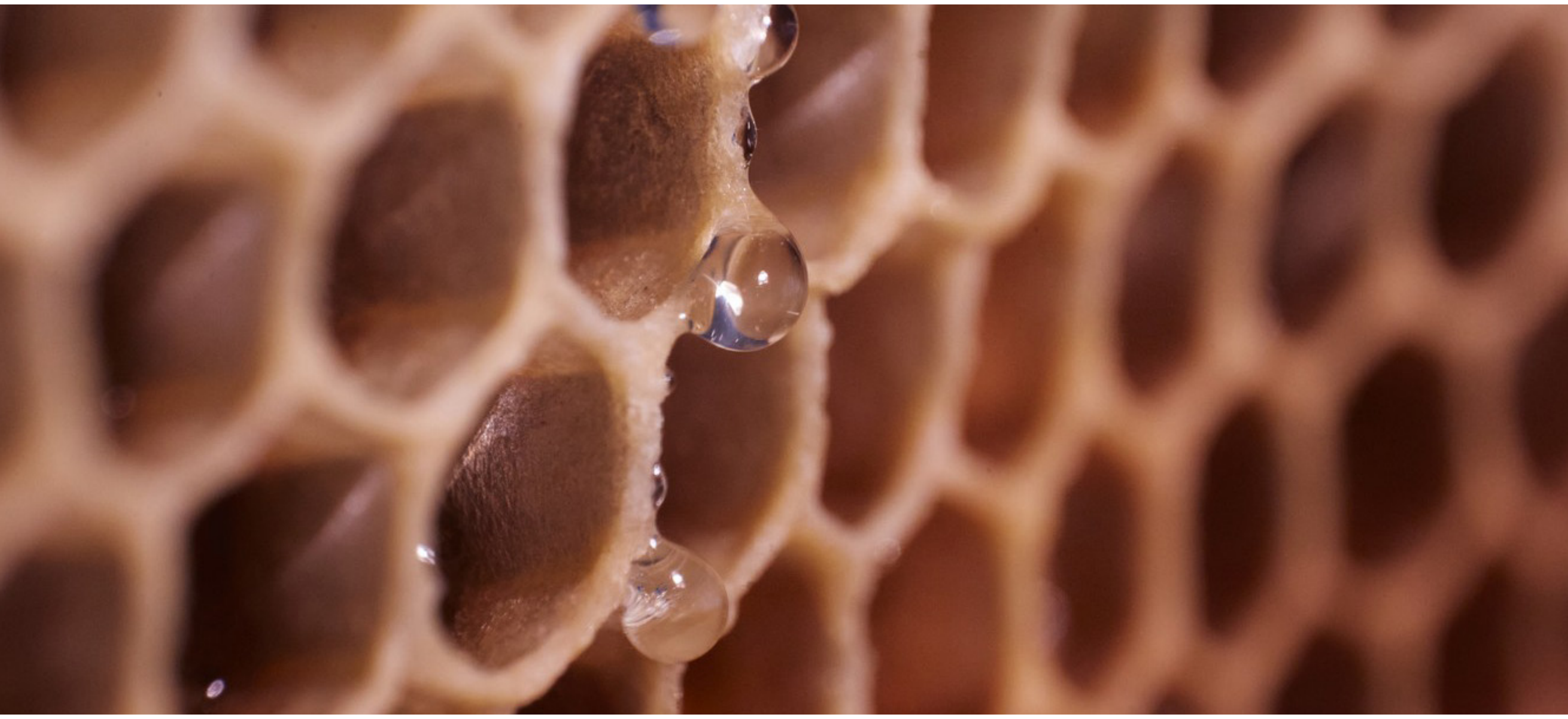
Application Note

Microscopic analysis of pollen in honey

Identification and documentation with ZEISS Axiolab 5



Seeing beyond

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Microscopic examination of honey and pollen (a field of study known as melissopalynology) allows researchers to identify the type of pollen, distinguish blossom and honeydew honey from each other, and detect any contaminations.

Introduction

The study of pollen, also known as palynology, has gained in importance in recent years:

- Around 30 million allergy sufferers in Germany are informed about the expected concentration of airborne pollen (Figure 1) by the German Pollen Information Service Foundation (Stiftung Deutscher Polleninformationsdienst, PID).
- Researchers from the fields of archaeology, forensics, and geoscience can draw valuable conclusions from pollen findings.
- The pollination of agricultural land can be verified by means of pollen analysis, early action initiated, and economical loss prevented.
- **Pollen analyses of honey are performed within the scope of consumer protection. The objective is to determine the quality, origin, and purity of source of the honey. This way, consumers are protected against false statements as well as any contamination or allergen content in honey products.**

Figure 2 *Pollen of the pussy willow*

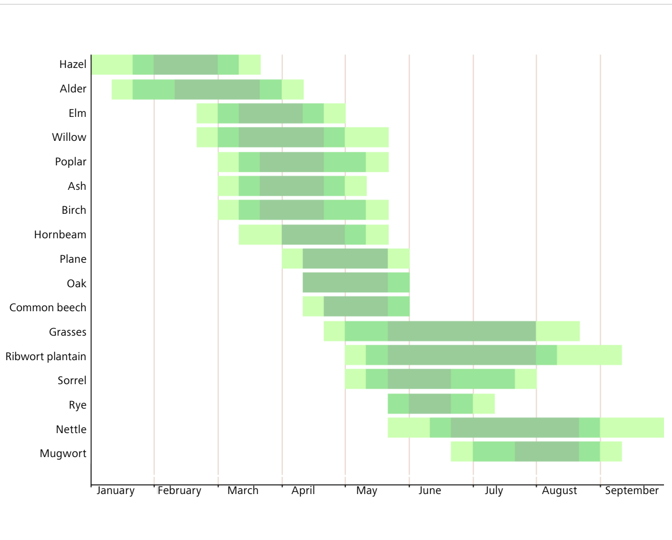

Figure 1 *Pollen calendar*

This Application Note is dedicated to this last point – namely melissopalynology.

The function and structure of pollen

Pollen grains, usually called pollen or farina, are the male germ cells of plants.

The stamen is the male organ of the flower (Figure 3). It consists of the filament and the anther. The anther usually contains four pollen sacs connected to each other, in which the pollen grains are formed. Once the pollen has ripened, the pollen sacs tear open and release it. As plants blossom at different times, their pollen can only be found in the air and honey at specific times.

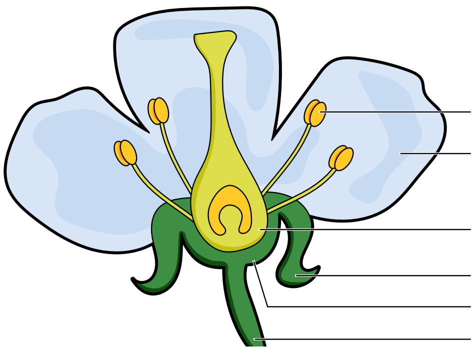

Figure 3 *Structure of the flower*

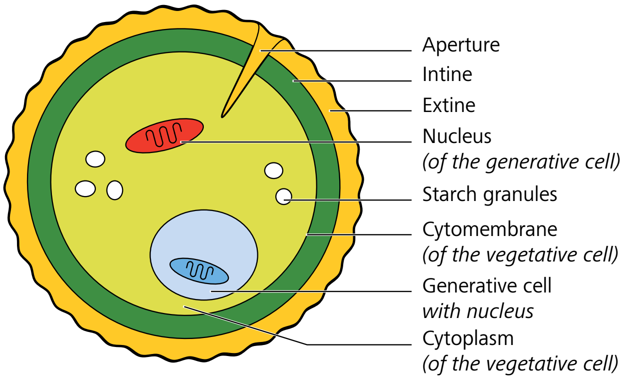

Figure 4 *Pollen structure*

The outer hull of the pollen grain is called the exine. It protects the pollen grain against disintegration due to biological and chemical factors, rendering it very durable. Pollen can thus survive and be detected for a long time in honey. Distinctive sculpture elements (such as spikes, warts, sticks, clubs, or grooves) sit on the outer hull. Most pollen grains have further characteristic features, such as germ folds or germ pores, which theoretically enable identification of the type of pollen. These are small openings in the pollen wall, from which the pollen tube can grow out of the pollen grain. The inner layer, the so-called intine, can be found under the exine. It encloses the cell plasma with the functional cell components (Figure 4).

Depending on their function, pollen grains can be dry or sticky. While wind-pollinated plants usually have dry pollen, sticky pollen grains target insects as a means of transport (Figure 5). The stickiness comes from a coating of oily lipids, the so-called pollenkitt, which ensures adhesion to bumblebees and bees.

Figure 5 *Pollen sticks to bees perfectly thanks to the pollenkitt.*
(Photo: József Szabó / pixabay.com)

From bees to honey

Honey is a natural product and has been a valuable foodstuff for thousands of years. The German Honey Ordinance defines honey as a naturally sweet substance, created by bees. Bees collect nectar or other secretions of plants, add their own specific substances such as enzymes, and store it all in honeycombs for ripening (Figure 6).

Honey therefore mainly consists of different types of sugar (fructose and glucose), organic acids, enzymes, and solid particles absorbed while collecting nectar.
Pollen primarily gets into the nectar while the honey bee collects it from the flowers. The pollen sticks to the legs and coat of the bees, and thus finds its way into the honeycomb, which has not yet been covered.

Different kinds of honey

In **monofloral honey**, such as rape honey, only one type of pollen can be detected for the most part. Pure unblended honey is produced when a bee colony mostly focuses on one type of plant when collecting the raw materials.

In contrast, many different types of pollen can be found in **forest and blossom honey**.

Blossom honey has a higher glucose content. Compared to fructose, glucose has a lower solubility in water, which is why blossom honey crystallizes quickly. The crystal structure can be seen clearly under the microscope.

In **forest honey**, the fructose content is generally higher, which means that forest honey usually stays liquid. In addition to pollen, other particles – such as spores of fungi, algae, and yeasts – can be found in forest honey. They mainly get into the honey through the honeydew.

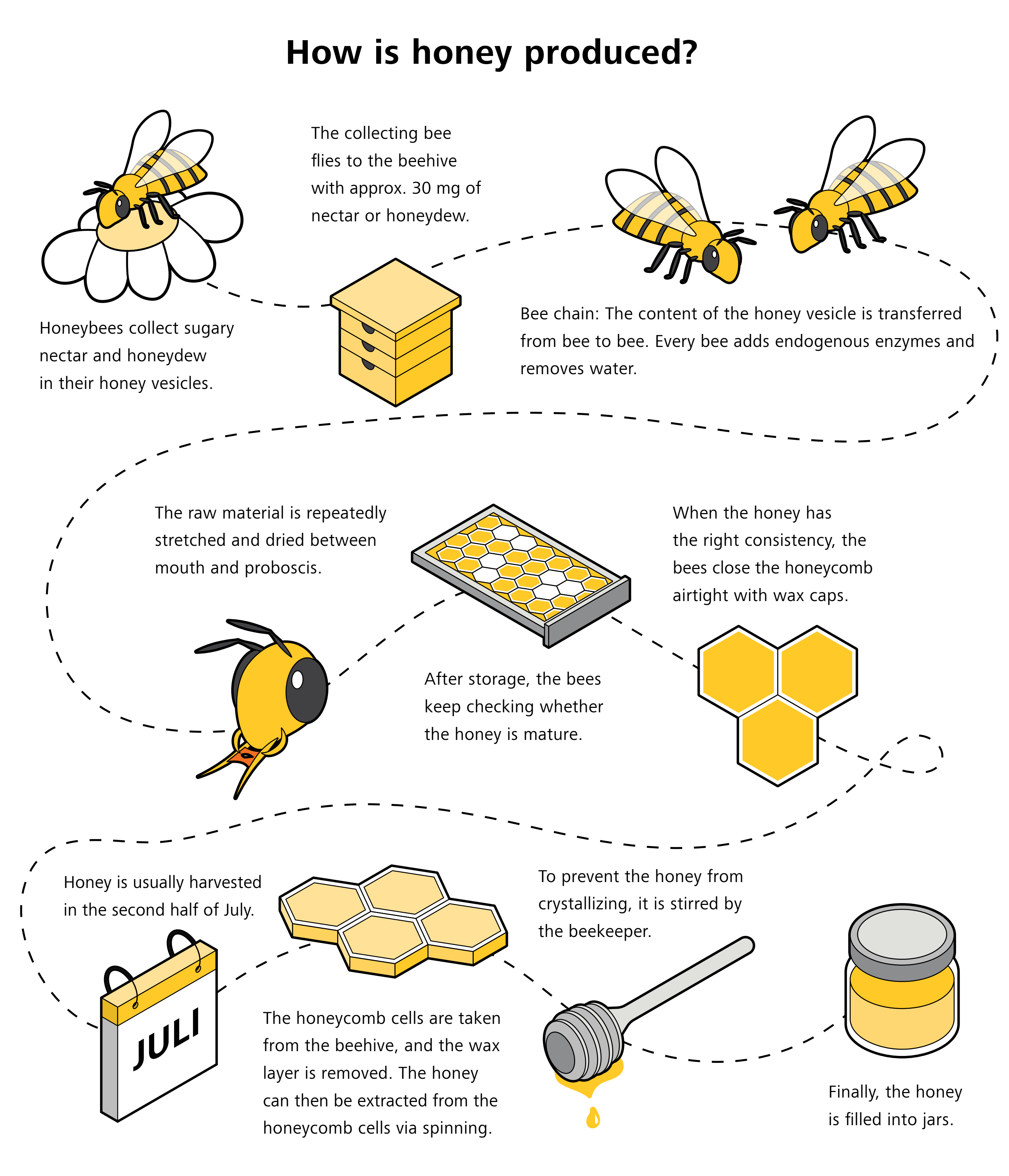

Figure 6 *From bees to honey*

Honeydew usually does not originate from flower nectar but from other plant and insect secretions.

Honey analysis

Why honey analysis is necessary

Below you will find some data and facts that highlight why honey analysis is necessary and useful.

Worldwide honey production totals more than 1.8 million tons. After China, the European Union is the second largest honey producer worldwide, producing around 250,000 tons of honey each year, followed by Turkey, Argentina, and Iran. In the EU, there are about 650,000 beekeepers and more than 18 million beehives. Fortunately, the number of beekeepers and beehives in the EU is increasing. With more than 125,000 beekeepers, Germany is leading the way. However, the honey produced in

Germany cannot meet local demand by far. Honey is very popular in Germany, which is why around 70% is imported from EU and non-EU countries. Almost 40% of honey is imported (largely from China, the Ukraine, and Latin America) to cover the demand for honey in the EU.

Honey thus represents a relevant economic factor – one that is at the center of food fraud scandals worldwide time and again. Food fraud is nothing new, but its intensity and frequency are increasing. There are reports about fake extra virgin olive oil and the deliberate adulteration of spices as well as the production of fake honey. In total, the economic damage caused by food fraud is estimated at USD 40 billion per year. According to some sources, fake honey is the most common type of food fraud (https://focusonfoodsafety.wordpress.com/2018/09/18/food-fraud-fake-honey/).

Fake honey is apparently big business. Honey is one of the most frequently incorrectly labeled foods around the world. As honey is declared as a pure natural product, additives are not permitted. For example, feeding the bees with sugar or syrup for the production of honey is prohibited. In the same way, no substances inherent to honey may be removed, and no other substances added. Honey labels, such as rape honey or sunflower honey, may only be used if the honey in question predominantly (>50%) originates from the named flowers or plants and has certain microscopic, organoleptic, and physical-chemical characteristics (source: German Honey Ordinance).

In reality, honey – in particular imported honey – is often mixed with cheap sweeteners such as rice syrup or corn syrup. This process, in which impure honey is passed off as real honey, is called honey laundering. The geographic origin is also often concealed in order to avoid import or punitive duties. In this case, the products are imported via third countries and brought into the target country under a false flag. Often, the pollen is removed from the honey through filtration, in order to make microscopic pollen analysis that traces the country of origin more difficult.

Figure 7 *Types of honey (Photo: Hans Braxmeier / pixabay.com)*

For example, food health and safety experts examined a large part of the honey sold in the United States and found that the honey mostly consisted of sweeteners, unrefined sugars, corn syrup, and only a tiny amount of real honey. A more recent scandal was exposed in mid-2018, when researchers found that almost half of the honey sold on Australian supermarket shelves was fake honey. Honey imported from Asia even contained metal toxins and antibiotics.

Honey fraud is demonstrably a worldwide phenomenon – which is not surprising in light of advancing globalization.

Source: https://www.europarl.europa.eu/news/en/headlines/economy/20180222STO98435/key-facts-about-europe-s-honey-market-infographic

Standardization

This is reason enough to regularly analyze the properties of honey in terms of quality and quantity based on specific standards, particularly with regard to food safety, and thus check the correctness of botanical or regional indications of origin. Organizations and authorities worldwide are addressing this issue. This is being done in the United States, for example, by the American Beekeeping Federation, which represents US producers of non-ultrafiltered honey. It asked the FDA to create a "standard of identity" for honey, which basically is a detailed definition specifying legal standards.

In Germany, beekeeping products are also subject to strict requirements and checks. Respective specifications can be found in DIN 10760 and the German Honey Ordinance. In accordance with the Honey Ordinance issued by the German Federal Ministry of Consumer Protection, Food, and Agriculture (which came into force on January 29, 2004), honey must undergo certain analyses to obtain information about ingredients as well as geographical and botanical origin. In Germany, the **Institutes for Apiology** (Länderinstitute für Bienenkunde, LIB) are responsible for the strict requirements and checks that beekeeping products are subject to. The pollen analyses with regard to quality and quantity that are carried out for this purpose are very costly and time-consuming, as trained employees need to manually count and identify pollen in samples.

How pollen analysis works

The different types of analysis required to fully characterize honey are divided into three categories:

- Sensory analysis: Sensory and organoleptic analysis includes the examination of color, smell, and taste.
- Chemical-physical analysis: During chemical-physical analysis, dimensions such as the water content, invertase activity, and sugar spectrum are analyzed in accordance with respective DIN standards.
- Microscopic analysis: Microscopic analysis primarily comprises pollen analysis and provides additional insight into sediment components, which also include yeasts and starch.

On average, 1 g of honey contains 5,000 pollen grains. This accounts for a medium share of approx. 0.02 g pollen per 100 g of honey, or 0.02%. The small size of pollen (mostly between 20 and 80 µm) means microscopic analysis is required.

Microscopic examination of honey also provides insight into contained particles and an idea of the different degrees of crystallization for different honeys.

Pollen analysis takes place in accordance with DIN 10760 and enables an assessment of the geographical and botanical origin of the honey. The determination of the geographical origin is particularly important for imported honey – not only to enable the detection of fakes like those described above, but also for certain designations like the quality feature "Real German Honey". Products that want to earn this label may not contain any pollen originating from outside Germany. The botanical identification verifies the purity of a honey's floral source. A certain percentage of pollen from a flower must be detected for the honey to receive the respective designation. For example, acacia honey only has to be made up of 20% robinia pollen. In contrast, rape honey must be made up of at least 80% rape pollen. The different percentage limits depend on how common a type of pollen is in nature. Robinia pollen is inherently underrepresented, which explains the seemingly low pollen frequency limit of 20%. For the pollen analysis of honey, the detection of primary pollen and secondary pollen is predominantly of interest.

Pollen group	Proportion of total pollen in the honey sample
Primary pollen	45%
Secondary pollen	16–45%
Single pollen	max. 16%

Overview of the characteristic pollen categories for honey analysis

In 2017, there were approx. **870,000 bee colonies** in Germany. Since 1985, approx. 25% of honey bee colonies have become extinct in Central Europe.

Worker bees cover a distance of **8,000 km** throughout their lifetime. They pollinate about **4,000 flowers** per day.

For the production of **1 kg of honey**, honey bees must collect approx. 3 kg of nectar. This corresponds to approx. 900,000 to 6,000,000 flower landings.

Modern agriculture is one of the main causes for the **decline in the insect population** worldwide (use of pesticides (12.6%) and fertilizers (10.1%)).

Bees pollinate around **71 of 100 crop plants**, which cover 90% of the worldwide food requirements.

A bee colony needs approx. 70 kg of honey for energy supply and approx. 25 kg of pollen for protein supply – the beekeeper only takes what the bees collect exceeding this, about 20 to 30 kg.

Source: www.statista.com

Sample preparation of centrifuged honey

The honey is weighed in with respect to its viscosity and dissolved in distilled water. As a general rule, each 10 g of a type of honey require 20 ml of distilled water. The solution is centrifuged for approx. 15 minutes at 3,500 revolutions per minute. Approx. 2 × 2 cm of the dregs are then applied to an object slide by means of a pipette. This should correspond to a maximum quantity of 0.5 ml. For a brief assessment, the edge of a cover glass is positioned at the edge of the water drop and laterally dropped on it. For a longer-term examination or storage of the sample, the last step is replaced with drying the sample on a heating plate and covering it with gelatine. Afterwards, the different honey samples can be examined under the microscope.

Microscopic analysis

For pollen identification, a light microscope (usually an upright microscope with bright field contrast) is used to analyze the pollen. The form, size, and surface structure of the pollen grains differ widely depending on the species or family, which is significant for the analytical differentiation of pollen types. Therefore, microscopic examination of the pollen grain can often be used to identify the related plant or at least its family. One challenge here is that the sculpture elements and other features such as apertures appear very differently under the light microscope depending on their position in the sample. Because of this, the sample must be examined at different focus levels to gain a complete picture of the pollen. In case of a honey sample, the pollen is usually examined and identified with 400× magnification in the first three to five object fields. Once the pollen has been identified, counting can begin. For this purpose, 200× or even 100× magnification is often used.

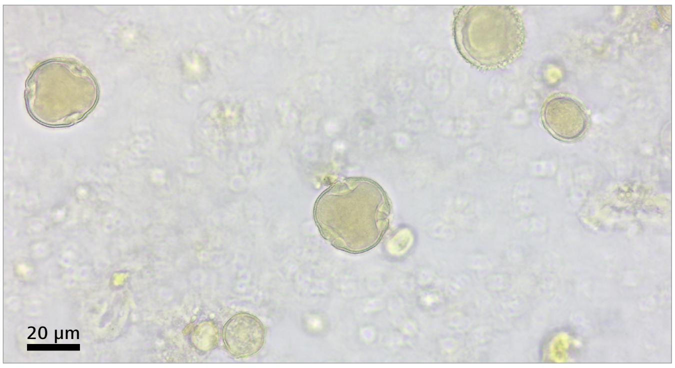

Figure 8 *Pollen of a linden, captured with ZEISS Axiolab 5 in brightfield mode, lens: 40×*

In case of unknown pollen or difficulties with identification, higher magnification with a 63× or 100× lens can be used. The DIN standard stipulates that at least 500 pollen grains must be counted, whereby a percentage distribution is usually calculated every 100 pollen grains. If the fluctuation in distribution is too great, more pollen might be counted if required. It is important to count and identify the pollen without nectar separately. Furthermore, the yeast and starch content is determined, as well as further structures or contaminations noted. This is a time-consuming procedure: The microscopic analysis of pollen can easily take one to two hours.

Recommended microscope equipment

ZEISS Axiolab 5

Objective lenses:
Plan Neofluar: 10×, 20×, 40×, 63×

Documentation:
ZEISS Axiocam 208 color
ZEISS Labscope

Summary

Honey is a natural product. The accuracy of botanical and regional indications of origin can be verified by means of pollen analysis because honey contains a proportion of pollen – which is visible in microscopic samples – that reflects the bees' collecting activities. A distinction can also be made between blossom honey and honeydew honey using microscopic analysis. Microscopy also reveals contaminations, such as too much yeast or starch, which do not belong in honey. The first step of microscopic analysis is to identify all pollen present and designate its plant of origin. Researchers require comprehensive knowledge of plants and their pollen to be able to distinguish different types of the latter. In-house pollen collections or pollen databases can be used for the purpose of comparison. In the second step, pollen is counted, and the percentage of the different pollen types is determined. This is used to define a honey's purity of source.

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