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MICROSCOPICAL TECHNIQUES USED IN MELISSOPALINOLOGY FOR BOTANICAL ORIGIN OF HONEY DETERMINATION

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Abstract

The paper aimed to present the microscopic techniques used in botanical origin determination of honey samples, shortly melissopalynology. This technique is used for microscopic examination of pollen grains to determine the botanical origin of honey. It is known that a certain number of pollen grains must be recovered from sediment of honey solution, and the presence of small amounts of pollen may be related to falsification. Identification of pollen structure is generally made using light microscopy; phase contrast microscopy may be also used. Fluorescence microscopy is also a powerful method in palynology. Confocal laser scanning microscopy is effective in revealing the ultrastructure of pollen outer layer and shape of the pollen. The determination of the botanical origin of honey using palynology is based on the relative frequency of the pollen belonging to nectariferous plants. Honey is considered monofloral if the pollen from the sediment comes predominantly from a named botanical origin and overpasses 45% from the total count of pollen grains counted on the microscopic slide.

Key words*: confocal microscopy, electron microscopy, light microscopy, palynology, pollen.*

INTRODUCTION

Honey represents one of the most used sweetening agents (Sakač et al., 2019). Honeybees produce this substance that exhibits a great impact for human health and beyond (Abdiniyazova et al., 2016; Nguyen et al., 2018). Nectar of blossoms, secretions of living plants, excretions of plant-sucking insects are some examples of substances that are collected by bees. These species have the ability to combine these substances with their own in order to produce honey (Simsek et al., 2012). The main compounds of honey consist in glucose and fructose, but amino acids, phenolic compounds, organic acids, vitamins, minerals, lipids, enzymes and other phytochemicals are also present in a smaller amount (Baltrušaityte et al., 2007). They depend on many factors such as plant species, climate and environmental conditions, respectively beekeeping practice (Silva et al., 2009). In order to determine the composition and geographical origin of honey, several analytical techniques and parameters combined with statistical methods are needed (Council Directive 2001/110/EC). Honey can be characterised by the aggregation state (liquid), color (light and dark) and can be classified as honeybee (*Apis mellifera*) and stingless bee (*Melipolini*) (da Silva et al., 2013). The produced amount and taste are the major difference between honeybee and stingless bee (Aziz et al., 2017). Different honey properties are related to its composition, especially the minor compounds and residual pollen. These aspects depend on the nectar and pollen of the original plants. Bee pollen possesses antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, antioxidant, antiatherosclerotic, antianaemia, antiallergic, antiosteoporosis and anti-prostatic effects demonstrated by several studies (Gomes et al., 2010; Akbulut et al., 2009; Theunissen et al., 2001). Also, honey is an essential source of polyphenols, flavonoids, sugars, proteins, amino acids, fatty acids, minerals and vitamins (Szczesna, 2006). This bee product can be used as functional food or as nutritional supplement and has an important role in determining the botanical origin.

Microscopic analysis of honey can be done because of the fact that it contains pollen particles, which are concentrated by

centrifugation of diluted honey. Melissopalynology is the main analysis that is used to detect the presence of pollen grains. Also, this type of analysis can identify the floral source and the dominant pollen. If there is more than 45% of one kind of pollen, than it is considerated monofloral honey (Escuredo et al., 2012; Soria et al., 2004). Melissopalynology plays a major role in quality control and origin of honey (von der Ohe et al. 2004; Bryant 2018). Pollen can be a great indicator of the local and regional plant and also can provide information about the floral group used by honeybees to produce honey (Russmann, 1998) Vegetation and climate can be essential factors which have influence above the quantity and diversity of pollen in honey.

Quantitative analysis of pollen in the sediment of honey is used to determine the pollen grain frequency and qualitative control in addition to physico-chemical analysis. If 45% of pollen grain is detected, it is considered predominant, an amount of 16-45% is considered frequent, secondary pollen, and respectively an amount of 3-15% or less is considered sporadic, minor pollen. Due to their pollen amounts, honey can be classified as unifloral or multifloral (polyfloral) (Louveaux et al., 1978). In order to evaluate both the geographical and the botanical origin of different honey types, optical microscopic analysis plays a key role. The present paper aimed to describe some of the microscopic techniques used in melissopalynology in order to denominate different botanical honey samples.

METHODS IN MELISSOPALYNOLOGY

Melissopalynology is a microscopic analysis of honey sediment used to detect the botanical and geographical origin of honey, the pollen types, respectively the source of the flowers (Rech & Absy, 2011). It is the first method which provides information about the botanical characterization of honey. This technique has some limitations such as a requirement of previous knowledge of pollen morphology and specialised employee (Cometto et al., 2003). Optical microscopy is the basic analysis for pollen determination, where a microscopic slide is prepared from honey (simple or using acetolysis), following the procedure described by Louvreaux et al. (1978). Identification of pollen types is made counting at least 500 grains from the slide, with the help of optical microscope, using 40x, 60x magnification, with the help of reference slides of known plants and electronic data bases (Layek & Karmakar, 2016; http://pollen.tstebler.ch).

As stated before, optical microscopy technique requires specialized personnel and time for sample preparation. The sediment of different honeys (10 g honey) is very different in respect of pollen number (due to botanical origin of the sample). It can vary between 1,000 and 10,000. Also the number of distinguishable pollen grains is different and can vary greatly. For this reason, a high percent of pollen grains cannot be determined exactly (down to the species level), and only the higher taxon or the family is determined (Vorwohl, 1967).

The morphological difference between the pollen grains can be determined by using contrast or phase contrast microscopy (Hochuli & Feist-Burkhardt, 2004). Electron microscopy is generally used to differentiate the fine structure of pollen grains (Holst et al., 2007). Fluorescence microscopy has a great impact above analysing cell physiology and it is considerate a common tool of modern cell biologists. One of its major physical limitations is the resolution, which is determined by image contrast and the diffraction of light (Hell, 2003). This method is based on absorption and emission of light energy with the aim to separate them. This process is generally achieved by using optical filters (Helmchen & Denk, 2005). Optical microscopy combined with digital video can quickly and efficiently detect thin optical sections. Wide-field microscopy is used to illuminate simultaneously all parts of the image which allows a faster acquisition. It is also used to analyze specimens in real time (Sheppard & Shotton, 1997). The low cost, simplicity and flexibility of the system are the major advantages of this type of microscope. Toward this, it also has disadvantages such as low image resolution and the possibility for shading artefacts.

Confocal microscopy is based on using spatial filtering to generate a focused spot of illumination with the aim to reject the background light from the image (Helmchen &

Denk, 2005). This process can be achieved with the use of a pinhole aperture which ensures that only the lights from a focused point can reach the detector. The main limiting factor is the speed of the laser scan. Excitation wavelengths on commercial confocal system can include 488, 534, 592 and 635 nm, which means that they are suitable for several fluorescent proteins (Drobizhev et al., 2011).

Electron microscopy is also used in the determination of pollen surface texture, since some decades ago (Laere et al., 1969; Dustmann & von der Ohe, 1993). Scanning electron microscopy is not a routine microscopic determination, because is more difficult to count the pollen grains. This technique is more used for identification of taxa, knowing that same plant family may have similar shape and size of pollen grain (Jones & Bryant, 2007).

RESULTS AND DISCUSSIONS

Pollen grains identification and counting present a huge challenge for the analyst. If simple optical microscopy is used, general information regarding the important plant families may be given (Bobiş et al., 2013; Corvicci et al., 2015; Maida & Özkök, 2020). Also, in bee collected pollen and beebread analysis (other two essential bee products), this method is also used (Mărgăoan et al., 2014; Bobiş et al., 2020; Urcan et al., 2021).

Generally, plant families have similar shapes of pollen, and for this reason using optical microscopy in most of the cases only the plant family may be determined. For more accuracy, other microscopic techniques are required.

Confocal scanning microscopy has proved to be effective in showing details of the fine-structure of pollen exine and more detailed information regarding the shape of pollen grains (Salih et al., 1997; Vitha et al., 2009). Confocal scanning microscopy is based on autofluorescence of the pollen grain (Driessen et al., 1989; Mitsumoto et al., 2009; Castro et al., 2010). In these studies, autofluorescence is used for taxonomical discrimination, on the basis of the intensity and the ratio of the blue to red spectra.

Autofluorescence imaging is considered a non-disruptive method, due to the fact that does not requires any treatment of fixation and staining of the sample. This method could be used in combination with other morphological parameters of the pollen grain in order to identify and quantify correctly the number and species of the grains from the sediment.

A comparation between optical microscopy and confocal microscopy is presented in Figure 1, images made in laboratories of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.

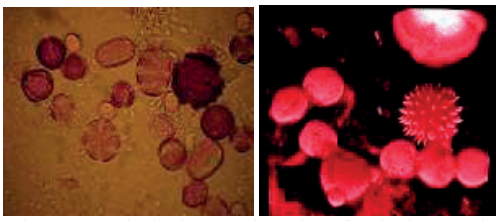

Figure 1. Optical microscopic and confocal microscopic images of multifloral honey sediment (original foto: Mărgăoan Rodica, Tărăban Flaviu)

Using in comparison light microscopy (LM) and scanning electron microscopy (SEM), Jones & Bryant (2007), made a study for morphological comparison of different pollen types and identification of their taxonomy. Although, significantly more taxa were found when using SEM method compared to LM, pollen grains viewed with SEM were divided into three categories: identifiable, obscured and virtually impossible to identify. Taking into consideration the advantages and disadvantages, the authors concluded that there was a minor difference between counting the pollen samples using the two microscopic methods. Every method has its advantages (LM is convenient, SEM have increased resolution of images and more taxa identification), and the final decision for the appropriate method is taken considering the sample, information needed and how much money are available for the study (Jones & Bryant, 2007).

An interesting study (Sivaguru et al., 2012), compared different microscopy techniques used in the analysis of pollen grains. These techniques provide informations on the shape and surface of different pollen types, which present different morphological aspects: widefield, apoptome, confocal, two-photon microscopy, brightfield and differential

interference contrast microscopy and super-resolution microscopy.
The obtained results show that no single optical microscopical techniques capture the pollen shape and the texture of its surface, and only a combination between reflected and transmitted light techniques may recover all morphological information of the pollen, for the exact identification.
A recent study uses three-dimensional (3D) refractive index maps and optical diffraction tomography (ODT) for morphological parameters of the pollen obtained from *Pinus* spp. (Kim et al., 2018).
Figure 2 presents comparatively, original images of different methods of microscopic analysis of pollen grains.

CONCLUSIONS

There appears to be no single optical microscopy technique that can satisfactorily show pollen shape and texture of the pollen surface. For this reason, a combination of reflected and transmitted light techniques is required to maximize the correct identification and quantification of pollen from both the sediment of different honey types, and from bee collected pollen or beebread.
The intraspecies pollen differences can be highlighted using different microscopic techniques and specialized personnel.
Although palynological analysis is apparently an easy determination, a high specialization is needed, both in microscopy and botany.

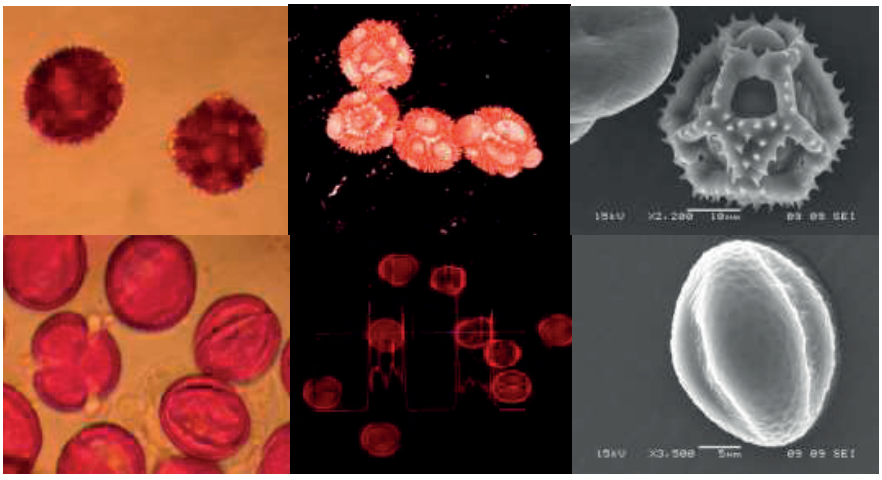

Figure 2. *Taraxacum officinale* and *Salix* spp. pollen optical, confocal and electron microscopic pollen images (original foto: Mărgăoan Rodica, Tărăban Flaviu, Varadi Alina)

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