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NUMERICAL METHODS AND IMAGE PROCESSING TECHNIQUES FOR MELISSOPALYNOLOGICAL HONEY ANALYSIS

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ABSTRACT
Pollen analysis is a method used for verification of the botanical and geographical honey origin. Currently, much effort is being made to introduce automated systems with the use of image analysis programs. The automatic analysis is impeded by the insufficient depth of field of objects when using a light microscope, however, this can be avoided by using image reconstruction from images obtained from different focal planes. In this method, testing was performed on the normal focus (NF) and extended-depth-of-focus (EDF) images. These two methods were compared and statistically evaluated. The number of pollen grains and selected morphometric characteristics were compared. For EDF images, a higher number of pollen grains was obtained for the analysis, and except for the length/width ratio, a statistically significant difference was observed in the characteristics of pollen grains between the compared NF and EDF methods.

Keywords: Bright field microscopy; extended depth of focus; length/width ratio pollen; morphometry

INTRODUCTION
One of the important pieces of information for consumers of honey is its geographical and botanical origin **(Švamberk, 2015)**. The way to obtain this information is pollen analysis which is one of the most important examinations of honey, especially in terms of quality control. Nevertheless, sensory and physicochemical analyses are not less important for the correct detection of botanical origin **(Von Der Ohe et al., 2004)**. Highly valued honey are mono-floral ones which, however, should contain at least 45% of the pollen grains of the specified plant species. Nevertheless, there are some exceptions, as some plants are less pollen-producing and therefore the amount of their pollen in honey is lower **(Kolayli et al., 2016)**. Conversely, other plants have a high pollen-producing capacity, thus the pollen content of the single species should be up to 90% **(Oddo et al., 1995)**. To verify the botanical and geographical origin of honey, qualitative pollen analysis has to be applied; while the quantitative analysis is suitable for assessing the extraction and processing of honey (or filtration) **(Von Der Ohe et al., 2004)**.

Pollen analysis is a very demanding method. The examiner performing it must be able to recognize the species or at least the genus of pollen grains contained in a microscopic sample of honey **(Punt et al., 2007)**. The method for the qualitative analysis of honey and its performance is described in the International Commission for Bee Botany of IUBS (International Union of Biological Sciences). According to these recommendations, at least 300 pollen grains should be examined and at least 500 to 1000 pollen grains should be examined to determine the frequency of individual pollen species **(Jones and Bryant, 2001; Von Der Ohe et al., 2004)**. This limit (500 pollen grains and more) is also recommended in the case of bee pollen examination **(Lau, Bryant and Rangel, 2018)**.

Pollen grains differ in appearance, color, and shape. Morphological characteristics of pollen grains can be divided into several groups, according to their shape, size, pollen unit, polarity, symmetry, number and size of apertures, pollen surface, stratification of sporoderms, or exine ornamentation **(Punt et al., 2007)**. Some of these parameters can be measured using an eyepiece scale, but image analysis is currently used more commonly **(Shafiee et al., 2013)**. The most suitable tool for this examination is an electron microscope which is, nonetheless, very expensive and difficult to control in comparison with an optical microscope. When using optical microscopy, a frequent problem is insufficient depth of focus, especially when the sample surface is uneven and part of the object lies outside the selected focal plane. To obtain a sharp image, we can use image reconstruction which involves obtaining images from different focal planes. It is creating a super-sharp image by merging images from multiple focal planes – EDF (extended depth of focus image) **(Wu and Wang, 2009)**. To do this, algorithms are currently used to increase the depth of focus of the image using digital processing. Digital methods of processing the extended depth of focus are based on a set of optical sections that fully cover the studied object. Only a small portion of each section is focused due to the limited depth of focus of the microscope. The purpose of the extended-depth-of-focus algorithms applied is to recover the in-focus pixels from each section

and to create the final single composite image from them. Also, if we record not only the values of the in-focus pixel (intensity) but also the section index from which the in-focus pixels were selected, the three-dimensional structure (3D) information can be preserved. Such extended depth of field algorithms allows the incorporation of 3D information into a single two-dimensional (2D) composite image **(Valdecasas et al., 2001)**.

Scientific Hypothesis

The scientific hypothesis was to verify whether using an EDF image allows for detecting and identifying more pollen grains than scanning a single focal plane image.

MATERIAL AND METHODOLOGY

Samples

In this work, a sample of honey from the market network, which came from the Czech Republic, was tested.

Description of the Experiment

The amount of 10.0 g of the sample measured to the nearest 0.1 g was used for the examination. This amount was dissolved in conical centrifuge tubes in 20 mL of distilled water tempered at 40 °C. Subsequently, the obtained solution was centrifuged in a centrifuge (Centric 322A, Technica, SLO) for 10 minutes at a speed corresponding to 1000 g. After the centrifugation, the supernatant was removed. Again, 20 mL of tempered distilled water was added and centrifugation was performed. After removing the supernatant again, the remaining sediment was transferred to a microscope slide using a Pasteur pipette and allowed to dry on a heated plate (Vezas spol. ltd, CZE) at 40 °C. Before placing the sample on the slide, a square of 22 × 22 mm had been drawn using a barrier marker (Elite Mini PAP Pen, USA) to prevent the sediment from spilling over a larger area. After the sediment had dried, the sample was mounted with Kaiser's glycerol gelatine.

Subsequent sample scanning was performed with a DFK 23U274 camera (Imaging Source, GER) using an Eclipse Ci-L microscope (Nikon, JPN) with a Prosca III motorized stage (Prior, USA). NIS-Elements AR 5.20 software (Laboratory Imaging, CZE) was used to scan the samples. The program performed a random selection of 100 fields of view, on which the automatic counting of pollen grains was performed. Scanning was performed in the acquisition mode in five focal planes. The distance between the individual focal planes was 8 μm. Thresholding, counting, and measurement of morphometric properties of pollen grains were performed in the ideal focal plane (NF) according to the evaluator's choice and also after merging images from 5 different focal planes after creating an extended depth of focus (EDF) image. The actual scanning and automatic counting of pollen grains were performed in ten replicates per 100 images for each repetition. The two individual methods were then compared and statistically evaluated.

In the next step, the following parameters were measured by the image analysis software of NIS-Elements AR 5.20 (Laboratory Imaging, CZE) for both methods: pollen grain area (basic quantity indicating the size of the object in μm^2), its length (calculation of the lengths of the central axes of thin objects is used and is given in μm), width (indicating the ratio between the area and length of the object in μm), and MaxFeret90 (indicating the projection length perpendicular to the maximum Feret's projection, Figure 1) **(Laboratory Imaging, 2019)**.

Statistical Analysis

The data were processed statistically using the MATLAB 2019b (MathWorks, USA). The Anderson-Darling test, Student's t-test, sign test, and Wilcoxon test, two-sample test, and nonparametric Mann-Whitney test were used to evaluate the obtained results.

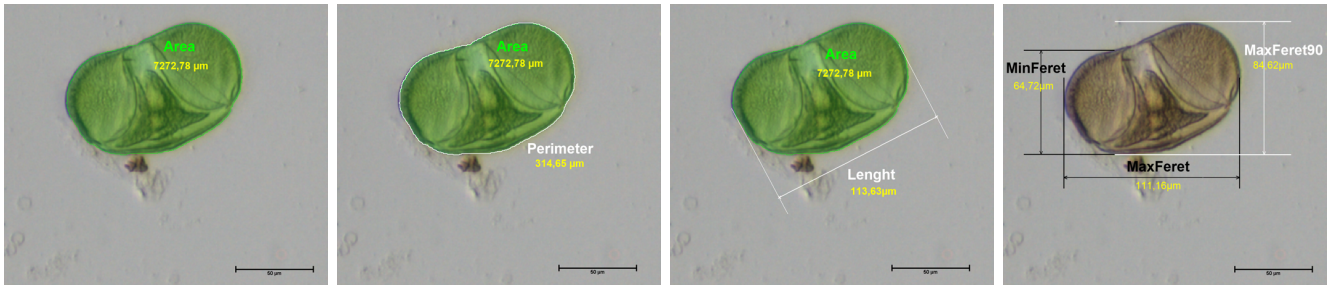
RESULTS AND DISCUSSION

The analyzed sample was scanned automatically in different focal planes. The sharpest image was selected for NF according to the experience of the human evaluator. For EDF, all the scanned Z levels were combined into one super-sharp image. Z-images were combined into one focused image by picking the focused regions from each frame and the pieces combined. In our study, balanced algorithms of Laboratory Imaging software were used. Algorithms for extended depth of focus were developed in the past 20 years. The primary application of EDF is for transmitted light microscopy systems **(Valdecasas et al., 2001; Tympel, 1997)**.

When merging multiple focal planes, a higher number of pollen grains was recorded in 80% of the measurements. 5397 pollen grains were counted in NF scanning and 5689 pollen grains in EDF scanning for all 10 repetitions on 100 randomly scanned images. This is consistent with the results of a study by **Valdecasas et al. (2001)** who argues that the use of algorithms in digital processing uses extended depths of focus and restores in-focus pixels, thus creating a final composite image from the selected in-focus pixels. The NF and EDF results for all repetitions are summarized in Figure 2.

The numbers of pollen grains recorded by both methods were compared using a paired test, using the difference in the results of individual methods for the same sites instead of the original data. However, the commonly used paired Student's t-test assumes a normal distribution of these differences. This assumption was tested by the Anderson-Darling test described in the study by **Tympel (1997)**. If the assumption of normality is not met, the differences must be tested by one of the nonparametric alternatives, such as the sign test or the Wilcoxon test **(Montgomery and Runger, 2011; Anděl, 2011)**.

By imperfect focusing of the evaluated image, especially smaller pollen grains can completely disappear from the image. A similar problem was addressed in the study by **France et al. (1997)** that focused on the automatic detection of pollen grains and their identification. The author also pointed out a situation whether or not it is a pollen grain or another unidentifiable object. The application of automatic systems and their use to filter objects based on their properties (size and shape) and other information in the image and possible errors are described in the paper by **Ranzato et al. (2007)**.



Area

$$Area = \sum pixel$$

Length

$$Length = \frac{Perimetr + sqrt(Perimetr^2 - 16 * Area)}{4}$$

Width

$$Width = \frac{Area}{\text{Length}}$$

MaxFeret90

Figure 1 Measured morphometrical criteria.

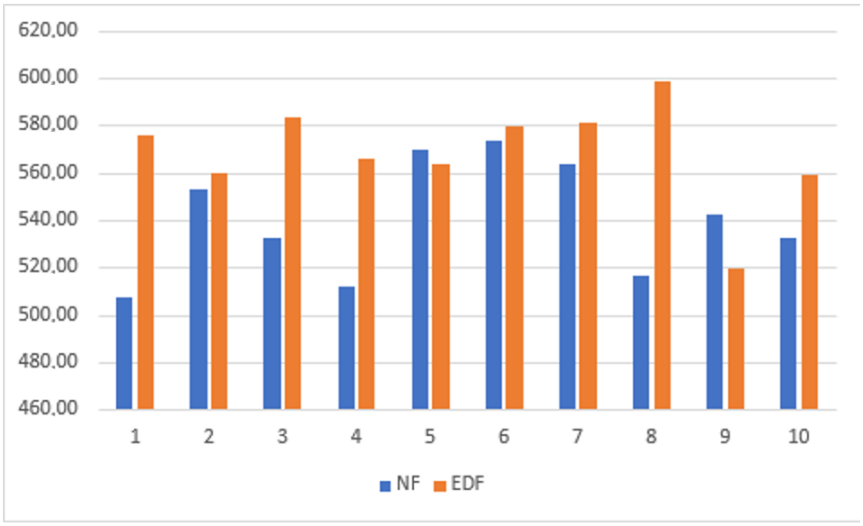


Figure 2 Number of pollen particles in NF and EDF images for each repetition.

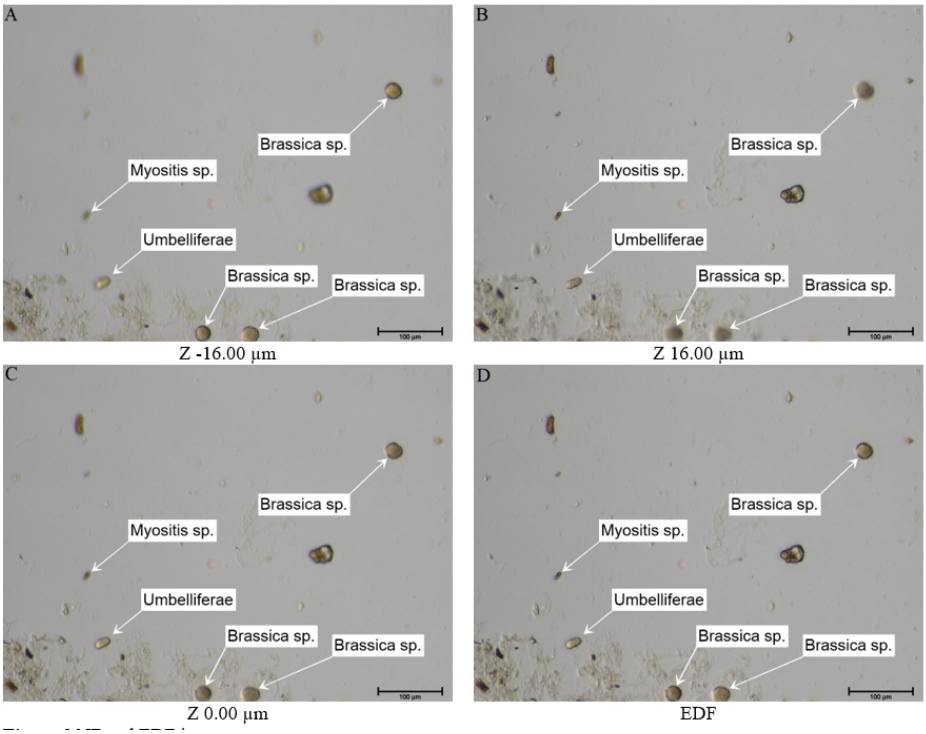


Figure 3 NF and EDF image. Note: A – C: NF scanning, D: EDF scanning.

In the case of melissopalynological analyses, it is common for both, small as well as large pollen grains, to be present in honey. Small pollen grains typically come from members of the *Myosotis* genus (4 – 10 μm), while large pollen grains are common for conifers (50 – 90 μm). However, the most abundant pollen grains in honey are medium-sized particles (30 – 50 μm). One of the many representatives is, for example, *Brassica napus* **(Oddo et al., 2004)**. If an image contains taxa with pollen grains of different sizes, certain pollen grains are lost in the evaluation from such an image. This error can be observed both when selecting a sharp image by a human evaluator as well as when using automatic focusing systems (Figure 3).

None of the images at different NF levels (Figure 3 C) provides a focused view of all pollen grains. In the case of focusing on the predominant pollen grains of *Brassica* sp. in the image, a strong blurring is evident in the pollen grain of *Myosotis* ssp. (Figure 3 A) and partial blurring in *Umbelliferae* (Figure 3 B) pollen.

As part of the statistical evaluation, it was first tested whether the images obtained by both methods (NF and EDF) detect the same number of pollen grains. For this comparison, all 100 x 10 measured images were considered as a single statistical file. Due to the availability of pairwise comparisons for the number of pollen grains detected by both methods (NF and EDF), it was possible to "filter out" inter-image variability using the paired statistical test. As shown by the histogram in Figure 4, the difference appears to be more pointed than the corresponding normal distribution should be.

A similar deficiency is indicated by the Normal Probability plot (Figure 5). The normal distribution of data was then definitively ruled out by the rejection of the Anderson-Darling test of normality on differences (p <0.001, A-D statistic 35.5644, at a critical value of 0.7513).

The rejection of normality meant that it was not possible to use the standard (parametric) paired variant of the Student's t-test. Thus, to demonstrate a statistically significant difference between the number of grains detected by both methods, it was necessary to use paired nonparametric tests (sign and Wilcoxon test, both with continuity correction). The null hypothesis of these tests in this case was the zero medians of differences (NF-EDF). The sign test rejected the null hypothesis (p-value which contained the first valid digit in the eighth decimal place, $8.5045*10^{-7}$). The result of the Wilcoxon test was then yet by two orders of magnitude stronger rejection of the null hypothesis (p <0.001). Hence, although the difference between the two methods used may seem negligible (EDF finds on average only 0.292 more pollen grains), it is undoubtedly statistically significant and the EDF method detects a higher number of pollen grains.

The amount of pollen grains in honey also varies naturally. Beekeeping practices, most importantly the method of honey extraction, are mainly responsible for the variability of the pollen grains amount in honey. As to concerns about honey adulteration, it is not common to filter honey through sieves with meshes smaller than 0.2 mm. This filtration is only allowed to remove foreign matter **(Ruoff and Bogdanov, 2004)**. However, even honey processed in this way must be designated as filtered honey **(Codex Alimentarius, 2001)**. Other technological steps, such as sedimentation and collection of floating particles on honey, also slightly affect the number of pollen grains in honey. Differences in the number of pollen grains in honey were also noted in a German study which confirmed that the amount of pollen grains in newly produced honey is lower than that in honeybee feed. The pollen concentration is decreased by the honey stomach filtration system of honeybees **(Bryant and Jones, 2001)**. Another reason for the different amounts of pollen grains in honey is also their botanical origin. Some taxa are strongly nectar-producing and poorly pollen-producing, such as Robinia pseudoacacia honey. These honey are naturally characterized by a low content of pollen grains. The opposite example is *Myosotis* honey, which has a significant pollen-producing capacity and low nectar-producing capacity. The content of pollen grains in these honey is therefore high **(Demianowicz, 1964)**.

Thus, the number of pollen grains usable for melissopalynological analysis is not the only criterion for evaluating the suitability of the compared methods. The pollen grains of each botanical taxon have their typical morphometric properties, which may vary according to the botanical species of the plants. The literature describes both, differences between plant species **(Evrenosoğlu and Misirli, 2009)** and between individual varieties **(Hebda and Chinnappa, 1994; Geraci et al., 2012)**. To evaluate the morphology, the parameter of total area, which represents the size of pollen grains **(Pospiech et al., 2019)**, and the parameter of length/width **(Nazeri Joneghani, 2008)** is recommended. Length of polar axis and equatorial diameter evaluations are also commonly used **(D'Albore, 1998)**. The use of these morphological characteristics has found application in both conventional melissopalynological analysis **(Al-Watban et al., 2013; Čeksterytė, Kurtinaitienė and Balžekas, 2013)** as well as in automatic melissopalynological systems based on image evaluation **(Treloar, Taylor and Flenley, 2004**; **Redondo et al., 2015)**. To compare the morphometric properties of the detected pollen grains, the morphometric characteristics of these grains were measured for the first iteration (100 images). The area was determined by the simple sum of the pixels of each pollen grain. Length and width here mean the size of the major (length) and minor (width) axis of the Legendre ellipse of the pollen grain. The construction of a Legendre ellipse is described in detail, for example, in the study by **Flusser, Suk and Zitová (2009)**. Since the number of detected pollen grains was not the same, it was necessary to use a two-sample test for statistical comparison of both methods in this case. Due to the limitations of all observed geometric characteristics, a normal distribution cannot be assumed for any of them (all are limited to 0 below). Therefore, the nonparametric Mann-Whitney test was consistently applied to compare the morphometric parameters **(Montgomery and Runger, 2011; Anděl, 2011)**.

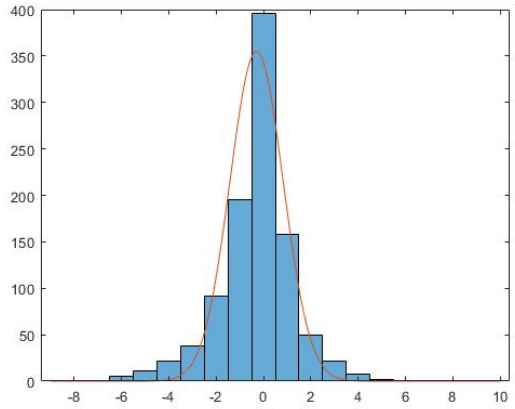


Figure 4 Histogram of differences in the number of pollen grains obtained by NF-EDF.

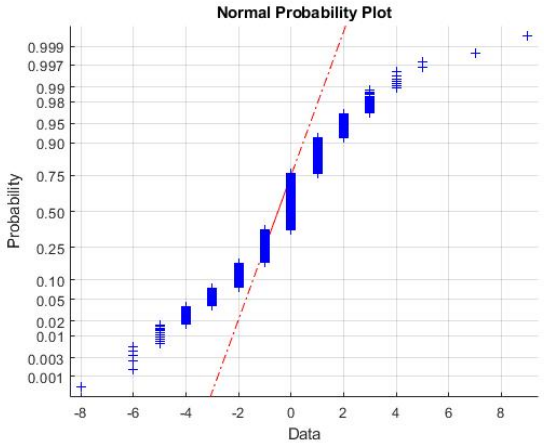


Figure 5 Normal probability plot of differences in the number of pollen grains NF-EDF.

Table 1 Medians of the observed geometric parameters of pollen grains.

Method	Area [µm^2]	Length [µm]	Width [µm]	MaxFeret90 [µm]	Length/Width Ratio
NF	531.24	28.39	18.78	25.96	1.52
EDF	490.68	27.31	18.00995	25.11	1.53
p-value	*p* <0.001	*p* <0.001	*p* <0.001	*p* <0.001	0.7851

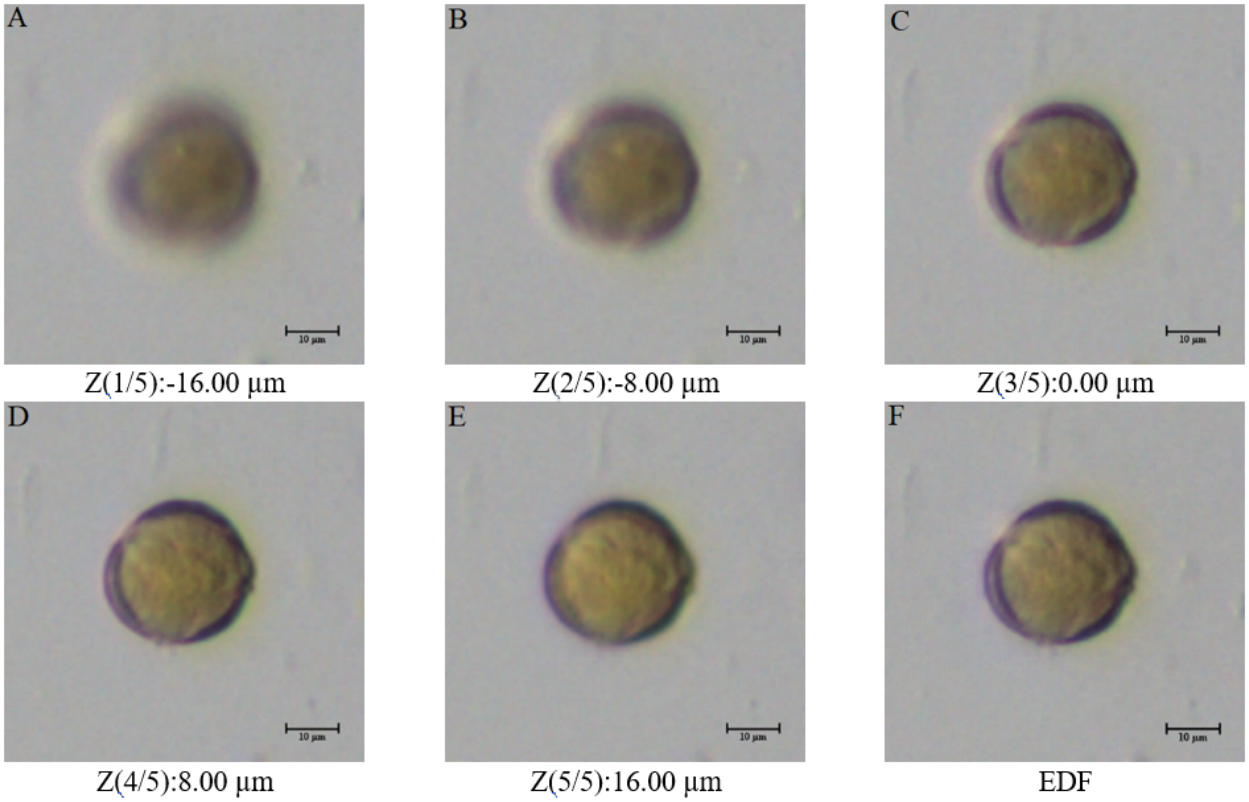


Figure 6 Pollen sample of Brassica napus. Note: A – E: NF scanning, F: EDF scanning.

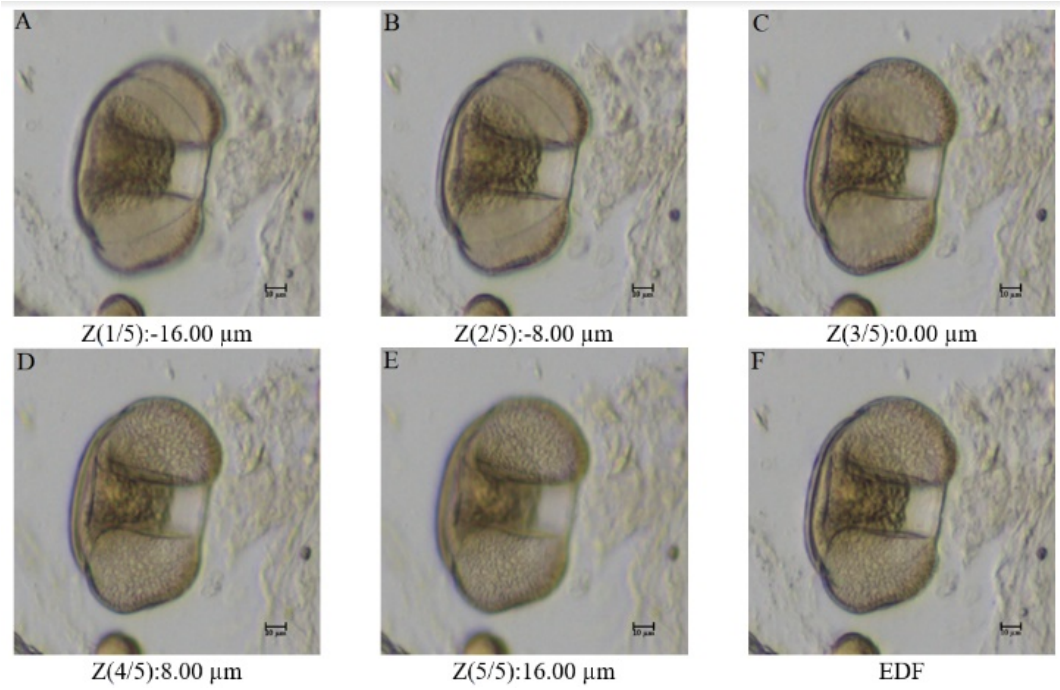


Figure 7 Pollen sample of Pinus sylvestris. Note: A – E: NF scanning, F: EDF scanning.

All the observed geometric parameters (except the length/width ratio) can be argued that the smaller median indicates that EDF is a better method. In the case of imperfect focusing on NF, the observed pollen grain may contain image information "mixed" with the background, which increases all the monitored morphometric parameters (Figure 6 and Figure 7). Another reason for using the EDF method is the ability of automatic analysis to distinguish pollen grains from other honey impurities. As stated by **France et al. (1997)** in their study, identification of whether it is a pollen grain or not is problematic in automatic processing. The results may be affected by textural properties such as homogeneity, contrast/roughness and grey scatter. These are not only morphometric criteria. Textural properties, including morphometric parameters, can then be used to create classification rules that utilize these texture characteristics, as described by **Li et al., (2004)**. In their study, **Currie et al. (1997)** used only the morphometric parameters (length, width, area, and length/width ratio) of pollen grains, exine, and pollen pores for the discriminant analysis of apple varieties.

Further reduction of the median is caused by more grains detected. The "lost" pollen grains (not detected by the NF method) can be expected to have a smaller length, width, and area.

To compare the suitability of morphometric parameters in NF and EDF, the null hypothesis of the median equality of two statistical sets was tested by the nonparametric Mann-Whitney test. The medians of all observed morphometric characteristics are summarized in Table 1, as well as the *p*-values corresponding to the hypothesis tests.

Hence, it is clear that except for the length/width ratio, there is a statistically significant difference between NF and EDF in all observed geometric characteristics of pollen grains. Since the medians of all statistically significantly different geometric characteristics of EDF are smaller than the medians of NF, it can be argued that the identification of pollen grains in EDF images will be more efficient than in NF images. This rule was not confirmed for the recalculated length/width factor. This result confirms that this factor is a suitable criterion especially for individual evaluation of pollen grains **(Nazeri Joneghani, 2008)** even in the case of differently focused pollen grains in the analyzed image. It can also be assumed that in the case of comparing different focal planes or EDF images, a more suitable parameter is a criterion that is not affected by the texture of the pollen grain. As reported by several authors, it is appropriate to use rather a higher number of measured parameters for the use of image analysis, which will allow more accurate classification of the analyzed pollen grains **(Holt et al., 2011)**, than to choose one or a smaller group of measured parameters only.

CONCLUSION

In this work, the extended depth of focus obtained from images from different focal planes and normal focused images was compared. The calculation and measurements of morphometric characteristics of pollen grains were used for comparison. The EDF method can detect a larger number of pollen grains in the evaluated images. A statistically significant difference between NF and EDF was demonstrated in all the observed morphometric characteristics of pollen grains except the length/width ratio. Identification of pollen grains in images based on morphometric criteria obtained by the EDF method is more efficient than the identification of pollen grains in images obtained by the NF method. Based on our results, the EDF scanning method is a method more suitable for image-based melissopalynological analysis, providing better results than the technique of scanning a single depth of field in NF.

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Conflict of Interest:

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