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METHODS OF MICROSCOPIC SLIDES PREPARATION TO IDENTIFY THE POLLEN GRAINS DERIVED FROM DIFFERENT BEE PRODUCTS

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Abstract

Palynological research has applicability in several scientific fields, the most important being taxonomy, plant evolution, medicine and the analysis of honey - melissopalynology. The palynology provides data regarding the botanical and geographical origin of bee products like bee pollen, bee bread, royal jelly and propolis, which is useful in establishing their provenance and correct labelling. This paper details the characteristics and investigation of several methods for obtaining microscope slides from different bee products. The microscopic examination represents an essential part of the palynological analysis. Several methods for the preparation of microscopic slides were selected and assessed regarding preparation time, costs, dangerous substances used and results. The methods were employed successfully for different bee products, but the non-acetolysis method published by Louveaux et al. (1978) offers several advantages in terms of ease of use, safety and efficiency. The results of our comparative analysis emphasize that some methods are recommended for the creation of reference libraries, while others are well suited for being used in routine analysis of bee products.

Key words*: bee products labelling, microscopic analysis, palynology, slide preparation.*

INTRODUCTION

Palynology is the science that deals with the study of pollen and spores, both viable and fossil. The most important object of study is the pollen grains, which are analyzed morphologically and botanically (Grant-Downton, 2010). The term palynology was coined by English botanists, Hyde and Williams, in 1944. "Palynology" is a term derived from two Greek words, "*palinos*" meaning "powder/fine particles" and "*logos*" meaning science. The study of pollen focuses on its morphological details, polarity, symmetry, size, shape and openings on its surface ("General Botany, Botany Subdisciplines, Palynology," 2019) (www.biocyclopedia.com, 2019).

The results find applicability in many fields such as plant taxonomy and evolution, ecology, beekeeping, plant culture, archaeology, medicine, entomology and forensics (Jarzen, 2023).

Another branch and one of the most important of palynology are melissopalynology. It deals with the study of pollen sediments in honey with the aim of identifying the botanical and geographical origin of the honey. It has a decisive role in the price of honey, establishing the name under which it can be sold and subsequently how the lots will be labelled. Following the microscopic analysis, the botanical species with the predominant pollen from the honey sample is determined (Ige & Obasanmi, 2014). It is checked whether or not it is an attempt at food fraud (Soares et al., 2017) or whether the composition contains *Echium vulgare* pollen, a species declared toxic, due to the alkaloids in its composition (Dübecke et al., 2011).

Palynology is a tool used to give the botanical and geographical origin information of bee products like bee pollen, bee bread, royal jelly and propolis (Rojo et al., 2023).

Many beekeepers currently rely heavily on the bee pollen trade as a significant source of revenue. Bee pollen's botanical origin has a significant role in defining its quality and is extremely important to its price. Therefore, it is especially crucial to standardize the procedures used to gather this data with accuracy. For assessing the botanical profile, there is a fair

number of research and a variety of methodologies utilized. The criteria used to categorize bee pollen as mono or multifloral is made in a lot of modes. The latter is particularly significant for businesses since it affects the bee pollen market price (Almeida et al., 2022).

In the case of propolis, the palynological analysis gives information regarding the botanical origin, the flora that can be found in the area during the picking and the difference between propolis (a product elaborated by honey bees (*Apis*) and geopropolis (a product elaborated by stingless bees) (Barth & Freitas, 2015).

Regarding RJ (royal jelly), palynological studies are useful for determining which plant species and vegetation types are of interest to bees (Barth, 2005), but the important for marketing is the determination of the botanical origin (Yil et al., 2022) or more correctly the determination of the pollen content of this beekeeping product.

Microscopic examination constitutes the essential part of the palynology analysis assessing both overall aspects (polarity, shape, color, apertures) as well as details (morphology of the exine and its ornamentation) of pollen grains. Several methods for the preparation of microscopic slides were selected from different references and assessed regarding preparation time, costs, dangerous substances used and results.

MATERIALS AND METHODS

A. Methods of obtaining palinotheque from freshly collected pollen

Sampling pollen from inflorescences

When collecting the pollen from the inflorescence, the samples are placed in plastic or paper envelopes and marked accordingly. In the case of unknown species, it is necessary to take a larger sample for further identification (Secomandi, 2018). Depending on the size and morphology of the flower, the entire stamen (Figure 1.a), individual anthers (Figure 1.b) or the entire flower (Figure 1.c) can be harvested.

I. Direct method

The gelatin-glycerin is dissolved on the induction hotplate at a temperature of 40ºC. A drop is placed on the slide, over which, with the help of a spatula, the pollen from the surface of the anther is transferred and homogenized with glycerine (Figure 2). Finally, a lamella is placed over the preparation.

Microscopic preparations made by this method contain fresh pollen with a viable cytoplasmic interior. For the microscopic analysis to be as precise as possible, a sufficiently large amount of pollen grains must be found on the slide so that they can be analyzed from all plans (Kisser, 1935; Secomandi, 2018).

II. Ether-based method

The present method requires particular materials and instruments, namely: watch glass, ethyl ether, pipettes, distilled water, tweezers, a cover and an induction hotplate. The working protocol according to (Secomandi, 2018) is presented in the following paragraphs.

The anthers or whole flowers are placed a watch bottle marked with an identification code or number and species name. Over the anthers, ether is added over the anthers until complete evaporation in the chemical niche, as shown in Figure 3.

Afterward, the remains of the anther are removed; distilled water is added and homogenized with a Pasteur pipette.

A drop of the resulted solution is taken and placed on the slide in a square shape that fits the lamella's dimension. The slide is left on the hotplate at a temperature of 40ºC, a drop of gelatin-glycerin is added and finally the lamella is placed over the preparation.

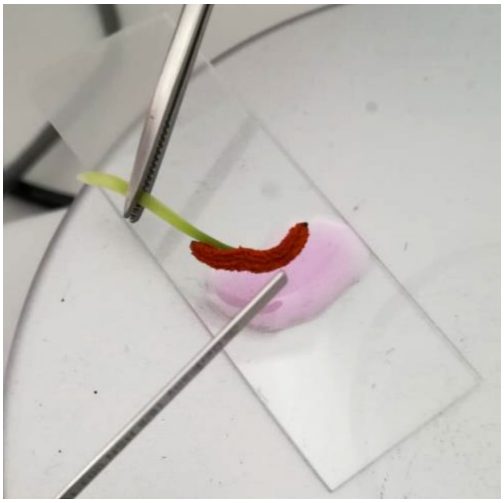
III. Acetolysis method

The acetolysis method was first published by (Erdtman, 1943) and is frequently used today (Hesse & Waha, 1989) It has undergone numerous improvements or modifications over time: (Lieux, 1980; Louveaux et al., 1970; Louveaux et al., 1978; Martins d'Alte, 1951; Maurizio, 1953).

Figure 1a. Sampling of the entire stamen

Figure 1b. Sampling individual anthers by sectioning of the flower

Figure 1c. Sampling the entire flower



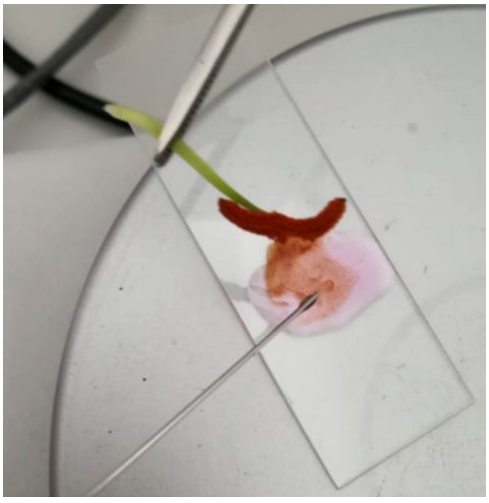


Figure 2. Preparation of microscopic slides based on gelatin-glycerin method

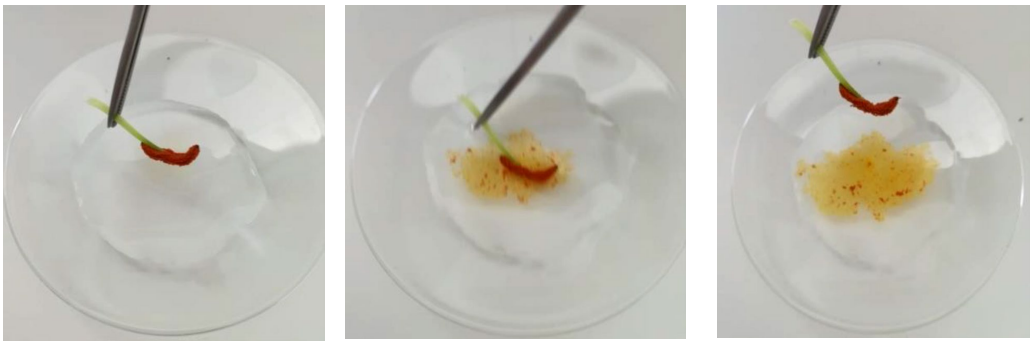


Figure 3. Preparation of ether-based microscopic slides

Figure 4. Required steps of the method without acetolysis

Acetolysis involves the dissolution of most tissues, organic debris and removes proteins, lipids and carbohydrates from the surface of pollen grains (Jones, 2014), leaving only pollenin, the material from which the exine of pollen grains is formed.

By completely removing non-pollen debris, such as intine, cytoplasm and genetic material, the grain structure is damaged and limits pollen analysis, changing morphology, shape and structure (Hesse & Waha, 1989).

A working protocol has been developed by (J. Louveaux et al., 1978) accordingly.

Anthers are mixed with 50 mL of warm distilled water (i.e. < 40ºC), and mix centrifuged for 10 minutes at 2500 rpm. Afterward, the remains of the anthers are separated and the supernatant is removed with a Pasteur pipette, with a final solution of 1 mL remaining.

Distilled water is added over the sediment, homogenized and transferred into two 10-20 mL micro centrifuge tubes in equal parts and finally centrifuged for 5' after which the

supernatant is separated. The remaining sediment is drained on a filter paper and left to dry. The second tube is separately placed.

Afterward, 10 mL of acetolizing mixture is prepared by adding H_2SO_4 and acetic anhydride (1:9). The used instruments need to be thoroughly well dried (Caution: Acetolysis fluid reacts quite vigorously with water and causes bubbling and splashing).

A drop of acetolytic mixture is added to the dried sediment. The sediment is homogenized thoroughly using a glass rod along with the remaining mixture. The tube is placed on a water bath at a temperature of 70°C, taking into account that the water will not come into contact with the acetolytic agent. After an incubation period of 5 minutes, the tube is centrifuged. The supernatant is transferred into a dry vessel and the amount of acetolyzed pollen is visibly noticeable lower compared with the amount from the second tube.

The tube is filled with distilled water and a drop of detergent and mixed vigorously and centrifuged once more for 5 minutes.

After the removal of the liquid supernatant, an important aspect is to observe the presence of pollen grains to the walls. In this case the washing procedure must be repeated with an increased centrifugation at 3500 rpm.

The entire sediment is placed on a glass slide in a 20 x 20 mm surface using a Pasteur pipette.

The blade is dried on a magnetic hotplate at 40ºC.

If the resulting sample contains a small amount of pollen, the acetolization process will be carried out for the second tube as well and afterward the obtained sediment quantities will be combined.

Acetolysis of pollen must be performed in a hood under laminar flow and laboratory equipment is mandatory, because the acetolytic mixture (consisting of sulfuric acid and acetic anhydride) is corrosive and reacts violently in contact with water (Jones, 2014).

IV. The rapid method with acetolysis

The quick and easier-to-implement variant of the previous method is to use the acetolizing mixture directly on the anthers of the flowers, placed on a watch glass. These are mixed and incubated until the pollen separates from the anther tissues, which are then removed. The rest of the mixture is transferred to a slide and dry it under the flame of a spirit bottle, avoiding contact with the direct flame, to prevent excessive blackening of the pollen. After complete evaporation, with the help of a spatula, the pollen is transferred to a new slide where a drop of glycerine is added (Grant-Downton, 2010; Halbritter, 2018).

V. Method without acetolysis

The method without acetolysis, according to the protocol described by (J. Louveaux et al., 1978) is as follows and presented in Figure 4.

A minimum of 7-8 harvested anthers are inserted into each Falcon tube and 10 mL H_2SO_4 5‰ is assed. The samples are centrifuged for 10 minutes at 3500 rpm. After centrifugation, the supernatant is harvested and discarded, double-distilled water is added and after vigorous shaking it is re-centrifuged, followed by removal of the supernatant.

Using a Pasteur pipette, the complete amount of pollen is collected which is present at the base of the tube in the form of sediment.

The collected sediment is placed on the microscope slide on a 20 x 20 mm surface, dried on an induction plate (that must not exceed 40ºC) or directly at room temperature.

B. Melissopalynology

The method is used as a tool for the determination of the botanical and geographical origin of honey.

Slide preparation without acetolysis

The protocol described by (Louveaux et al., 1978) is as follows. A quantity of 20 mL of distilled water which is not exceeding 40°C is used to dissolve 10 g of honey. The honey sample is dissolved with diluted sulphuric acid or diluted potassium hydroxide (5 g H_2SO_4 or 100 g KOH to 1 liter of water) in cases of honey samples high in colloidal particles. The liquid supernatant is discarded from the solution after centrifuging for 10 minutes at roughly 2500 r/min. For a thoroughly removal of honey sugars, the previous step is repeated, accordingly; the sediment is mixed with approximately 10 mL of distilled water and centrifuged for 5 min. The whole amount of the sediment is spread out over a slide in a 20 mm x 20 mm. In most cases it is recommended to

use the Pasteur pipettes for transferring the sediment from the centrifuge tube to the slide. The sediment is mounted after drying with glycerine gelatine (optional fuchsine) which has been liquefied by heating in a water bath at a temperature of 40°C.

Slide preparation with acetolysis

The sample weight is the same as the previous method (e.g. 10 g) and the steps are presented in section A.III. Methods of obtaining palinotheque from freshly collected pollen, acetolysis method.

C. Bee pollen and bee bread analysis

The bee pollen and bee bread samples must be ground and well homogenized, following the protocol described by (Louveaux et al., 1978), with slight modifications. According to (Almeida-Muradian et al., 2005) the sample to be analyzed must weigh 2 g. The initial suggested approach underwent various modifications to increase repeatability within and across laboratories and to evaluate some of the probable factors that could generate the variability of the findings obtained. According to preliminary findings, the pollen sample size must be increased to 5 g in order to improve its representativeness (Almeida et al., 2022). The steps followed in preparing the microscopic preparation are the same as for the honey sample (see Section B. Melissopalynoloy).

D. Pollen analysis of royal jelly (RJ)

The slide preparation to assess the qualitative analysis of RJ is realized after (d'Albore & Bernardini, 1978; Von Der Ohe et al., 2004).

A conical-bottom centrifuge tube of 50 g is used after 1 g of RJ is weighted with the addition of 10 mL of KOH 1% and vortexed. The sample is centrifuged for 10 minutes at 2500 rpm and the supernatant is discarded. A quantity of 10 mL distilled water is added, thoroughly (vortex) mixed, following the complete fill of the test tube with the remaining distilled water and reaching a total volume of about 45 mL. The resulting mixture is centrifuged for a second time for 10 minutes at 2500 rpm and the finally the supernatant is discarded. The sediment is transferred from the centrifuge tube to the slide drawing this time a much smaller surface (10 x10 mm), compared to the one from the honey analysis.

A different technique performed by (Barth, 2005) involves the dilution of a quantity of 1g of RJ in 10 mL of distilled water with the addition of a single tablet of alien spores (*Lycopodium clavatum*; 10679 spores per tablet) to the mixture (Stockmarr, 1971). The sediment is centrifuged, re-suspended in 10 mL of acetic acid, and allowed to sit overnight to dehydrate. The sediment is acetolyzed in the following day (see the method A.III.)

E. Pollen analysis from propolis

Propolis

The protocol proposed by (Barth, 1998) as follows: a quantity of 0.5 g of the scraped propolis extract is mixed with 15 mL of ethanol overnight or for a couple of days with occasional shaking of the tube. The suspension is divided into two tubes and centrifuged. After centrifugation the decanted sediment is re-suspended with 13 mL ethanol. Over the sediment 12 mL of KOH (10%) is added and brought to boiling point for at least 2' in a water bath. After this step, the tubes are placed in an ultrasonic agitator for 5', centrifuged and decanted. A quantity of 13 mL of distilled water is added, the sediment is transferred to another centrifuge tube, throughout a 0.3 mm mesh sieve for the removal of large organic particles, followed by centrifugation, decantation. Finally, a slide of the sediment is prepared using glycerine-jelly.

The use of acetolysis in other methods requires the following steps. Over the propolis sample a quantity of 5 ml of glacial acetic acid is added and left overnight. Afterward, the sample is centrifuged and decanted, and the acetolysis mixture composed of acetic anhydride and concentrated sulphuric acid (9:1) is added. The mixture is left for 3 minutes at 80°C in a water bath, centrifuged and decanted. The resulting sediment is washed with distilled water and after a mixture of glycerine and water (1:1) is added and left to rest for at least 30', followed by centrifugation and decantation of the sediment. The final slides are prepared, one with unstained glycerin jelly and another with basic fuchsin-stained glycerine-jelly.

RESULTS AND DISCUSSIONS

Due to their beneficial effects on human health, apicultural products have been utilized in phytotherapy and nutrition for a long time. The presence of bioactive chemicals has been thought to provide health benefits, they are becoming more popular nowadays (Ares et al., 2018). The bioactive compounds are in close correlation with the botanical and geographical origin. Due to the growing consumer desire for distinctive products with a sense of place, the use of geographical indication (GI) for bee products is necessary to obtain consumer confidence, leading to market recognition and a premium price (Sattler et al., 2015).

By the use of palynological or melissopalynological examination, the bee product can be labelled according to its botanical and/or geographic origin. The quality of bee products is correlated with their botanical and geographic provenance, which can serve as indicator in their identification of origin and labelling (Mădaş et al., 2020).

Since palynological analysis often gives a subjective result, it must be complemented or correlated with other determinations that justify the obtained results. Among the quality criteria Bogdanov assessed several constituents, including: electrical conductivity, reducing sugars, sucrose concentration, free acid, proline and protein content, hydroxymethylfurfural (HMF), and diastase activity (Bogdanov et al., 2002). Additionally, other quality markers used are color, phytochemicals, and sensorial characteristics of honey (Thrasyvoulou et al., 2018).

To define honey from different areas or to detect a mixture of honey from different locations some researchers, have established a model for predicting the geographic origin of honey using pollen grains frequency from the same type of honey samples produced in different areas or combining this strategy with cluster and correlation statistical analysis was effective for describing honey samples of different geographical and botanical origins (Herrero et al., 2002; Karabournioti et al., 2006).

The melissopalynological analysis may be influenced by the flower's morphology and physiology (Louveaux et al., 1978; Todd & Vansell, 1942), but also according to the foraging bee activity or hive contamination (Molan, 1998). There are several unifloral honey types with underrepresented pollen, such as Citrus, Lavandula, Rosmarinus and Salvia between 10 and 20%, and Tilia, Medicago, and Robinia with pollen grains between 20 and 30%. On the contrary, Brassica (>60%), Castanea (>90%) and Eucalyptus (>80%) honey types contain overrepresented pollen (Von Der Ohe et al., 2004). In this context, mellisopalynology coupled with other analysis may provide reliable results in terms of authenticity and discrimination of honey samples (Corvucci et al., 2015). a number of honey sample quality variables were examined in relation to the prevalence of overrepresented pollen grains. Several methods were examined, mainly: melissopalynological, organoleptic, physicochemical (water content, electrical conductivity, color), and volatile properties of the blends of overrepresented mixed honeys, specifically eucalyptus and chestnut honeys with thyme honeys in various analogies. The most sensitive metrics were microscopic features, followed by organoleptic (Rodopoulou et al., 2018).

The combination of these analysis with multivariate analysis techniques (e.g. hierarchical cluster analysis (HCA) and principal component analysis (PCA)) provides a more precise classification of the honey samples (Geana & Ciucure, 2020). Authenticity of multiple monofloral and multifloral honey samples has been assessed using the method described by Louveaux without acetolysis (Louveaux et al., 1978), along with SDS-PAGE and combined with multivariate analysis. This novel methodology has been evaluated to provide a simple, low-cost method for identifying honey sources, as well as new opportunities to detect honey adulteration. The generated heatmap depicted the link between melissopalynological analysis and protein pattern providing a reliable method in honey authentification (Mureșan et al., 2022).

The method with acetolysis has been successfully used to characterize the pollen grains from different species of the genus Anthemis (Dauti et al., 2014). In a different study, the same method was implemented to evaluate the differences in

palynomorphological characteristics of different plants found in different areas in Albania. Furthermore, the colored method of basic fuchsine and fixation with gelatine-glycerin were also used (Kallajxhiu et al., 2015).

The method without acetolysis firstly introduced by (Erdtman, 1943) and modified by (Louveaux et al., 1978) was used to assess the foraging preferences of bees from different tropical regions. In the context of plant-pollinator interactions, this study determined the variations in pollen contents of honey across time and distance, even within the restricted landscape mosaic (Ponnuchamy et al., 2014).

The mellisopalynological analysis using the ether-based method was evaluated to recognize the pollen granules of Mediterranean floral species from multiple honey samples. In order to evaluate the pollen grains in their natural color, which can vary depending on the botanical family, microscopic observations were first done without the addition of any coloring additives. A colored preparation with fuchsin was used in order to improve the mellisopalynological recognition. Furthermore, in order to perform a more thorough study DNA extraction was also carried out. The microscopic analysis of the pollens removed from certain commercially available unifloral honeys was supported by the results of the molecular study, which was conducted using RT-PCR. The microscopic examination revealed the presence of pollen grains from both the species listed on the honey label and other species. It was possible to conduct an analysis on the pollen composition of some honey that was marketed and designated unifloral due to the application of microscopic and biomolecular analysis techniques in melissopalynology (Vancheri et al., 2019).

In a different study, the techniques reported by (Almeida et al., 2022; Louveaux et al., 1978; Morais et al., 2011) modified for pollen load formed the basis of palynological study (Margaoan et al., 2014). The palynological analysis was complemented with individual carotenoids and fatty acids identification. The levels of lipids and carotenoids in bee-collected pollen varied greatly between samples, which can be explained by the different botanical sources of those samples.

CONCLUSIONS

The findings of our comparative study demonstrate that while some strategies are recommended to be used in the development of reference libraries, while others are best suited for use in bee product analysis. A high level of proficiency in both microscopy and botany are necessary for palynology analysis. Furthermore, the use of different methods to identify the pollen types and characteristics are significant to assess the origin and even authenticity of the samples when coupled with other analysis.

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