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Quantitative melissopalynological analysis of bee honey using a Bürker chamber

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Abstract

The article describes a new proposal for quantitative melissopalynological analysis of pollen grains in 10 g bee honey, using a Bürker chamber for peripheral white blood cells counting. The research related to the development of the method and its algorithm are presented. Further investigations in certified laboratories are required to evaluate the repeatability and reproducibility of the method for "under-represented", "normal-represented" and "over-represented" bee honeys.

Introduction

Bee honey contains numerous pollen grains (mainly from plants bees feed on) and honeydew elements (like wax tubes, algae, fungal spores), both of which provide exact information for the environment honey originates from. Melissopalynology is a branch of palynology (science of pollen and spores) dealing with microscopic investigation of bee honey. Therefore, melissopalynological analysis, sensory and physico-chemical analysis are used simultaneously to determine and control the geographical and botanical origin of honey (von der Ohe *et al.*, 2004). The method provides essential information about the hygienic aspects of bee honey production, its contamination with mineral dust, soot, starch particles (Louveaux *et al.*, 1978), filtration, fermentation (Russmann, 1998), and attempts for bee honey adulteration (Kerkvliet et al., 1995). The approved melissopalynological method was developed and proposed by the International Commission for Bee Botany in 1978 (Louveaux *et al*., 1978). The method was validated in 2004 (von der Ohe *et al*., 2004), and afterwards, widely used in European laboratories for bee honey analysis. It is among the methods used for description of European honey types in the beginning of the 21st century (Persano Oddo and Piro, 2004). The combination of sensory, physiochemical and microscopic analysis is a widely accepted assumption by many scientists (Persano Oddo and Piro, 2004; Piana *et al.*, 2004).

It should be outlined that the current normative framework that sets the quality parameters of bee honey, does not reflect latest trends for implementation of new filtration systems for mechanical removal of product's contaminants. Thus, a larger part of pollen grains could be possibly removed and hence obtaining an inaccurate result from the determination of the geographical and botanical origin of honey. With regard to the preservation of the natural specific number of pollen grains in the different produced honey types, the EC has established that mesh size of filters used for honey filtration should be larger than 0.2 mm (Bogdanov, 2009).

In Bulgaria, Vitkov (1980) has outlined in the early 1980s that the lack of any objective parameter for the accurate determination of bee honey type except for pollen analysis requires more detailed research on the subject. The current requirements about the percent content of pollen grains in monofloral honey in Bulgaria (Bulgarian State Standard 3050, 1980; Bulgarian State Standard 2673, 1989; Ordinance of the Ministry of Health on requirements for honey intended for human consumption, 2002; Ordinance No 48, 2003), are not compliant, in both methodological and regulating aspects the modern requirements for determination of honey geographical and botanical origin (von der Ohe *et al*., 2004). Bulgarian normatives do not specify a requirement for quantitative analysis of the number of pollen grains in 10 g honey as contemporary European criteria (von der Ohe *et al*., 2004).

By the end of the first decade of 21st century, the area of Bulgarian land cultivated with honey plants

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has dramatically changed. The traditional sunflower is gradually replaced with rapeseed, due to the increasing interest of farmers to this crop species. With regard to the production of culinary herbs and spices, the cultivation of some plant species non-typical for Bulgarian flora has recently started. An example is the coriander, cultivated on large areas of land in the region of Yambol and the fennel, encountered in the region of Razgrad. In some parts of the country (Strandzha, Region of Haskovo, Stara Zagora, Smolyan etc.), the once rarely encountered honeydew honey is more frequently seen. Often, some monofloral honey types are mixed with honeydew honey (Dinkov, 2014).

The analysis of data about standard deviation of pollen grain counts in 10 g of the different types of honey determined by the approved EC method (von der Ohe *et al.*, 2004) in Italy, the parameter was found to vary within a wide range (Persano Oddo *et al.*, 2000). The data further confirm the significant variability in pollen grain concentrations in the different types of honey in support of the necessity for further research in this direction.

Haemocytometers is used for counting pollen grains from plants (Delaplane *et al*., 2013), and to evaluate the number of pollen grains attached to bees (Human *et al*., 2013). All aforementioned facts motivated to investigate the potential of the Bürker chamber, used in routine laboratory practice for counting peripheral blood leukocytes (Heldrup *et al*., 1992), to develop and validate a new method for quantitative melissopalynological analysis of pollen grains in 10 g honey. The research conducted for development of the method, the algorithm of the analysis and preliminary studies on method's repeatability are described. The proposed new method aims at more rapid determination of the number of pollen grains in 10 g honey and differs considerably both from the original (Louveaux *et al*., 1978), as well as from the modified and approved (von der Ohe *et al*., 2004) methods for quantitative melissopalynological microscopic analysis of bee honey.

Material and Methods

Method for quantitative melissopalynological analysis of pollen grains in 10 g bee honey

The 10 g honey was weighed in a graduated cylinder. The method is with 10 g because of such quantity used for referent method (von der Ohe *et al*., 2004). Distilled water was added to a total volume of 20 cm^3.The obtained honey solution was divided into two aliquots of 10 cm^3 in graduated centrifuge tubes. The tubes were centrifuged at 2000×g for 10 min. With an automated pipette, 9 cm^3 of the supernatant were discarded from each tube. To the remaining amount of 1 cm^3 in each of the two tubes, 5 cm^3 distilled water were added to a total volume of 6 cm^3. Thus, the volume of the solution containing 10 g honey with the additional dilution was 30 cm^3. The two tubes were centrifuged at 2000×g for 5 min. With an automated pipette, 5 cm^3 of the supernatant were discarded from each tube. From the remaining 1 cm^3 honey solution, after a thorough mixing with a glass rod, 9 mm^3 (µl) samples were taken with automated pipettes from the bottom of each tube. The amount was chosen due to the experimentally established fact that a volume of 9 µl fills entirely without leaking the two semi-reflective segments of the Bürker's chamber after placing the cover glass.

Under direct light, the position of the gridded areas were identified and the 9 µl samples were pipetted in the middle of grids, resulting in two drops on each grid. A thin coverslip (Cover glass, made in China, 24 x 32 mm, thickness 0.13-0.17 mm), was then carefully placed perpendicularly to the wide side of the chamber to cover entirely the gridded areas and to contact tightly the chamber edges (Figure 1). At the time of coverslip contact with honey solution, the latter should fill entirely the respective segment of the chamber.

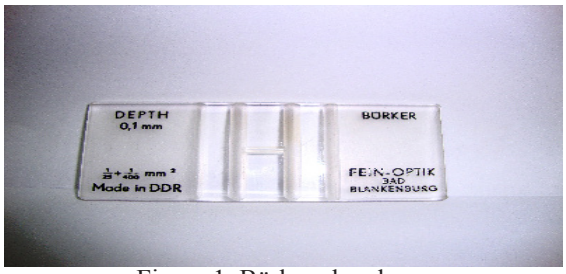

Figure 1. Bürker chamber

Using a light microscope, eyepiece lens 10x, objective lens 10x/0.24, the two grids of the chamber were brought into focus and all pollen grains within them were counted, including those grains which cross or touch the outer borders and angles of grids. Pollen grains in both segments of the chamber were counted and the arithmetic mean (A) was calculated.

The number of pollen grains in 10 g honey is calculated by the equation:

$$X = A \times 3703.7037$$

where:

X – number of pollen grains in 10 g honey;

A – arithmetic mean of pollen grains counted in two grids of the chamber.

3703.7037– coefficient for calculation of honey solution volume (30 cm^3, 10 g respectively).

Algorithm for obtaining the coefficient 3703.7037:

The volume of the solution containing 10 g honey with the additional dilution is 30 cm^3 . Burker's chamber has 9 large squares (Figure 2), and each of them could hold 0.9 mm^3 of honey solution (Bürker's chamber, 2006). Therefore, $9 \times 0.9 = 8.1$ mm^3 (0,0081 cm^3) represents the volume of honey solution in each of two chamber grids (volume in each of the grids). The counted pollen grains is the number for 0.0081 cm^3. When the total volume of 30 cm^3 is divided to solution volume in each of grids , we obtain the coefficient 3703.7037 used to obtain the total number of pollen grains in 30 cm^3 solution, i.e. 10 g honey.

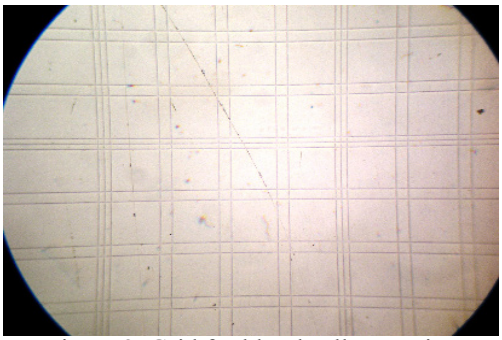

Figure 2. Grid for blood cells counting (Bürker chamber)

Example: If the average number of pollen grains in the chamber is 8 (A=8), the total number X would be $8 \times 3703.7037 = 29629.6296$ in 30 cm^3 (10 g) honey. we propose to round the result up or down to the nearest whole number. In our example, pollen grains number would be 29 630 in 30 cm^3 (10 g) honey. The results could be expressed in thousands (10 to the powder 3) – thus, the result could be given as 29.63×10^3 pollen grains / 10 g honey.

For method repeatability, the relative standard deviation (coefficient of variation) - RSD% (CV%), was calculated as per Westgard *et al.* (1998): RSD% (CV%) =SD / X × 100,

where: SD – standard deviation; X – $A_{1\text{-}10}$ (arithmetic mean of pollen grains in both chamber grids during the 10 tests) or $X_{1\text{-}10}$ (calculated number of pollen grains in 10 g honey for each determination).

Results

The results from counting pollen grains in bee honey samples of different geographic and botanical origin are presented in Table 1.

Determination of repeatability of the method

According to Bulgarian State Standard 17397-1(2005): "Repeatability is the agreement of results from repeated measurements of the same item under the same conditions (same procedure, same observer, same instrument used under the same conditions, same place, within a short time period)". The palynological analysis is a specific analysis and determination of the method repeatability according to requirements recommended for chemical analysis does not prove correct. The successive readings of the same microscopic slide should be done in longer time intervals to prevent being influenced by the previous readings.

According to these requirements, 10 readings done in longer time intervals (1 month), were made from a coriander standardized honey sample, collected from experimental apiary from Yambol region, Bulgaria, produced in 2000. The pollen grains of anemophilous plants and honeydew elements were not present in the sample. The results for pollen grains number in both chamber grids for the ten tests ($A_{1\text{-}10}$) were as followed:

A1 = 16 / 2 (number of chamber grids) = 8
A2 = 15 / 2 = 7.5
A3 = 15 / 2 = 7.5
A4 = 16 / 2 = 8
A5 = 15 / 2 = 7.5
A6 = 15 / 2 = 7.5
A7 = 16 / 2 = 8
A8 = 15 / 2 = 7.5
A9 = 16 /2 = 8
A10 =16 / 2 = 8
X = 7.75
Min = 7.5
Max = 8
SD = 0.2635147
RSD% (CV %) = 0.2635147 / 7.75 x 100 = 3.4 %

The calculated pollen grain numbers in 10 g honey for each test run according to the formula ($X_{1\text{-}10}$) was as followed:

X = 8 x 3703.7037 = 29629.6296 pollen grains / 10 g
X = 7,5 x 3703.7037 = 27777.77775 pollen grains / 10 g
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X = 8 x 3703.7037 = 29629.6296 pollen grains / 10 g
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X = 8 x 3703.7037 = 29629.6296 pollen grains / 10 g
X = 8 x 3703.7037 = 29629.6296 pollen grains / 10 g
X =28703.5
Min =27777
Max =29630
SD (standard deviation) =976.01
RSD% (CV %) = 976.01 / 28703.5 x 100 = 3.4 %

Table 1. Pollen grains number in 10 g standardized bee honey samples, determined by the newly proposed quantitative melissopalyngological analysis method

Kind of honey	Geografical origin	Year of harvesting	Pollen grains in 10 g honey
Organic multifloral honey	Kalofer, Bulgaria	2010	$29,63 \times 10^3$
Organic multifloral honey	Kalofer, Bulgaria	2010	$44,44 \times 10^3$
Multifloral honey	Cherven Briag, Bulgaria	2005	$81,4 \times 10^3$
Organic acacia honey	Kalofer, Bulgaria	2010	$18,51 \times 10^3$
Rape honey	Stara Zagora, Bulgaria	2010	$177,8 \times 10^3$
Scotch thistle honey (Onopordum acanthium)	Montana, Bulgaria	2009	$29,63 \times 10^3$
Fennel bee honey (Foeniculum vulgare Mill.)	vil. Ostrovo, Bulgaria	2009	$81,4 \times 10^3$
Fir honey	Switzerland	Sample presented from "1-st Honeydew Honey Symposium", Tzarevo, Bulgaria, 2008	$66,6 \times 10^3$
Chestnut honey	Switzerland	Sample presented from "1-st Honeydew Honey Symposium", Tzarevo, Bulgaria, 2008	293×10^3

Discussion

Our results for SD in coriander honey (Yambol region, produced in 2000) – 976.01 are also comparable to values of Italian honeys (Persano Oddo *et al*., 1997). The average pollen grain counts using the proposed method in a samples of Scotch thistle honey *(Onopordum acanthium)* – 29.63×10^3/10 g honey (Table 1), are similar to the maximum value reported by Italian researchers for this honey type – 20×10^3/10 g (Persano Oddo *et al.*, 1997).

For precise HPLC analyses RSD% (CV %) values under 10% are required for a reliable result (Ubaldi *et al*., 2005). The authors give an example with RSD% from 4.1 to 8.8 for 5 tests of the same sample and concluded that the method's repeatability was acceptable (Ubaldi *et al*., 2005). In our experiments, the ten tests of pollen grain numbers performed for the same sample resulted in relative standard deviation RSD% (CV %) of 3.4 %. This value is lower even compared to the lowest one reported in HPLC analyses (4.1%) in the cited research work (Ubaldi *et al.*, 2005). We could therefore conclude that the repeatability of results during the preliminary validation of our proposed method is satisfactory.

The RSD% (CV%) values obtained by us is also lower that the recommended threshold of 5%, ensuring acceptable test-retest reliability of the laboratory analysis (Westgard *et al*., 1998). If honey with crystallization samples should be prepared, as described in referent method (von der Ohe *et al.,* 2004), but this steps as also accetolysis have to be done in the future in connection with validation procedures of the proposed method. The fact that the quantitative melissopalynological analysis requires specific filter with pores 3 μm and diameter

25–47 mm and the necessity for counting a large number of pollen grains for an objective evaluation (from 500 to 1000), (von der Ohe *et al*., 2004), impede the wide implementation of the quantitative melissopalynological analysis.

An advantage of the quantitative melissopalynological analysis method proposed by us is the lack of specialised labware except for the Bürker's chamber, tubes, pipettes and centrifuge. Another plus of the method is its promptness – during experiments it was demonstrated that the enumeration of pollen grains in one sample took about 15 min.

Conclusions

A new method for quantitative melissopalynological analysis of pollen grains in 10 g bee honey, using a Bürker chamber for peripheral blood white blood cells counting (Heldrup *et al*., 1992), is described. The research related to the development of the method and its algorithm are presented. Investigations on method's repeatability have shown a coefficient of variation RSD% (CV %) of 3.4%, which agrees with laboratory analysis reliability criteria proposed by other authors (Westgard *et al*., 1998).

Further investigations in certified laboratories are required to evaluate the repeatability and reproducibility of the method, according to the requirements of ISO 5725-2 (1994). The validation should be conducted for, at least, three levels (so-called "under-represented" honey, e.g., *Tilia, Robinia*, "normal-represented" honey. e.g., *Fagopyrum*, *Trifolium repens*, and "over-represented" honey, e.g., *Castanea, Eucalyptus*). In the future comparable analysis have to be performed with the other methods that are used for the quantitative melissopalynogical analysis (von der Ohe *et al.*, 2004).

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