.78±3.22
Sucrose (g/100g)	0.23±0.04	3.36±0.65
F/G ratio	1.42± 0.21	1.31± 0.03
Turanose (g/100g)	2.43±0.15	1.07±0.04
Maltose (g/100g)	1.54±0.04	2.67±0.12
Trehalose (g/100g)	0.44±0.00	0.55±0.01
Erlose (g/100g)	0.98±0.01	1.71±0.21
Diastase activity (DN)	24.6±3.24	14.2±1.55
HMF (mg/kg)	3.03±0.56	2.17±0.23
Electrical conductivity (µS/cm)	324±21.24	486±31.15

As far as it is known, *Filipendula* is a non-nectariferous plant, so we could not have meadowsweet (*Filipendula ulmaria*) honey. For this reason, a second count was performed, excluding the pollen from the nectarless plant (*Filipendula* sp.). Following this procedure, low amount of pollen grains was counted, and *Robinia pseudoacacia* was a percent lower than the limits for monofloral

acacia honey in Romania is established by standard (Table 1), respectively (<5%).

Other pollen types, present in the sediment were: *Cornaceae* – *Cornus sanguinea*; *Rosaceae* – *Prunus* spp.; *Brassicaceae* – *Brassica* spp.; *Gramineae*; *Plantaginaceae* – *Plantago*; *Salicaceae* – *Salix* spp. (Figure 2).

The second acacia sample analyzed in the present study, presented a high amount of fungus and

Figure 1. Pollen grains from *Filipendula ulmaria*
(source: <http://pollen.tstebler.ch/MediaWiki/index>)

Figure 2. Pollen sediment of first declared acacia honey sample

hyphae (Figure 3). The origin of the fungus, may be from non-compliance of the good beekeeping practices, or together with a high conductivity, show another denomination for the honey sample (honeydew, not nectar honey) (Seijo *et al.*, 2011). Different types of hyphae were identified in the analyzed sample (Figure 3). These were *Metschnikowia* cells (this yeast come from nectar itself and there is an interaction of nectar producing plants and flower visiting insects) (Magyar *et*

al., 2005; Herrera *et al.*, 2009; Seijo *et al.*, 2011). Other fungal cells identified in the analysed honey sample was *Cladosporium* and *Alternaria* (Seijo *et al.*, 2011). As can be seen in Figure 3, the most abundant fungus is *Cladosporium*, some conidia found indoor and outdoor environments (Fernández-González *et al.*, 1998; Sabariego *et al.*, 2004). This myxomycete is a saprophytic fungus found also in the alimentary tract of worker honey bees, and probably it came into honey due to second-

Figure 3. Pollen sediment of the second declared acacia honey sample (2)

Figure 4. Pollen grains from *Plantago lanceolata* and *Rumex obtusifolius* (source: <http://pollen.tstebler.ch/MediaWiki/index>)

ary contamination, which may be due to bad bee-keeping practices. Also these types of fungus and hyphae were identified by Dimou *et al.* (2006) in Greek honey and Özkök *et al.* (2016) in Turkish honeydew honey. The pollen grains identified in this honey belong to: *Brassicaceae* - *Brassica* spp.; *Polygonaceae* - *Rumex* spp.; *Plantaginaceae* - *Plantago* spp.; *Caryophyllaceae*, *Rosaceae* - *Filipendula* spp.; *Loranthaceae* - *Loranthus*, *Gramineae*, *Tiliaceae* - *Tilia* spp., *Cornaceae* - *Cornus sanguinea* and *Fabaceae* (Figure 3).

Also in the sediment of the second acacia declared honey, pollen from non-nectariferous plants such as *Rumex* and *Plantago* were present (Figure 4).

Conclusion

Monofloral honeys are consumer preferable and more expensive, and for this reason the determination of botanical origin using pollen analysis is very important. The presence of some nectarless pollen in monofloral honeys which are generally from under-represented pollen flowers, may decrease its marketing value.

Also in microscopic analysis, different fungal elements may be identified, being an important parameter for honey origin source determination. If *Metschnikowia* cells are related to flower honey, different other fungal spores (*Cladosporium* and *Alternaria*) are related to honeydew. The presence of these honeydew elements in monofloral spring honey is due to the climatic conditions of the respective beekeeping area, knowing that a high temperature and lack of precipitations, favors the honeydew production.

In conclusion, our study demonstrated, once again, that the combination of microscopic analysis, sensorial and physico-chemical determinations, must be applied as a battery of tests for the correct determination of the botanical origin of monofloral honeys.

Acknowledgments. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

- Balkanska R, Ignatovas M (2013). Physicochemical parameters of Bulgarian rape honey (*Brassica* spp.) and coriander honey (*Coriandrum sativum* L.). Proceedings of the 10th International Symposium Modern Trends in Livestock Production, 618–626.
- Bayram NE (2019). Quality evaluation and pollen profile of honey samples from different locations. Progress in Nutrition 21(4): 928–934. doi: 10.23751/pn.v21i4.8862.
- Bogdanov S (2002). Harmonised Methods of the International Honey Commission.
- Council Directive 2001/110/EC relating to honey. Official Journal of European Commission L 164: 1–5.
- Devillers J, Morlot M, Pham-Delègue MH, Doré JC (2004). Classification of monofloral honeys based on their quality control data. Food Chemistry, 86(2): 305–312. doi: 10.1016/j.foodchem.2003.09.029.
- Dimou M, Katsaros J, Klonari KT, Thrasyvoulou A (2006). Discriminating pine and fir honeydew honeys by microscopic characteristics. Journal of Apicultural Research, 45: 16–21. doi: 10.1080/00218839.2006.11101319.
- Geana EI, Ciucure CT (2020). Establishing authenticity of honey via comprehensive Romanian honey analysis. Foods Chemistry, 306, 125595. doi: 10.1016/j.foodchem.2019.125595.
- Herrera CM, de Vega C, Canto A, Pozo MI (2009). Yeasts in floral nectar: a quantitative survey. Annals of Botany, 103: 1415–1423. doi: 10.1093/aob/mcp026.
- Karabagias IK, Louppis AP, Kontakos S, Drouza C, Papastephano C (2018). Characterization and Botanical Differentiation of Monofloral and Multifloral Honeys Produced in Cyprus, Greece and Egypt Using Physicochemical Parameter Analysis and Mineral Content in Conjunction with Supervised Statistical Techniques. Journal of Analytical Methods in Chemistry, ID 7698251. doi: 10.1155/2018/7698251.
- Khalil MI, Moniruzzaman M, Boukraâ L, Benhanifia M, Islam MA, Islam MN, Sulaiman SA, Gan SH (2012). Physicochemical and antioxidant properties of Algerian honey. Molecules, 17: 11199–11215. doi: 10.3390/molecules170911199.
- Larran S, Ringuet JA, Carranza MR, Henning CP, Re MS, Cerimele EL, Urrutia M (2001). In vitro fungistic effect of essential oils against *Ascosphaera apis*. Journal of Essential Oil Research, 13(2): 122–124.
- Louvreux J, Maurizio A, Vorwohl G (1978). Methods of Melissopalynology. Journal Bee World, 59(4): 139–157. doi: 10.1080/0005772x.1978.11097714.
- Machado AM, Miguel MG, Vilas-Boas M, Figueiredo AC (2020). Honey Volatiles as a Fingerprint for Botanical Origin – A Review on their Occurrence on Monofloral Honeys. Molecules, 25: 374. doi: 10.3390/molecules25020374.
- Mădaş NM, Mărghitaş LA, Dezmiorean DS, Bobiş O, Ouissam A, Danthine S, Francis F, Haubruge E, Nguyen BK (2019). Labelling Regulations and Quality Control of Honey Origin: A Review. Food Reviews International, 36: 215–240. doi: 10.1080/87559129.2019.1636063.
- Maghraby AS, Hassan (2005). Effect of antioxidative properties of honey on *Schistosoma mansoni*-infected mice. Polish Journal of Food Nutrition Science, 14/55(3): 323–326.
- Magyar D, Gönczöl J, Révay A, Grillenzoni F, Seijo-Coello MC (2005). Stauro-and scoleococonidia in floral and honeydew honeys. Fungal Diversity, 20: 103–120.

17. Oroian M, Ropciuc S, Buculei A, Pădureț S, Todosi E (2016). Phenolic Profile of Honeydew Honeys from the North-East Part of Romania. *Bulletin UASVM Food Science and Technology* 73(2): 105–110. doi: 10.15835/buasvmcnfst:12316.
18. Özkök A, Sorkun K, Salih B (2016). The Microscopic and GC-MS Analysis of Turkish Honeydew (Pine) Honey. *Hacettepe Journal of Biology and Chemistry*, 44(4): 375–383. doi: 10.15671/HJBC.2016.117.
19. Pita-Calvo C, Vázquez M (2016). Difference between honeydew and blossom honeys: A review. *Trends in Food Science and Technology*, 59: 79–87. doi: 10.1016/j.tifs.2016.11.015.
20. Rodopoulou MA, Tananaki C, Dimou M, Liolios V, Kanelis D, Goras G, Thrasyvoulou A (2017). The determination of the botanical origin in honeys with over-represented pollen: combination of melissopalynological, sensory and physicochemical analysis. *Journal of the Science of Food and Agriculture*, 98: 2705–2712. doi: 10.1002/jsfa.8764.
21. Seijo MC, Escuredo O, Fernández-González M (2011). Fungal diversity in honeys from northwest Spain and their relationship to the ecological origin of the product. *Grana*, 50(1): 55–62. doi: 10.1080/00173134.2011.559555.
22. Soares S, Amaral JS, Oliveira MBPP, Mafra I (2017). A Comprehensive Review on the Main Honey Authentication Issues: Production and Origin. *Comprehensive Reviews in Food Science and Food Safety*, 16: 1072–1100. doi: 10.1111/1541-4337.122787.
23. SR-784/2009 Miere de albine.
24. Tanleque-Alberto F, Juan-Borras M, Escheriche I (2019). Quality parameters, pollen and volatile profiles of honey from North and Central Mozambique. *Food Chemistry*, 277: 543–553. doi: 10.1016/j.foodchem.2018.11.007.
25. Thrasyvoulou A, Tananaki C, Goras G, Karazafiris E, Dimou M, Liolios V, Kanelis D, Gounari S (2018). Legislation of honey criteria and standards. *Journal of Apicultural Research*, 57(1): 88–96. doi: 10.1080/00218839.2017.1411181.
26. von der Ohe W, Persano-Oddo LP, Piana ML, Morlot M, Martin P (2004). Harmonized methods of melissopalynology. *Apidologie*, 35: 18–25. doi: 10.1051/apido:2004050.