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Melissopalynological methodologies for investigating honey samples – a critical approach

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Abstract: Melissopalynological techniques are used in the analysis of pollen grains. However, the adoption of methodologies considering cost-effectiveness, shorter preparation time and lesser toxic procedures for researchers is relevant. Thus, this study aimed to analyze different melissopalynological methodologies in polyfloral honey samples. Three melissopalynological protocols were applied to the samples using alcian blue dye, basic fuchsin, and the traditional acetolysis methodology without dye. After preparing the pollen samples, and analyzing them under an optical microscope, six botanical families were recorded, e.g., Amaranthaceae, Asteraceae, Bignoniaceae, Fabaceae, Malvaceae, and Myrtaceae. The methodologies investigated proved to be efficient for detecting pollen structures and identifying botanical families thereof. The alcian blue dye-based protocol allowed a greater separation and discrimination of pollen grains as compared to the basic fuchsin and acetolysis ones, where pollen conglomerates were often identified. Even though acetolysis has been the most used method in melissopalynological studies, it has been claimed to offer risks to users due to the manipulation of corrosive and toxic solvents (i.e., H_2SO_4), also being lesser cost-effective and more time-consuming. Thus, considering the cost-effectiveness, the alcian blue dye and basic fuchsin-based methods seem to be preferred, being as efficient as acetolysis for identifying pollen grains.

Key words: acetolysis, alcian blue, basic fuchsin, pollen grains.

INTRODUCTION

Honey is a natural food consumed both as a sweetener and for its therapeutic properties (Sohaimy et al. 2015). It presents high nutritional value, containing sugars, vitamins, enzymes, proteins, minerals, organic acids, carotenoids, polyphenols, and amino acids (Miguel et al. 2017).

Bees produce honey from the nectar collected of plants and by doing so they also harvest pollen grains that are stored in the corbicula in the posterior tibia (Engel & Rasmussen 2019). Such pollen grains collected by worker bees after undergoing biochemical transformations become a mass called bee bread, which will serve as food for adult bees and larvae (Gilliam 1979). Some pollen grains stick to the bee body and fall into the combs still uncapped (Jones & Bryant 1996). These pollen grains end up immersed in honey and can be used for typification of that food, determining its geographic origin according to the families and plant species sources of pollen, in addition to serving to identify frauds in honey (Aronne & De Micco 2010, Sniderman et al. 2018).

The pollen grains analysis of honeys is called melissopalynology and can be performed following diverse methodologies. Melissopalynology is one of the approaches used to detect adulteration in honey, as it allows

the detection of its botanical and geographic origins based on the type of vegetation found around the hives. Additionally, it also allows one classifying honey as unifloral or multifloral (Selvaraju et al. 2019, Mureşan et al. 2022), a trait with relevant meaning in the quality and market price of that food. Honey is among the most counterfeit products in the world, due to the high demand and prices paid in the market, currently being the third more frequently adulterated food (García 2018). Therefore, improving melissopalynological techniques is essential and urgent, as well as applying them to poorly studied regions.

Acetolysis, proposed by Erdtman (1952), is commonly used for melissopalynological analysis, considering that it allows the removal of non-pollen materials, e.g., sugar grains. However, using this methodology requires dangerous and high-cost solvents to monitor the quality of honey.

In this sense, it is relevant to investigate melissopalynological protocols regarding cost-effectiveness, the time required for sample preparation, and the use of hazardous chemicals during sample handling. In addition, it is necessary to consider that in countries with a great diversity of plant species like Brazil (MMA 2021) that offers a large number of pollen donor plants to bees, these techniques need to allow one identifying botanical families without cause deformation, breakage or agglomeration of the pollen grains. In fact, pollen damage might happen in acetolysis analysis due to the use of strong acids, compromising the quali/quantitative pollen's traits (Özkök et al. 2022, Wrońska-Pilarek et al. 2023).

An alternative methodology for pollen grain analysis is the use of dyes commonly adopted in chemistry and biology investigations, due to their low health risk, lower cost, and effectiveness in staining pollen structures, separating the grains from other elements, such as dust (Oliveri et al. 2019, Jia et al. 2021, Viertel & König 2022). Thus, this study aimed to evaluate three methodologies for pollen detection and identification regarding its botanical origin in floral honey samples, based on acetolysis reaction and the use of the dyes basic fuchsin and alcian blue. These dyes were chosen due to their widespread industrial use, cost-effectiveness, and capability to react with pollen grains (Faegri & Iversen 1964, Rowley & Nilsson 1972). As a working hypothesis, it is proposed that by using the dyes basic fuchsin and alcian blue it will be possible to accurately detect and identify the botanical origin of the pollen present in floral honey samples, compared to the method traditionally used for this purpose.

MATERIALS AND METHODS

Sample collection

Floral honey samples were collected in the 2019-2020 production season in eleven agroecological zones of the State of Santa Catarina (southern Brazil - Figure 1). Ten samples were randomly chosen from the sample set in order to perform melissopalynological analysis through two dye-based protocols (e.g., alcian blue and basic fuchsin) and by the acetolysis method without staining.

Analysis of pollen grains via acetolysis

The melissopalynological analysis was performed by adapting the acetolysis method described by Erdtman (1952). The acetolysis solution was prepared by adding 9 mL acetic anhydride to 1 mL sulfuric acid. In a plastic test tube, 1 g honey was added to 1 mL glacial acetic acid, allowing to stand for 24 h. The sample was centrifuged (2000 rpm, 10 min), the supernatant discarded, and 5 mL acetolysis solution were added to the pellet, after which the samples

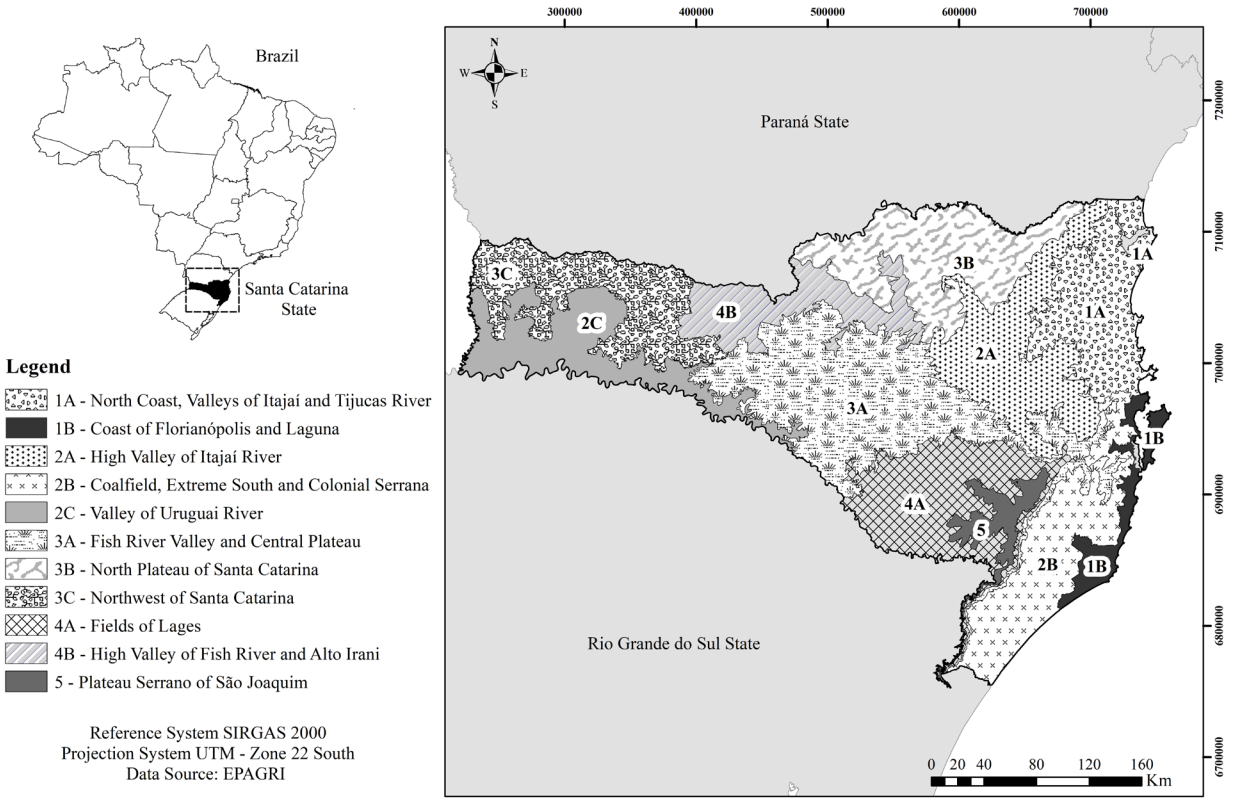

Figure 1. Agroecological zones of the State of Santa Catarina, Brazil. Source: Prepared by the author (2022).

were incubated in a water bath at 80 °C, for 2 min. The sample was centrifuged (2000 rpm, 5 min) again, the pellet recovered, 10 mL distilled water were added and briefly stirred. In the case of foam formation, 2-4 drops 70% alcohol were added to the samples, following centrifuging (2000 rpm, 5 min) and discarding the supernatant. A 1: 1 distilled water - glycerin solution (v/v) was added to the sample and after 12 h resting the centrifuging step was repeated. The solids deposited on the bottom of the eppendorf tube were recovered with a Pasteur pipette and transferred to a microscope glass slide and covered with a coverslip.

Analysis of pollen grains using basic fuchsin and alcian blue stains

The floral honey samples were heated in a water bath (25 min, 60 °C) for de-crystallization and homogenization. After that, an aliquot (1g) of honey was solubilized into 2 mL distilled water, vortexed, and centrifuged (2000 rpm, 10 min). The pellet was recovered and a washing process was carried out to concentrate the pollen grains prior to the assembly of the slides for microscopic analysis. In the first wash, 1 mL distilled water was added to the sample, following homogenization (vortex) and centrifuging (2000 rpm, 10 min). For the second washing step, the supernatant was removed, 1 mL 96% ethyl alcohol was added, and the sample centrifuged again (2000 rpm, 10 min). After decanting the supernatant, an 1: 1 (v/v) mixture of glycerin and basic fuchsin or alcian blue dyes was added to pollen sample. To ensure the effectiveness of the coloring of pollen grains and no other materials such as sugar crystals, a last washing step was performed with 90% ethyl alcohol. The

pollen sample (5 µL) was transferred to a glass slide onto which was previously added 10 µL bi-distilled glycerin and covered with a coverslip for microscopic analysis.

Image capture and identification of pollen grains

The analysis of pollen grains was performed under an optical microscope (Opton®) and the images were captured with the aid of a Tucsen digital camera. The recorded images were digitized using the software provided by Tucsen® (Scientific Camera Tucsen Photonics Co., Ltd.). All samples were analyzed in triplicate, with microscopic visualization being performed at 10x and 40x magnification. Identification of the pollen type, i.e., botanical family, was carried out with support of the Rede de Catálogos Polínicos Online database (available at http://rcpol.org.br/pt/home/ – (RCPol 2022), and also by consulting the Pollen Catalog (Silva 2014).

Five glass slides of each tested methodology were chosen to perform the image treatment process. For each sample investigated, it was possible to visualize a single pollen grain in the frame to assist the botany identification and the pollen grains did not undergo any type of adjustment, whether in terms of color, light, or temperature. Thus, only the background of the images was modified by the temperature balance, i.e., changing to a white background, making the pollen grain more visible. The correction of the image background (white balance) was performed manually pixel by pixel, so that any part of the pollen grain was lost. The images recorded were analyzed with the support of the open-source software GNU Image Manipulation Program (GIMP, Version 2.10.8) and further added to the pollen database of the State of Santa Catarina, Brazil.

RESULTS AND DISCUSSION

Pollen grains of several botanical families were identified in the Brazilian floral honey samples investigated. This finding results from the great floral diversity typically found in the country, implicating in a polyfloral composition of pollen grains in honey samples (Cardoso 2016). Due to this factor, testing different melissopalynological methodologies can be decisive and illustrative for the characterization of the meaningful diversity of species sources of pollen grains present in honey.

The image analysis of pollen grains treated with the alcian blue and basic fuchsin dyes, as well as with acetolysis revealed donor plant species belonging to six botanical families as follows: Amaranthaceae, Asteraceae, Bignoniaceae, Fabaceae, Malvaceae, and Myrtaceae. All methodologies investigated allowed identifying such botanical families, what demonstrates the efficiency of these methods.

Most of the botanical families identified in this study have previous records in the RCPol (2022) database, except for Bignoniaceae. In southern Brazil, pollen from Amaranthaceae, Asteraceae, and Malvaceae families have been more frequently found compared to Fabaceae and Myrtaceae (RCPol 2022). Similar results were found by Osterkamp & Jasper (2013) in floral honey from Rio Grande do Sul State, southern Brazil. The authors were able to identify pollen grains of 15 botanical families, with a predominance of Arecaceae and Asteraceae. Barth & Luz (2022) analyzed honey samples collected in sandbank areas spread over the southeastern, northeastern, and southern regions of Brazil and found in the latter the Myrtaceae and Solanaceae families as the main pollen sources. Bosco & Luz (2018) identified 26 botanical families of nectariferous species donors of pollen, in honey produced in southeastern Brazil (São Paulo State), with

higher frequencies to the Fabaceae, Sapindaceae, Asteraceae, and Euphorbiaceae. Santos et al. (2019) described 27 pollen types from 19 botanical families, mostly the Fabaceae, Rubiaceae, Asteraceae, and Sapindaceae, in northeastern Brazilian honey (Bahia State).

Souza et al. (2018) reviewed the melissopalynological studies carried out between 2005 and 2017 in Brazil and described that among the most frequent pollen types found in honey belong to the Fabaceae, Asteraceae, Euphorbiaceae, Rubiaceae, Myrtaceae, Malvaceae, Bignoniaceae, and Arecaceae. The authors note that fewer studies have been conducted on honey collected in the central-west and southern regions of Brazil, indicating a need for more research to characterize the flora foraged by bees in these areas and the impacts on honey quality and provenance.

In this study, the alcian blue dye-stained sugar crystals present in the honey samples, implying the presence of several blue dots in the micrographies recorded. However, the dye changed the tone of the pollen grains to green, which allowed their identification without major difficulties. Importantly, following the exposure to that dye, the pollen grains did not agglomerate, with separation on the glass slide prior to the analysis by optical microscopy (Figure 2a, b, c and d).

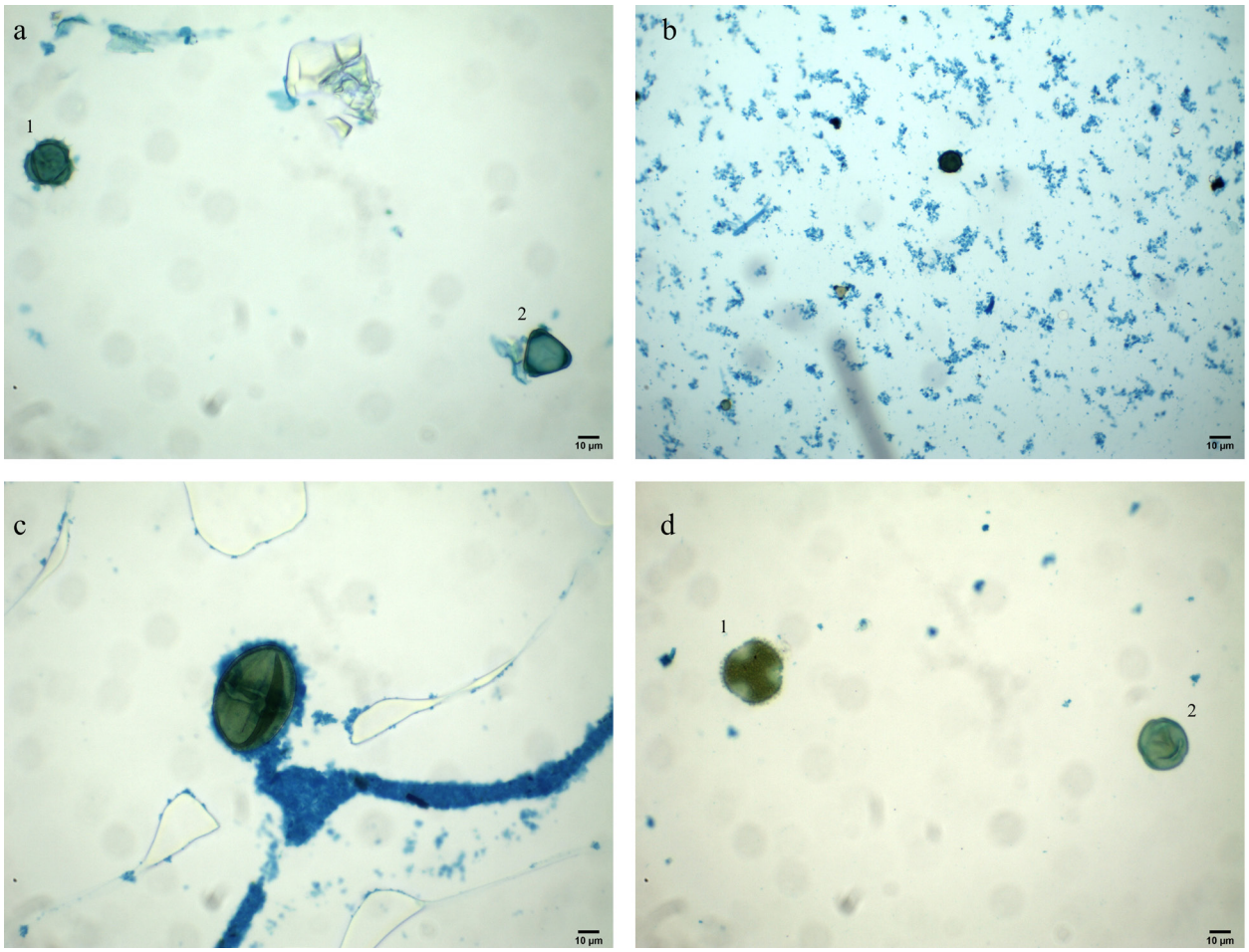

Figure 2. Pollen grain structures belonging to the Asteraceae (a1), Myrtaceae (a2), Asteraceae (b), Fabaceae (c), Bignoniaceae (d1), and Asteraceae (d2) botanical families, stained with alcian blue dye. Source: Prepared by the author (2022).

Alcian blue is a basic polyvalent dye commonly used in histochemistry and cytochemistry protocols (Hayat 1993). This dye colors acidic polysaccharides (Demarco 2017), mucins (Steedman 1950), proteoglycans (Bjornsson 1993), nanoparticles (Carton et al. 2019), and plant cell walls (Beneš 1968). Beneš (1968) and Zhao et al. (2019) describe that alcian blue specifically binds to the dissociated carboxyl acid groups of pectins in the plant cell walls, resulting in strong staining. Rowley & Nilsson (1972) note that alcian blue can be used to color pollen grains, which may react to give the appearance of fresh pollen. However, this may depend on the pH of the reaction medium used for coloring.

By using basic fuchsin dye, the pollen grains showed a very strong hue and some structures could not be accurately differentiated or measured, such as openings and small ornaments. It was also noticed that the pollen grains became quite agglomerated, with smaller ones being covered by the larger grains, hindering the identification of the donor botanical families (Figure 3a, b, c and d).

Basic fuchsin is a dye from the triaminotriphenylmethane group, widely used in the textile industry. In cell biology, this

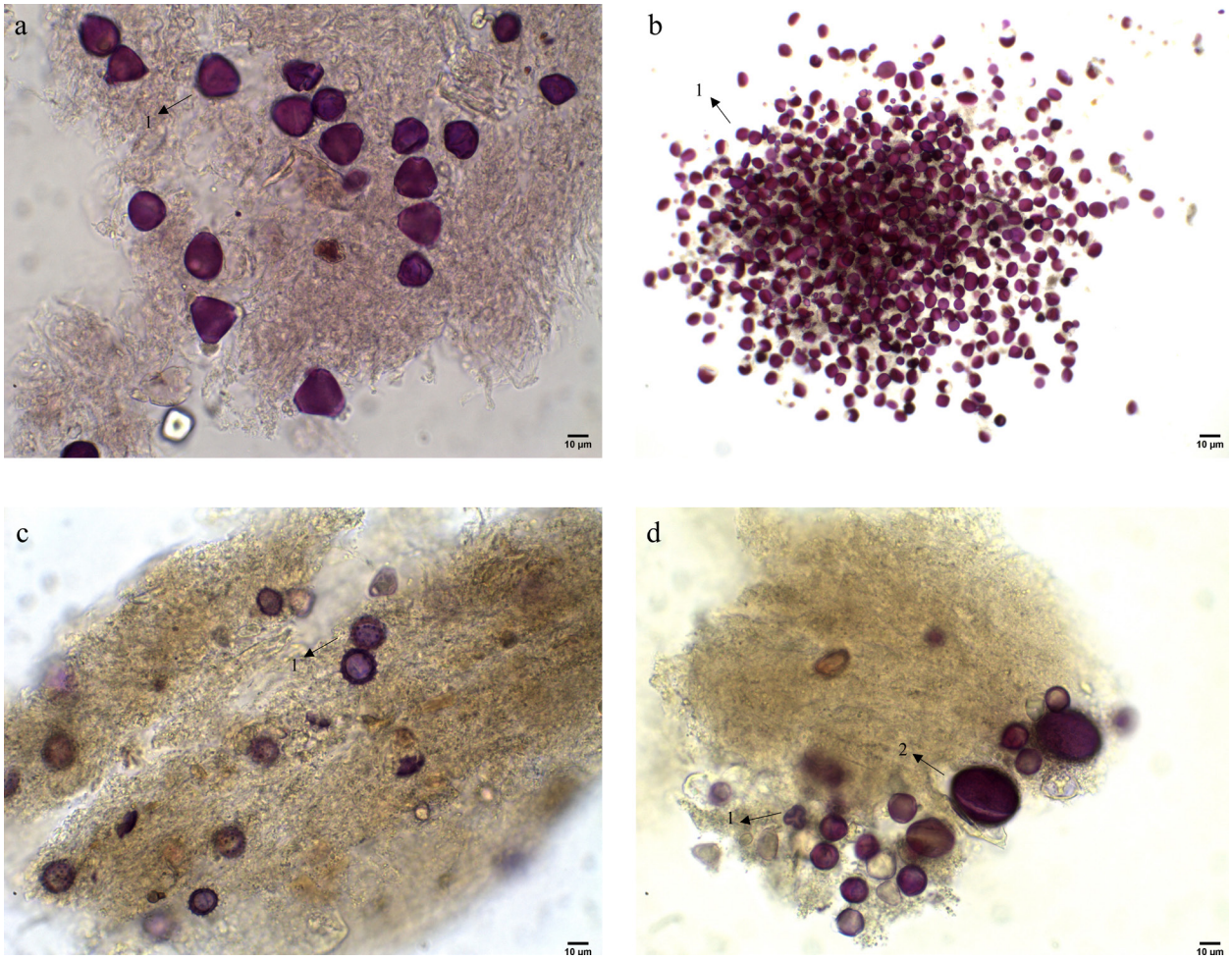

Figure 3. Pollen grain structures identified in floral honey samples typical of the Myrtaceae (a1 e d1), Fabaceae (b1), Malvaceae (c1), and Fabaceae (d2) botanical families, stained with basic fuchsin. Source: Prepared by the author (2022).

compound has been used to stain collagen, muscle, mitochondria, and other kind of samples. It has been reported that it can also be used to stain plant's cell walls (Kapp et al. 2015). According to Kraus et al. (1998), basic fuchsin has affinity with lignified, suberized, or cutinized structures of plant tissues, and even chloroplasts (an organelle devoid of lignin) are stained by this substance.

In the case of viable pollen grains, basic fuchsin acts by staining the protoplasm with a pink color, as in non-viable ones no color is developed due to the absence of the nucleus (Jesus et al. 2018). Faegri & Iversen (1964) point out that by using basic fuchsin to differentiate exine layers, a rapid and deep ektexine staining is achieved, while endexine stains weakly or has no action.

The acetolysis methodology, more commonly used in melissopalynological studies in comparison with the other staining techniques herein investigated, presented a hue that allowed easy identification of pollen grain structures, such as openings, colpo, and exine ornamentation. However, in some micrographies the formation of pollen agglomerates was noted, hindering the botanical identification (Figure 4a, b, c and d).

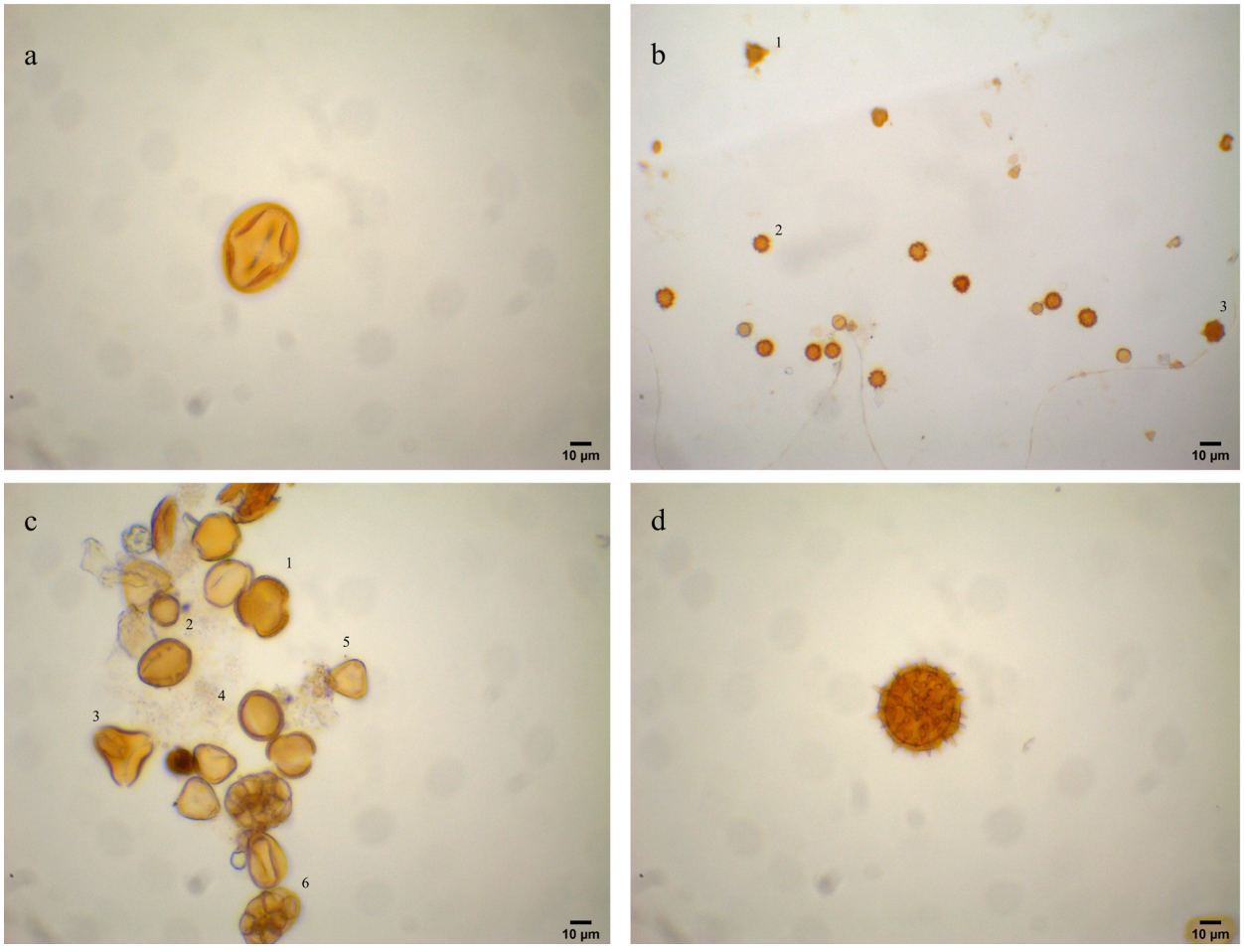

Figure 4. Pollen grains of floral honey samples identified according to the acetolysis protocol. The most common botanical families detected were Fabaceae (a, c2, c4, c6), Myrtaceae (b1, c3, c5), Malvaceae (b2, d), Amaranthaceae (b3), and Bignoniaceae (c1). Source: Prepared by the author (2022).

Acetolysis is a classical method developed by Erdtman in 1952 and has been used worldwide in palynology studies. In this process, the pollen is heated in a mixture of sulfuric acid and anhydrous acetic acid, destroying all non-sporopollenin substances. As the pollen grain's external wall is made of sporopollenin, acetolysis allows an excellent visualization of the grains (Hesse & Waha 1989). The cleaning of the pollen wall enables clear identification of external structures and botanical families (Basarkar 2017, Correia et al. 2017). However, acetolysis poses risks of toxicity and damage to the user, due to the solvents used. It can also interfere with the structure of the pollen grains, compromising their sizes or selectively showing certain botanical families (O'keefe & Wymer 2015).

The cost of performing melissopalynological analyses using the different methodologies was also investigated. Three budgets obtained from various companies were considered, listing the main products used, excluding distilled water, microscope slides, and coverslips. Operational costs associated with equipment were not included. Among the methodologies, the basic fuchsin dye was the cheapest, costing US$ 2.00 for 100 samples (Table I).

In addition, it is worth noting that the simple staining method using basic fuchsin and alcian blue does not require the use of a fume hood, which is essential for the acetolysis methodology, due to the use of hazardous chemicals (e.g., sulfuric acid and anhydride acetic). Thus, laboratories that do not have a fume hood are able to carry out the melissopalynology analysis without risk to the operator and would save between US$ 1,980.00 plus US$ 3,960.00 with the purchase and maintenance of that apparatus, which must occur every year.

By comparing the analytical protocols herein described, the basic fuchsin method resulted in a cost/sample 6.5x and 8x lower than verified with the use of alcian blue dye and acetolysis, respectively. In addition, sulfuric acid and acetic anhydride are required to carry out the acetolysis solution, being reagents undergoing controlled access and purchase by authorities in several countries, such as in Brazil (Federal Police - Ordinance n° 240/2019, Ministry of Justice and Public Security - Brazil 2019). In several cases, such a legal demand makes it difficult to have enough reagents for routine melissopalynological analyses.

Image treatment of microscopic slides was performed in order to separate the pollen grains, which provided better visualization of pollen structures since the white background eliminates conglomerates, sugar crystals, and/or other impurities present in the honey (Figure 5a, b, c, d, e, f, g, h, i, j, k, l, m, n and o). It is important to emphasize that the image treatment did not interfere with the matrix, that is, the pollen grains, being extremely important for the inclusion of images in a database that helps in the identification of floral species used by bees for honey production in southern Brazil and elsewhere.

The search for new melissopalynological methodologies, or even the improvement of

Table I. Cost per melissopalynological analysis.

Methodology	Unit cost US$	Total cost (100 samples) US$
Basic fuchsin dye	0.02	2.00
Alcian blue dye	0.13	13.00
Acetolysis	0.16	16.00

those protocols already used is mandatory, because the most frequent technique used, i.e., acetolysis, is time consuming, as well as demand for specialized personnel to carry out the analysis. In fact, acetolysis allows very clear identification of pollen grains, however, for that it is necessary to stand for at least 12h to start the image visualization, while the other methods herein investigated allowed the analyzes to be performed immediately after mounting the slides for microscopic visualization. In practical terms, this scenario has obvious implications in routine analysis, besides the higher costs associated, as herein described.

Colorimetric tests using alcian blue and basic fuchsin have proved to be viable methodologies for the analysis of pollen grains in floral honey samples, being fast and safe techniques. In

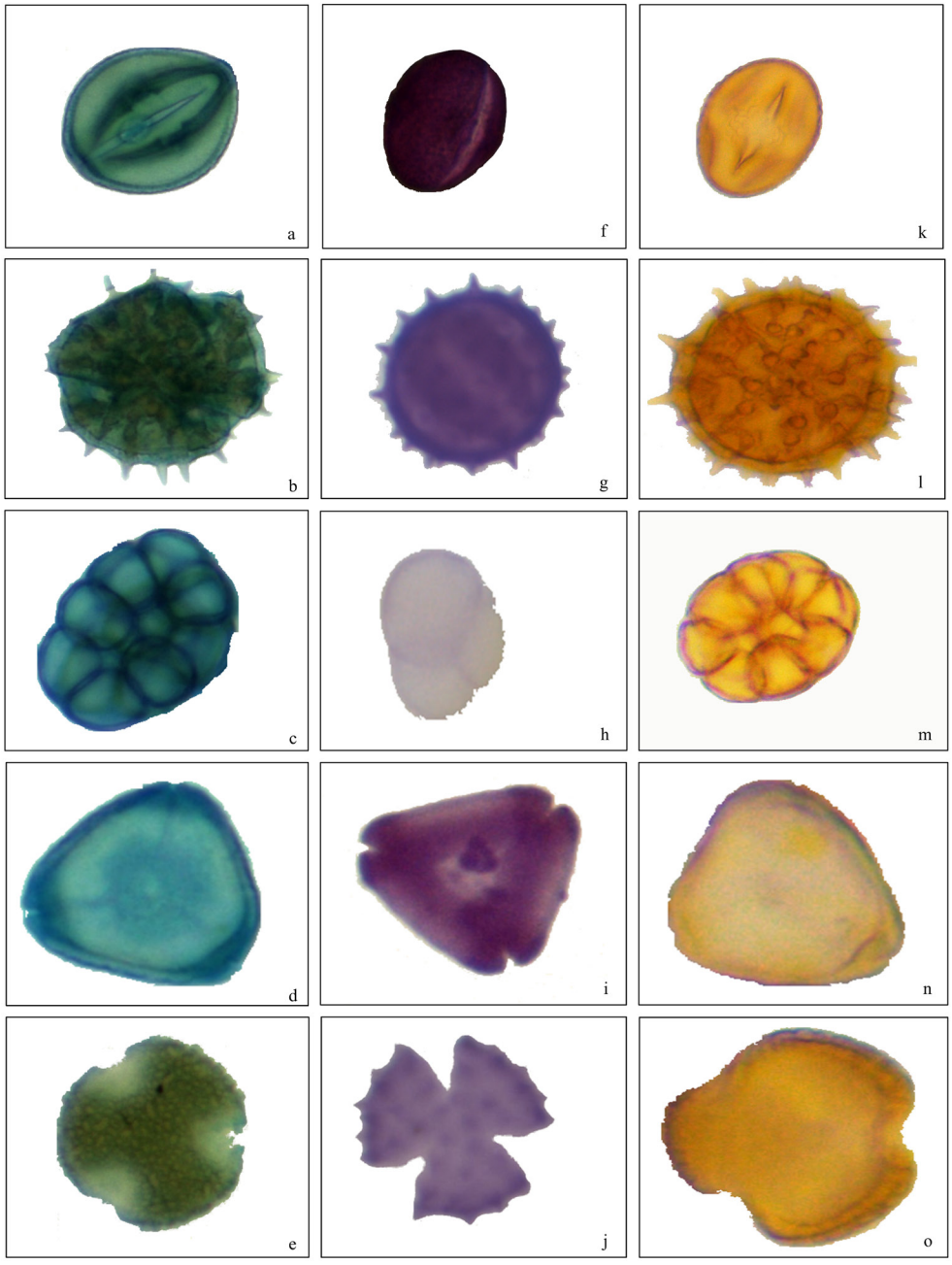

Figure 5. Pollen grains after background treatment of microscopic images. Alcian blue staining (a, b, c, d, and e), basic fuchsin staining (f, g, h, i, and j), and acetolysis (k, l, m, n, and o). Source: Prepared by the author (2022).

addition, the cost analysis showed that the simple methodology using dyes presented lower values, which allows analyzing a large number of samples at reasonable costs compared to the acetolysis protocol. Colorimetric techniques are less likely to harm the user, as they do not use strong acid (i.e., sulfuric acid) for sample preparation.

Regarding plant species identification, dye-based methodologies can be used for this purpose, not just for family-level identification. However, as with the acetolysis method, the analysis of pollen grains requires a trained person due to the diversity of Brazilian plant species, which makes identification challenging.

The importance of utilizing a larger number of samples from various Brazilian regions, as well as honey from other countries, is emphasized to enhance understanding of these colorimetric techniques. This would provide greater robustness to the methodology or promote improvements for a secure, reliable, and cost-effective analysis.

CONCLUSIONS

All the methodologies herein adopted in the melissopalynological studies proved to be efficient to verify the structures of pollen grains and, thus, identifying their donor botanical families. Among the techniques applied, acetolysis is the most frequently used, but the solvents offer higher cost and risks to the operator's health, important factors to be considered, especially when there are large amounts of honey samples in routine analysis. Thus, by applying the simpler methodologies with the alcian blue and basic fuchsin dyes, it was possible to reduce analysis costs, with less time for sample preparation, and without involving risks to users' health. Therefore, the alcian blue dye protocol seems to be plenty enough to identify pollen types in honey, accurately determining their botanical families of origin and also affording information to be added to databases to support Brazilian agencies in monitoring and inspecting honey quality and origin.

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