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REVIEW ARTICLE

Standard methods for *Apis mellifera* beeswax research

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Due to its multifunctional and complex role in the honey bee colony functioning and health (construction material allowing food storage, brood rearing, thermoregulation, mediation in chemical and mechanical communication, substrate for pathogens, toxins and waste), *Apis mellifera* beeswax has been widely studied over the last five decades. This is supported by a comprehensive set of scientific reports covering different aspects of beeswax research. In this article, we present an overview of the methods for studying chemical, biological, constructional, and quality aspects of beeswax. We provide a detailed description of the methods for investigating wax scales, comb construction and growth pattern, cell properties, chemical composition of beeswax using different analytical tools, as well as the analytical procedures for provenancing beeswax and beeswax-derived compounds based on the hydrogen isotope ratio (IRMS). Along with classical physico-chemical and sensory analysis, we describe more precise and accurate methods for detection of adulterants in beeswax (GC-MS and FTIR-ATR). Moreover, we present methods for studying the influence of beeswax (comb foundation) adulteration on comb construction. Analytical protocols for determining the pesticide residues using different chromatographic and spectroscopic techniques are also described. As beeswax is an agent of high risk for the transmission of bee diseases, we present methods for detection of pathogens in beeswax. To ensure the reproducibility of experiments and results, we present best practice approaches and detailed protocols for all methods described, as well as their advantages and disadvantages.

Métodos estándar para la investigación de la cera de abejas *Apis mellifera*

Debido a su papel multifuncional y complejo en el funcionamiento y la salud de la colonia de abejas melíferas (material de construcción que permite el almacenamiento de alimentos, la cría, la termorregulación, la mediación en la comunicación química y mecánica, el sustrato para patógenos, toxinas y desechos), la cera de las abejas *Apis mellifera* ha sido ampliamente estudiada en las últimas cinco décadas. Esto se apoya en un amplio conjunto de informes científicos que abarcan diferentes aspectos de la investigación sobre la cera de abejas. En este artículo presentamos una visión general de los métodos que permiten el estudio químico, biológico, estructural y cualitativo de dicha cera. Proporcionamos una descripción detallada de los métodos usados para la investigación de las escamas de cera, la construcción del panal y su patrón de crecimiento, las propiedades de las celdas, y la composición química de la cera utilizando diferentes herramientas analíticas; así como los procedimientos analíticos basados en el ratio de isótopos de hidrógeno (IRMS) que permiten determinar el origen de la cera y los compuestos derivados de ella. Junto con el análisis físico-químico y sensorial clásico, describimos métodos más precisos y exactos para la detección de adulterantes en cera de abejas (GC-MS y FTIR-ATR). Además, presentamos métodos para estudiar la influencia de la adulteración de la cera de abejas (base del panal) en la construcción del panal. También se describen protocolos analíticos para determinar los residuos de pesticidas utilizando diferentes técnicas cromatográficas y espectroscópicas. Dado que la cera de abejas es un agente de alto riesgo en la transmisión de enfermedades de abejas, presentamos métodos para la detección de patógenos en dicha cera. Para garantizar la reproducibilidad de los experimentos y los resultados, presentamos modelos de buenas prácticas y protocolos detallados para todos los métodos descritos, así como sus ventajas y desventajas.

西方蜜蜂蜂蜡研究标准方法

蜂蜡在蜂群功能和健康中具有多种功能和复杂作用（食物储存、哺育幼虫、温度调节的筑巢材料，化学和机械通讯的中介，以及病原体、毒素和废物的基质），因此西方蜜蜂蜂蜡在过去五十年中被广泛研究。这得到了一套全面科学报告的支持，涵盖了蜂蜡研究的不同领域。本文概述了蜂蜡的化学、生物、结构和质量的研究方法。本文详细介绍了利用不同分析工具研究蜡鳞、筑巢，蜂蜡的生长方式、细胞特性和化学成分的方法，以及基于氢同位素比值(IRMS)的蜂蜡和蜂蜡衍生化合物的来源分析方法。除了经典的物理化学和感官分析，我们还描述了更精准的检测蜂蜡掺假的方法（GC-MS和FTIR-ATR）。此外，我们还研究了蜂蜡（巢础）造假对蜂巢结构的影响。还介绍了使用不同色谱和光谱技术测定农药残留的分析流程。由于蜂蜡是传播蜜蜂疾病的高危因素，我们提出了检测蜂蜡中病原体的方法。为了确保实验和结果的可重复性，我们为所描述的所有方法提供了最佳实践方法和详细流程，以及它们的优缺点。

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Keywords: *Apis mellifera*; beeswax; wax scales; comb construction; cell properties; chemical composition; beeswax provenancing; adulterants; pesticide residues; pathogens

1. Introduction

The wax of the western honey bee (*Apis mellifera* L.) is a complex lipid-based organic compound (natural wax) produced by worker bees using four pairs of specialized wax glands located on the inner side of the 4th to 7th abdominal sternites. Beeswax is secreted in the form of wax scales which honey bees transfer with the forelegs to their mandibles, where wax scales are chewed (salivary secretions added), and then added to the comb being constructed. The main effect of this mandibulation process is a transformation of the texturally anisotropic scale wax into isotropic comb wax (Hepburn, Pirk, & Duangphakdee, 2014). Chemically, beeswax represents a complex organic mixture of more than 300 compounds, of which the fatty acid esters (~67%), hydrocarbons (~14%), and free fatty acids (~13%) predominate (Tulloch, 1980). There are no significant differences in the basic chemical composition of wax originating from different *A. mellifera* subspecies, only small variations related to the proportion of the above mentioned predominant compounds (Beverly, Kay, & Voorhees, 1995; Fröhlich, Riederer, & Tautz, 2000; Tulloch, 1980).

The outstanding hexagonal cell structure of the honeycomb has fascinated scientists and the general public worldwide for centuries. This characteristic architectural structure reflects a complex behaviour of the honey bees in terms of their self-organization, as well as chemical and mechanical modifications of the wax during the wax scale-to-comb transformation pathway (Pirk, Hepburn, Radloff, & Tautz, 2004). Honey bees use beeswax as a construction material for building the comb (also known as honeycomb or comb wax) that serves as food (honey, pollen) storage and as brood-rearing compartment (provides infrastructure for rearing brood). Beeswax is also important for the chemical communication within a honey bee colony; its characteristic chemical composition plays an important role as a source of nest-mate recognition cues (Breed, Williams, & Fewell, 1988, et seq., D'ettorre et al., 2006; Fröhlich, Tautz, & Riederer, 2000). Furthermore, comb mediates pheromonal cues for cell capping, repairs and queen cell construction, nectar forage, colony defence and colony odour (Hepburn, 1998). It has an important role in thermoregulation and colony waste management, and serves as a humidity buffer in honey bee nests (Ellis, Nicolson, Crewe, & Dietemann, 2010; Jay, 1964). Mechanically, the comb transmits vibrational signals during the waggle dance (Kirchner, 1993; Michelsen, Kirchner, Andersen, & Lindauer, 1986; Michelsen, Kirchner, & Lindauer,

1986; Sandeman, Tautz, & Lindauer, 1996), and recruitment of new foragers (Tautz, 1996; Tautz, Casas, & Sandeman, 2001; Tautz & Lindauer, 1997).

Various practical uses of beeswax (e.g., for embalming, preserving the papyrus, protection of paintings, making of candles, figures, cult objects and ancient seals, as medicinal ingredient, and adhesive material) reach back into ancient history (Bogdanov, 2016b). Nowadays, the majority of beeswax is used for the production of comb foundations, and thus re-enters the beekeeping industry. Although it is difficult to obtain reliable figures on beeswax production and international trade statistics for products made of beeswax, it can be stated that comb foundation production is probably the major use of beeswax (Crane, 1990; Bogdanov, 2016b). Beeswax is also widely used in pharmacy, cosmetics, and in food industry as food additive E901 (Directive 2009/10/EC, 2009; EFSA, 2007; FAO, 2005; JECFA, 2005; ISO TC34/SC19, 2017).

Given the complex and important role of beeswax in the honey bee colony, it is of crucial importance that the comb foundation on which the honey bees are building their comb is genuine and uncontaminated. Nevertheless, the major beeswax quality issues nowadays include contamination of beeswax with adulterants (natural and/or synthetic substances that are deliberately added to beeswax for economic gain, such as paraffin wax as the most commonly used adulterant), and pesticide residues, as reported in numerous studies (e.g., Bernal, Jiménez, del Nozal, Toribio, & Martín, 2005; Bogdanov, 2006; Bogdanov et al., 2004; Chauzat & Faucon, 2007; Maia, Barros, & Nunes, 2013; Maia & Nunes, 2013; Ravoet, Reybroeck, & de Graaf, 2015; Serra Bonvehí & Orantes Bermejo, 2010, 2012; Svečnjak, Baranović et al., 2015; Svečnjak, Prđun, Bubalo, Matošević, & Car, 2016; Svečnjak, Prđun, Baranović, Damić, & Rogina, 2018; Wallner, 1992, 1997, 2000; Waś, Szczęsna, & Rybak-Chmielewska, 2016). In apiculture, these agents are primarily being transmitted through the comb foundation trade, as there are no regulations controlling their quality or authenticity. Additionally, commercially made comb foundation has not been incriminated as an agent for the dissemination of any bee disease although it poses a high risk for the transmission of various diseases and, to some extent, parasitic mites (Mutinelli, 2011).

In this article, we present an overview of the methods for studying beeswax in different aspects. We provide a detailed description of the methods for investigating wax scales, comb construction and its growth pattern, cell properties, chemical composition of beeswax using different analytical tools, as well as the analytical procedure for provenancing beeswax and beeswax-derived compounds based on the hydrogen isotope ratio. Along with classical physico-chemical and sensory analysis, we describe various analytical tools for determining beeswax authenticity (detection of adulterants by GC-MS and FTIR-ATR). Moreover, we present methods for studying the effects of beeswax (comb foundation) adulteration on comb construction. Methods for determination of pesticide residues using different analytical techniques are also described. As beeswax is an agent of high risk for the transmission of bee diseases, we present methods for detection of pathogens in beeswax [American Foulbrood (AFB), European Foulbrood (EFB), Small Hive Beetle (SHB), *Nosema* sp., *Ascosphera apis*]. To ensure the reproducibility of experiments and results, we present the best practice approach and detailed protocol steps for all methods described.

2. Beeswax sampling, processing, and storage

Sampling of the beeswax is strongly dependent on the aim of the study and the method intended to be used for its investigation. Therefore, we here provide general guidelines for collecting different types of beeswax samples from individual honey bees, from the hive or from the market (comb wax in Section 2.1.1, comb foundation in Section 2.2.2, and beeswax blocks in Section 2.2.3.) that can be followed for most of analytical methods described in this article. Specific sampling requirements related to particular methods are described in detail in corresponding sections (wax scale in Sections 3 and 4, wax caps and wax in honey in Section 5.5, and wax debris and old comb in Section 7). Beeswax samples are usually subjected to the melting (refining) prior to analysis. Thus, we also present the procedures for beeswax melting in Section 2.2, along with the requirements on beeswax storage in Section 2.3.

2.1. Collection of different types of beeswax specimens

Different types of beeswax specimens can be collected for analysis. We describe the procedures for collecting the beeswax samples from the hive (comb wax, for details see Section 2.1.1), and from the market (comb foundation, see Section 2.1.2.; beeswax block, see Section 2.1.3).

2.1.1. Collection of comb wax

When aiming to analyze genuine beeswax, it is best to collect the samples of wild-built combs, i.e., newly built combs that are not constructed upon comb foundation but in empty space in the hive, as described below (steps 1–4). This allows obtaining virgin beeswax samples containing no impurities or residual contaminants that might be present when comb is constructed on the comb foundations (their origin is often questionable). To achieve this, sampling of the comb wax (wild-built combs) should be performed as follows:

1. Take out 1 or 2 frames from the hive to make space for inserting empty frames.
2. Put 1 or 2 empty frames (unwired, without comb foundation or starter) in the prepared empty space in the hive during the wax production season (spring and early summer).
3. Allow time for bees to build the required amount of comb. Note: this period can vary in duration according to local conditions.

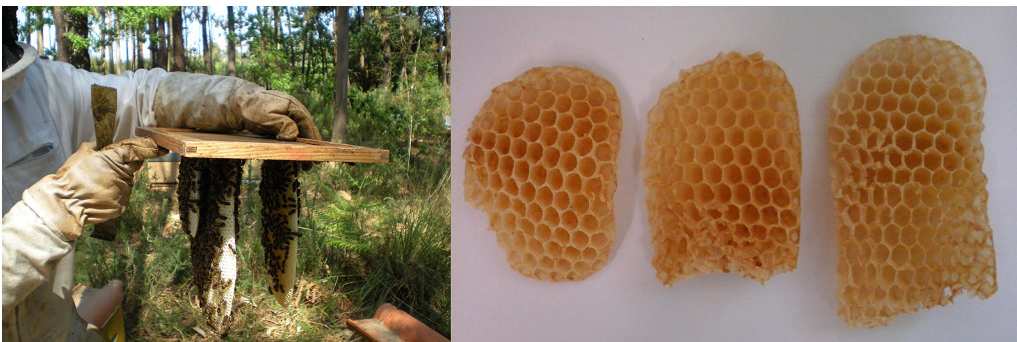


Figure 1. Wild-built combs constructed by fulfilling an empty space in the hive after removing two frames (left) and combs collected from the hive cover board (right). Photo: Maia M. (left) and Svečnjak L. (right).

Alternatively, after taking out the frames from the hive (step 1), the empty space can be left vacant (without putting the empty frames); honey bees will build the combs in this vacant space and the comb wax samples can easily be collected (Figure 1). Also, smaller wild-build combs from the hive walls may also be collected, as presented in Figure 1.

4. Collect wild-built combs from the frames immediately after construction using beekeeping gloves (the use of laboratory gloves is required if potential contaminations need to be excluded).

Colonies used for wild-built combs collection should not have been treated with any kind of veterinary medicinal preparation previously to ensure the acquisition of pure beeswax samples, without residues of potential contaminants (unless the aim of the study requires different approach).

Make sure there are no physical impurities (propolis, pollen, nectar/honey or other hive material) on the comb wax samples before analysis. If an intact comb wax in genuine form is not required for analysis, the best way to remove impurities is melting of the comb wax that can be carried out as described below in Section 2.2. An alternative is to cut away manually the section of comb with impurities.

Old comb wax can also be collected for analysis, but it should be noted that it may contain significant amounts of other hive-derived substances (cocoons, faeces, propolis, pollen, etc.) that need to be removed by melting prior to analysis.

For the purpose of bee pathogens investigation (Section 7), (old) comb wax and wax (hive) debris can be collected. The collection of debris is an easy and non-invasive method of obtaining biological samples from apiaries during the quiet part of the year. This type of sample can be used to predict the possible occurrence of AFB in the next spring-summer.

2.1.2. Comb foundation sampling

Sampling of the comb foundations can be performed by collecting the entire foundation sheet or a part of it, depending on the quantity needed for the analysis. Comb foundations are commonly purchased directly from the market (specialized beekeeping shops and/or fairs) or collected from the beekeepers. There are no particular requirements for comb foundation refining as commercial comb foundation beeswax is homogenized and free from physical impurities.

2.1.3. Sampling the beeswax blocks

Beeswax blocks are obtained by melting the old combs and/or cappings. Different types of beeswax blocks (of various sizes, shapes and structures) can be found on the market, or can be collected from the beekeepers. Sampling of wax blocks depends on the sample type; the samples of wax blocks may be homogenous, or in layers/different phases, i.e., heterogeneous (this often appears due to the beeswax tendency to stratify).

- When the beeswax block is *homogeneous*, collect 3–4 aliquots from different locations, and pool all aliquots into one sample.
- When the beeswax block is stratified (*heterogeneous*), collect aliquots from the bottom, middle and upper phase, and merge all aliquots into one sample (i.e., mix the three phases collected).

When collecting aliquots, take into consideration to collect sufficient material for the analysis intended. Use a metal spatula or a spiral for sample collection. Clean the spatula with ethanol (96%) between uses to avoid contamination.

2.2. Beeswax melting

The simplest way to homogenize and purify the beeswax sample before analysis is melting by boiling water. This is primarily referring to the comb wax samples that usually contain hive-originating residues that have to be removed prior to analysis. Melting by boiling water will remove physical impurities from the comb wax (propolis, pollen, nectar/honey or other hive material). If the sample has already been refined (e.g., comb foundation, wax block), melting (heating) of

beeswax without the water is performed (melting is required for most analyses).

The melting procedure with boiling water is performed as follows:

1. Place the beeswax sample in a glass (thermostable) or stainless steel beaker with distilled water as much to cover the sample (beakers made of other metals should not be used for melting purposes as they may darken the beeswax colour and/or contaminate it).
2. Heat at a temperature of 70–90 °C until the sample is completely molten.
3. After beeswax melted, filter the solution (or melted beeswax) through gauze or similar filtering materials, such as tightly woven cotton cloth, canvas or paper filters.
4. Leave to cool at room temperature.
5. Collect the beeswax (as a solid) from the upper layer of floating beeswax to obtain clean sample (beeswax is lighter than water and floats on its surface, and impurities can accumulate at the top of the water).
6. Dry the sample at room temperature for several hours before analysis (to remove droplets from the surface).

The melting procedure without water is performed as follows:

1. Place the beeswax sample in a glass (thermostable) or stainless steel beaker.
2. Place the beaker in an electric plate, water bath, oven or a temperature chamber.
3. Heat at a temperature of 70–90 °C until the sample is completely molten.
4. Leave to cool at room temperature.

Beeswax should not be heated at a temperature higher than 140 °C as volatile fractions begin to evaporate at this temperature (Bogdanov, 2016a). Moreover, temperatures above 150 °C may significantly affect beeswax composition (i.e., the content of hydrocarbons, free fatty acids, and long chain esters), and analytical values of beeswax (physico-chemical parameters); a prolonged heating (>24 h) at a temperature higher than 100 °C may cause the same effect (Tulloch, 1973), and it is therefore not recommended. Melting and other refining procedures commonly applied to beeswax in the beekeeping industry have been reviewed by Bogdanov (2016a).

Additional protocols for beeswax extraction and purification required for detection of pesticide residues in beeswax by LC-MS and GC-MS techniques are described in detail in Sections 6.5.2 and 6.5.3.

2.3. Beeswax storage

In case of performing the melting procedure, the samples must be completely dried before storage. It is not necessary to immerse the sample in any kind of preservation medium due to the natural beeswax stability. All types of beeswax specimens can be stored in the same way, as follows:

1. Store the beeswax samples in adequate packaging. Samples can be stored in one of the following packing materials:
   - plastic (polypropylene) containers or bags
   - glass containers
   - stainless steel (airtight) containers (containers made of other metals such as lead, iron, zinc, brass or copper should not be used for storage as they can make the beeswax turn dark)
   - wrapping paper or paper envelopes (suitable for storage of larger wax blocks or comb foundations)
2. Store the beeswax samples in a dark place at a temperature between 10 °C and 23 °C (note: it is advisable to store samples of brood combs or combs with bee bread below 9 °C to avoid wax moth problems).

Beeswax samples can be stored indefinitely.

3. Standard methods for research on wax gland cells and production of wax scales

3.1. Wax scales production

There were many hypotheses about the origin of beeswax throughout history, including the one of Aristotle who believed that it originates from flowers. The first correct description of beeswax origin dates back to 1744 when the German scientist Hornbostel described bees wax scales, their probable origin and use. These observations were further elaborated by Hunter in 1792, who gave additional notes on the background of beeswax secretion and comb construction (Bogdanov, 2016a; Hepburn, 1986; Hepburn et al., 2014).

Western honey bees produce wax in the form of scales used to construct their nest combs (Hunter, 1792). Secretion of wax scales is an age-related physiological process that may occur in adult honey bees at any time from day 3 to day 21 post emergence (Hepburn et al., 1991). The wax production phase primarily occurs from day 9 to 21 post emergence, peaking at day 12 (Hepburn, Hugo, Mitchell, Nijland, & Scrimgeour, 1984; Hepburn et al., 2014). In addition to age, the onset and duration of this phase is influenced by multiple biological and ecological factors including season, comb/storage space needs, honey and pollen availability, and brood-care needs (Boehm, 1965; Goetze & Bessling, 1959; Hepburn & Magnuson, 1988; Muller & Hepburn, 1992). There are eight ventrally paired wax mirrors (extremely thin sections of cuticle located underneath overlapping sternite segments on the underside of the bee's abdomen; they join adjacent sternites) on sternites 4–7, allowing bees to secrete up

to eight wax scales at a time (Cassier & Lensky, 1995; Locke, 1961). Underlying fat body cells associated with the wax gland, including oenocytes and adipocytes, increase in size and undergo physiological changes prior to wax synthesis (Boehm, 1965; Claus, 1867; Hepburn, 1986; Hepburn et al., 1991). Fatty acids and hydrocarbons are taken up by oenocytes of the wax gland to produce wax precursor materials; the role of wax gland adipocytes is uncertain, but they are thought to be an important source of lipids (Hepburn et al., 1991, 2014; Piek, 1964; Sanford & Dietz, 1976). The wax precursor materials pass through the microtubule pore canals of wax mirror epithelial cells as liquid wax fractions that coalesce at the surface of the cuticle (Cassier & Lensky, 1995; Locke, 1961; Hepburn, 1986; Hepburn et al., 1991; Sanford & Dietz, 1976). At the wax mirror, subsequent layers of liquid wax exude and harden to eventually form a scale (Cassier & Lensky, 1995; Dietz & Humphreys, 1970; Hepburn et al., 1991).

3.2. Collection of wax scales

When observable, wax scales protrude from between sternite segments. When this is not the case, a pair of forceps can be used to pull back the preceding sternite to expose the wax scale underneath. Wax scales may be collected directly from individual bees (3–21 days post emergence) or from the floor of an experimental cage after they fall from the bee.

3.2.1. Collecting workers from hives to study wax scales

Within bee colonies, adult workers with wax scales may be found on brood frames and where new comb is being built. Please see *BEEBOOK* vol I article on standard methods for maintaining adult *A. mellifera* in cages under *in vitro* laboratory conditions, Section 4 “Obtaining adult workers for laboratory experiments” by Human et al. (2013), for more information. Caging of bees is performed to limit the influence of external factors and numerous variables that might impact the study (weather, forage resources, water availability, pests, pathogens, robbing, potential nearby pesticide use, beekeeping practice, etc.). The wax scales can be collected from bees of unknown or known age, depending on the aim of the study.

Obtaining adult bees of unknown age

If cohorts of known age are not needed, adult bees are removed from a hive and placed into cages as follows:

1. Removing a frame from the hive.
2. Check the frame for the queen to ensure her safety.
3. Brush bees gently off the frame into a sealable cup to bring into the lab.
4. Maintain adult bees in rearing cages in the incubator until sampling according to standard rearing methods, please see *BEEBOOK* vol I article for maintaining adult *A. mellifera* in cages under *in vitro* laboratory conditions Sections 5–7 by Williams et al. (2013).
5. Proceed to Section 3.2.2.

Obtaining adult bees of known age

This method is more difficult and time consuming compared to the collection of the wax scales from bees of unknown age, but provides information on the age if necessary for the research intended to be carried out. Knowing the age of bees may be important as honey bees go through significant physiological and behavioral changes in their adult lifetimes; this also includes changes in metabolism of fat stores near the cuticle and the extrusion of liquid wax. Wax scales can be collected from individual bees starting from day 3 after emergence until day 21. In addition, experience-based observations indicate that it is best to collect wax scales as early as days 5 and 6 post emergence.

If cohorts of known age are needed, please see the *BEEBOOK* vol I article on miscellaneous honey bee research methods, Section 2.5.6 “Obtaining workers of known age,” by Human et al. (2013). The following is a summary of *BEEBOOK* vol I article on miscellaneous honey bee research methods, Section 2.5.6 “Obtaining workers of known age,” by Human et al. (2013) and *BEEBOOK* vol I article on behavioral methods, Section 2.3 “Marking individual bees” by Scheiner et al. (2013), with modifications to aid the researcher conducting research on wax scales.

A brood frame is collected, incubated in the lab, and emerged bees are marked and returned to the hive to obtain adult bees of known age as follows:

1. Remove a frame of ample (mostly capped) brood.
2. Check the frame for the queen; if she is present, either choose another brood frame or very carefully move her to another frame.
3. Brush off adult bees back into the hive.
4. Place the selected frame into a frame cage and place in a rearing incubator (incubator settings: temp, 34.5 °C; humidity, 70%).
5. Collect and mark eclosed bees daily and return to hive according to procedures to mark bees with paint described in *BEEBOOK* vol I article on miscellaneous honey bee research methods, Section 2.5.6 “Obtaining workers of known age,” by Human et al. (2013), or by following procedures for gluing tags to the bee thorax described in *BEEBOOK* vol I article on behavioral methods, Section 2.3 “Marking individual bees,” Scheiner et al. (2013).
6. Come back to the hive at desired time points and collect marked bees with wide-tip featherweight forceps, gently pinching their wings, legs, or thorax, and place into a sealable container.

3.2.2. Collecting wax scales from individual bees

Wax scales can be collected directly from individual bees by using a probe or forceps to check underneath overlapping ventral sternites of the bee abdomen. Until

selecting individuals for wax scales analysis, maintain bees according to standard methods found in *BEEBOOK* vol I article on maintaining adult *A. mellifera* in cages under *in vitro* laboratory conditions by Williams et al. (2013).

3.2.2.1. Selecting and anesthetizing individual bees. Bees are selected through a small opening in the container or cage, and then subjected to cold- anesthesia as follows:

1. Grab individual bees by the leg using wide tip forceps through a small opening in the cage and quickly remove.
2. Deposit bee into a 15 mL tube.
3. Cap the tube and place in freezer to cold-anesthetize (−2 °C for 2.5–3 min), see the *BEEBOOK* Vol I article on miscellaneous honey bee research methods, Section 2.1.2.2 "Immobilizing adults, chemical and physical immobilization" by Human et al. (2013).

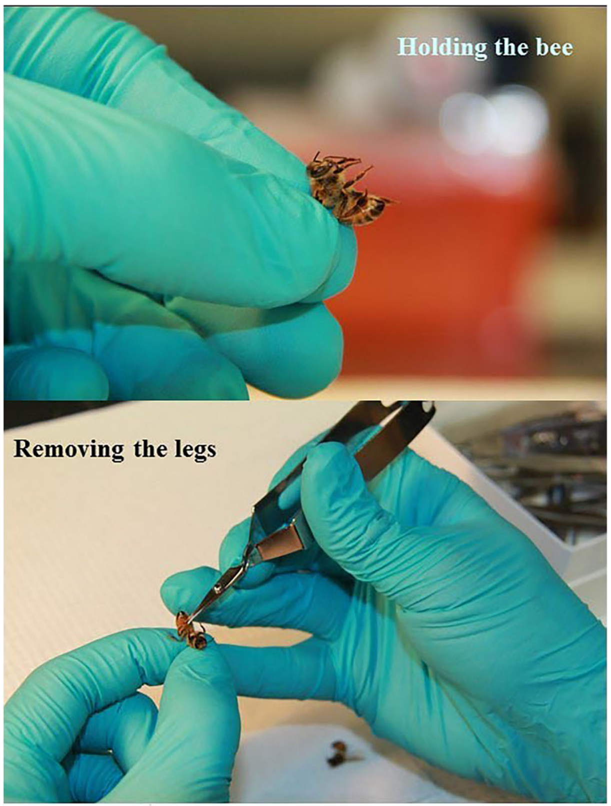
If an entire cage or container of adults needs to be anesthetized to select and remove individuals for collecting wax scales,

1. Set cage in the freezer for 5 min to slow them down before selecting individuals, or
2. Place the cage in the refrigerator (4 °C) for 10–15 min.

The more bees in a cage, the longer it will take to lower their body temperatures (for a cage of 30 bees, it will take about 15 min at 4 °C). Be careful not to kill bees by leaving them in the cold for too long; check on them every 2–3 min to observe effect. Bees can also be anesthetized with carbon dioxide; however, they recover more quickly.

3.2.2.2. Collecting wax scales from the wax mirror. Wax scales are collected from underneath overlapping sternites of the honey bee abdomen of anaesthetized bees as follows:

1. Pinch the wings of the anesthetized bee carefully between the forefinger and thumb.
2. Pivot to display the ventral surface of the bee (Figure 2).
3. If the bee is to be killed anyway, remove the legs to the femur.
4. Prod underneath the overlapping ventral sternites with a hooked probe or fine-tip forceps to observe wax scales.
5. Rate the size of wax scales relative to the wax mirror before removal (see Section 3.3.1).
6. If using forceps, grip the sternites gently and pull outwards (away from the bee) slowly and gently to remove wax scales; this will cause enough friction between the scale and the end of the forceps to pull out the wax scale without ripping the abdomen.
7. Grab the protruding wax scale with forceps and place on a slide or into a 1.5 mL microcentrifuge tube labelled with the date, treatment group, cage number, and bee number.
8. Repeat for all eight areas where wax scales can be found: sternites 4–7, and on either side of the centerline bisecting the ventral-side of the abdomen.



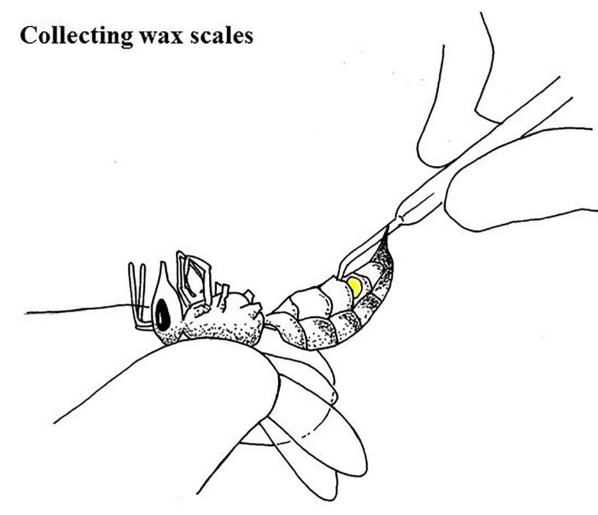


Figure 2. Demonstrates how to hold the bee, remove legs, and use a hooked probe to remove wax scales (high-lighted yellow) from the wax mirror on the underside of a honey bee abdomen. Photos and Illustration: Waters T.

9. Record the mass of collected wax scales (see Section 3.3.2).

Removing the legs is optional and this procedure may expose wax scales to the risk of contamination from hemolymph. Collecting wax scales from individual bees is more time consuming than retrieving fallen scales in cages; however, there is a decreased chance of contamination with cage debris.

3.2.3. Collecting fallen wax scales from cages

The easiest way to collect wax scales is to wait until after they have fallen off the bees, using wooden cages or plastic cup cages with raised wire mesh floors. The mesh needs to be sturdy enough to serve as a stable enclosure for the bees but large enough to allow wax scales to fall through (#6, 3 mm opening, steel wire mesh).

3.2.3.1. Modifying plastic cup cages. Procedure to modify plastic cup cages to insert a wire mesh false floor:

1. Cut a circle out of a Solo Cup® lid leaving the rim intact (Figure 3(a,b)).
2. Use cuticle scissors to remove the bottom of the cup (Figure 3(c)).
3. Cut steel wire mesh into a circle approximately 7 cm in diameter for a 473 mL clear Solo cup (this just so happens to be the same diameter as a wide-mouth Ball® mason jar lid that can be used as a model/gauge, Figure 3(d,e)).
4. Place wire mesh circle into the cup – the mesh circle should be small enough to fit snugly in the cup leaving about 2 cm of space between the mesh and the bottom of the cup as shown (Figure 3(f,g)).
5. Cut approximately 15 cm × 15 cm piece of fine-mesh cloth.
6. Place fine-mesh cloth square over cup and snap lid on top to secure (Figure 3(h)).
7. Poke a hole in the fine mesh with an razor blade or snip with dissection scissors, to allow top feeder to be placed through hole without falling through (Figure 3(h)).
8. Set the cup onto a petri dish to collect fallen scales without disturbing the bees (Figure 4).

3.2.3.2. Collecting fallen wax scales from cages designed with wire mesh floors. For *in vitro* adult rearing cages designed with wire mesh floors, remove fallen wax scales as follows:

1. Place a flat removable surface underneath the cage to collect fallen wax scales (such as a strip of plastic from a notebook divider, with edges bent up to form a rim) (Figure 5).
2. Collect wax scales at set time-course.
3. Record the number of wax scales collected from each cage.
4. Measure the mass of wax scales (see Section 3.3.2).

Feeder drips, bee feces, mold, and other contaminants are a problem in most caged experiments. Compared to rearing cages with closed or solid floors, the wire-mesh floors appear to improve cage ventilation and prevent food and feces build-up on the floor of the cage, reducing mold problems and improving cage health. Additionally, the wire mesh floors facilitate wax collection while minimizing disturbance of bees. Despite the raised floor, wax scales may still be contaminated by cage debris.

3.3. Wax scale measurements

3.3.1. Recording scale size

Wax scales are flat oval-shaped flakes of raw wax (Figure 6) that can be up to 3 mm wide. When collected directly from bees, their size can be compared to the wax mirror as described in Jordan (1962) (as cited in Ferguson & Winston, 1988; Ledoux et al., 2001; Otis, Winston, & Taylor, 1981). In this way, scale size can be rated as follows:

0, no wax
1, very small scales, not easily removable
2, medium scales, not extending beyond the overlapping sternite
4, very large scale or irregularly shaped clumps of wax (covering the entire wax mirror and extending well beyond the overlapping sternite).

Described rating method represents a simplified procedure for measuring the wax scales collected directly from the bees. However, if wax scales need to be sized or when fallen wax scales are collected from the cages, their size (mm) can be determined using an eye-piece ocular micrometer fixed within a stereomicroscope (alternative: digital photographs in combination with various image analysis programs).

3.3.2. Measuring scale mass

The relative quantity of wax secreted by different age groups can be estimated by measuring the weight of wax scales recovered per bee or per group. For measuring the mass of wax secreted by cohorts of bees (e.g., Hepburn et al., 1991), a microscale readable to 1 mg should be sufficient when large numbers (dozens) of wax scales are pooled together. To weigh wax scales of individual bees (e.g., Hepburn et al., 1991) a microscale readable to 0.01 mg will be needed:

1. Tare microscale with clean, small (0.4–1.5 mL), microcentrifuge tube.

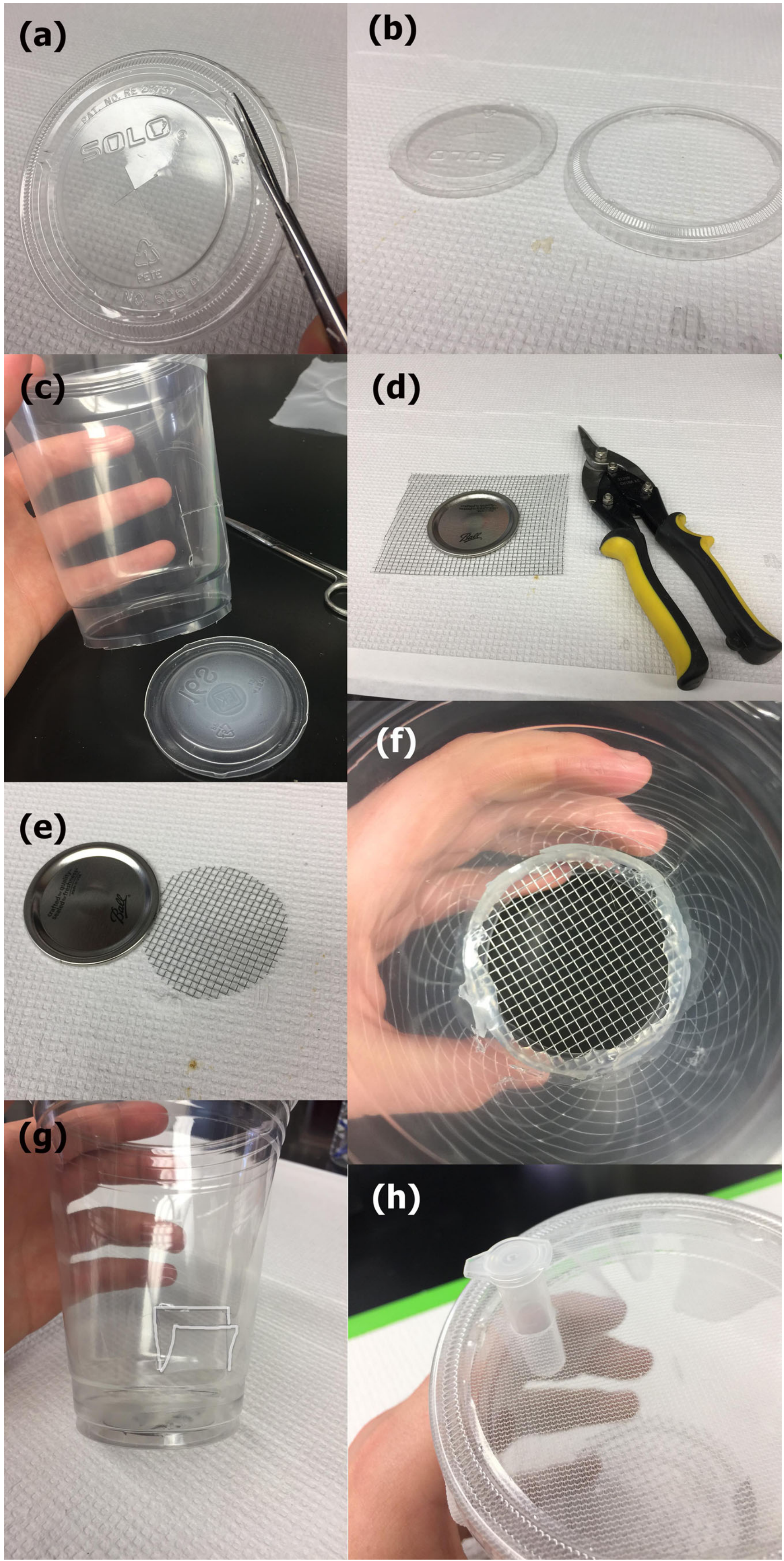


Figure 3. Steps to modify plastic cup rearing cage to collect wax scales (a–h). Photo: O'Grady M.

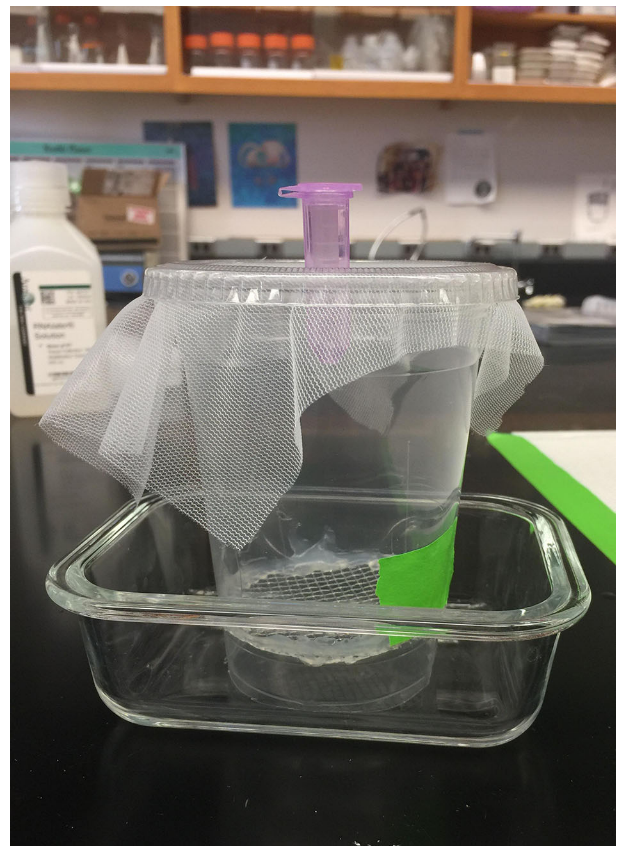


Figure 4. Modifying a Solo Cup® rearing cage to collect wax scales by inserting a wire mesh false floor, removing the bottom of the cup, and placing over dish. Photos: O'Grady M.

2. Place wax scales in small microcentrifuge tube.
3. Measure and record the weight of the scales.
4. Freeze at $-20\,^{\circ}$C to preserve until analysis.

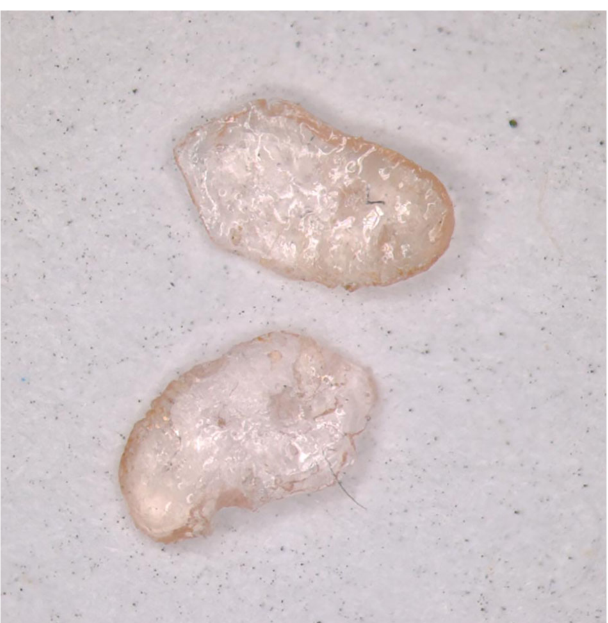


Figure 6. Wax scales viewed under a dissection scope. Photo: Waters T.

4. Methods for investigating honeycomb cell properties and comb construction

4.1. The two-dimensional structure of the hexagonal cell

Despite the fact that the honeybee cells might originate from initially circular tubes, cells exhibiting a hexagonal structure as a final result remains universally accepted. Stating this assertion is essential for the rest of the chapter, since adequate measurements depend crucially on the model of the cell that we adopt. From a

Figure 5. Plastic placed under rearing cages with wire mesh floors to collect wax scales. Photo: Waters T.

geometrical point of view, the honey bee cell can be described as follows (Figure 7, Saucy, 2014).

1. The dimensions of a regular hexagon can be approached by two circles, i.e., an inscribed circle (i) and a circumscribing circle (c), of radius r_i (the apothem) and r_c, respectively.
2. The hexagon can be decomposed in 6 isosceles triangles, i.e., triangles with edges of equal lengths.
3. The small diameter of the inscribed circle d_i is $d_i = 2*r_i$.
4. The large diameter d_c of the circumscribed circle is: $d_c = 2*r_c$.
5. Since the triangles are isosceles, the length of each edge of the hexagon is equal to the radius of the circumscribed circle r_c.
6. If follows that the height of each triangle is equal to r_i.
7. The area of each isosceles triangle is given by: $A = r_c*r_i/2$.
8. The area of a cell is therefore: $A_{cell} = 6*r_c*r_i/2 = 3*r_c*r_i$.
9. Each of the six triangles of the hexagon being also equilateral triangles, the length of each side of the hexagon is equal to the radius of the circumscribed circle r_c, while the height of each triangle is equal to r_i. Applying the Pythagorean theorem we deduce $r_i^2 = r_c^2 - (r_c/2)^2 = 3r_c^2/4$, from which the following relationships between r_i and r_c:

$$r_i = \frac{\sqrt{3}}{2} r_c \text{ and } r_c = \frac{2}{\sqrt{3}} r_i,$$

10. Therefore, the area of a cell can be expressed as:

$$A_{cell} = 3*\frac{2}{\sqrt{3}} r_i * r_i = 2\sqrt{3} r_i^2$$

11. Or, expressed in d_i units: $A_{cell} = \frac{\sqrt{3}}{2} d_i^2$
12. Finally, the cell density (expressed as the number of cells per area unit) is given by: $D_{(1side)} = \frac{1}{\frac{\sqrt{3}}{2} d_i^2}$
13. Taking the two sides of the comb into account yields: $D_{(2sides)} = \frac{4}{\sqrt{3} d_i^2}$

4.1.1. Linear measurements along the small diameters (d_i)

The linear measurements were formalized by Baudoux (1933). His results have been summarized as follows in de Meyer (1938): "wax foundation should be measured in three directions and each time along the double apothem of the cells. To minimize error measurements, they should be conducted in several series of 10 cells" (Figure 8). To estimate cell densities, Baudeaux developed a simplified formula for field work (de Meyer, 1938):

1. take several measurements of 10 cells (in mm)
2. calculate the average of these measurements

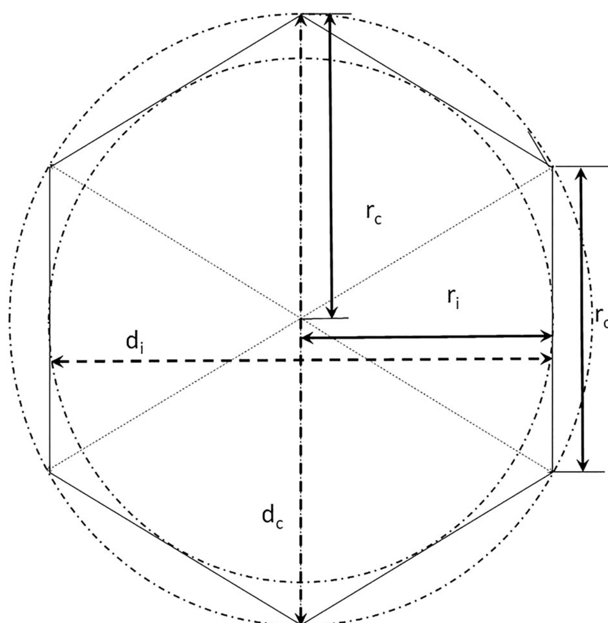


Figure 7. Two-dimensional model of the cell as a regular hexagon with respect to its inscribed (i) and circumscribing (c) circles. r_i, radius of the inscribed circle (apothem); d_i, diameter of the inscribed circle; r_c, radius of the circumscribing circle; d_c, diameter of the circumscribing circle.

3. divide the number 2,309,467 by the square of the averaged measurements

The number 2,309,467 corresponds to the square of a measurement of 10 cells in a comb of cell density = 1000 cells/dm^2, which in turn corresponds to a cell width of 4.805691417 mm. Beaudoux's simplified formula is correct, still valid and in full accordance with the geometrical and mathematical properties of a two-sided comb made of hexagonal cells, as described in the formula $D_{(2sides)} = \frac{4}{\sqrt{3} d_i^2}$.

Figures 8–10 illustrate various ways of taking measurements of 10 cells in different configurations, for instance within a hexagon encompassing exactly 100 full cells (Figure 9). Figure 10 shows how the same hexagon of small diameter 10d_i can be transformed into a rhomb of same area $A_{hexagon} = A_{rhomb} = 100\frac{\sqrt{3}}{2} d_i^2$.

Linear measurements should be taken along rows of a minimum of 10 aligned cells through their small diameters, i.e., through the diameters of their inscribed circles (d_i), either horizontally, either laterally at 45° angles (Figure 8), through the middles of the external walls of the most extreme cells of the row:

1. Identify an area of cells homogenous in size and shape (worker cells, drone cells, honey cells).
2. Identify a row of n aligned cells (at least 10).
3. Mark a cell, then count n cells and mark the next cell.
4. Measure the distance from the 1st to the nth cell, from the middle of the external walls of the 1st and nth cell using a calliper (Figure 11).
5. Calculate the average cell width (d_i) by dividing the measurement by *n*, the number of cells of the measurement.

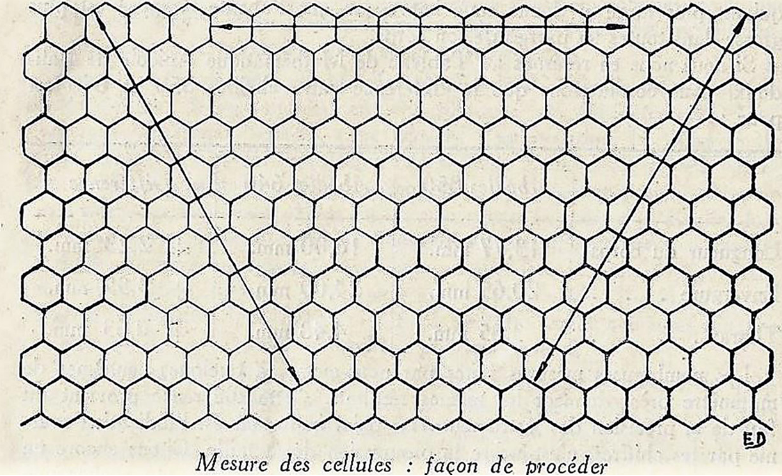


Figure 8. Measurement of the cells: way to proceed according to Baudoux (De Meyer, 1938).

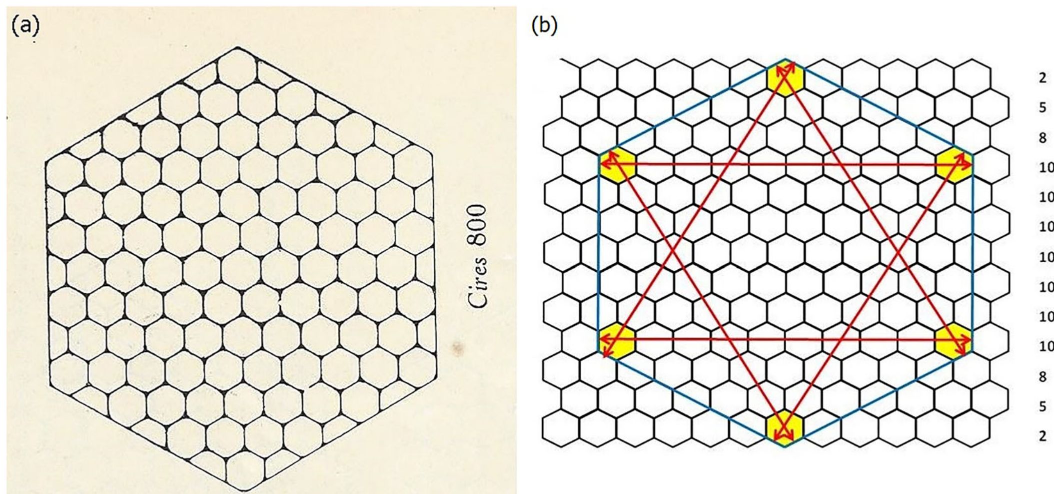


Figure 9. (a) Example of a hexagonal subset of wax foundation with a density of 800 cells/dm^2 encompassing the area of hundred full cells (according to Baudoux, in De Meyer, 1938); (b) diagram of the same hexagonal subset of a comb exhibiting that 7 rows encompass an area of 10 full cells in 3 directions. The red lines indicate 6 possible measurements of rows of 10 cells within the hexagon. The figures on the right give the area (in full hexagonal cells) of each horizontal row.

6. Calculate cell density using formula (13): $D_{(2sides)} = \frac{4}{\sqrt{3}d_i^2}$, or read it from Table 1.
7. Replicate the same measurements to get statistical estimates of measure errors.
8. Repeat measurements at regular intervals on the comb to get statistical estimates of variability of cell sizes.

4.1.2. Sampling the comb

4.1.2.1. Sampling the comb using linear measurements along the small diameters (d_i). Since a comb may contain different kinds of cells of different sizes (worker, drone or honey cells), sampling the comb is usually necessary to seize its cell size diversity.

The method of linear measurements can be extended in different ways:

1. Three linear measurements of 10 cells in a triangular pattern around a topic cell (Figure 13(a)).
2. Three linear measurements of 10 cells crossing a topic cell in a star pattern (Figure 13(b)).
   In both cases (a and b), the average of the three measurements gives an estimate of cell sizes over approximately 1/4 of dm^2. Five such measurements on each side of the comb should be sufficient to assess the comb's diversity.
3. Linear measurements at regular intervals (e.g., every each 5th row) from the top to the bottom of the comb or from the right to the left of the comb.

4.1.2.2. Sampling the comb using rectangular measurements along the small and large diameters. Combs built on artificial foundation are expected to exhibit little variability, but sampling the comb using rectangular measurements may be a useful approach to assess the regularity of wax foundation sheets. Measurements should be conducted along both dimensions of the cells, i.e., along the

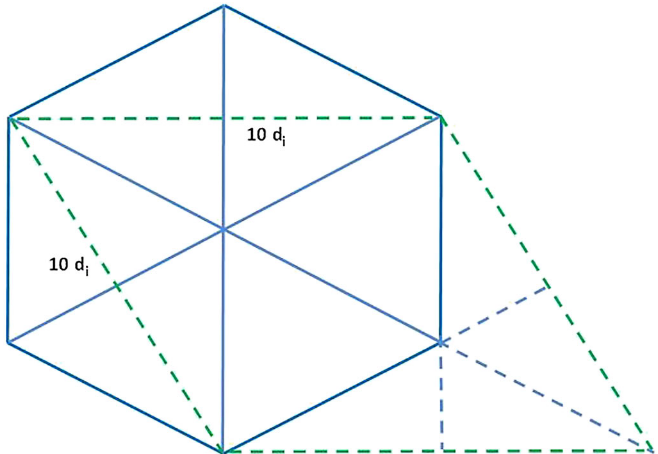


Figure 10. Picture showing how a hexagon of diameter $10d_i$ can be transformed into a rhomb of length $10d_i$ and of same area as the hexagon, i.e., $A_{hexagon} = A_{rhomb} = 100\frac{\sqrt{3}}{2}{d_i}^2$. Note that each of the 6 isocele triangles of the hexagon can be divided in 2 right triangles, yielding 12 such triangles of same area for both the hexagon and the rhomb. A square of side $10d_i$, of area $100{d_i}^2$, would encompass 16 such rright triangles. The ratio $H/S = \frac{\text{area of the hexagon}}{\text{area of the square}}$ is therefore $\frac{12}{16} = \frac{3}{4} = \frac{\sqrt{3}}{2}$.

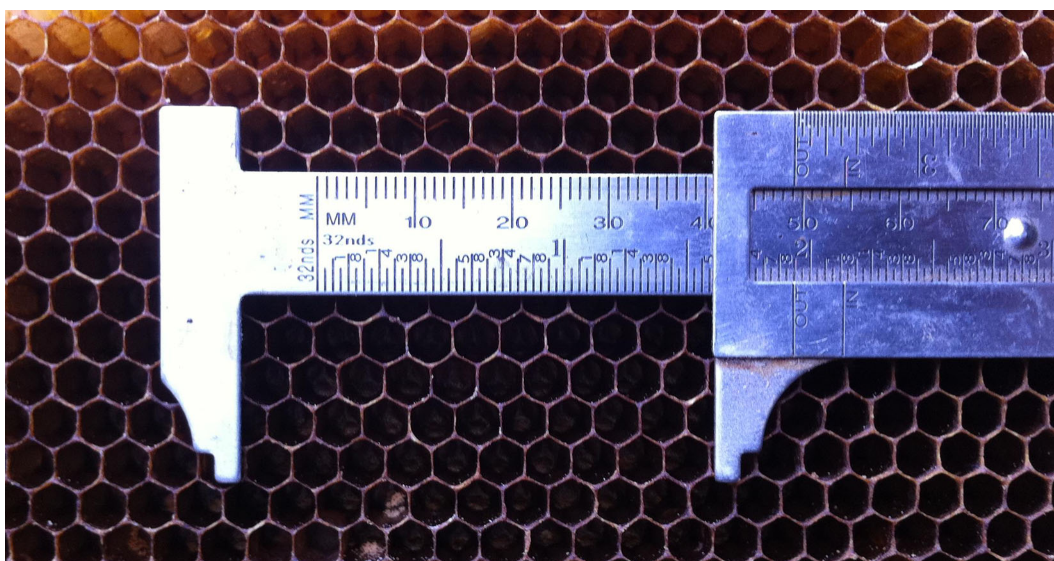


Figure 11. Measuring 10 cells in a row using a caliper. (Source: http://parkerbees.com/beespace.html)

small and large diameters, d_i and d_c (Figure 12). The measurements and calculations are slightly more complicated, since linear measurements through rows of cells pass alternatively through large diameters and large radii of the cells. The average height (h) of a row is therefore:

$$h_{1row} = \frac{3}{4}d_c = \frac{\sqrt{3}}{2}d_i.$$

In practice, it is recommended to measure an even number of rows (e.g., 10), so that the average height is estimated based on an equal number of large diameters and large radii.

1. Mark a row, count n (10) rows vertically along the comb, mark the $n+1$th row (11th row).
2. Take a measurement of the n (10) rows.
3. Divide your measurement by n (10) to get h_{1row}.
4. Calculate $d_c = \frac{4}{3}h_{1row}$ and $d_i = \frac{\sqrt{3}}{2}d_c$.

On this basis, it is therefore easy to estimate the regularity of the cells. For instance, a linear horizontal measurement of 53 mm along the small diameters (d_i) of 10 cells corresponds to an average 5.3 mm cell width. If cells are regular, we expect for a vertical measurement of 10 rows a figure of $10*\frac{\sqrt{3}}{2}d_i$, i.e., 45.9 mm. A larger figure indicates a vertical distortion of the hexagons, with d_c larger than expected from d_i, while a smaller figure is indicative of a horizontal distortion of the cell.

Table 2 gives examples of such linear cell measurements conducted on wax and plastic foundation. In this case, the longest possible rows of full cells were measured in both horizontal and vertical directions and compared to samples of 10-cell measurements using a ruler

Table 1. Cell density estimates based on measures of the diameter of the inscribed circle d_i, according to the formula: $D_{(2sides)} = \frac{4}{\sqrt{3}d_i^2}$.

Cell width (diameter d_i in mm)	Density (number of cells/dm^2)
4.5	1140.4
4.6	1091.4
4.7	1045.5
4.8	1002.3
4.9	961.8
5.0	923.8
5.1	887.9
5.2	854.1
5.3	822.1
5.4	792.0
5.5	763.4
5.6	736.4
5.7	710.8
5.8	686.5
5.9	663.4
6.0	641.5
6.1	620.6
6.2	600.8
6.3	581.9
6.4	563.8
6.5	546.6
6.6	530.2
6.7	514.5
6.8	499.4
6.9	485.1
7.0	471.3

or a calliper. The results indicate that using callipers (Column J; Table 2) yields closer estimates of cell widths than using a ruler (I) for rows of 10 cells with regard to cell width estimated using measurements of whole cell rows (L). Columns P and Q give estimates of horizontal and vertical distortion ranging from 0.2% for plastic foundation (third row) to 1.1% for wax foundation (first row).

4.1.2.3. Measurements and analyses conducted on photographs. Taking measurements on combs is not only a time-consuming task which may be postponed for further analysis in the laboratory, but may also be complex because of the bees activity during field work, as well as fragility of the wax. Taking pictures of the combs is an alternative and efficient way of speeding up field work. In all cases, one should label the subject and use reference objects (e.g., a ruler) to be able to calibrate all further measurements. Such reference tags should be located as close as possible of the areas of interest and should be disposed at different locations of the comb to be able to assess possible distortions due resulting from different angles of view of the camera with respect to the picture's subject. Taking measurements on photographs has the advantage that measurements can be postponed, can be replicated and that pictures can be shared with colleagues everywhere in the world. In addition, pictures may be enlarged, allowing increased precision measurements. They are particularly suited if the subject has to be enlarged, e.g., in the case of cell width measurements. Various software are available for the analyses. Examples given in this section were operated using ImageJ (Fereira & Rasband, 2012), an open source software widely used among scientists:

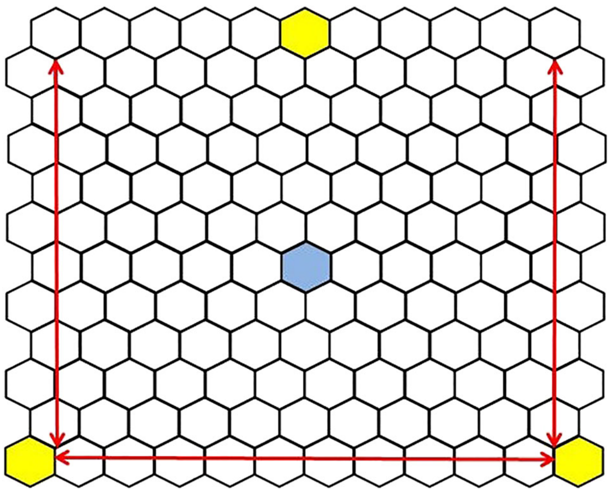


Figure 12. Rectangular measurements of 10 rows of cells to assess the two-dimensional regularity of the cells.

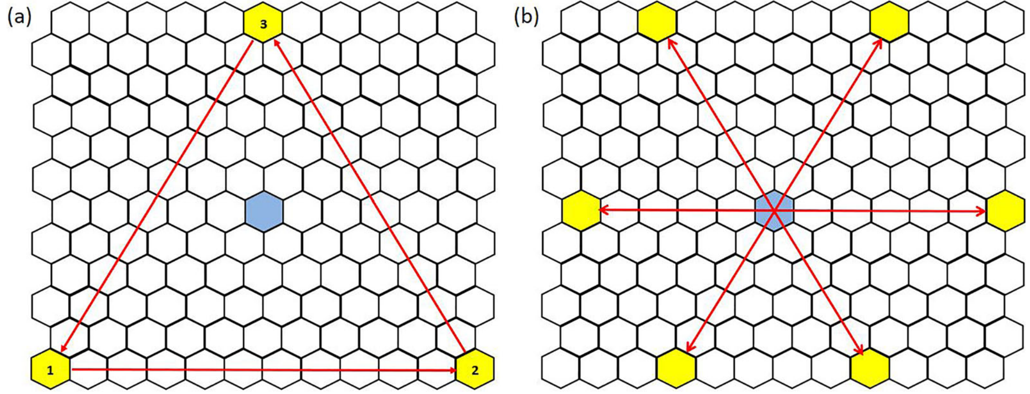


Figure 13. Linear measurements of three rows of: (a) 10 cells in a triangle pattern and (b) of three rows of 10 cells in a star pattern (blue: topic cell; yellow: cells adjacent to each row of 10 cells). Note that the triangle pattern is easier to implement since it only requires marking 3 cells, instead of 7 (including the topic cell) in the star pattern. Moreover, the latter displays an asymmetrical pattern with either 4 or 5 cells on each side of the topic cell for each measurement.

Table 2. Examples of linear measurements conducted on artificial foundation from three different suppliers.

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Type of foundation	Number of cells per (horizontal) row	Number of (horizontal) rows	Length of (horizontal) rows (mm)	Height of (horizontal) rows (mm)	Area (calculated from col. D & E) (dm^2)	Number of cells on measured area (calculated from col. B & C)	Cell density (number of cells/dm^2 calculated from col. F & G) (2 sides)	Measurement of 10 cells using a ruler along the small diameter d_i (cm)	Measurement of 10 cells using a caliper along the small diameter d_i (cm)	Average cell width d_i (calculated from col. D & B) (mm)	Average row height h_{row} (calculated from col. E & C) (mm)	verage cell large diameter d_c (calculated as dc= $h_{row}*4/3$ (mm)	d_i estimated from $d_i = d_c*\sqrt{3}/2$	d_c estimated from $d_c = 2d_i/\sqrt{3}$	Horizontal distortion (ratio d_i measured/d_i estimated after d_c (col. K & N)	Vertical distortion (ratio d_{ic} measured/d_c estimated after d_i (col. M & O)
Dadant (body) wax	76	56	407	257	10.5	4 256	814	5.5	5.4	5.355	4.589	6.119	5.299	6.184	1.011	0.990
Dadant (super) wax	77	28	408	128	5.2	2 156	826	5.3	5.3	5.299	4.571	6.095	5.279	6.118	1.004	0.996
Dadant (super) plasic	72	27	410	135	5.5	1 944	702	5.8	5.7	5.694	5.000	6.585	5.703	6.575	0.998	1.002

1. Tag the comb using a reference for measurements (e.g., a ruler).
2. Take pictures at different time intervals.
3. Document pictures (number, label, date and time, remarks).
4. Measure reference tags.
5. Set the scale (e.g., transform pixels in mm).
6. Take measurements of comb features (cell widths, wall widths, series of cells, etc).
7. Save measurements.

4.1.2.4. Addressing the regularity of the cell. The honey bee comb, composed of hexagonal cells, is commonly viewed as a model of regularity and perfection in nature. This comes both from an idealization of the honey bee construction abilities and from the fascination for the comb's two-dimensional structure. However, this regularity partly results from visual impressions and is enforced by the use of wax foundation. However, it is often challenged and is rarely observed on natural combs. On the contrary, the combs exhibit many irregularities with cells of different shapes, often far from regular hexagons, including squared, pentagonal and heptagonal cells (Figure 15). Irregularities may result from different processes:

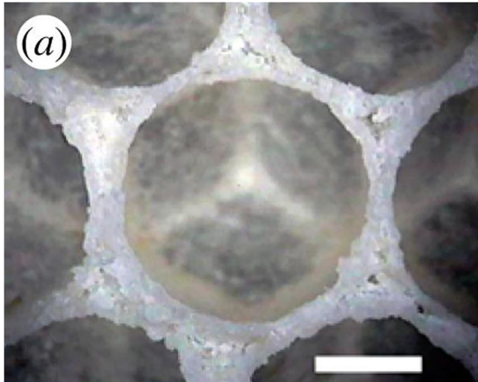
a. Transition from worker to drone cells during comb construction.
b. Junction from comb sections started at different distances during the construction process.
c. Repair of damaged combs.

Examining how bees handle transitions from worker to drone cells and junctions of combs started at different locations or how they repair damaged combs is perhaps even more fascinating than understanding how they build apparently regular cells. During these processes, bees may exhibit solutions which preserve the function and the overall integrity of the comb, generating numerous irregularities. On some combs, it is hardly possible to find a typical hexagonal structure.

Heaf (2012) has published several pictures and cell size measurements from natural combs (http://www.dheaf.plus.com/warrebeekeeping/cell_size_measurements.htm). As Figure 16 shows, trying to find regular rows of cells as they commonly appear on wax foundation is a hopeless goal. Analyses of Heaf's measurements display a clear trend of decreasing cell size from the top to the bottom of the comb (Figure 17(a)), as well as little correlation between the two sides of the comb with respect to cell size (Figure 17(b)).

To take measurements addressing the regularity of the comb:

1. Apply a 10 cm × 10 cm grid on the top row centred on the middle of the comb.
2. Measure the length and the height of the comb along the horizontal and vertical lines of the grid.



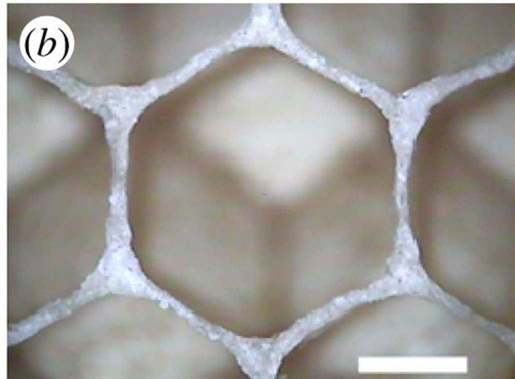


Figure 14. Photographs showing the process of construction of the honey bee cells exhibiting different wall widths during the "maturation" process of the cells: (a) cell at "birth", (b) 2-days old cell. The scale is given by the reference tag (115 pixels = 2 mm), which for the 2-days old cell yields a width of 5.32 mm between wall centers (306 pixels) (from Karihaloo et al., 2013).

Figure 15. Picture of a natural comb exhibiting irregularities; note a heptagonal cell in the centre and "filling" cells. A regular hexagonal cell can hardly be found.

3. Count the total number of rows from the top to the bottom along the vertical lines of the grid.
4. Count the total number of cells from the right to the left along the horizontal lines of the grid.
5. Take measurements of rows of 10 cells at regular intervals (e.g., every single, 2nd or 5th row) along the vertical lines of the grid (or at each node of the grid) from the top to the bottom on both sides of the comb.

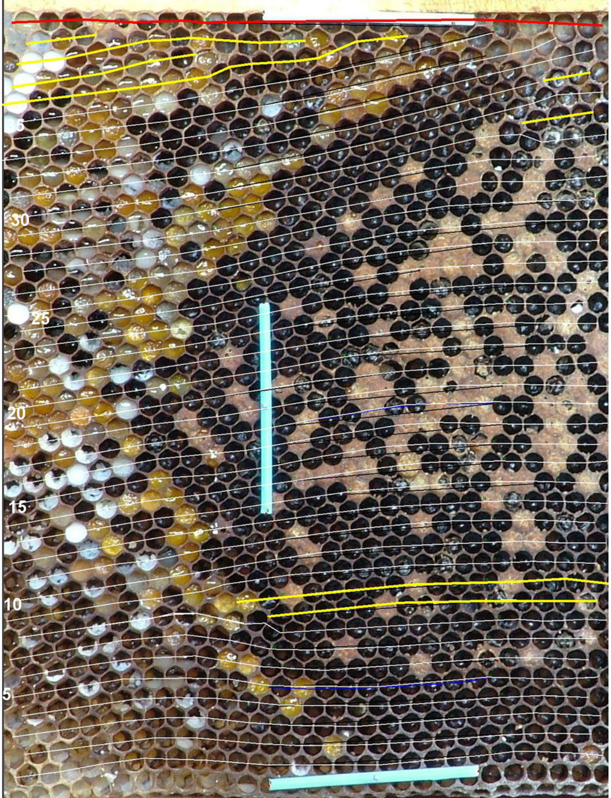
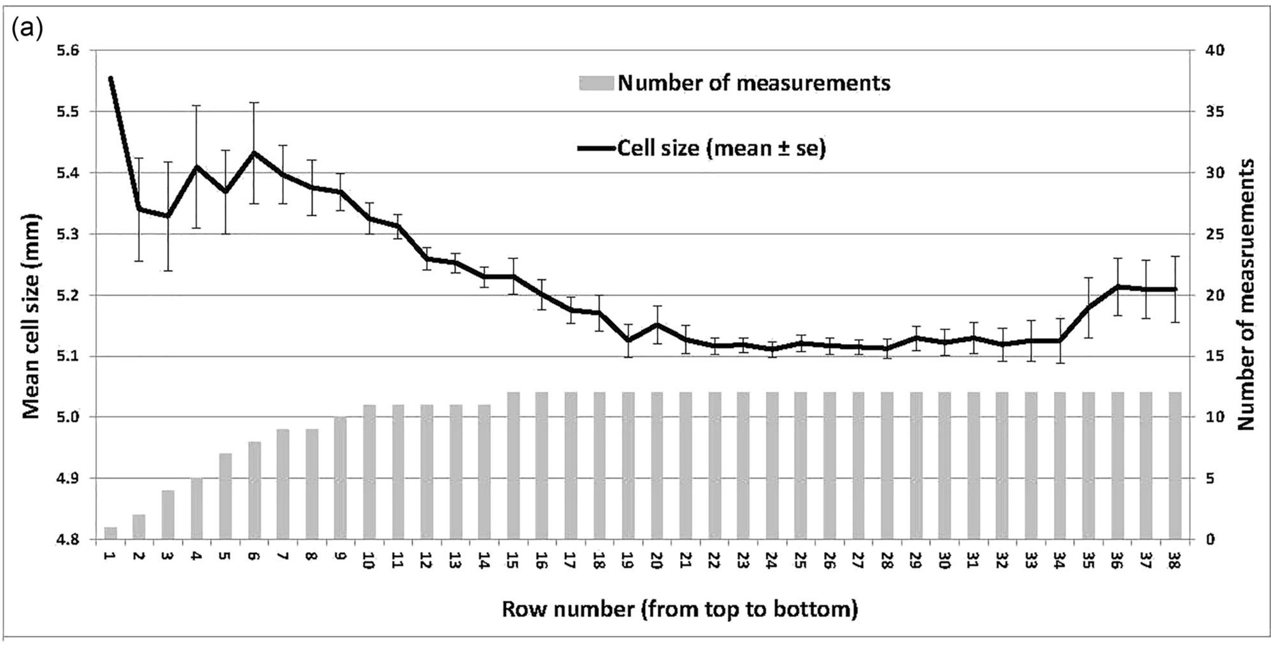


Figure 16. Identification of "horizontal" rows of cells on a natural comb exhibiting a large variability of the two-dimensional cell structure.

Recent methods of photograph analyses allow much more sophisticated analyses of the two-dimensional structure of the comb than simple lengths measurements. For instance, Kaatz, Bultheel, and Egami (2008) conducted a study in which they compared natural combs and combs built on wax foundation against an ideal hexagonal structure (Figure 18).

4.1.2.5. Advantages and disadvantages of different measurement techniques. Table 3 gives a summary of the principal characteristics, strengths and weaknesses of the different methods and techniques of measurements described in this section. For instance, measurements of rows of 10 cells are clearly best suited for field work, because the method is simple and measurements quick to conduct. Linear measurements of all full cells in a row are particularly well suited for artificial foundation: it is much quicker than repeated measures of series of 10 cells in the same row. While there is only one approach suited for estimating cell distortion in horizontal or vertical direction, among the various approaches to sample the comb in different directions, the triangular pattern is clearly the quickest. If measurements in a



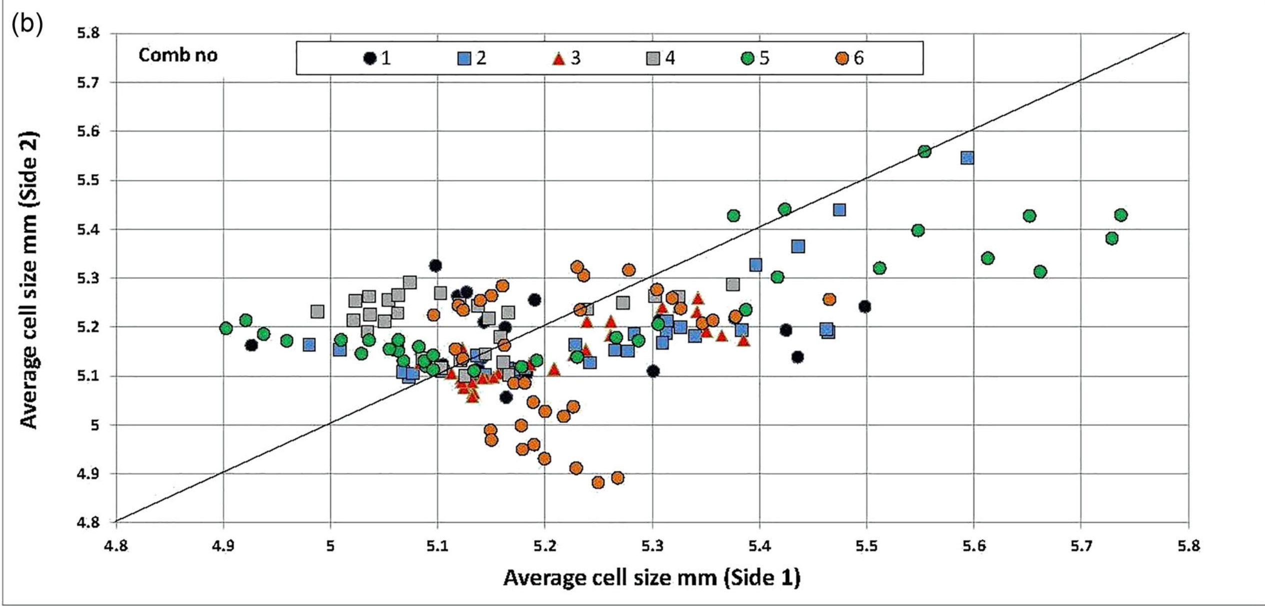


Figure 17. Example of natural cell size variations among combs. Measurements of 6 combs (12 sides) from one Warré box (topbar hive without wax foundation; data from David Heaf: http://www.dheaf.plus.com/warrebeekeeping/cell_size_measurements.htm; Warré 1, measured 04.12.2011). (a) Average cell size (±SE) from top to bottom in the middle of the comb (average of twelve measurement of 10 cells per row; missing data correspond to accidentally damaged top of combs); (b) scatterplot of cell size measurements (mm) for corresponding rows on both sides of the combs (same data as above) exhibiting variation between and within combs. The oblique line corresponds to equal cell size measurements on both sides of the comb.

star pattern are basically equivalent, the approach is more time consuming (7 cells to identify). In addition, from an esthetical point of view, it is geometrically unsatisfactory and would be best suited for measurements of rows of odd numbers of cells (e.g., 9 or 11). In terms of precision, callipers should be preferred to rulers. As long as optical distortions are controlled, taking measurements on pictures is a satisfying approach. It is a quick method in field work and measurements can be reproduced. In conclusion, the best approach depends on the goals, means and resources of the study. What really matters is to take into account the peculiar properties of the hexagonal cell properly, to avoid crude errors (Saucy, 2014).

4.1.2.6. Investigating interspecific and intraspecific cell size variability. Cell size varies between *Apis* species and is in direct relationship with the size of the adult workers. It also varies within species, between subspecies, but also within subspecies. It has long been known that smaller

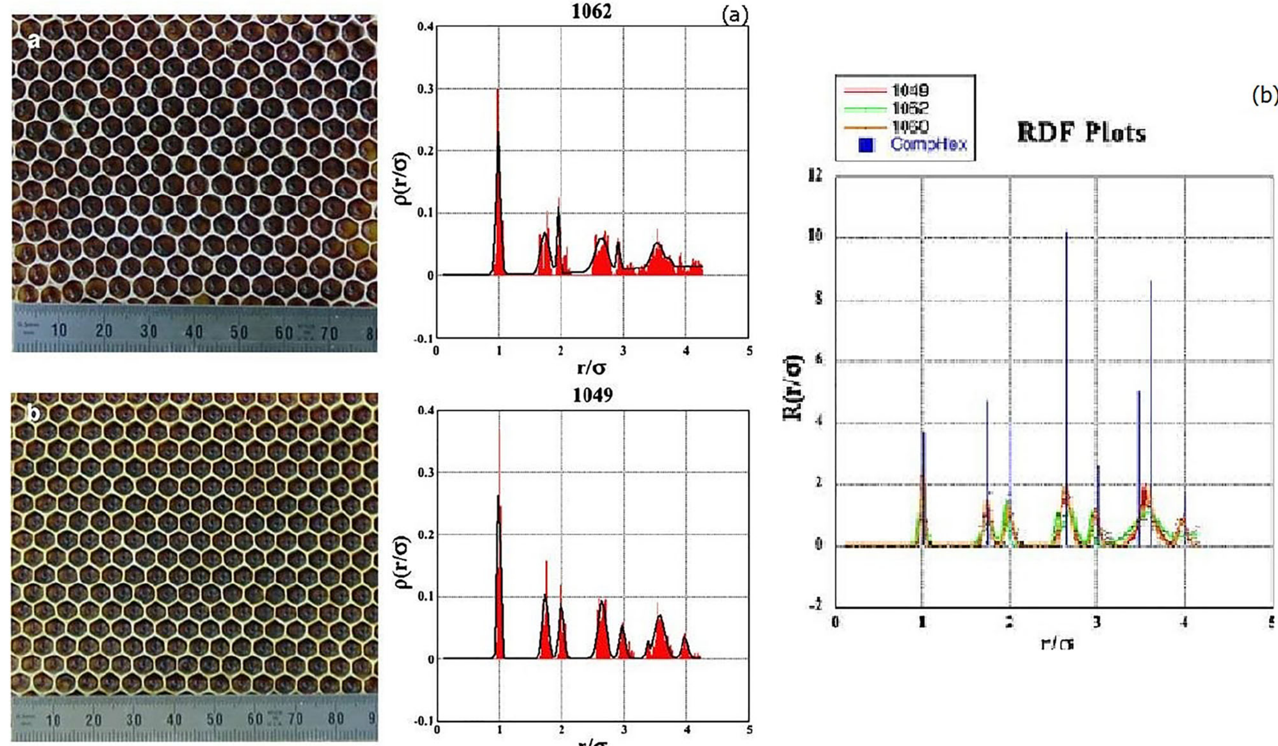


Figure 18. (a) Analysis of the comb structure (from Kaatz et al., 2008): left: comb built on artificial foundation (bottom) and natural comb (top), right: plots of radial diffusion functions; (b) plots of radial diffusion functions (RDF) of 3 combs compared to a perfect hexagonal array (blue lines).

workers emerge from old brood comb because silk residues accumulate in the cells and that smaller or larger workers can be produced depending on the size of wax foundation (Baudoux, 1933; Honegger, 1937; Vogt, 1911). In the early 1990s, uncertainties arose regarding the natural size of cells (Erickson, Lusby, Hoffmann, & Lusby, 1990a, 1990b) that led to a debate with repercussions on beekeeping management, honeybee biology, physiology and health issues (Heaf, 2011, 2012; Saucy, 2014; Stever, 2003; Zeissloff, 2007), an issue which highlighted the need for proper methods.

4.1.2.7. Interpreting historical data on cell size. To interpret historical data on cell width properly, the following steps should be conducted:

1. Find a copy of the original publication.
2. Read the publication in the original language or get a certified translation.
3. Correctly identify the unit(s) used for the measurements.
4. Establish the correspondence between the historical measure units and metric units.
5. Convert the measurements of the publication in metric units.

Point 3 is of particular importance. Before the introduction of the metric system, the same words were often used to refer to locally different units of measure. For instance, foot and inch were different in Great Britain, France, some provinces of Germany, Switzerland, the Netherlands and Eastern European countries. Definitions might also change with time. Failing to understanding these peculiarities properly may lead to errors of interpretation (Saucy, 2014 for a review).

Several examples of erroneous interpretations have been reported (Saucy, 2014). For instance, Huber (1814), probably based on the measurements of de Castillon presented in 1781 at the Academy of Sciences of Berlin and which were the only replicated measurements avaible at Huber's times, gives the same cell size as de Castillon, i.e., an average cell size of 2 2/5 lines. Properly interpreted from the Parisian foot used by the French speaking authors before the introduction of the metric system (de Castillon, 1781; Huber, 1814; Maraldi, 1712), this corresponds to a cell size of 5.41 mm, and not to 5.08 as incorrectly transposed from the modern Anglo-American foot defined in the 19th century.

4.1.3. The three-dimensional structure of the comb

With its hexagonal cells, the bottom of each facing three cells on the other side (Figures 19 and 20), understanding the three-dimensional structure of the honeybee comb has been a practical and theroretical issue since the antiquity (review in Saucy, 2014). The three-rhombic configuration of the bottom of the cell,

Table 3. Comparison of the different approaches to measuring various comb parameters.

Characteristics	Speed	Use in field work	Precision	Natural comb	Artificial foundation	Reproducibility of measures	Remarks, advantages and recommendations
Linear measurements of (horizontal) rows of 10 cells	G	G	S	S	S	S	Best approach for field work
Linear measurements of all cells in a row	Nr	Nr	B	Na	B	S	Best approach for artificial foundation
Linear measurements of the whole comb	R	R	G	G	G	G	Best for estimates of whole comb area
Three measurements of 10 cells in a triangle pattern	R	R	R	R	R	G	Best for comb sampling in field work
Three measurements of 10 cells in a star pattern	Nr	S	S	S	G	G	Good for comb sampling in field work
Measurements in horizontal and vertical directions	Nr	S	S	Nr	G	G	For estimates of cell distortion
Ruler	S	S	S	S	S	S	Lowest precision
Caliper	S	G	G	G	G	G	Best precision in field
Picture	B	B	B	B	B	B	Best reproducibility

B: best; G: good; Na: not applicable; Nr: not recommended; R: recommended; S: satisfactory.

widely accepted since Maraldi (1712), has been questioned by Pirk et al. (2004), but later confirmed by Hepburn et al. on the basis of polyester resin mouldings. It can therefore be accepted as an established fact.

4.1.3.1. Measuring the depth and estimating the internal volume of the cell. Because of its particular geometry, measuring the depth of the honey bee cell is not an easy task. Three different measurements can be taken, depending on the purposes of the study:

a. along JR (the longest possible measurement: h_1) (Figure 19),
b. along CO, AN or EP (the smallest side lengths: h_2) and
c. along BH, DK or FM (the longest side lengths: h_3).

From the geometry, $h_3 = (h_1 + h_2)/2$. The volume of the cell is therefore given by:

$$V_{cell} = A_{cell} * h_3 = h_3 * \frac{\sqrt{3}}{2} d_i^2$$

Although cell depth is commonly measured as h_1, one should measure h_3 to compute estimates of the cell volume. In addition, one should also take into account the effect of the 10–14 cell's angle with the horizontal line perpendicular to the comb's vertical direction, which makes measurements quite complicated.

In practice, cell depth is measured as h_1, from the top to the bottom of the cell using the calliper's depth probe.

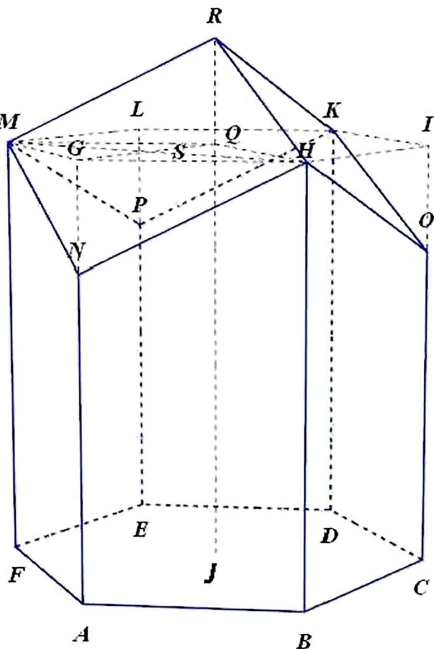


Figure 19. A three-dimensional view of the honeycomb cell (from Huber, 1814).

Alternatively, one could fill a row of 10 cells and measure their capacity using a pipette or weighing their water content. A proper method with estimations of sources of error is still to be developed.

4.1.3.2. Investigating the bottom of the cells. As already mentioned (Section 4.1.3; Figure 20), the bottoms of the cells are made of three rhombs connected to each

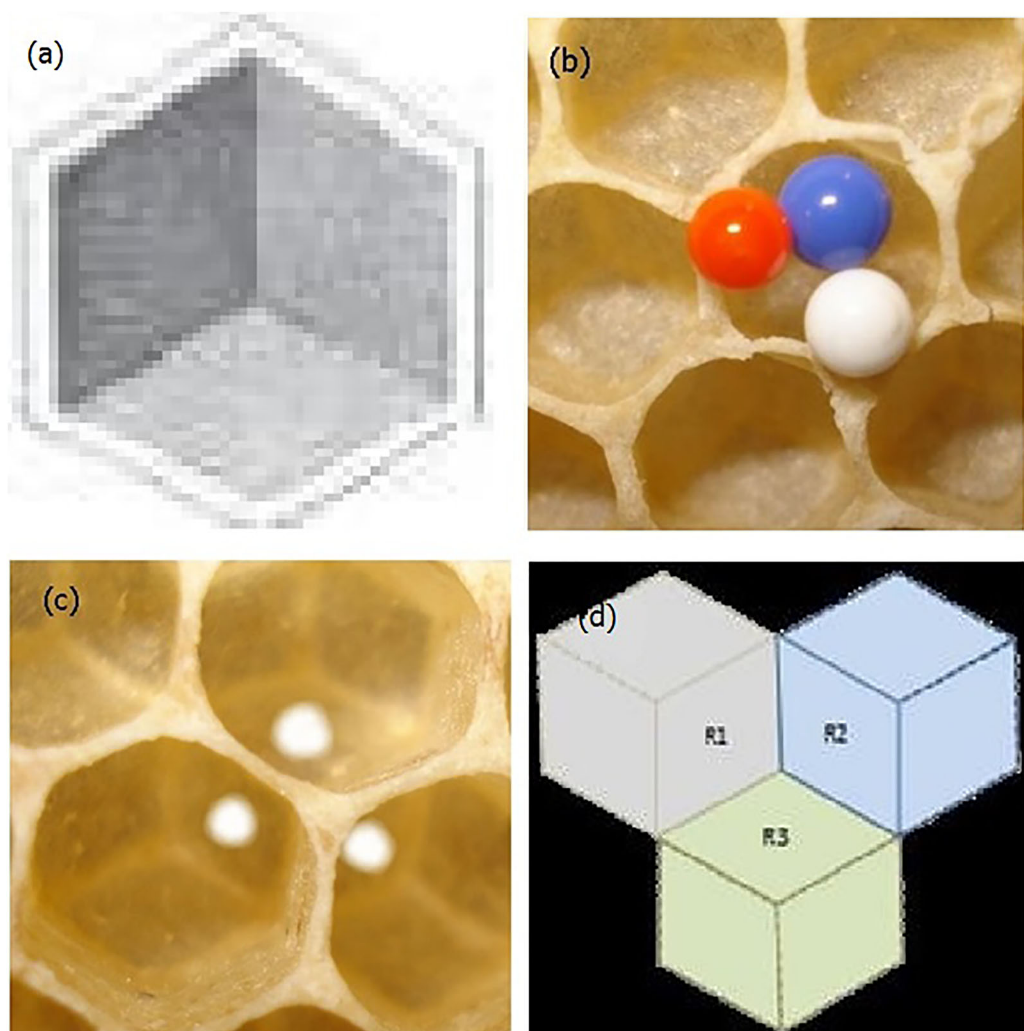


Figure 20. (a) Bottom of the cell showing the three rhombs and the reversed *Y* (*λ*) pattern resulting from their connections; (b) three pins in each rhomb of one cell; (c) produce 1 hole in 3 different cells on the other side of the comb; (d) three-dimensional model showing the reversed *Y* (*λ*) at the junction of R1, R2, and R3 and the upside *Y* in each of the three cells on the other side of the cell (from Saucy, 2014).

other on one edge and facing each other at angles of approximately 120°. This structure can be studied using mouldings with plaster or artificial resins (Hepburn, Muerrle, & Radloff, 2007; Vogt, 1911). Measurements can then be conducted on the dry moldings according to Vogt (1911).

4.1.3.3. Estimating the width and the external volume of the comb. In practice, since estimations refer to both sides of the comb, the difficulty of measuring the cell's depth is overcome using a measure of the average width of the comb based on several measurements (e.g., 1/4, 1/2, 3/4 of the length of the comb from the top to the bottom) using a calliper. The volume of the comb is then obtained as the area of the comb multiplied by its average width.

4.1.3.4. Estimating the capacity of the comb. The effective capacity of the comb, i.e., its internal volume excluding the volume of the wax, is of course smaller than its external volume. Moreover, it is reduced in old brood combs in which cocoons accumulate. Content can be estimated by filling them with water:

1. Clean the comb in a water bath (4 h; pollen might be difficult to remove).
2. Remove water from the comb.
3. Dry the comb overnight in a ventilated oven at 25–30 °C.
4. Weigh the comb.
5. Fill both sides with water using a shower.
6. Weigh the filled comb.
7. Subtract the weight of the dry comb from the weight of the filled comb to obtain the weight of the water.
8. Transform weight of the water into volume (1 kg = 1 L).

4.1.3.5. Documenting transitions between different cell types or junctions of combs. Transitions from one type of cell to

another (e.g., from worker to drone cells) appears not only from the two dimensional perspective, but also affect the three-dimensional aspects of the comb (Hepburn et al., 2014). Such transitions have already long been described (Huber, 1814) and documented as natural processes maintaining the function and integrity of the comb. They are difficult to quantify and should be described qualitatively. Some examples are depicted in Figure 21. Figure 21(a) shows the transition from worker to drone cells. Transitions as changes in cell size, bottom cell structure, as well as numbers of rows affected should be documented. For instance, Figure 21(b) shows a complete transition from the *Y* to the λ pattern across a range of 11 adjacent cells, with intermediate cells displaying four bottom plates facing 4 cells (instead of three) on the opposite side of the comb, while cell size remains unaffected.

4.2. Perspectives for methods in comb construction research

The architecture of the comb has fascinated the best human minds for centuries (reviews in Hales, 2001; Hepburn et al., 2014; Huber, 1814; Réaumur, 1742; Saucy, 2014; Thompson, 1945). According to some authors, bees are seen as architects able to master the geometry of the hexagonal cell and able to measure distances and angles (e.g., Bauer & Bienefeld, 2013; Huber, 1814; Nazzi, 2016; Réaumur, 1742), while others suggested that the hexagonal structure results from self-organizing processes depending on physical forces applied on originally cylindrical cells built in a malleable medium (e.g., Buffon, 1753; Darwin, 1859; Hepburn et al., 2007; Karihaloo, Zhang, & Wang, 2013; Pirk et al., 2004; Figure 14).

Understanding the structure of the comb and its building rules has practical consequences, e.g., for the production of comb foundation, as well as in health issues of the honey bee. For instance, the significance of the bees forming living chains while building comb (known as festoons) remains poorly understood. Despite interesting and promising studies, no standard methods have emerged yet.

4.2.1. Investigating comb construction and its growth pattern

To be able to observe every single bee active in the building process, Huber (1814) induced the bees to construct their combs from the bottom to the top in an upward direction. Although such combs seem undistinguishable from those built downwards, Huber's approach cannot be recommended as a standard method for other purposes. Usually researchers install and observe bees building downwards from the top. They smoke them out at times to observe and periodically record the progress of their work. Investigations the comb's growth pattern have been reviewed in great detail by Hepburn et al. (2014). The many experimental approaches reported suggest that the methodology should be developed according to the questions under scrutiny. For instance, honey bee combs may exhibit various cell shapes (e.g., pentagons, squares, heptagons) aside from the typical hexagonal cell, various sizes as well as amazing plasticity in their organization (horizontal, oblique, vertical rows, rosettes, etc.). Investigating these patterns involves experimentally manipulate cell sizes or patterns on embossed wax foundation, submit bees to restrictive conditions to assess their plasticity limits (Hepburn et al., 2014).

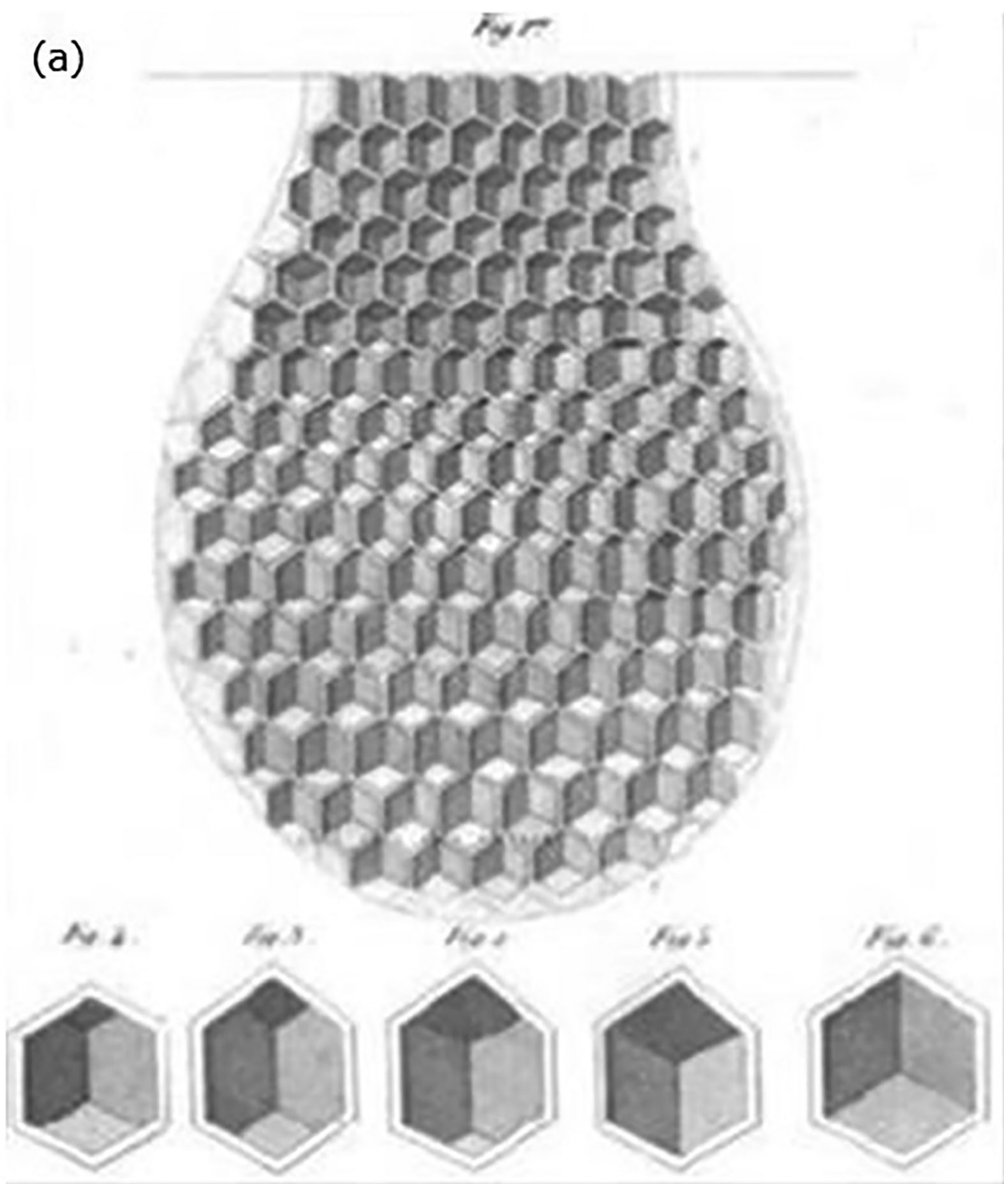
4.2.2. Investigating timing and type of comb construction

Under natural conditions, comb construction depends strongly on the time of the year and on the availability of nectar, with wax production reaching its climax when vegetation blooms and nectar becomes superabundant (Bogdanov, 2016a, 2016b). Many other factors may affect or stimulate wax production, such as outside temperature, the presence/absence of an egg-laying queen, pollen availability, ratio of brood combs, ratio of unoccupied combs, population of workers or drones, as well as available space in the nest cavity (Hepburn et al., 2014). These factors are often intermingled and correlated (Pratt, 2004). For instance, in temperate climates of both southern and northern hemispheres, the vegetation bloom is correlated with increasing sunlight and average temperature, as well as abundance of pollen and nectar. To address the importance and the relative contribution of these factors, we recommend apply classical methodological approaches involving a strong experimental design linked to appropriate statistical analyses according to Pirk et al. (2013). Construction may be measured counting the number of built cells of each type (worker, drones, honey), measuring comb area and weighing comb produced at the end of the experiment. Timing can be investigated experimentally manipulating external factors (such as day duration, food abundance) under laboratory conditions or replicating the experiments at various times of the year, or at different locations. The following approach should be applied:

1. Clearly define the goals of the study and identify the factors to test.
2. Identify major possible confounding factors to control or to monitor.
3. Build a solid experimental design taking account of all these factors.
4. Define the statistical methods to use before starting the experiments.
5. Conduct the experiments, collect the data and perform the statistical analyses.

5. Standard methods for beeswax chemical characterization

Numerous studies employing different gas chromatography (GC) and/or gas chromatography–mass



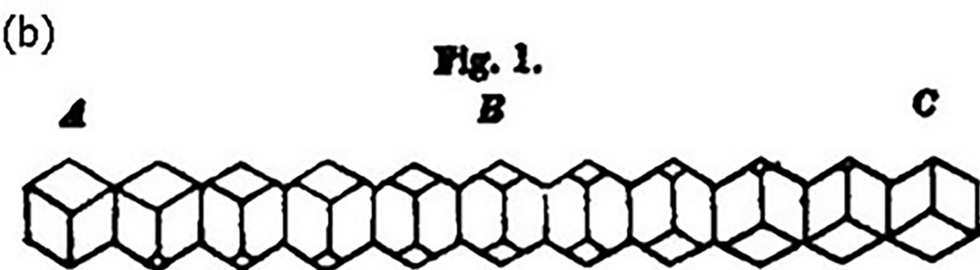


Figure 21. (a) Transition from worker to drone cells (Huber, 1814); (b) transition from an upside *Y* to a reversed *Y* (λ) cell pattern over 11 cell rows (Wyman, 1866).

spectrometry (GC-MS)-coupled analytical methods allowed detailed insight into beeswax chemical composition (Aichholz & Lorbeer, 1999, 2000; Jiménez, Bernal, Aumente, Toribio, & Bernal, 2003; Jiménez, Bernal, Aumente et al., 2004; Jiménez, Bernal, del Nozal, Martín, & Bernal, 2006; Maia & Nunes, 2013; Serra Bonvehí & Ornantes Bermejo, 2012; Tulloch, 1980; Waś, Szczęsna, & Rybak-Chmielewska, 2014a, 2014b).

Besides GC-coupled techniques, other analytical tools have also been employed for investigating beeswax, but focusing on more specific aspects of beeswax research. Hence, liquid chromatography (LC) techniques

were mostly used for determination of pesticide residues in beeswax (Bernal, del Nozal, Toribio, Jimènez, & Atienza, 1997; Jiménez, Bernal, del Nozal, & Alonso, 2004; Pirard et al., 2007; Yáñez, Martín, Bernal, Nozal, & Bernal, 2014), while Fourier transform infrared spectroscopy (FTIR) was employed for the chemical fingerprinting of beeswax, as well as successful detection of adulterants in beeswax (Maia et al., 2013; Svečnjak, Baranović et al., 2015). Furthermore, isotope ratio mass spectrometry (IRMS) was found to be very useful in establishing the geographical origin of beeswax (Chesson, Tipple, Erkkila, Cerling, & Ehleringer, 2011; Tipple, Chesson, Erkkila, Cerling, & Ehleringer, 2012). All of the above methods are described in the following sub-sections of Sections 5 and 6. Due to their complexity and very recent application for beeswax analysis, FTIR-ATR and IRMS methods are described in more detail in Sections 5.3 and 5.5, respectively.

5.1. Chemistry of beeswax

Understanding the chemical composition of beeswax is of crucial importance when applying chromatographic and spectroscopic analytical methods, which are commonly used for the chemical investigation of beeswax. Here, we present an overview of the major constituents of beeswax to enable easier interpretation of results obtained by the methods described in Sections 5 and 6.

5.1.1. Beeswax constituents

Beeswax derived from *A. mellifera* comb wax is a lipid-based complex mixture of more than 300 constituent fractions; it consists mainly of esters (67%), hydrocarbons (14%), free acids (12%), free alcohols (1%), and other (unidentified) constituents (6%), as presented in Table 4 (Tulloch, 1980). Specific chemical composition of beeswax holds a significant importance for a honey bee colony given that comb wax mediates the acquisition of nestmate recognition cues in honey bees (Breed, Williams et al., 1988, et seq.), and due to its capturing of toxins. The major compound families in beeswax are defined as those exceeding 5% (w/w) of the total beeswax composition (alkanes, alkenes, free fatty acids, monoesters, diesters and hydroxymonoesters); those of lesser abundance are regarded as minor constituents representing <5% of the total composition (fatty alcohols) (Aichholz & Lorbeer, 1999, 2000; Hepburn et al., 2014). However, according to Tulloch (1980), only several individual constituent fractions constitute more than 5% of the total beeswax composition, namely C40 monoester (6%), C46 monoester (8%), C48 monoester (6%) and C24 (lignoceric/tetracosanoic) acid (6%). Aichholz and Lorbeer (1999, 2000) also observed that each compound family in beeswax represents a series of homologues differing in chain length by two carbon atoms. The list of major and minor compound families, and their chain lengths (number of carbon atoms), are summarized in Table 5.

Hydrocarbons. The total content of hydrocarbons determined in beeswax ranges between 12.3 and 17.8%, as summarized by Waś et al. (2014b). Beeswax is mostly comprised of straight chain (unbranched) saturated hydrocarbons (12.8%), with a predominant chain length of C27–C31. More specifically, the most numerous group of beeswax hydrocarbons are linear saturated hydrocarbons (n-alkanes), accounting for ca. 67% of all hydrocarbons occurring in beeswax, while branched alkanes occur in much smaller amounts (0.2%) (Streibl et al., 1966). Moreover, the percentage share of the odd-numbered n-alkanes is significantly higher in comparison to even-numbered alkanes and amounts to about 95% (Waś et al., 2014a, 2014b).

Numerous studies revealed that the number of carbon atoms in unbranched alkanes in beeswax is generally ranging from C17 to C35 (Aichholz & Lorbeer, 1999, 2000; Downing, Kranz, Lamberton, Murray, & Redcliffe, 1961; Jiménez, Bernal, Aumente et al., 2004; Maia & Nunes, 2013; Serra Bonvehí and Ornantes Bermejo (2012; Tulloch, 1980; Waś et al., 2014a, 2014b; White, Reader, & Riethof, 1960).

The alkane C27 (heptacosane) is the most abundant hydrocarbon in beeswax, and comprises 6.2% of the total composition of *A. mellifera* wax (Aichholz & Lorbeer, 1999). The presence of even higher amounts of heptacosane was reported in recent study by Maia and Nunes (2013); the average percentage of C27 alkane determined in virgin beeswax was 13%, ranging from 9.19 to 16.8%, followed by nonacosane (C29) and hentriacontane (C31). The most recent study investigating hydrocarbon composition of *A. mellifera* beeswax collected from light and dark coloured combs (Waś et al., 2014b), confirms previously reported results; the highest contents were determined for C27, C29, C31, and C25, with an average of 34.0, 23.5, 20.3, and 8.6%, respectively, of the total content of unbranched alkanes in the light virgin beeswax (dark comb wax showed slightly different values, 34.5, 22.5, 18.4, and 10.1%, respectively). The proportion of less abundant (2.9%)

Table 4. Chemical composition of European *A. mellifera* comb wax (Tulloch, 1980).

Constituent fraction	% (w/w)
Hydrocarbons	14
Monoesters	35
Diesters	14
Triesters	3
Hydroxy monoesters	4
Hydroxy polyesters	8
Acid esters	1
Acid polyesters	2
Free acids	12
Free alcohols	1
Unidentified	6
Total	100

Table 5. Compound families identified in *A. mellifera* comb wax (Aichholz & Lorbeer, 1999, 2000).

Compound family	% (w/w)	Chain length (number of C atoms)
Hydrocarbons	15.7	C23–C35
Saturated (alkanes)	(12.8)	C23–C35
Unsaturated (alkenes)	(2.9)	C29–C33
Fatty acids	18	C20–C36
Lignoceric (tetracosanoic) acid	(5.8)	C24:0 (saturated)
		C14–C36[a]
Fatty alcohols	0.6	C33, C35
		C24–C34[a]
Monoesters (palmitates, oleates)	40.8	C38–C54
	(6.6)	C40
	(11.9)	C46
	(9)	C48
Hydroxymonoesters	9.2	C40–C50
Diesters	7.4	C54–C64
Esters total	57.4	C38–C64
Total	91.7	C14–C64

[a]Reported by other authors: Serra Bonvehí and Orantes Bermejo (2012) and Jiménez et al. (2003, Jiménez, Bernal, Aumente et al., 2004, Jiménez et al. 2006)

unsaturated hydrocarbons (alkenes) increases with the chain length (>C29), while dienes (C31–C35) were reported at only low, almost insignificant levels (Giumanini, Verardo, Strazzolini, & Hepburn, 1995; Jiménez, Bernal, Aumente et al., 2004, Jiménez et al., 2006; Jiménez, Bernal, del Nozal, Toribio, & Bernal, 2007; Maia & Nunes, 2013; Waś et al., 2014a, 2014b).

None of the major structural hydrocarbons of honey bees (i.e., n-alkanes) produces a positive result in a recognition bioassay, nor do these compounds differ significantly in relative concentration among families of bees. However, hydrocarbons that are present in smaller quantities, alkanes hexadecane and octadecane, and alkenes heneicosene and tricosenee, yielded positive results as nestmate recognition signals (Breed, 1998; Breed, Garry et al., 1995).

Fatty acids. A. mellifera beeswax contains remarkably high levels of free fatty acids (18%) in comparison to beeswaxes of other species studied, *A. cerana* (3.6%), *A. florea* (0.8%), *A. andreniformis* (2.6%), *A. dorsata* (4.9%), *A. laboriosa* (4.3%), respectively (Aichholz & Lorbeer, 1999). It mostly contains saturated, unbranched long-chain fatty acids (ca. 85%) with an even number of carbon atoms (C14–C36). The most abundant free fatty acid of *A. mellifera* beeswax is tetracosanoic (lignoceric) acid (Aichholz & Lorbeer, 1999, 2000; Jiménez et al., 2003; Serra Bonvehí and Ornantes Bermejo (2012; Tulloch, 1980), comprising more than 5% of the total beeswax composition (Aichholz & Lorbeer, 1999; Tulloch, 1980). Serra Bonvehí and Ornantes Bermejo (2012) gave a detailed overview of beeswax fatty acids profile and reported C16 (palmitic) as the main acid among saturated group, with a concentration of about 14.8%, followed by tetracosanoic (3.24%) and octacosanoic (C28) acid (1.80%). In relation to the unsaturated acids, C18:1 (oleic) represents major compound (3.90%), followed by C18:2 (linoleic). Lower quantities of other unsaturated fatty acids (primarily C16:1–C36:1), such as hexadecenoic C16:1 acid (palmitoleic), docosenoic C22:1 and eicosenoic C20:1, were also reported (Jiménez et al., 2003).

Fatty acids play an important role in honey bee colony as a source of nestmate recognition cues, thus allowing a discrimination of nestmate vs. non-nestmate individuals (along with other, queen derived and environmentally acquired "colony odours") (Breed, Williams et al., 1988, et seq.; Hepburn et al., 2014). The pattern of nestmate recognition within a honey bee colony has been widely studied by Breed, Williams et al. (1988), Breed, Stiller, and Moor (1988), Breed and Stiller (1992), Breed, Garry et al. (1995), Breed, Page, Hibbard, and Bjostad (1995), Breed, Leger, Pearce, and Wang (1998), and Breed, Diaz, and Lucero (2004) in a series of bioassays. The results of these studies revealed that fatty acids, rather than hydrocarbons, represent a key element in honey bee nestmate recognition which was also confirmed by other authors (Brockmann, Groh, & Fröhlich, 2003; D'ettorre et al., 2006; Fröhlich, Riederer et al., 2000). Accordingly, fatty acid recognition cues include four unsaturated acids (palmitoleic, oleic, linoleic, and linolenic) and two saturated fatty acids (palmitic and lignoceric). These acids are present in beeswax in substantial quantities and provide a chemical signature on the surface of bees that identifies to which colony they belong. Although very abundant in the free fatty acid fraction of beeswax, stearic acid proved to be inactive as a recognition cue (Breed, 1998). The role of fatty acids in the mechanical properties of beeswax (particularly resilience and stiffness) confirmed by Buchwald, Greenberg, and Breed (2005), Buchwald, Breed, Greenberg, and Otis (2006), and Buchwald, Breed, Bjostad, Hibbard, and Greenberg (2009), should also be mentioned here.

Fatty alcohols. Free fatty alcohols (~1% of the total beeswax composition) with a chain length of C28–C35 are minor compounds. Saturated fatty alcohols C30 and

C32 have been reported in higher concentrations by Serra Bonvehí and Ornantes Bermejo (2012), while Aichholz and Lorbeer (1999, 2000) identified C33 and C35 fatty alcohols in *A. mellifera* alcohol fraction.

Esters. Monoesters are the most abundant fraction of beeswax. The total content of the monoesters comprises more than 35% of *A. mellifera* beeswax. Beeswax monoesters are linear wax esters almost entirely derived from palmitic and oleic acid (palmitates, oleates), and comprise two main types of structures: saturated esters, which are predominantly alkylpalmitates (C38–C52), and unsaturated esters, mostly alkyloleates (C46–C54) (Aichholz & Lorbeer, 1999, 2000; Tulloch, 1980). These findings are consistent with results reported in other studies (Jiménez et al., 2003; Jiménez, Bernal, Aumente et al., 2004; Maia & Nunes, 2013; Serra Bonvehí and Ornantes Bermejo (2012). The content of hydroxymonoesters and diesters is significantly smaller in comparison to monoesters, 9.2 and 7.4%, respectively (Aichholz & Lorbeer, 1999). Two types of hydroxyesters structure dominate in beeswax: long chain alcohols, esterified by a hydroxy acid (mainly 15-hydroxypalmitic acid), and diols esterified with palmitic acid (Aichholz & Lorbeer, 1999, 2000; Maia & Nunes, 2013; Tulloch, 1971). The presence of smaller quantities of diesters, triesters and higher esters was determined by the same authors.

5.2. Investigation of beeswax composition by gas chromatography-mass spectrometry (GC-MS) and other GC-coupled techniques

Definitions, acronyms:

- GC: gas chromatography
- MS: mass spectrometry
- GC-MS: gas chromatography–mass spectrometry (gas chromatography with mass detector technique)
- IS: internal standard
- SPE: solid phase extraction
- FID: flame ionization detector
- GC-FID: gas chromatography coupled with flame ionization detector
- EI: electron impact ionization

Gas chromatography with different detectors (FID, MS) is commonly used in studies of beeswax composition (Aichholz & Lorbeer, 1999, 2000; Giumanini et al., 1995; Jiménez et al., 2003, 2004, 2006, 2007; Maia & Nunes, 2013; Namdar, Neuman, Sladezki, Haddad, & Weiner, 2007; Serra Bonvehí and Ornantes Bermejo (2012; Waś et al. 2014a, 2014b). The majority of compounds belonging to certain homologous series have been characterized by different researchers (Table 6). The GC-MS is one of the latest techniques used in testing the chemical composition of beeswax. In many cases GC-MS and GC-FID complement each other. The GC-MS allows identification of unknown compounds based on the mass spectra without using standards, as described by Torto et al. (2013) in the *BEEBOOK* chapter on chemical ecology. It is mainly used for qualitative analysis of beeswax composition (Jiménez et al., 2003, Jiménez, Bernal, Aumente et al., 2004; Maia & Nunes, 2013; Serra Bonvehí and Ornantes Bermejo (2012), while GC-FID is preferable for quantification (Jiménez, Bernal, Aumente et al., 2004, Jiménez et al., 2007; Maia & Nunes, 2013; Serra Bonvehí and Ornantes Bermejo (2012).

The GC-MS method elaborated by Waś et al. (2014a) allows in a single run for identification of beeswax hydrocarbons (alkanes, alkenes and dienes) as well as for quantification of n-alkanes, the biggest group of hydrocarbons occurring in beeswax.

This method is described in Section 5.2.1 and is recommended as standard method for analysis of beeswax hydrocarbons. However, for determination of other beeswax constituents such as esters, acids and alcohols, other methods elaborated by different authors should be taken into consideration (Jiménez et al., 2003; Jiménez, Bernal, Aumente et al., 2004; Jiménez et al., 2006; Maia & Nunes, 2013; Serra Bonvehí and Ornantes Bermejo (2012).

In the following subsections we also present the methods for simultaneous determination of hydrocarbons and monoesters (see Section 5.2.2), as well as fatty acids and fatty alcohols (see Section 5.2.3) by GC-MS and GC-FID according to Maia and Nunes (2013).

5.2.1. Determination of beeswax hydrocarbons by GC-MS

The most important advantage of the proposed GC-MS method according to Waś et al. (2014a) is that it allows the identification of beeswax hydrocarbons (alkanes, alkenes and dienes) and quantification of n-alkanes in a single run. Quantitative analysis of n-alkanes is conducted by the method of internal standard with squalane used as the internal standard. For extraction of hydrocarbons from beeswax the SPE technique with columns filled with neutral aluminum oxide is used (Waś et al., 2014a). There are many advantages of using SPE, which include mainly decreasing the time of chromatographic analysis and substantial simplification of the qualitative and quantitative analysis by improving detection and increasing selectivity of the determined compounds. Applying the SPE have the most significant effect for determining n-alkanes with even numbers of carbon atoms in a molecule, which are difficult to detect because they occur in beeswax in a very small amounts. In the case of determination of hydrocarbons without the preliminary purification of a sample, the n-alkanes peaks, especially even-numbered n-alkanes, overlap with the peaks of esters. This overlap can lead to inaccurate results of the quantitative analysis. Application of GC-MS method in analysis of beeswax hydrocarbons according to Waś et al. (2014a) allowed

Table 6. Investigation of beeswax composition with gas chromatography methods.

Identified groups of compounds	Method	Quantitative analysis	References
Hydrocarbons (dienes)	GC-MS (EI)	Relative abundances of parent ion	Giumanini et al. (1995)
Hydrocarbons (alkanes, alkenes, dienes), free fatty acids, monoesters, hydroxy monoesters, diesters, hydoxy diesters, fatty alcohols	GC-FID, GC-MS (CI)	Estimation of share (%) of individual compounds based on the ratio of the certain peak area to the sum of the areas of peaks for all of analyzed compounds (method of internal normalization)	Aichholz and Lorbeer (1999, 2000)
Hydrocarbons (alkanes, alkenes, dienes), monoesters, alcohols (monoalcohols, diols, propanetriols), fatty acids (saturated, unsaturated), hydroxyacids	GC-MS (EI)	Relative amounts (%) calculated from the peak areas and in relation to the peak of the most abundant compound	Jiménez et al. (2003, Jiménez, Bernal, Aumente et al., 2004)
Hydrocarbons (alkanes, alkenes, dienes), esters, acids, hydroxyacids, alcohols, monoesterified propanetriols	GC-FID	Expression in weight percentage referred to one standard	Jiménez et al. (2006, 2007)
Hydrocarbons (n-alkanes)	GC-FID, GC-MS (EI)	Relative peak areas normalized to the most abundant alkane	Namdar et al. (2007)
Hydrocarbons (alkanes, alkenes), monoesters, acids, alcohols	HTGC-FID/MS (EI)	Comparisons peaks areas with those of a reference external standard (eicosane) with the assumption the response of all compounds is equal	Serra Bonvehí and Orantes Bermejo (2012)
Hydrocarbons (alkanes, alkenes, dienes), monoesters	GC-FID, GC-MS (EI)	Percentage area method	Maia and Nunes (2013)
Hydrocarbons (alkanes, alkenes, dienes)	GC-MS (EI)	Internal standard method with using standard mixture of n-alkanes and squalane used a IS	Waś et al. (2014a, 2014b)

EI, electron ionization; CI, chemical ionization.

identifying these compounds and determining the ranges of their amounts in natural beeswax (Waś et al., 2014b). These values can be used as concentration guide-value to distinguish between pure and adulterated beeswax. The GC-MS method presented here is characterized by good linearity as well as satisfactory repeatability and within-laboratory reproducibility (Waś et al., 2014a). The disadvantage of proposed method may be that it only allows the analysis of hydrocarbons, but from the point of view of detection of beeswax adulteration with hydrocarbons of foreign origin, e.g., paraffin or ceresin, substances the most frequently used for this purpose, the method is very important and helpful (Waś, Szczęsna, & Rybak-Chmielewska, 2015; Waś et al., 2016).

The procedure for determining hydrocarbons in beeswax according to Waś et al. (2014a) is described in the following subections. Typical GC-MS chromatograms of beeswax hydrocarbons are presented in Figure 22(a–c).

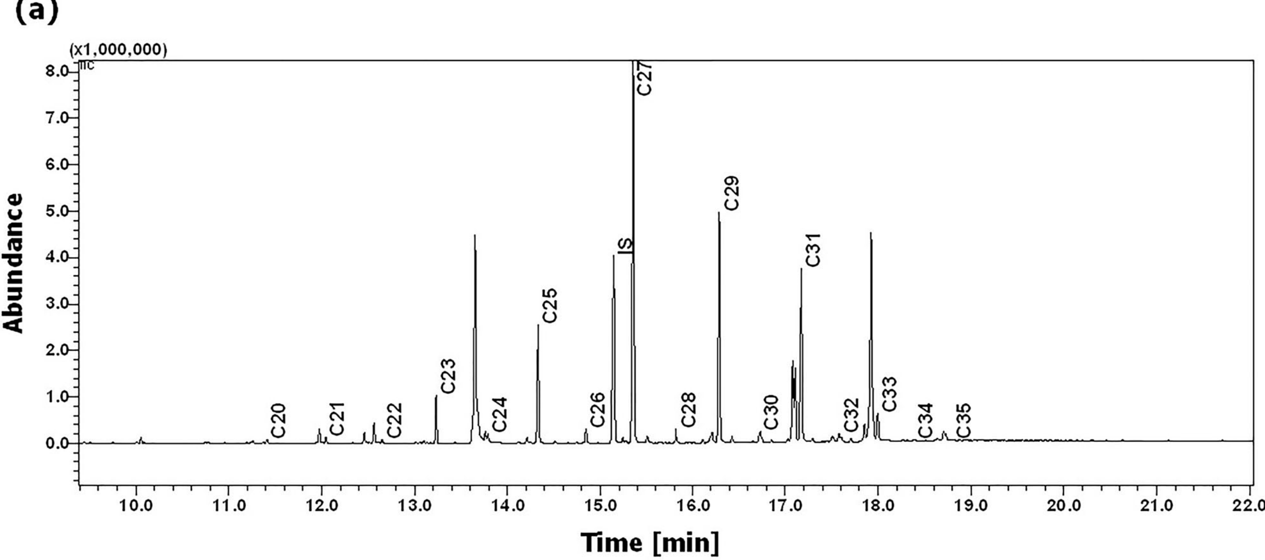
Equipment required:

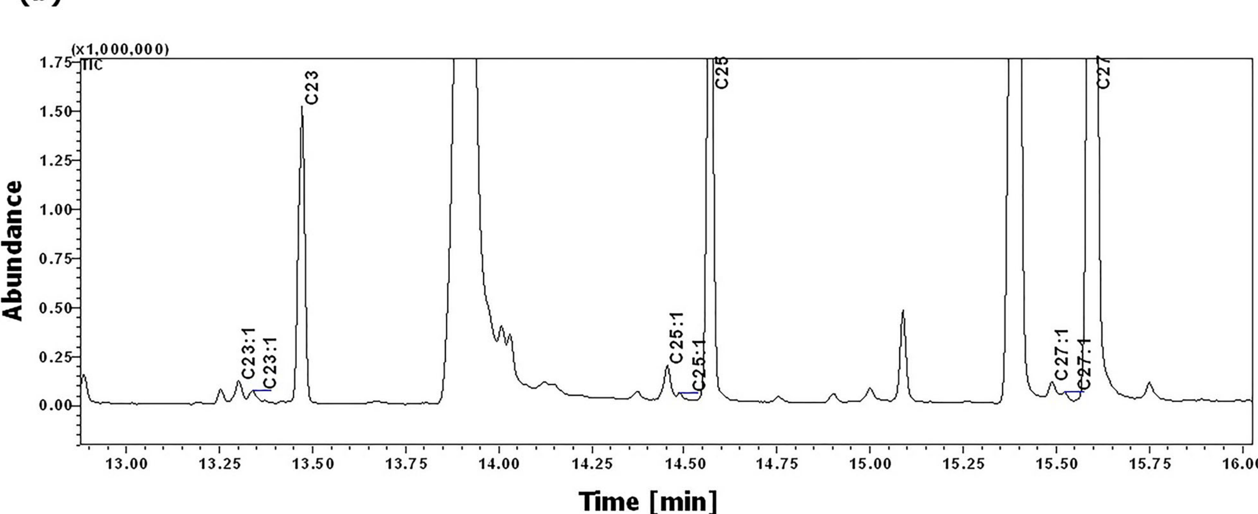
- GC-MS instrument such as Shimadzu GCMS-QP2010 Plus system, optionally equipped with autosampler
- Non-polar column ZB-5HT INFERNO (20 m × 0.18 mm × 0.18 µm, Phenomenex) or other with similar characteristics
- Incubtaor Shaker such as Innowa 40 (New Brunsfic Scientific)
- SPE Column Processor (J.T. Baker)
- Vortex
- Analytical balance (with accuracy ±0.001 g)
- Erlenmayers flasks of 100 mL with PTFE stopper
- Volumetric flasks of 10 and 100 mL
- Vials of 3 mL
- Autosampler vials of 2 mL
- Automatic pipettes (20–200 µL, 100–1000 µL, 1000–5000 µL).

Materials and reagents required:

All analytical standards and reagents used must be for gas chromatography (≥98% purity). The all reagents listed below comparable purity can be used from other companies.

- Standard mixtures of n-alkanes C_8H_{18}–$C_{20}H_{42}$ in hexane and $C_{21}H_{44}$–$C_{40}H_{82}$ in toluene at 40 mg/l





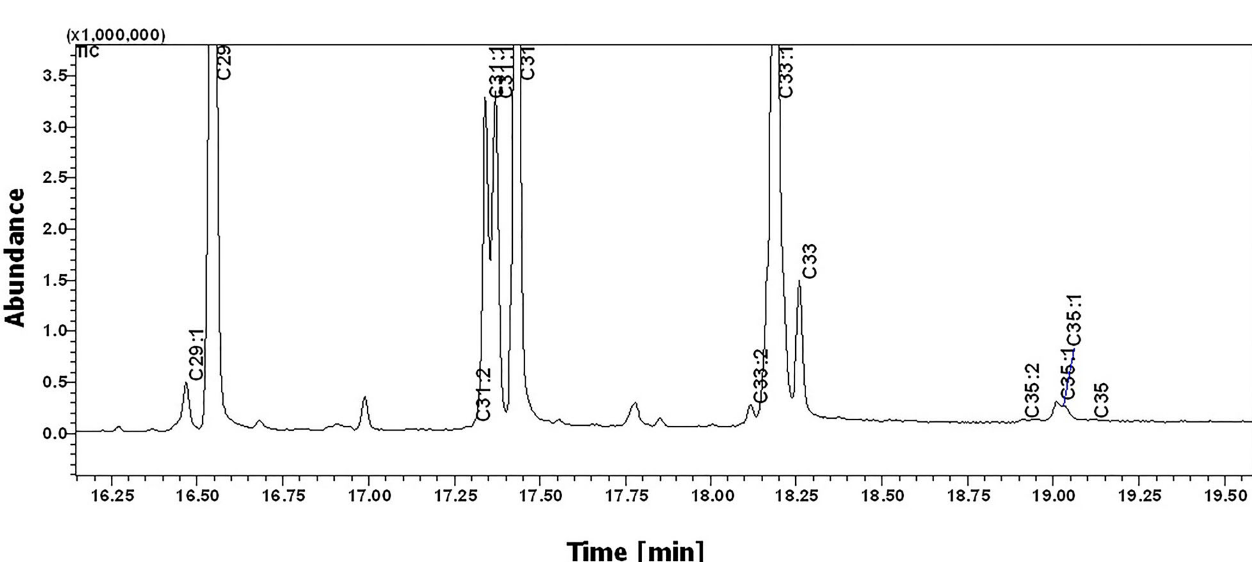


Figure 22. GC-MS chromatogram of hydrocarbons in beeswax: (a) saturated hydrocarbons, C20–C35 – n-alkanes with the formula $C_{20}H_{42}$–$C_{40}H_{82}$; IS – internal standard ($C_{30}H_{62}$); (b, c) unsaturated hydrocarbons (alkenes and dienes): 1,2 – number of double bonds in the molecule.

concentration of each (Fluka, Buchs, Switzerland; Saint Louis, MO, USA)

- Squalane, 99.9% purity (Supelco, Bellefonte, PA, USA)
- Hexane SupraSolv®, ≥98% purity (Merck, Darmstadt, Germany)
- Heptane anhydrous, ≥98.5% purity (Sigma-Aldrich, Steinheim, Germany)
- SPE cartridges (Alumina–N, 1000 mg, 6 mL (Agela Technologies, Wilmington, DE, USA).
- Helium, 99.9999% purity (Air Products, Warsaw, Poland).

5.2.1.1. Preparation of standard solution.

1. Prepare the stock solution of squalane ($c = 400$ mg/L).
   a. Weight the 40 mg of squalane ($C_{30}H_{62}$) with an accuracy of 0.2 mg.
   b. Dissolve it in a small amount (about 10 mL) of heptane.
   c. Transfer to a volumetric flask of 100 mL.
   d. Fill the flask to a 100 mL volume with heptane.
2. Prepare the solution of squalane ($c = 20$ mg/L).
   a. Pipette of 1 mL of squalane stock solution ($c = 400$ mg/L) into a volumetric flask of 10 mL.
   b. Fill to a 10 mL volume with heptane.
   c. Pipette of 1 mL solution into a vial of 3 mL.
   d. Add 1 mL heptane.
   e. Mix the solution on vortex.
3. Prepare the standard solution of 10 mg/L of n-alkanes (C_8H_{18}–$C_{40}H_{82}$) with IS ($C_{30}H_{62}$).
   a. Use a commercially available reference mixtures of n-alkanes (C_8H_{18}–$C_{20}H_{42}$) and ($C_{21}H_{44}$–$C_{40}H_{82}$) at 40 mg/L concentration of each.
   b. Pipette of 200 μL of each above standard mixtures of n-alkanes to the autosampler vial.
   c. Add of 400 μL of IS solution at 20 mg/L concentration.
   d. Mix the standard solution on vortex.

The standard solution of n-alkanes (C_8H_{18}–$C_{40}H_{82}$) with IS ($C_{30}H_{62}$) at 10 mg/L concentration is used for further GC-MS analysis.

5.2.1.2. Beeswax sample preparation. The preparation procedure for the samples of beeswax combs should begin with melting down the pieces of beeswax combs at a temperature of 70–75 °C and purifying on a filter made of gauze. Homogenized and free from mechanical impurities, beeswax should be kept in a dry and dark place (as described in Section 2.3) until further analysis. For the other beeswax samples (wax blocks or comb foundations), the sample preparation can be started from the preparation of beeswax solution for solid phase extraction (SPE) which is described below.

1. Prepare the beeswax solution ($c = 5$ mg/mL) for SPE.
   a. Weigh the 0.05 ± 0.001 g of beeswax.
   b. Add 7.5 mL of heptane.
   c. Shake the solution at 50 °C for about 12 min until the beeswax dissolved.
   d. Cool the solution to room temperature.
   e. Add 2.5 mL of squalane (IS) at 400 mg/L concentration.
   f. Mix a beeswax solution with IS on vortex.
2. Perform the SPE extraction of hydrocarbons from beeswax with neutral aluminum oxide (Alumina-N, 1000 mg, 6 mL).
   a. Wash the sorbent with 2 mL of hexane.
   b. Inject of 1 mL of beeswax solution in heptane ($c = 5$ mg/mL).
   c. Eluate of hydrocarbons from the intergrain spaces with 3 mL of hexane (in 3 portions of 1 mL).
   d. Collect the hydrocarbon fraction into a 10 mL flask.
   e. Fill the flask to a 10 mL volume with heptane.
   f. Transfer the solution to the autosampler vial.
   g. Use the solution to carry out chromatography analysis (see Section 5.2.1.3).

The SPE procedure used for isolation of hydrocarbons from beeswax is also schematically illustrated by Waś et al. (2014a).

5.2.1.3. Conditions of GC-MS analysis. The hydrocarbon composition of beeswax is analyzed by GC-MS after extraction using SPE described in Section 5.2.1.2. The GC-MS analysis should be conducted with appropriate instrument such as Shimadzu GCMS-QP2010 Plus system equipped with turbo molecular pomp, the GCMSsolution software, a non-polar column ZB-5HT INFERNO (20 m × 0.18 mm × 0.18 μm, Phenomenex) or other with similar characteristics, and optionally equipped with autosampler. The following steps describe in detail the parameters of GC-MS that could be applied for the hydrocarbon analysis of beeswax. The GC-MS condition settings can depend on the instrument with consideration to the optimization of the chromatographic separation.

1. Set the initial temperature of column at 80 °C (1 min), and then apply a temperature gradient of 15 °C/min until 340 °C, at which further separation of hydrocarbons is carried out.
2. Use the helium as the carrier gas, and set its flow through the column at 1.0 mL/min.
3. Set the temperature of the injector at 320 °C.
4. Inject the sample of 1 μL in the splitless mode and under 300 kPa (High Pressure Injection Mode).
5. Use the electron source (EI) with a standard ionization energy (70 eV), and the temperature of the ion source adjusted at 250 °C.
6. Set the interface temperature at 348 °C.
7. Use the value of voltage on the detector equal to the voltage obtained during autotune.
8. Set the range of mass scanning at 50–700 U.

5.2.1.4. Qualitative analysis of beeswax hydrocarbons. Identification of individual hydrocarbons of beeswax (n-

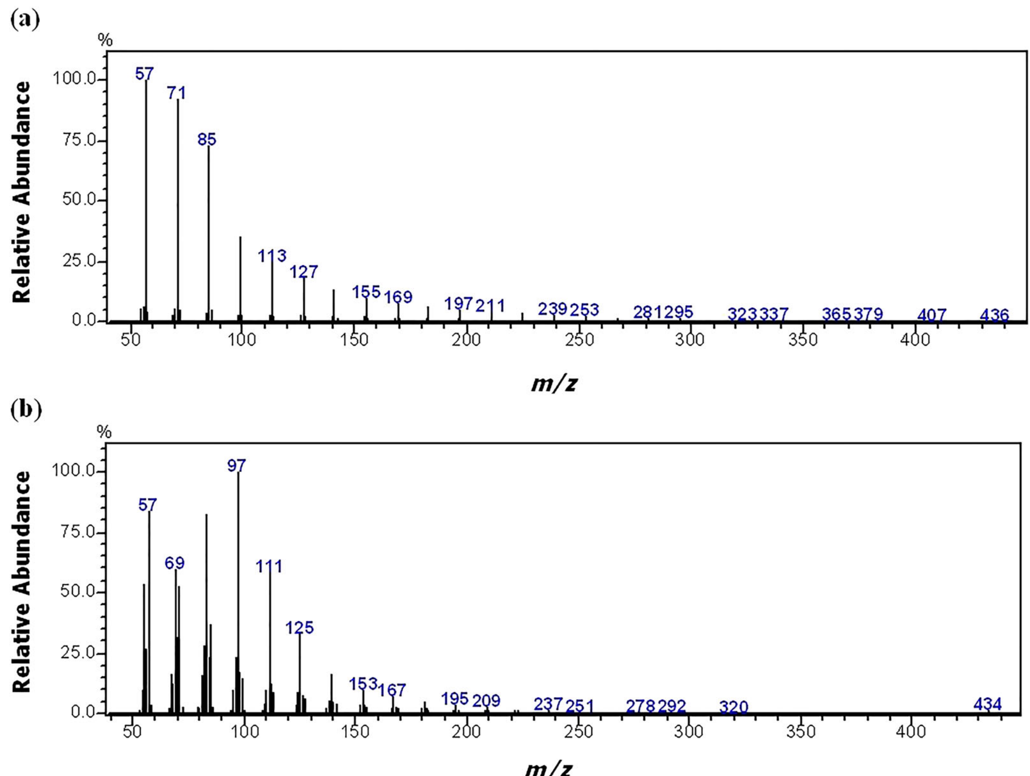


Figure 23. Electron impact spectrum of hydrocarbons: (a) hentriacontane ($C_{31}H_{64}$) with molecular ion of $m/z = 436$; (b) heneitriacontene ($C_{31}H_{62}$) with molecular ion of $m/z = 434$.

alkanes, alkenes and dienes) can be performed by comparison of the obtained mass spectra of n-alkanes with the mass spectra collected in commercial libraries (e.g., NIST 05 Mass Spectra Library). If no reference spectra are available, identification can be performed based on the fragmentation pathway and the presence of ions characteristic for the particular group of hydrocarbons in the following way. In mass spectra of n-alkanes and alkenes, certain groups of peaks should be identified when the *m/z* values differed by 14 Da. The peak of the highest intensity occurred in every group. In the case of n-alkanes, the peak corresponded to the ions $[C_nH_{2n+1}]^+$ and occurred when $m/z = 57$, 71, 85, 99, etc.; in the case of alkenes, the highest intensity had the peaks of the C_nH_{2n} and C_nH_{2n-1} ions. In addition, mass spectra of these compounds differ in molecular ions. Examples of the mass spectra of n-alkanes and alkenes are presented in Figure 23(a,b).

The qualitative analysis of n-alkenes can also be performed by comparison of the retention times of individual n-alkanes in the standard solution and the analyzed beeswax solution. If the retention time of the compound in beeswax sample corresponds to the retention time of the compound in the standard solution, it should be identified as the same compound.

The rules of identification of unknown compounds using GC-MS were also described in details by Torto et al. (2013).

5.2.1.5. Quantitative analysis of n-alkanes in beeswax. The quantitative analysis of n-alkanes is conducted using an internal standard method. General advantages resulting from using of internal standard in quantitative analysis are listed by Torto et al. (2013). The standard mixture of n-alkanes (C_8H_{18}–$C_{40}H_{82}$) is used with the internal standard - squalane ($C_{30}H_{62}$) at 10 mg/L concentration. Preparation of the standard solution is described in details in Section 5.2.1.1.

5.2.1.6. Advantages and disadvantages of GC-MS anylsis.

Advantages:

- precise, accurate and selective method
- good linearity as well as satisfactory repeatability and reproducibility
- suitable for the characteristics of beeswax hydrocarbons
- allows identification of the beeswax hydrocarbons (alkanes, alkenes and dienes) and quantification of n-alkanes (the most numerous group of hydrocarbons occurring in beeswax) in a single run
- preliminary purification of a sample with SPE, eliminates the overlapping of peaks, especially even-numbered n-alkanes with the peaks of esters

Disadvantages:

- allows only analysis of hydrocarbons
- expensive due to a very specialized equipment

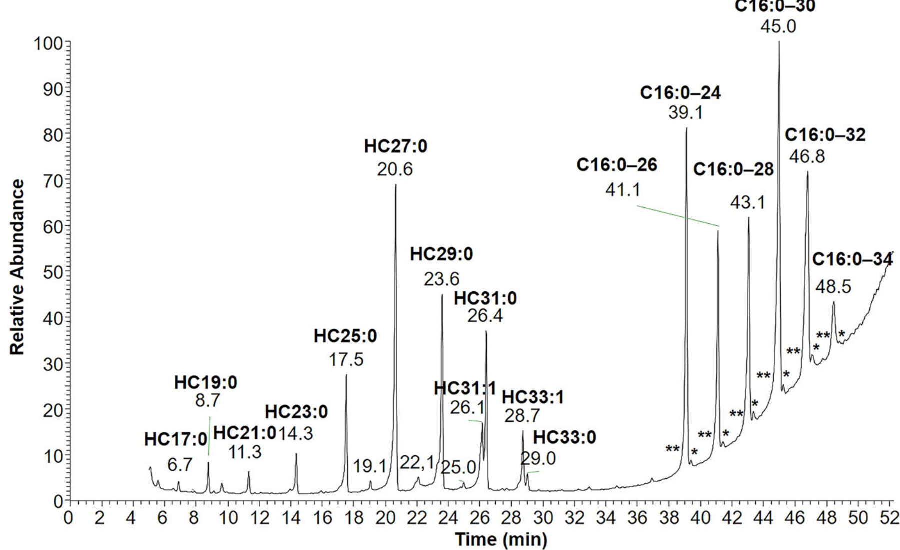


Figure 24. Typical GC-MS chromatogram of beeswax odd number hydrocarbons HC, even number hydrocarbons (unlabeled peaks between odd number hydrocarbons), palmitate monoesters C, oleate monoesters ** and 15-hydroxypalmitate monoesters * (Nunes & Maia, 2017, unpublished data).

- time-consuming at initial stage (preparation of sample using SPE)

5.2.2. Simultaneous analysis of monoesters and hydrocarbons by GC-MS and GC-FID

GC coupled with Flame Ionization Detector (GC-FID) is commonly used method for detection of volatile organic compounds. General analytical properties of CG-FID technique is provided in the *BEEBOOK* chapter on chemical ecology research in *A. mellifera*; for details see Section 2.2.4. "Detection and analysis of volatiles" (Torto et al., 2013). Using the methods described by Jiménez et al. (2004), and Maia and Nunes (2013) for the analysis of beeswax by GC-MS and GC-FID, odd number hydrocarbons (with 17–35 carbon atoms), even number hydrocarbons (with 22–34 carbons), and monounsaturated hydrocarbons with an odd number of carbon atoms (from 21 to 35) containing isomers with a different position of the double bond (although from the mass spectra the position of the double bond cannot be identified) can be determined qualitatively and quantitatively in a single run. Also, the highly abundant monoesters with carbon numbers from 34 to 50 corresponding to the esterification of palmitic acid (C16) with fatty alcohols containing 18 (1-octadecanol) to 34 (1-tetratriacontanol) carbon atoms) can be identified (Figure 24). Not all of the monoester components can be completely resolved under these chromatographic conditions, but their presence can be detected using the extracted ion chromatograms with specific ions for each monoester series: *m*/*z* 256 for the palmitate monoesters, *m*/*z* 264 for the oleate monoesters, *m*/*z* 236 for the hydroxypalmitate monoesters. The characteristic EI mass spectrum of palmitate monoesters is shown in Figure 25(a) for triacontyl palmitate, the most abundant palmitate ester found in beeswax, and in Figure 25(b) for the hydroxypalmitate monoester.

Equipment required:

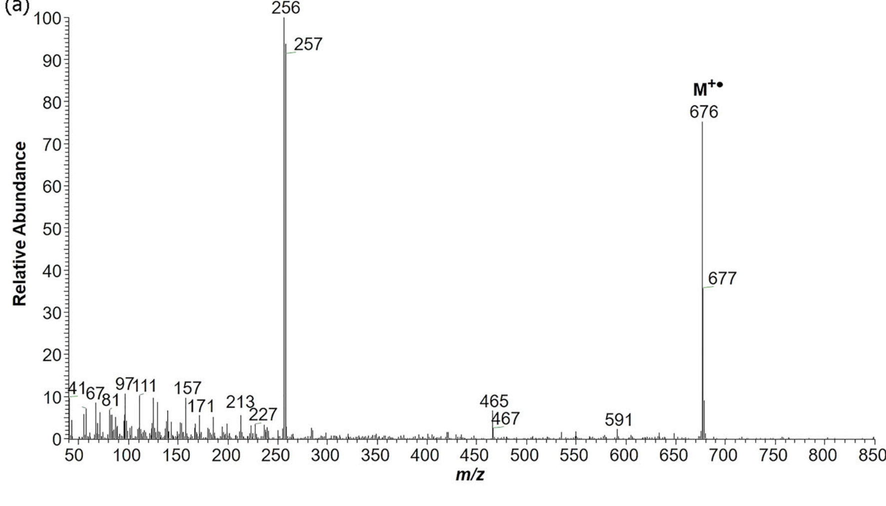
- Gas-chromatograph (GC) equiped with a mass spectrometer (MS) with electron impact ionization (EI)
- Gas-chromatograph (GC) equiped with a flame ionization detector (FID)
- Balance with a 0.01 mg precision
- Horinzontal shaker

Materials and reagents required:

- mL glass tube with screw cap with a teflon faced rubber
- Chloroform p.a.
- Stearylstearate p.a.
- High temperature resistant apolar column 30 m column with 0.25 mm ID and 0.25 μm film thickness (for example, ZB-5 Inferno column, Phenomenex, or an equivalent high temperature resistant apolar column)
- μL syringe
- Helium 99.999%
- Air 99.999%
- Hydrogen 99.999%

5.2.2.1. Preparation of beeswax solution.

1. Weigh 3 mg of beeswax.
2. Add 4 mL of chloroform (if quantitative analysis is needed, replace 2 mL of chloroform with 2 mL of the internal standard solution of stearylstearate at 2 mg/mL).
3. Shake the solution mechanically until all the sample is dissolved.



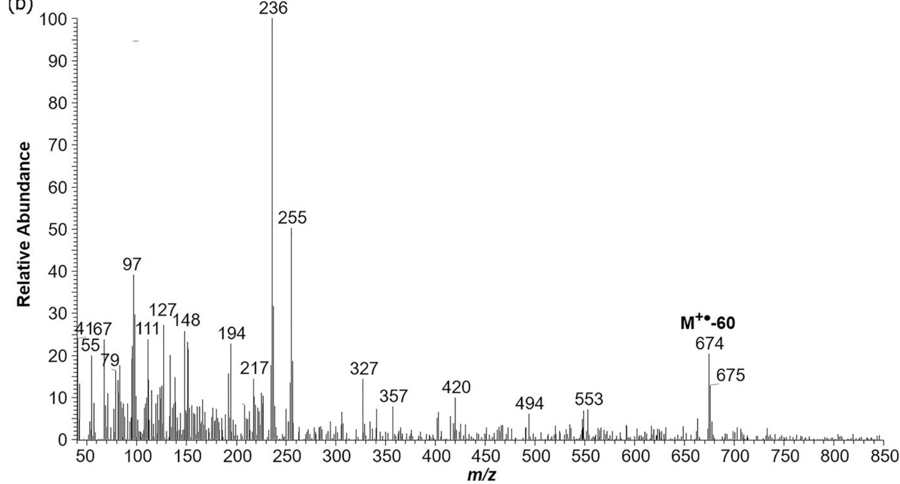


Figure 25. EI-MS of (a) triacontyl palmitate; (b) triacontyl hydroxypalmitate (Nunes & Maia, 2017, unpublished data).

4. Analyze the sample by GC-MS for qualitative analysis and GC-FID for quantitative analysis according to Sections 5.2.2.2 and 5.2.2.3 described below (for hydrocarbons analysis only see Section 5.2.1).

5.2.2.2. Qualitative analysis by GC-MS.

1. Use a 30 m ZB-5 Inferno column (Phenomenex) with 0.25 mm ID and 0.25 lm film thickness or an equivalent high temperature resistant apolar column.
2. Set the injector temperature at 325 °C.
3. Inject 1 µL of sample in a splitless mode with 2 min splitless time.
4. Use the following oven temperature program: initial temperature 50 °C, held for 3 min, a 50 °C/min ramp to 180 °C and held for 1 min, then a 3 °C/min ramp to 390 °C held for 5 min.
5. Use the carrier gas (He) at constant flow-rate of 1 mL/min.
6. Set the transfer line temperature at 350 °C.
7. Set the MS ion source temperature to 220 °C.
8. Set the scan range to *m*/*z* 40–850 with a total scan time of 0.72 s with a maximum ion time of 25 ms.
9. Use a solvent delay of 5 min.

5.2.2.3. Quantitative analysis by GC-FID.

1. Use a 30 m ZB-5 Inferno column (Phenomenex) with 0.25 mm ID and 0.25 µm film thickness or equivalent high temperature resistant apolar column for beeswax analysis.
2. Set the injector temperature at 325 °C.
3. Inject 1 µL sample in a splitless mode with 2 min splitless time.
4. Use the following oven temperature program: initial temperature 50 °C, held for 3 min, a 50 °C/min ramp to 180 °C and held for 1 min, then a 3 °C/min ramp to 390 °C held for 5 min.
5. Use the carrier gas (He) at a constant flow-rate of 1 mL/min.
6. Set the detector temperature at 400 °C.
7. Use hydrogen (20 mL/min) and synthetic air (200 mL/min) as auxiliary gases for the flame ionization detector.

After integration of all hydrocarbons peaks and internal standard in the obtained chromatogram, perform semi-quantification of each peak using the response factor calculated for the internal standard (IS) as:

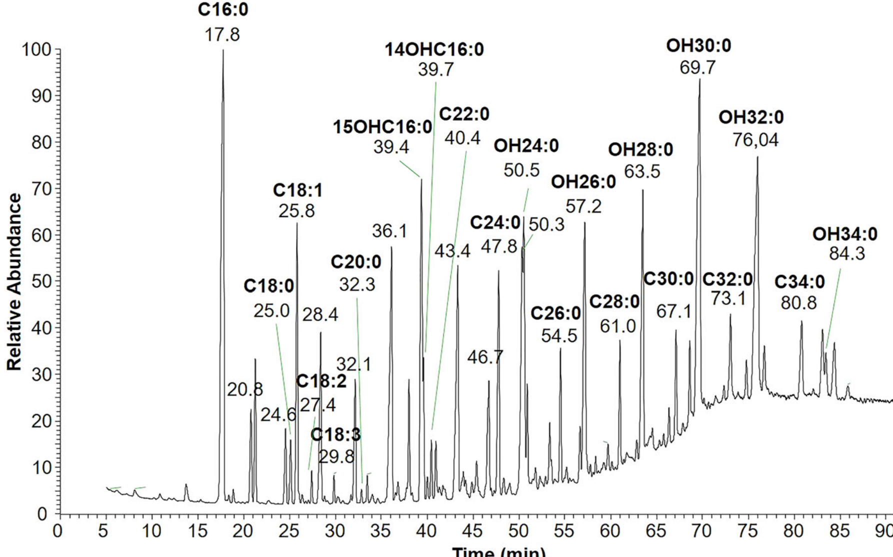


Figure 26. Typical GC-MS chromatogram of beeswax fatty acids and fatty alcohols analyzed by GC-MS after derivatization: fatty acids C; hydroxyl-fatty acids OHC; fatty alcohols OH. Unlabeled peeks correspond to hydrocarbons (Nunes & Maia, 2017, unpublished data).

$$\text{Response factor of IS} = \frac{\text{IS area}}{\text{weight of IS in sample injected}}$$

5.2.3. Simultaneous analysis of fatty acids and fatty alcohols by GC-MS and GC-FID

The fatty acids and fatty alcohols are two of the main beeswax components. Fatty acids are present in beeswax in the free form or esterified with fatty alcohols in the monoesters, mainly palmitates. Fatty alcohols are mainly present in monoesters with low amount present in the free form (Figure 26). For their characterization in beeswax, the determination of the total amount of fatty acids and fatty alcohols can be performed by GC-MS for qualitative analysis, and by GC-FID for quantitative analysis (Jiménez et al., 2003). According to the procedure described below, the method employed involves an esterification with methanol of the carboxyl groups of fatty acids followed by acetylation of the free hydroxyl groups of fatty alcohols and also of the hdroxyl fatty acids.

Equipment required:

- Gas-chromatograph (GC) equiped with a mass spectrometer (MS) with electron impact ionization (EI)
- Gas- chromatograph (GC) equiped with a flame ionization detector (FID)
- Balance with a 0.01 mg precision
- Dry-block heater
- Horinzontal shaker
- Centrifuge

Materials and reagents required:

- mL glass tube with screw cap with a teflon faced rubber
- Chloroform p.a.
- Methanol p.a.
- 14% BF_3 (boron trifluoride) solution in methanol p.a.
- Heptadecanoic acid p.a.
- Acetic anhydride p.a.
- Pyridine p.a.
- 30 m polar column with 0.25 mm ID and 0.25 µm film thickness (for example Supelcowax 10M column, Supelco).
- µL syringe
- Helium 99.999%
- Air 99.999%
- Hydrogen 99.999%

5.2.3.1. Preparation of beeswax solution and derivatization.

1. Weight 30 mg of beeswax for a 10 mL glass tube with screw cap with a teflon faced rubber.
2. Add 4 mL of chloroform containing 2 mg/mL of internal standard (Heptadecanoic acid C17:0).
3. Add 2 mL of methanol and 2 mL of a 14% BF_3 solution in methanol as catalyst (commercially available).
4. Close tightly the screw cap and heat during 1h at 90 °C in a dry-block heater.
5. After cooling to room temperature, transfer 2 mL of the solution to a new glass tube.
6. Add 2 mL of acetic anhydride and 0.2 mL of pyridine.
7. Close tightly the screw cap and heat during 2 h at 90 °C in a dry-block heater.
8. Cool the tubes in an ice bath during 15 min.
9. Add 2 mL of water and mix the contents thoroughly.
10. Centrifuge at 3000 rpm during 5 min to separate the phase and discard the upper aqueous phase.
11. Repeat steps 9 and 10 twice.
12. Analyze the sample by GC-MS for qualitative analysis and GC-FID for quantitative analysis according to Sections 5.2.3.2 and 5.2.3.3 described below.

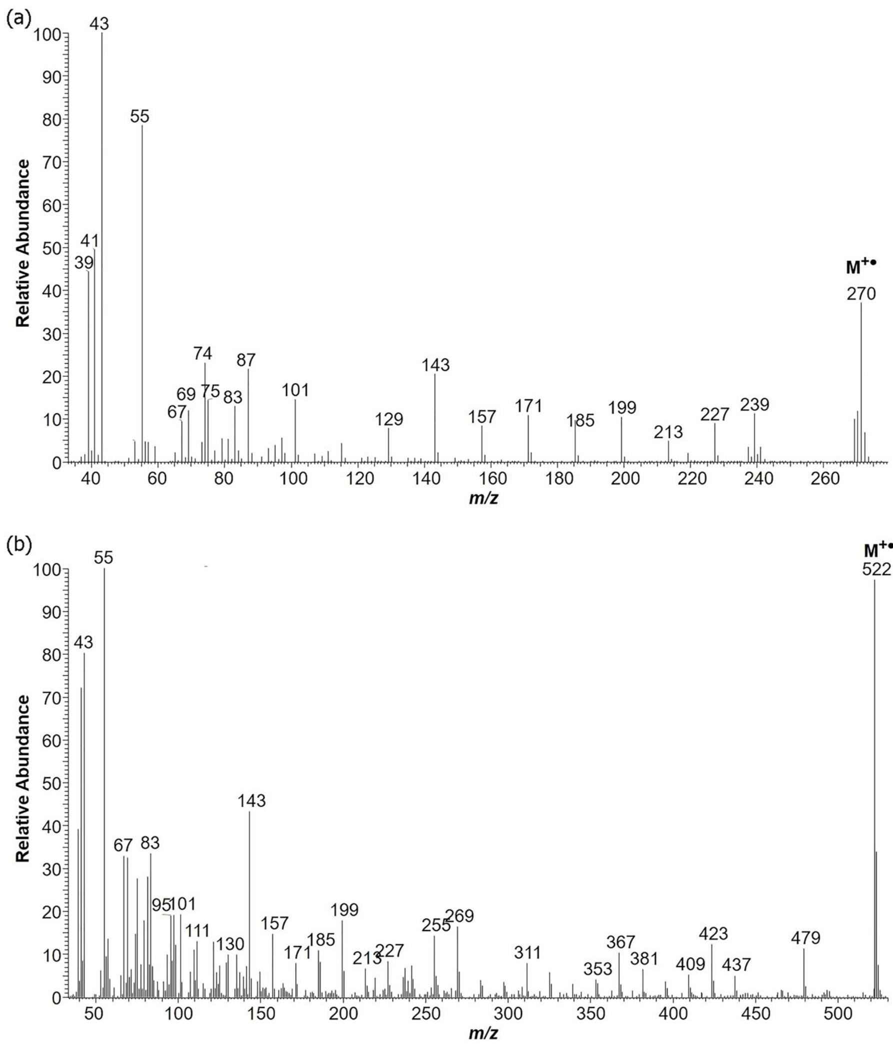


Figure 27. EI-MS of (a) palmitic acid methyl ester (C16:0), and (b) tetratriacontanoic acid methyl ester (C34:0) (Nunes & Maia, 2017, unpublished data).

The fatty acids methyl esters and acetyl fatty acids can be readily identified using an available standard or alternatively by comparison of the mass spectra available in several data bases. Fatty acids methyl esters can be easily identified by the presence of the molecular ion in their spectra (Figure 27). For the fatty alcohols the spectra are less characteristic and contain less distinguishing elements, contrary to fatty acids (Figure 28). The acetyl hydroxyl fatty acids methyl esters also present the molecular ion on their mass spectra although with a lower intensity, being also present the ion at *m*/*z* corresponding to the loss of an acetyl group (−60 Da) (Figure 29). For the acetyl fatty alcohols, the molecular ion is of very low intensity or absent for all practical purposes. Also present with low intensity is the ion resulting from the loss of an acetic acid (−60 Da). One of the most distinctive ions present in the mass spectra of acetyl fatty acids is the ion at *m*/*z* 61 presumed to be a protonated acetate moiety (Figure 28).

5.2.3.2. Qualitative analysis by GC-MS.

1. Use a 30 m Supelcowax 10M column (Supelco) with 0.25 mm ID and 0.25 μm film thickness or equivalent polar column for beeswax analysis.
2. Set the injector temperature at 280 °C.
3. Inject 1 μL of sample in splitless mode with a 2 min splitless time.
4. Use the following oven temperature program: initial temperature 140 °C, held for 1 min, a 2 °C/min ramp to 280 °C and held for 20 min.
5. Use a constant carrier gas (He) flow-rate at 1 mL/min.
6. Use a transfer line temperature of 280 °C.
7. Set the MS ion source temperature to 220 °C.
8. Set the scan range from *m*/*z* 33–650 with a total scan time of 0.72 s with a maximum ion time of 25 ms.
9. Use a solvent delay of 5 min.

5.2.3.3. Quantitative analysis by GC-FID.

1. Use a 30 m Supelcowax 10M column (Supelco) with 0.25 mm ID and 0.25 μm film thickness or equivalent polar column

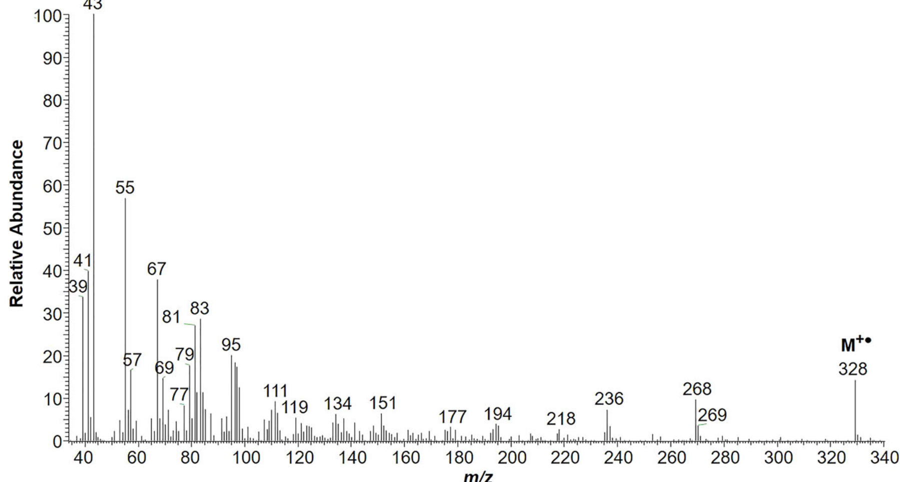


Figure 28. EI-MS of acetyl 1-triacontanol (OH30:0) (Nunes & Maia, 2017, unpublished data).

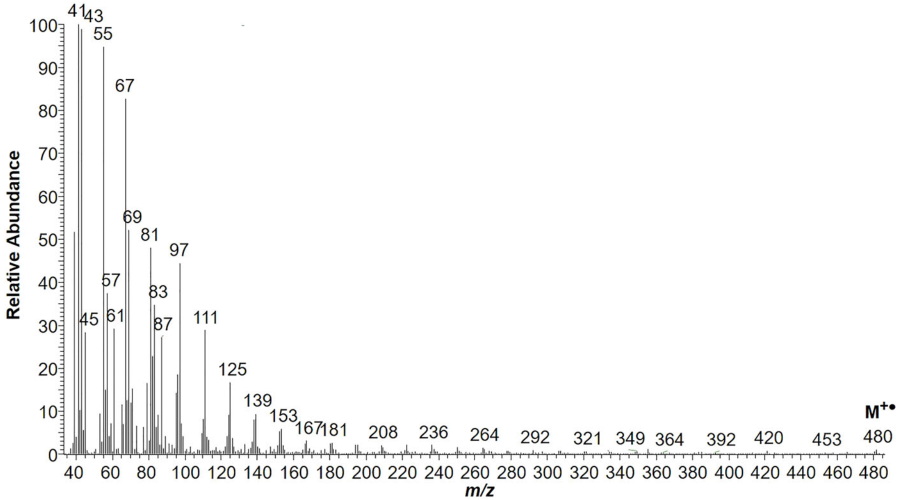


Figure 29. EI-MS of 15-hydroxypalmitic acid methyl ester (15OHC16:0) (Nunes & Maia, 2017, unpublished data).

2. Set the injector temperature at 280 °C.
3. Inject 1 μL of sample in a splitless mode with 2 min splitless time.
4. Use the following oven temperature program: initial temperature 140 °C, held for 1 min, a 2 °C/min ramp to 280 °C and held for 20 min.
5. The carrier gas (He) flow-rate is constant at 1 mL/min.
6. Set the detector temperature at 350 °C.
7. Use hydrogen (20 mL/min) and synthetic air (200 mL/min) as auxiliary gases for the flame ionization detector.

After integration of all fatty acids and fatty alcohols peaks and internal standard in the obtained chromatogram, perform semi-quantification of each peak using the response factor calculated for the internal standard (IS) as:

$$\text{Response factor of IS} = \frac{\text{IS area}}{\text{weight of IS in sample injected}}$$

5.2.4. Advantages and disadvantages of simultaneous analyses by GC-MS and GC-FID

Advantages:

- provide broader chemical characterization of the chemical composition of beeswax
- simultaneous analysis of hydrocarbons and esters or fatty acids and fatty alcohols in a single run
- minimum sample preparation requirments
- sensitive and selective method

Disadvantages:

- expensive due to a very specialized equipment
- require advanced chemical analysis training

5.3. Investigation of beeswax by infrared (IR) spectroscopy

Definitions, acronyms:

- IR: infrared
- λ: wavelenght
- ν: frequency
- cm^{-1}: reciprocal centimeter
- $\tilde{\nu}$: wavenumber
- mid-IR (mid-infrared region): spectral region between 4000 and 400 cm^{-1}
- fingerprint region: spectral region between 1500 and 500 cm^{-1} (approximatelly)
- FTIR: Fourier transform infrared (spectroscopy/spectrometer)
- ATR: Attenuated Total Reflectance (recording technique/accessory)
- T: transmittance (the amount of IR radiation passed through the sample)
- *A*: absorbance (the amount of IR radiation absorbed by the sample)
- a.u.: absorbance units
- DTGS: deuterated triglycine sulfate (detector)
- CsI: sesium iodide
- ZnSe: zinc selenide

5.3.1. Interaction of IR radiation with the sample

Infrared (IR) spectroscopy is a method that utilizes infrared radiation as a physical medium for investigating the molecular structure of the sample. The basic principle of IR spectroscopy involves an interaction of IR radiation with the sample where molecules are being excited by the radiation. When IR radiation is absorbed by the sample, the molecules begin to vibrate with greater amplitudes (molecules gain energy) and experience a wide variety of vibrational motions characteristic for their component atoms and functional groups.

In the spectrum of electromagnetic radiation, an infrared portion (invisible, harmless thermal radiation) ranges from 0.8 to 1000 μm (800 nm–1 mm) in the wavelength (λ), i.e., from the nominal red edge of the visible spectrum to the microwave radiation. Frequency (ν) is another parameter often encountered in IR spectroscopy, and represents the number of cycles/waves per second; in this context, the infrared portion extends from 375 THz to 300 GHz. However, a third unit, wavenumber ($\tilde{\nu}$), is the most widely used in IR spectroscopy. Wavenumber is the reciprocal of the wavelength ($1/\lambda$) expressed in reciprocal centimetres (cm^{-1}), which represents the spatial frequency of a wave, more specifically, the number of waves in a length of one centimetre. The most important argument for the application of the wavenumber in IR spectroscopy is its proportionality with the frequency of the electromagnetic field, and thus, its linearity with energy (the energy associated with a photon of IR light is directly proportional to its frequency). IR radiation induces vibrational excitation of covalently bonded atoms and groups within a molecule which helps in identifying the sample compounds due to strong relationship between vibrational energy and molecular structure.

According to the wavenumber, the infrared portion of the electromagnetic radiation covers a range from 12,500 to 10 cm^{-1}. The IR spectrum is further divided into three main regions: the far-infrared (<400 cm^{-1}), the mid-infrared (4000–400 cm^{-1}), and the near-infrared (12,500–4000 cm^{-1}), named after their relation to the visible spectrum. Mid-IR is most frequently used for biological applications as the majority of organic compounds exhibits characteristic absorption bands in this region. This is particularly referring to the spectral region between ~1500 and 500 cm^{-1} (considered as the *fingerprint region*) which typically contains a series of absorption bands appearing in the IR spectrum mostly due to bending and skeletal vibrations of atoms within a molecule.

IR spectroscopy represents a valuable analytical tool for identification and analysis of numerous compounds in various organic specimens. As a type of vibrational (molecular) spectroscopy, where molecular vibrations are being analyzed, IR spectroscopy provides information on the total chemical composition of a sample, and permits acquisition of the absorption IR spectra of compounds that are a unique reflection of their molecular structure. IR spectrum, therefore, represents a unique chemical "fingerprint" of a sample, which is readily distinguished from the absorption patterns of all other compounds; only optical isomers absorb IR radiation in exactly the same way (Skoog & Leary, 1992). Interaction of vibrations of all atoms in the sample gives extremely complex but very characteristic absorption of IR radiation which prominent in the IR spectrum. Intensive IR signals, as well as weak bands, indicate a specific functional group positions and intensities associated with specific molecules in the specimen being analyzed. Details on the mechanisms of IR spectroscopy can be found in numerous contemporary scientific literature, such as Stuart (2004), Günzler and Gremlich (2002), and Barth and Haris (2009).

5.3.2. Analysis of beeswax by IR spectroscopy

The utilization of IR spectroscopy for the beeswax analysis was first employed by Birshtein and Tul'chinskii (1977), who investigated basic beeswax composition and associated impurities. Further investigations of molecular structure and dynamics in beeswax, as well as general interpretation of beeswax IR spectrum, were reported in only several spectroscopic studies (Edwards, Farwel, & Daffner, 1996; Muscat, Tobin, Guo, & Adhikari, 2014; Zimnicka & Hacura, 2006). A notable progress in spectroscopic investigation of beeswax was brought by Maia et al. (2013), who introduced a single-reflection attenuated total reflectance (ATR) recording technique (coupled with FTIR spectrometer) in beeswax analysis, aiming to detect adulterants in beeswax. The application of FTIR-ATR method was further improved and modified by Svečnjak, Baranović et al. (2015) who

demonstrated an analytical procedure for reliable routine detection of beeswax adulteration.

5.3.2.1. FTIR-ATR recording technique. Due to the numerous benefits elaborated further in this section, FTIR spectroscopy coupled with a diamond ATR accessory (FTIR-ATR recording technique) has become an important analytical tool for beeswax research as it was successfully applied for the chemical characterization of beeswax, as well as the qualitative and quantitative detection of adulterants in beeswax (Maia et al., 2013; Svečnjak, Baranović et al., 2015; Svečnjak, Prđun et al., 2015; Svečnjak et al., 2016, 2018).

Fourier transform infrared spectrometers (FTIR) are mainly used to measure absorption of the mid-infrared radiation (4000–400 cm^{-1}). FTIR spectroscopy has many advantages over conventional (dispersive) spectroscopy. Most importantly, FTIR spectrometers employ an interferometer based on the mathematical process of Fourier-transformation, and thus, reduce the measuring time and provide an acquisition of high quality spectra within seconds.

The IR spectrum of a sample can be measured either as transmittance (*T*, expressed as percent transmittance – %*T*) or absorbance (*A*, expressed in absorbance units – a.u.) which are commonly labelled on the *y*-axis of the IR spectrum, while the wavenumbers (cm^{-1}) that measure the position of an infrared absorption, are labelled on the *x*-axis. Absorbance is defined as the amount of IR radiation absorbed by a sample, and transmittance as the amount of IR radiation passed through that sample. Researchers use absorbance mode rather than transmittance, especially when it comes to quantitative analysis in biological applications. For the same reason, the absorbance mode is preferable for investigating beeswax samples.

An addition of ATR instrumental accessory further simplifies the measurement procedure, i.e., enables samples to be examined directly as obtained (in the solid or liquid state), without previous sample preparation. Unlike conventional transmittance measurements, ATR measurements are not dependent on the Beer–Lambert law which expresses the relationship of the IR radiation absorbed by the sample to the concentration of the targeted component and to the path length of the sample. ATR technique involves low and effective depth of penetration of the IR beam into the sample, typically between 0.5 and 5 µm, regardless of the sample thickness. This also ensures that only a small amount of the sample is sufficient for analysis. ATR spectroscopy utilizes the phenomenon of total internal reflection; a beam of radiation entering a crystal undergoes total internal reflection when the angle of incidence at the interface between the sample and crystal is greater than the critical angle, where the latter is a function of the refractive indices of the two surfaces (Stuart, 2004). This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the sample being held in contact with the crystal. The crystals used for ATR accessories are made from optically dense materials with a high refractive index [such as diamond, germanium (Ge) or zinc selenide (ZnSe)]. Diamond is considered the best ATR crystal material due to its robustness, durability and chemical inertness.

5.3.2.2. FTIR-ATR spectra acquisition. Here, we describe a method for investigating beeswax samples by FTIR-ATR spectroscopy introduced by Maia et al. (2013) and modified by Svečnjak, Baranović et al. (2015). Different types of beeswax specimens can be analyzed by FTIR-ATR spectroscopy (wax scales, comb wax, comb foundations, beeswax blocks) depending on the aim of the study; sampling and refining should be carried out as described in Sections 2.1 and 2.2., respectively. Beeswax samples should be analyzed directly as obtained, without sample pre-preparation (purification or the usage of any kind of chemical reagents). The IR spectra of beeswax samples should be recorded using FTIR spectrometer coupled with a single-reflection high temperature heated diamond ATR system (alternatively, a heated germanium crystal plate can be used).

We present the method using the example of Cary 660 Fourier transform mid-infrared spectrometer (Agilent Technologies) with a DTGS detector and CsI optics, coupled with Golden Gate high temperature (200 °C) heated single-reflection ATR accessory (Specac) with a diamond as internal reflection element (the depth of the beam penetration into the sample is ∼2 µm).

Equipment required:

- FTIR spectrometer with accompanying software (spectrometers with a DTGS detector and CsI optics are recommended, such as Agilent Technologies's Cary 660 FTIR spectrometer)
- High temperature (200 °C) heated single-reflection ATR accessory (ATR accessory with a diamond as internal reflection element is recommended, such as Specac's Golden Gate ATR)
- ATR thermocontroller (comes as a supplement to the ATR accessory listed above)
- Analytical balance (accuracy ±0.001 g)
- Laboratory (nitrile) gloves
- Metal spatula
- 96% ethanol (alternatively, acetone or isopropanol)
- Cotton wool
- Soft paper tissue

Procedure:

IR spectra of the beeswax samples are acquired as follows:

1. Heat the ATR plate to 75 °C (by adjusting ATR thermocontroller).

Figure 30. Beeswax sample on the ATR crystal (diamond) prepared for the acquisition of its IR spectrum (after melting at 75 °C). Photo: Svečnjak L.

2. Collect a background spectrum (spectrum of blank ATR crystal).
3. Place a small amount of a sample (~0.03 g; 0.02–0.05 g) directly onto the ATR sampling crystal using laboratory gloves or metal spatula, depending on the type of the specimen investigated (this amount of the sample should be sufficient to cover the internal reflection element/ATR crystal.
4. Leave the sample on the ATR crystal for 1 min to allow melting, homogenisation, and stabilisation of the sample (samples are analyzed in the liquid state) (Figure 30).
5. Collect sample IR spectrum (acquire two replicate spectra of each sample using the same aliquot).
   The following instrumentation parameters should be used to acquire the spectra:
   - *spectral mode*: absorbance
   - *spectral range*: mid-infrared spectral region (4000–400 cm^{-1})
   - *resolution*: 4cm^{-1} (gives a distance between two points in the resulting spectrum $\Delta\tilde{\nu} = 2cm^{-1}$)
   - *number of scans per spectrum*: 64

6. Store raw spectral data as SPC or ASCII file (this enables easier spectral data manipulation given that classical spectral formats are acceptable for numerous commercial software products commonly used for spectral data analysis (such as Origin, Matlab, The Unscrambler, Spekwin 32, etc), unlike instrument software-specific file formats, such as BSP in case of ResolutionsPro.
7. Clean the ATR sampling crystal thoroughly using 96% ethanol (it is best to use cotton wool soaked in alcohol for this purpose).
   Alternatively, other organic solvents can be used for cleaning the plate: acetone or isopropanol.
8. Dry the crystal with soft paper tissue.
9. Ensure the ATR crystal is completely dry and clean before the acquisition of another spectrum (scan an empty ATR crystal to confirm the absence of sample residues).

The analytical procedure for acquiring spectra described above is optimized by means of both time efficiency and reproducibility; analysis of beeswax samples in liquid state directly on the heated ATR plate using above listed instrumentation parameters enables easy and quick analysis and obtaining of high quality spectra.

After cleaning, the ATR plate is ready for the collection of another spectrum, starting with the background scan (new background spectrum should be acquired before each sample). It is recommended to use the live spectrum monitoring option (if possible) during the whole period of spectra processing, including cleaning of the ATR element. This enables an observation of the potential presence of impurities or other deviations, and ensures a clean ATR plate prior to the collection of the following spectrum.

Modern FTIR spectrometers ensure reproducible and accurate measurements. However, to validate the accuracy of measurement data obtained by specific methodology, a statistical analysis that provides information on the repeatability and reproducibility of the method can be performed by following ISO 5725-2,6:1994 standards (Accuracy (trueness and precision) of measurement methods and results – Part 2, Part 6, 1994). The repeatability of the IR measurements should be determined by ten-fold measurement of different aliquots of one beeswax sample. For this, the mean absorbance, standard deviation, coefficient of variation (c_v) and/or repeatability limit (95% confidence limits)

using the absorbance at 2921 cm^{-1} (band with the highest absorption intensity) can be calculated. Examples for calculating c_v/repeatability are provided in sections on detecting the adulterants in beeswax, i.e., Sections 6.2.5.3.3.1 and 6.2.5.3.3.2.

5.3.2.3. Spectral data processing. Raw spectral data can be stored and pre-analyzed using different software packages. Any software suitable for analysis of spectral data can be used. We present a procedure where spectra are recorded and stored using Resolutions Pro version 5.3.0 software package (Agilent Technologies):

1. Import spectral data in favourable software.
2. Exclude the noisy parts of the spectrum by cutting the spectrum to 3200–650 cm^{-1} (use simple *cut* or *truncate* options).
3. Select the region of interest for further qualitative, quantitative, or chemometric and statistical modeling.

Given that the most characteristic spectral features in beeswax sample appear in the fingerprint region (1800–900 cm^{-1}), this region is usually being studied. Selection of the spectral region of interest and appropriate chemomeric models for investigating the beeswax spectra primarily depend on the aim and research design (qualitative or quantitative IR analysis, investigation of spectral variations in beeswax, detection of adulterants, monitoring of temperature-depended structural changes in beeswax, etc.).

5.3.3. Characteristic FTIR-ATR spectrum of beeswax

When analysing beeswax by FTIR-ATR spectroscopy, the first step represents qualitative interpretation of spectral features (positions and intensities of absorption bands) appearing in the beeswax IR spectrum. Visual inspection can be performed using different software packages specialized for spectral data analysis (e.g., Origin version 8.1 or other/Origin Lab Corporation, Spekwin 32), and complemented with the use of electronic spectral libraries, spectral atlases or books (e.g., Socrates, 2001), and/or available scientific literature. Contrary to the simplicity from an analytical point of view, the interpretation of IR spectrum (assignation of underlying molecular vibrations) is demanding and time consuming process.

The characteristic FTIR-ATR spectrum of *A. mellifera* beeswax is dominated by the spectral features due to esters (mainly monoesters), free fatty acids and hydrocarbons (Figure 31). The other less abundant components can be considered here as traces. In Section 5.3.3.1, we provide a detailed assignation of molecular vibrations occurring in FTIR-ATR spectrum of genuine *A. mellifera* beeswax sample (derived from melted wild-built comb wax, as described in Sections 2.1.1 and 2.2). As demonstrated below in Section 5.3.3.1, IR spectrum of *A. mellifera* beeswax should be characterized by unique spectral features; an appearance of absorption bands other than listed below indicate a questionable genuinity of the sample, i.e., spectral effects related to the presence of adulterants (see Section 6.2.5).

Minor hemical variability occurring in different specimens of *A. mellifera* beeswax should be considered when analysing beeswax by FTIR-ATR spectroscopy (this may be important for other methods as well, depending on the aim of the study). There are several biological factors affecting chemical differences that naturally occur among different types of beeswax samples. These chemical variations are summarized by Hepburn et al. (2014) presenting comparative compositional changes (in hydrocarbon and fatty acid content) observed in oenocytes, epidermis, scale wax and comb wax samples of *A. mellifera capensis*. Notable species-related differences were observed in beeswaxes originating from different species (Aichholz & Lorbeer, 1999). However, there are no significant differences in the chemical composition of wax originating from different *A. mellifera* subspecies; it has the same basic composition with only small variations related to the proportion of particular constituents, i.e., hydrocarbons, esters and fatty acids (Beverly et al., 1995; Fröhlich, Riederer et al., 2000; Tulloch, 1980). As reported by Beverly et al. (1995), African, Africanized, and European waxes showed differences in the relative amounts of several compounds; but no unique biomolecules were found that were distinctive for a particular type of wax. Chemical composition of comb waxes varies between colonies; small interfamily variations are mostly related to the content of hydrocarbons, esters and alcohols (Breed, Page et al., 1995, Svečnjak, Baranović et al., 2015; Svečnjak, 2017, unpublished data). An investigation of the differences between the waxes of different age (wax scales, new, middle aged, and old waxes) within the same honey bee colony, also revealed small variations; analyzed samples could only be distinguished after chemometric treatment (based on the content of hydrocarbons and esters) (Fröhlich, Riederer et al., 2000).

5.3.3.1. Assignation of underlying molecular vibrations in beeswax IR spectrum. When exposed to IR radiation (mid-IR spectral range), beeswax exhibits characteristic molecular (functional group) vibrations that can be identified and assigned to particular compounds. We present an assignation of major and minor molecular vibrations (synonyms: analyte signal, absorption band, absorption, vibration, peak) observed in the characteristic IR spectrum of beeswax. Assignation was conducted based on Socrates's tables and charts of IR spectra/characteristic group frequencies (2001), and scientific literature (Maia et al., 2013; Pielichowska et al., 2008; Svečnjak, Baranović et al., 2015; Zimnicka & Hacura,

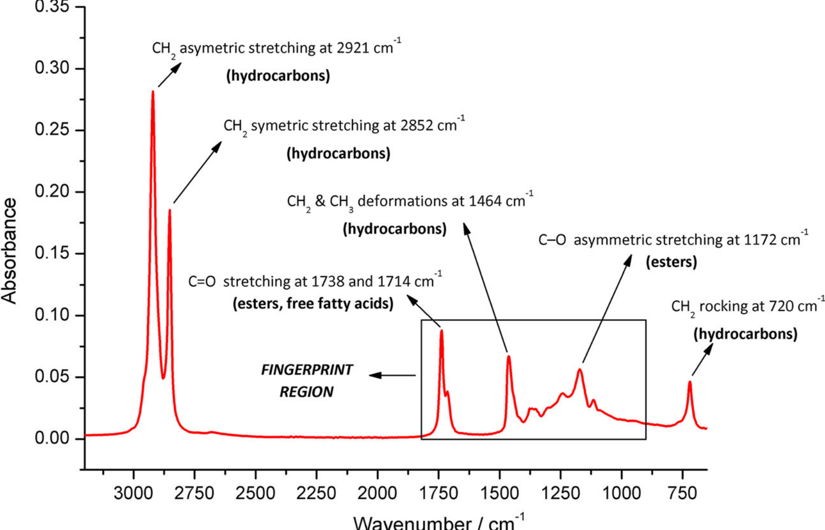


Figure 31. Characteristic FTIR-ATR spectrum of beeswax with assigned underlying absorption bands (whole spectrum – spectral region 3200–650 cm^{-1}) (modified from Svečnjak, Baranović et al., 2015).

2006). Respective molecular vibrations are given in Table 7 and various figures (noted below).

As shown in Figure 31, hydrocarbons are represented by very intensive absorptions observed at 2921 and 2852 cm^{-1}, medium band at 1464 cm^{-1}, and the weaker one arising at 720 cm^{-1}. These four bands are characteristic for all types of wax samples, being similar or almost of equal intensity and position.

Spectral region between 1800 and 800 cm^{-1} (fingerprint region) is populated by a number of absorption bands. The most prominent spectral feature in the fingerprint region is the absorption band occurring at 1738 cm^{-1} due to the carbonyl group (C = O) stretching vibrations of the of the ester bond. Given that saturated aliphatic esters typically absorb at 1750–1725 cm^{-1}, this absorption is attributed to the beeswax monoesters, which are the major ester component of beeswax (~40%). A weaker band peaking at 1714 cm^{-1} is assigned to the C = O stretching vibrations of the carboxyl groups of free fatty acids present in beeswax (Figure 32).

The most complex absorption envelope of the IR spectrum of beeswax arises in the region between 1400 and 970 cm^{-1}. The most prominent absorption in this region is a broad band in the 1150–1050 cm^{-1} with absorption maximum observed at 1172 cm^{-1}. This absorption corresponds to the C–O asymmetric stretching vibration of esters related to long-chain aliphatic acids (oleates, palmitates, stearates, etc.).

The spectral region between 1100 and 950 cm^{-1} is characterized by broad signal (1090–1020 cm^{-1}) due to a number of overlapping bands which can be attributed to several vibrations; this spectral envelope is also the most variable part of beeswax spectra. For details on molecular vibrations observed in the fingerprint region, see Table 7.

For assays where researchers are focused on determining the chemical alterations and corresponding spectral variations naturally occurring in various beeswax samples, we present an example of IR spectra of 20 different beeswax samples (wild-built combs) collected from different *A. mellifera* colonies situated in different geographical regions in Croatia. It is observable that integral spectral features appear to be very similar among different samples; only small changes in absorbance intensities are noticeable (Figure 33(a)). An exception is spectral region between 1090 and 1015 cm^{-1} characterised by different absorbance intensities, which indicate variability related to hydrocarbons (skeletal vibrations), esters and alcohols (Figure 33(b)). This region can be further subjected to spectral data mining and chemometric modelling, as discussed in Section 5.3.3.2.

5.3.3.2. Spectral data preprocessing and chemometric modelling. Along with basic investigation of beeswax molecular structure, the proposed FTIR-ATR methodology may also serve as an analytical tool for identifying chemical alterations and associated spectral variations occurring in beeswax due to different factors (such as different geographical origin, *A. mellifera* subspecies, comb age, type of beeswax specimen, etc.). However, such investigations are strongly dependent on the aim of the study, as well as the structure and complexity of the spectral data set attempted to be investigated. They also require more demanding analytical approach and employment of comprehensive chemometric assessment involving spectral data preprocessing and transformation (such as normalization, alignment, Savitzky–Golay filtering/smooting – SG, deconvolution, standard normal variate – SNV, multiplicative signal correction – MSC, and others), and multivariate statistical analyses (such as PCA, LDA, PLS-DA, FDA, ANN, etc.) to enhance the spectral variations between beeswax samples by

Table 7. Beeswax IR absorption bands with assignation of underlying molecular vibrations.

Band position (wavenumber, cm^{-1})	Functional group assignment and type of vibration	Absorption intensity	Associated beeswax chemical compound
2957	CH_3 stretching, asymmetric	Weak, shoulder	Hydrocarbons
2921	CH_2 stretching, asymetric	Strong, sharp	Hydrocarbons
2852	CH_2 stretching, symetric	Medium-strong, sharp	Hydrocarbons
1738	C = O stretching	Medium	Monoesters – saturated aliphatic esters, saturated free fatty acids
1714	C = O stretching	Weak, shoulder	Free fatty acids
1464	CH_2 scissoring, CH_3 asymmetric deformation (overlapped)	Medium	Hydrocarbons
1420	CH_2 deformation	Weak, shoulder	Esters
1376	CH_3 symmetric deformation	Weak	Hydrocarbons
1368	C–H deformation	Weak	Hydrocarbons
1353	C–H deformation	Weak	Hydrocarbons
1302	CH_2 wagging	Weak	hydrOcarbons
1242	C–O–C asymmetric stretching	Medium-weak, broad	Aliphatic esters
1172	C–O asymmetric stretching	Medium, broad	Esters of long-chain aliphatic acids (stearates, oleates, etc.)
1115	C–O–C symmetric stretching	Weak	Aliphatic esters
1080–1020	CH_3 rocking	Weak-to-medium, broad	Esters
1065	(unsat.)–CH_3		Hydrocarbons
1050	CCO stretching		Alcohols
720	CH_2 rocking	Medium-weak,	Hydrocarbons

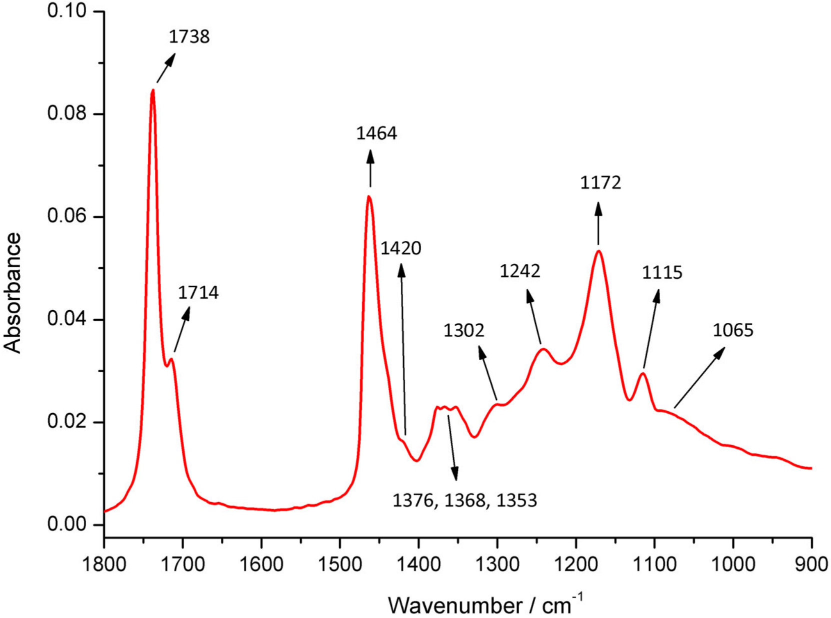


Figure 32. Fingerprint region (1800–900 cm^{-1}) of beeswax FTIR-ATR spectrum and underlying absorption bands.

enhancing the analyte signals. Therefore, a methodology for the chemometric modelling cannot be standardized. To determine the best chemometric model for particular data set, refer to the recent research notes on the optimization of SG parameters for improving spectral resolution and quantification reported by Zimmermann and Kohler (2013). As emphasised by the authors, calculating derivatives of spectral data by the SG numerical algorithm can be used as a preliminary pre-processing step to resolve overlapping signals, enhance signal properties, and suppress unwanted spectral features that arise due to nonideal instrument and sample properties. However, make sure to use the proper data treatment (preprocessing) and appropriate statistical models given that model with large number of latent variables (most often involved in spectral data mining) may lead to overfitting and perplexity of the statistical model (Toher, Downey, & Murphy, 2007). For general multivariate statistical approaches, refer to the respective Sections 5.4, 5.5, 6, 7, and 8 of the *BEEBOOK* paper on statistics (Pirk et al., 2013).

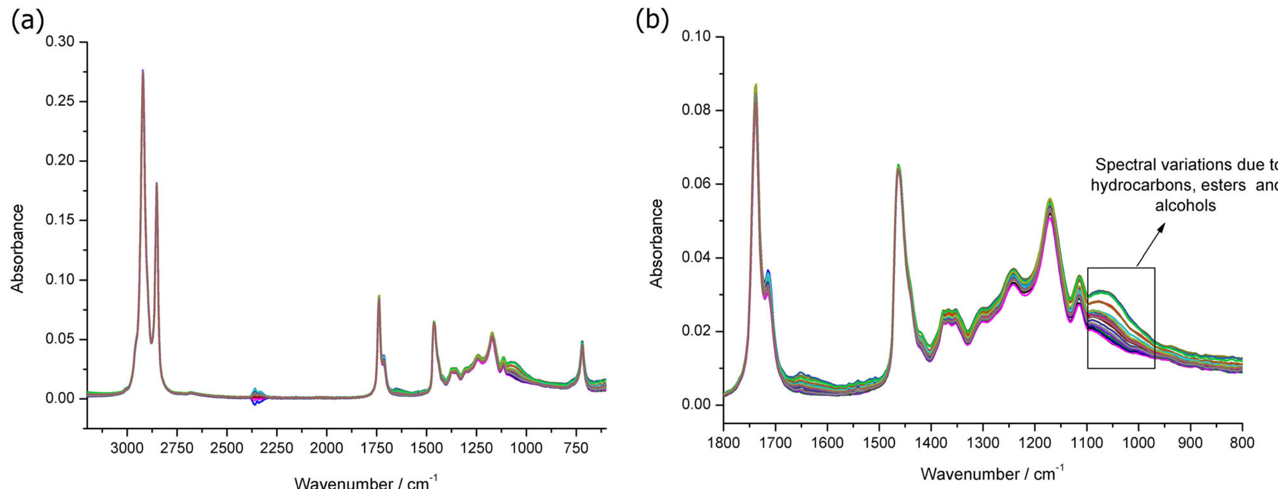


Figure 33. IR spectra and spectral variations between different beeswax samples ($n = 20$) originating from different *A. mellifera* colonies: whole spectra (3200–650 cm^{-1}) (a); fingerprint region (1800–900 cm^{-1}) (b) (modified from Svečnjak, Baranović et al., 2015).

5.3.4. Advantages and disadvantages of FTIR-ATR measurements

Advantages:

- Provide information on the total chemical composition of a sample.
- Speed advantage (after optimization of the analytical procedure as described above, it is possible to obtain the results in only few minutes).
- Cheap (consumables required only for the cleaning of ATR crystal) and easy-to-use (undemanding from the analytical point of view).
- Non-destructive and reagent-free method (requires no sample preparation).
- Only a small amount of a sample is sufficient to acquire the IR spectra (<0.5 g).
- Good accuracy, repeatability, and reproducibility of measurement results.
- Suitable for analysis of different types of beeswax specimens (wax scales, comb wax, comb foundations).
- Accompanying software and optional accessories for FTIR spectrometers (e.g., different ATR plates, liquid and gas transmission cells, FTIR microscope) enable implementation of various experimental designs using different measurement and chemometric techniques that can be used to complement and/or expand the beeswax research.

Disadvantages:

- Minor beeswax compounds cannot be detected easily by FTIR-ATR spectroscopy due to an overlap with the absorption bands of predominant beeswax compounds (esters, hydrocarbons and fatty acids).
- Only suitable for qualitative analysis since quantitative methods (analytical procedures for quantification of individual beeswax compounds) still have to be developed.

5.4. Determination of ash content and mineral composition

The ash content and mineral composition of beeswax have generally been poorly studied. Ash content is a measure of the total amount of minerals present in the wax, whereas mineral content is a measure of the amount of specific inorganic components present in it. Determination of the ash and mineral content of wax may be useful to evaluate the composition and quality of the product (Bernal et al., 2005; KEBS, 2013), and its age (Taha, Manosur, & Shawer, 2010).

5.4.1. Determination of ash content

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. The most widely used laboratory methods rely on the fact that minerals are not destroyed by heating, and that they have a low volatility compared to other components.

Equipment required:

- Muffle furnace
- Porcelain capsules
- Desiccator
- Analytical balance (accuracy ±0.001 g)
- Bunsen burner

Procedure:

1. Heat the porcelain capsules for 30 min in the muffle furnace set at 600 °C.
2. Allow the capsules to cool in a desiccator to room temperature.
3. Determine their weight on analytical balance to the nearest 0.001 g.

4. Transfer 2 of wax in a capsule.
5. Weigh on analytical balance to the nearest 0.001 g.
6. Heat gently on a Bunsen burner till the material is completely charred.
7. Transfer and heat in muffle at 600 °C for 1.5 h.
8. Transfer and cool the capsule with the ashes in a desiccator until constant weight.
9. Repeat incineration, cooling and weighing until the difference between two successive weighing is less than one milligram.

The ash percentage was determined as follows:

$$\text{Ash }\% = 100 \times \frac{W_1 - W_2}{w}$$

where W_1 is the mass, in g, of the porcelain capsule with the ash content; W_2 is the mass, in g, of the empty capsule; and w is the mass, in g, of the beeswax sample.

5.4.2. Determination of mineral composition

Various spectroscopic techniques may be used to determine the mineral content in the beeswax.

The older techniques, but still widely used, are atomic absorption spectroscopy (AAS), to determine metallic elements, and atomic emission spectroscopy (AES), to determine alkaline and alkaline-earth elements. The most recent and performing technique is inductively coupled plasma combined with mass spectrometry (ICP-MS), which allows the simultaneous determination of many elements, replacing both AES and AAS.

Whatever the instrumental technique, beeswax has to be wet digested to eliminate the organic part with the following procedure:

1. Weight 0.5 g of beeswax into a burning cup.
2. Add 15 mL of pure HNO_3.
3. Digest the sample in a microwave oven at 200 °C.
4. Dilute the solution to the desired volume with water.
5. Determine the concentrations with a spectroscopic technique.

To determine the amount of an element present in the wax, a calibration function will be calculated for each analyte using the relative values of the signals generated by the analysis of standard solutions with known and increasing concentration of these elements.

5.5. Standard methods for sampling and analysing beeswax for hydrogen isotope ratios

Definitions, acronyms:

- ^{1}H: hydrogen-1
- ^{2}H: hydrogen-2 or deuterium
- δ^2H: stable hydrogen isotope value
- IRMS: Isotope ratio mass spectrometer
- *R:* ratio of a less common isotope form to the more common isotope form (^{2}H/^{1}H)
- δ-notation: parts per thousand (‰) difference from an accepted standard reference point; $\delta = (R_{sample}/R_{standard} - 1)$
- V-SMOW: Vienna Standard Mean Ocean Water
- QA: quality assurance (QA standard)
- QC: quality control (QC standard)
- RM: reference material

The δ^2H values of beeswax are related to hydrogen incorporated by a honey bee through consumption of foods and water (Chesson et al., 2011; Tipple et al., 2012). The most significant source of hydrogen to animals is meteoric waters (Bowen, Wassenaar, & Hobson, 2005) and the δ^2H values of meteoric waters vary in systematic and highly predictable patterns across land surfaces (Bowen & Revenaugh, 2003; Craig, 1961). In general, more positive δ^2H values of meteoric waters are observed at lower elevations and locations nearer to the coasts, while more negative δ^2H values are observed at higher elevations and inland areas. Geographic patterns in water hydrogen isotope signatures create *isotope landscapes* or *isoscapes* (Bowen et al., 2009). These spatial patterns in the δ^2H values of waters are subsequently transferred to organisms, as organisms incorporate isotopic signals from their local waters. Thus, the δ^2H values of beeswax can be useful in identifying the region-of-origin (Figure 34). Stable isotope analysis and the isoscape method have been applied to bee products (Chesson et al., 2011, 2014a; Cho et al., 2012; Schellenberg et al., 2010; Tipple et al., 2012).

5.5.1. Methods for sampling and handling beeswax for hydrogen isotope analysis

Here, we present the methods applied by Chesson et al. (2011, 2014b). These methods are relatively standard procedures and can be modified for particular laboratory settings. Different types of beeswax can be analyzed for stable hydrogen isotope ratios. Sampling beeswax for hydrogen isotope analysis may include:

1. Wax scales (as described in Section 3.2)
2. Comb wax (as described in Section 2.1.1)
3. Wax caps (as described below in Section 5.5.1.1)
4. Dissolved wax in liquid honey (as described below in Section 5.5.1.2).

5.5.1.1. Sampling honeycomb wax caps. In both commercial beekeeping, as well as in the natural environment, the interior wax that makes up the cell walls of the honeycomb is often recycled year after year. For this reason, the wax cap covering the honey within the honeycomb is preferable for studies investigating the provenance of honey and honeycomb using isotope

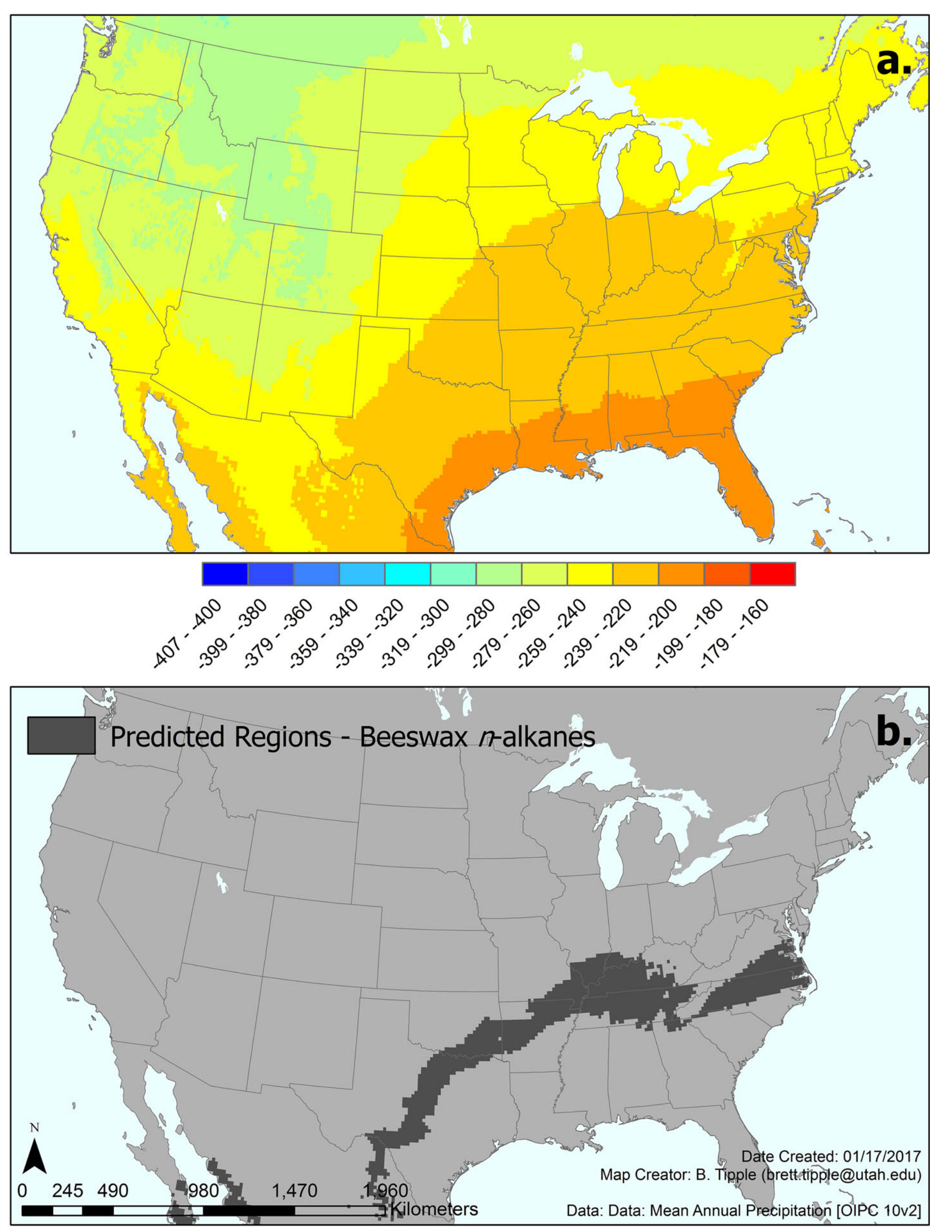


Figure 34. (a) Isoscape projection of beeswax wax cap *n*-alkanes as described by relationship from Tipple et al. (2012). (b) Regions consistent with a beeswax wax cap *n*-alkane δ^2H value of $-227 \pm 2.5‰$.

analysis. Here, the exclusive use of wax caps ensures that the associated honey and waxes reflect the same production season and geographic location. Thus, prior to any isotopic analysis, the wax caps must be isolated from the comb and free of any residual honey. The method below outlines the use of forceps and razor blades to isolate wax caps from the surrounding cells of the honeycomb (Figure 35). Following isolation from the honeycomb, techniques are described to clean any residual honey from the wax cap sample.

Equipment required:

- Ashed (i.e., baked at 500 °C for 4 h) 4-mL glass vial with Teflon-lined caps
- Balance with precision of ±1 mg
- Nitrile gloves
- Metal spatula
- Metal forceps
- Razor blade
- "Solvent residue" waste container
- Sonicator
- 50-mL glass beaker
- Ashed aluminium foil
- Drying apparatus

Reagents required:

- Laboratory soap
- Deionised or distilled water in squeeze bottle
- HPLC-grade (or better) methanol in Teflon® squeeze bottle

Figure 35. Representative honey and beeswax samples in ashed canning jars fitted with ashed aluminium foil cap liners. From left to right, orange, tupelo, sage, wildflower, and sourwood honeys.

Procedure:

1. Prepare a 4-mL vial (also known as a 1-dram vial) by weighing empty vial and cap while working in a fume hood and wearing nitrile gloves.
2. Wash spatula, forceps, and razor blade with laboratory soap and water: Rinse three times with tap water, followed by three rinses with deionised or distilled water from squeeze bottle.
   This step removes surface contaminants and soap residue from the tools.
3. Squirt clean spatula, forceps, and razor blade with methanol three times within a fume hood. Collect residual methanol in solvent waste container. This step removes any organic contamination from the tools.
4. Remove the honeycomb from storage container using forceps to a clean surface, such as a sheet of aluminium foil.
5. Remove 2–5 honeycomb cell caps using forceps and razor blade
6. Place in the pre-weighed 4-mL vial.
7. Label vial.
8. Fill 4-mL vial containing wax caps with ~3.5 mL deionised or distilled water.
   This is the *wash* water.
9. Cap vial.
10. Sonicate vial containing wax caps for 10 min at room temperature.
11. Decant and discard *wash* water from vial.
12. Repeat steps 8 and 10 two additional times, for a total of three washes.
13. Decant final *wash* water.
14. Dry wax caps within vial under a gentle stream of nitrogen gas or purified air at room temperature.
15. Cap vial once washed wax caps and vial are completely dry.
16. Determine weight of wax caps by weighing vial with cap and subtracting the mass of the empty vial with cap.
17. Place in dry storage area (see Section 2.3 on beeswax storage for details). Wax cap samples can be stored indefinitely.

While these steps describe sampling and cleaning wax caps from honeycomb, this method could be easily modified for sampling and cleaning of interior cells of a honeycomb.

5.5.1.2. Extraction of beeswax from liquid honey. Here, we present methods applied by Tipple et al. (2012). Solvent-extractable organic compounds can be isolated from liquid honey using a two-phase liquid-liquid extraction. Liquid-liquid extraction methods are particularly useful when visible wax is not present in a honey sample, such as filtered honeys (Tipple et al., 2012). The procedures presented here are those used for isotope analysis of *normal*-alkanes (*n*-alkanes) and different liquid-liquid extraction methods may be used for other analytical techniques.

Equipment required:

- Nitrile gloves
- Ashed aluminium foil
- Balance with precision of ±1 mg
- Ashed 20-mL glass scintillation vial with Teflon-lined cap
- 5.75" ashed, borosilicate glass Pasteur pipettes
- Vortex mixer (optional, but recommended)
- Ashed round-bottom culture tube
- Drying apparatus
- Ashed 4-mL glass vial with Teflon-lined cap

Reagents required:

- Solvent-extracted deionised or distilled water in squeeze bottle
- HPLC-grade (or better) hexanes in Teflon® squeeze bottle

Procedure:

1. Select a liquid honey sample.
   Either filtered honey or comb-honey can be used.
2. Weigh out 10 g of honey in a 20-mL glass scintillation vial while wearing nitrile gloves. Record honey mass.
3. Label vial.

4. Add 5 mL of distilled and deionised water to the scintillation vial with honey sample with a pipette.
5. Gently agitate to dissolve honey.
6. Add 10 mL of hexane to scintillation vial.
7. Seal vial with solvent-rinsed Teflon-lined cap.
   Close cap tightly.
8. Agitate vial for 2 min either by shaking in hand or using a vortex mixer.
9. Allow solvent and honey/water layers to separate (~10–20 min).
10. Remove top layer (i.e., hexanes) using a pipette to a culture tube.
11. Label tube.
12. Repeat steps 6–11 two additional times, combining extracts in culture tube, for a total of three extractions.
13. Dry extract under a gentle stream of nitrogen gas or purified air at room temperature using a drying apparatus. Concentrate to ~2–3 mL.
14. Transfer concentrate to 4-mL glass vial.
15. Label vial.
16. Dry concentrate completely under a gentle stream of nitrogen gas or purified air at room temperature using a drying apparatus.
17. Cap with Teflon-lined cap.
18. Dried extracts can be stored at room temperature indefinitely (see Section 2.3 for details).

5.5.2. Methods for isolating beeswax compounds for compound-specific hydrogen isotope ratio analysis

Here, we apply the methods of Tipple et al. (2012). Prior to hydrogen isotope analysis of specific compounds from beeswax, organic compounds must be isolated from the complex mixtures of bulk beeswax or beeswax extracted from liquid honey.

5.5.2.1. Dissolution of bulk beeswax. A mixture of 2:1 dichloromethane:methanol is used to dissolve sample wax in a sonicator. When the sample has dried, the remaining residue is the total lipid extract.

Equipment required:

- Nitrile gloves
- Metal spatula
- Metal forceps
- Razor blade
- Ashed aluminium foil
- Balance with precision of ±1 mg
- Ashed 4-mL glass vial with Teflon-lined cap
- 50-mL glass beaker
- Sonicator
- Drying apparatus

Reagents required:

- HPLC-grade (or better) dichloromethane
- HPLC-grade (or better) methanol in Teflon® squeeze bottle
- Deionised or distilled water

Procedure:

1. Select a clean and dry wax cap.
2. Remove from storage vial with spatula or forceps
3. Place on aluminium foil.
4. Cut a single wax cap into quarters with a clean and solvent-rinsed razor blade.
5. Weigh an empty 4-mL borosilicate glass vial.
6. Label vial.
7. Place a 1/4 of wax cap into the weighed, labelled vial.
8. Add additional pieces of wax cup until mass is approximately 5–10 mg.
9. Record final mass.
10. Mix a 2:1 solution of dichloromethane:methanol.
11. Add 0.5 mL 2:1 dichloromethane:methanol to the 4-mL vial containing wax cap sample of known weight.
12. Seal vial with solvent-rinsed, Teflon-lined cap.

    Close cap tightly.

13. Fill a 50-mL beaker with ~25 mL deionised water.
14. Place wax-containing vial in beaker.

    Ensure water in the beaker does not reach the threads of the capped vial.

15. Sonicate beaker and vial for 10–15 min or until all wax is dissolved.
16. Remove vial from beaker and uncap.

    At this point the dissolved wax cap can be quantitatively divided into separate aliquots using a graduated syringe. Aliquots can either be used at this point or dried for storage.

17. Dry dissolved wax within vial under a gentle stream of nitrogen gas or purified air at room temperature.
18. Cap vial once sample is completely dry.
19. Place in cool, dry storage area. Wax samples can be stored indefinitely (see Section 2.3 on beeswax storage for details).

5.5.2.2. Silica gel column chromatography. The preferred method for preparation of beeswax for compound specific isotope analysis follows Torto et al. (2013). This method uses Pasteur pipette-packed silica gel columns (i.e., the stationary phase) with organic solvents (i.e., the mobile phase) to separate organic compounds from beeswax into multiple fractions (see Section 4.2.2. in the *BEEBOOK* article on standard methods for chemical ecology research in *A. mellifera*, Torto et al., 2013). The separation of the fractions is based on compound and solvent polarity. This method can be used prior to any gas chromatographic analysis to simplify chromatogram

baseline. This is particularly important when using gas chromatography-isotope ratio mass spectrometry of beeswax compounds, as a clean baseline is mandatory (see Section 5.5.3). Here, dissolved wax from Section 5.5.2.1 is added to the top of the Pasteur pipette column as described in Torto et al. (2013).

5.5.3. Isotope ratio mass spectrometry and measurement systems

Here, we provide methods applied in Chesson et al. (2011, 2014a), Cho et al. (2012), Schellenberg et al. (2010), and Tipple et al. (2012). Isotope ratio mass spectrometry (IRMS) is used to analyze the stable hydrogen isotope abundance at natural levels (Hoef, 2004). With an IRMS instrument, materials must be converted to H_2 gas prior to analysis. The most common conversion methods are either offline pyrolysis or the use of an online pyrolysis system. Traditionally, offline methods were applied where organic material, such as beeswax, was reacted to produce H_2 gas initially, and then analyzed with a duel-inlet IRMS system (Bigeleisen, Perlman, & Prosser, 1952; Friedman, 1953; Schimmelmann & DeNiro, 1993; Sofer & Schiefelbein, 1986; Stuermer, Peters, & Kaplan, 1978; Wong & Klein, 1986). The main advantage of offline methods to measure the stable hydrogen isotope ratio of beeswax is high precision. The disadvantages include low sample throughput, long instrument dwell times, and numerous and costly consumables. Another significant disadvantage for offline methods is the requirement for highly-trained IRMS technicians. In addition, offline methods are typically non-standard and must be tailored to specific laboratories.

Most laboratories now perform stable hydrogen isotope measurements using continuous-flow IRMS systems with online preparative methods to produce H_2 gas (Begley & Scrimgeour, 1996; Burgoyne & Hayes, 1998; Hilkert, Douthitt, Schluter, & Brand, 1999; Prosser & Scrimgeour, 1995; Tobias & Brenna, 1997). This online H_2 gas preparation occurs by thermal decomposition (i.e., pyrolysis) and the H_2 gas is swept into the IRMS instrument with helium (He) as a carrier gas. The main advantages of continuous-flow systems are increased throughput, fewer consumable at lower costs, and relatively standard methodologies across laboratories. The main disadvantage is slightly less precise measurements.

5.5.3.1. Hydrogen isotope analysis of bulk beeswax and specific compounds. The relative abundances of stable isotopes within a material are described as the ratio (R) of a less common isotope form to the more common isotope form (i.e., $R = {}^2H/{}^1H$). Since these ratios are very small numbers, values are expressed in δ-notation, which is the parts per thousand (‰) difference from an accepted standard reference point, where:

$$\delta = \left(R_{sample}/R_{standard} - 1\right) \quad (5.5.1)$$

As defined in Equation (5.5.1), the stable hydrogen isotope value (δ^2H) is:

$$\delta^2H = \left[\left({}^2H/{}^1H_{sample}\right)/\left({}^2H/{}^1H_{standard}\right) - 1\right] \quad (5.5.2)$$

The standards used to express isotope abundances in δ-notation are internationally recognized reference materials. Primary reference materials have absolute isotope compositions. In the case of hydrogen, δ^2H values are reported relative to Vienna Standard Mean Ocean Water (V-SMOW), which has a ${}^2H/{}^1H$ of 0.00015576.

Laboratories also have access to a variety of secondary reference materials that have been calibrated to the primary reference material (Table 8). A recent effort to develop reference materials has specified 16 new or updated secondary reference materials for hydrogen isotope ratio measurements (Schimmelmann et al., 2016). Often, secondary reference materials are used to calibrate in-house reference materials or standards (Dunn & Carter, 2018). In-house standards can be produced in significant quantities to allow for day-to-day analysis and long-term usage. The "principle of identical treatment" requires in-house standards to be chemically matched to the samples being analyzed to produce robust hydrogen isotope ratios. Two-point normalisation of measured data to the international δ scale is also a requisite to report accurate δ^2H values (Coplen, 1988). In-house standards of contrasting isotope ratios are used in daily operation both for normalising results to the international δ scale, but also for accumulating long-term quality assurance data to assess system performance. A variety of in-house standards can be used for normalising measured sample data and compiling quality assurance information (e.g., Table 9).

The following descriptions are general considerations for hydrogen isotope ratio measurement of beeswax using either online or offline IRMS systems. We strongly advocate working with an established laboratory with the knowledge of and facilities in the area of stable hydrogen isotope ratio analysis (see list in Schimmelmann et al., 2016), as the specific methods described below may slightly vary between laboratories. Detailed procedures for making hydrogen isotope ratio measurements are beyond the scope of this contribution and chapter; however, specific IRMS guidelines and methodologies are thoroughly discussed elsewhere (Dunn & Carter, 2018). Detailed procedures for compound-specific IRMS operations are also available (e.g., Burgoyne & Hayes, 1998; Hilkert et al., 1999). Here, we briefly discuss quality assurance and quality control methods and data normalisation procedures.

5.5.4. Quality assurance and quality control reference materials

Both quality assurance (QA) and quality control (QC) standards or reference materials (RMs) must be

Table 8. Some commercially available secondary reference materials for hydrogen isotope measurement (compiled October 2016).

Reference material	Description	Material	δ^2H_{VSMOW}
NBS-30	Lakeview Tonalite	Biotite	−65.7
USGS-42	Human Hair from India	Keratin	−78.5
USGS-43	Human Hair from Tibet	Keratin	−50.3
USGS-67	n-C_{16}, Hexadecane	*n*-alkane	−166.2
USGS-68	n-C_{16}, Hexadecane	*n*-alkane	−10.2
USGS-69	n-C_{16}, Hexadecane	*n*-alkane	+381.4
NBS-22	Mineral oil	Oil	−117.2
IAEA-CH-7	Polyethylene Foil (formerly PEF-1)	Polyethylene	−99.2
GISP	Greenland Ice Sheet Precipitation	Water	−189.7
V-SLAP	Vienna Standard Light Antarctic Precipitation	Water	−428

Table 9. In-house standards and hydrogen isotope values used for hydrogen isotope measurement (as of October 2016).

Reference material	Description	Material	δ^2H_{VSMOW}
SQ	Squalane	Isoprenoid	−177
C28	n-C_{28}, Octacosane	*n*-alkane	−251
C36	n-C_{36}, Hexatriacontane	*n*-alkane	−240
C24	n-C_{24}, Tetracosane	*n*-alkane	−36
SM	Arndt Schimmelmann's Mix A (n-C_{16}, n-C_{24} … n-C_{29}, n-C_{30})	*n*-alkanes	Varies by compound and production run
PARA	Parafilm M®	Paraffin wax film	−101
ANDRO	5a-Androstane	Steroid	−253

analyzed alongside beeswax samples analyzed for δ^2H values to ensure reproducible and accurate measurements. Primary and secondary RMs are used to calibrate in-house RMs (see discussion above in Section 5.5.3.1), which are in turn used for isotope data normalisation (i.e., QA reference materials, see Section 5.5.4.1) and quality control (i.e., QC reference materials, see Section 5.5.4.2) step. All reputable stable isotope analysis laboratories maintain primary and secondary reference materials from NIST and IAEA as a means of initially calibrating in-house RMs to the international δ scales, as well as to periodically reconfirm those isotope values.

QA RMs are the basis upon which measured isotope data for beeswax samples are normalised to international δ scale (i.e., V-SMOW for δ^2H values). QA RMs must be analyzed alongside samples or in samples, in the case of compound-specific measurements, within a given analytical sequence. This allows for systematic in-run variations to be captured, monitored, and corrected as needed. Further, QA RMs should have a wide isotopic range that brackets the expected isotopic composition of the beeswax samples (Table 9).

QC RMs must always be included in an analytical sequence to assess the accuracy of the isotopic normalisation (see Sections 5.5.4.1 and 5.5.4.2) generated by QA RMs. QC RMs should ideally match the chemical matrix of the type of sample being analyzed (Table 9).

5.5.4.1. Normalisation of hydrogen isotope values to international scale. Normalisation is the process of converting measured hydrogen isotope values of a sample to the international δ scale. For hydrogen isotope measurements, all δ^2H values are reported relative to V-SMOW. Normalisation of hydrogen isotope values is compulsory as it is the only method to ensure δ^2H values from different laboratories and instruments are comparable. Normalisation should be done in each analytical sequence. The normalised δ^2H value of a sample is calculated using:

$$\delta^2H_{Normalised} = m * \delta^2H_{Measured} + b \qquad (5.5.3)$$

The slope (m) and intercept (b) are determined by comparing the measured and known δ^2H values of two QA RMs. Table 10 shows an example normalisation of measured δ^2H values using in-house RMs.

5.5.4.2. Additional quality control metrics. Normalised δ^2H values of QC materials (see Section 5.5.4.1) are compared to the known δ^2H values of those materials. Before any normalised δ^2H data of beeswax samples can be accepted, the normalised δ^2H values of the QC RMs must meet accepted limits. Typically, for bulk hydrogen isotope analysis this limit is ±2‰ (Chesson et al., 2011). If the QC RMs results do not meet this limit, δ^2H values from the beeswax samples are deemed unacceptable and must be re-analyzed.

Using best practices, each sample should be analyzed in replicate to provide robust metrics for reproducibility (Dunn & Carter, 2018). However, this is not always possible, particularly in commercial laboratories, due to the cost of preparing and analysing these additional samples. Alternatively, several samples within an analytical sequence may be analyzed multiple times and the reproducibility of these selected samples may be applied to other samples in the sequence. Nonetheless, either of these approaches to determine reproducibility is acceptable.

Table 10. Example two-point δ^2H normalization using in-house RMs.

ID	$\delta^2H_{Measured}$	δ^2H_{Known}	$\delta^2H_{Normalized}$
C24	−30	−36	–
C28	−244	−251	–
PARA	−94.7	−101	−101
Sample 1	−151	–	−158
Sample 2	−178	–	−185
Sample 3	−203	–	−210

6. Standard methods for beeswax authenticity and quality control

6.1. Legislation and quality control of beeswax

The quality control of beeswax worldwide includes official statutory documents referring to beeswax used in pharmaceutical and food industry, as pharmaceutical grade beeswax and food additive E901, respectively (national Pharmacopoeias, FAO legislation, EU regulations, U.S. FDA regulations, etc.).

There are different international and national Pharmacopeias (International Pharmacopeia, European Pharmacopeia, United States Pharmacopeia, British, French, German, Swiss, Slovak, etc.; with only small differences regarding beeswax criteria values) that give a definition of beeswax, and provides reference measurement guidelines for determination of its general physical and chemical characteristics.

Alongside Pharmacopeias where beeswax is described in terms of a pharmaceutical substance specification (yellow beeswax – *cera flava*, white beeswax – *cera alba*), beeswax is also an authorised food additive. Identified as additive E901, beeswax is permitted as a glazing agent, and as carrier for colours and flavours (Directive 95/2/EC, 2005; Directive 2009/10/EC, 2009; EFSA, 2007; FAO, 2005; ISO TC34/SC19, 2017; JECFA, 2005); two types of beeswax are specified: yellow beeswax (C.A.S No. 8006-40-4), and white beeswax (C.A.S. No. 8012-89-3).

The methods defining pure beeswax quality criteria based on 10 physico-chemical parameters determined in accordance with European Pharmacopoeia (EP, Council of Europe, 2007), and the Methods of German Society for Fat Science (Methods of Deutsche Gesellschaft für Fettwissenschaft: DGF-M-V2,3,6, 1957), have been proposed as general quality criteria for beeswax by the International Honey Commission (IHC) (Bogdanov, 2016a), as presented in Table 11 (comparative overview of beeswax quality parameters according to FAO, European legislation and IHC are presented in Table 12). However, the IHC material remained in the form of a proposal to date. As beeswax material used in the apiculture sector (primarily referring to the wax blocks and comb foundations) is considered as apiculture by-product not intended for human consumption, it is still not covered by any effective legislation nor subjected to any kind of authenticity or quality control prior to its

Table 11. Quality criteria parameters for routine authenticity testing of beeswax and reference range values defining pure beeswax according to the methods prescribed by European Pharmacopoeia (2007/2008) and the Methods of German Society for Fat Science (DGF, 1957) – proposal of the International Honey Commission (IHV) (Bogdanov, 2016a).

Quality criteria	Value	Method
Water content	<1%	DGF-M-V-2
Refractive index (75 °C)	0.4398–1.4451	EP
Melting point	61–65 °C	EP
Acid number	17–22	EP
Ester number	70–90	EP
Ester/acid ratio	3.3–4.3	EP
Saponification number	87–102	EP
Mechanical impurities, additives	Absent	DGF-M-V-3
Glycerols, polyols, fatty acids fats	Absent	EP
Hydrocarbons max.	14.5%[a]	DGF-M-V-6

DGV, V2,3,6 – Methods of Deutsche Gesellschaft für Fettwissenschaft.
EP – European Pharmacopoeia 7th Edition, 2008, 56.
[a]Wax from African and Africanized bees: max. 13.8%

placement on the market. This has resulted in a growing beeswax adulteration trend (Maia et al., 2013; Serra Bonvehí & Orantes Bermejo, 2012; Svečnjak, Baranović et al., 2015; Svečnjak et al., 2016, 2018; Waś et al., 2016). The quality of beeswax is further jeopardized by residues that mostly originate from the pesticides commonly used in the beekeeping technology (primarily acaricides), as well as environmental commercial pesticides widely utilized in agriculture (Bogdanov, 2006; Boi et al., 2016; Bonzini, Tremolada, Bernardinelli, Colombo, & Vighi, 2011; Chauzat & Faucon, 2007; Jiménez, Bernal, del Nozal, & Martín, 2005; Ravoet et al., 2015; Serra Bonvehí & Orantes Bermejo, 2010; Tremolada, Bernardinelli, Colombo, Spreafico, & Vighi, 2004; Wallner, 1992, 1993, 1997, 1999, 2000). This implies an urgent need for the implementation of mandatory regulations defining the beeswax quality criteria and standardized analytical methods for its testing, as well as routine quality/authenticity control of beeswax prior to its placement on the market. The methods for detection of adulterants and pesticide residues in beeswax are therefore described in the following sections.

6.2. Standard methods for beeswax adulteration detection

6.2.1. Beeswax adulteration

Adulteration has been one of the main quality issues of beeswax production and represents a long-term and increasing problem worldwide. Nevertheless, there are still no internationally standardised analytical methods for routine authenticity control of beeswax used in the apiculture sector (honeycombs, wax blocks and comb foundations). Moreover, beeswax and its products are classified as animal by-products (ABP) not intended for human consumption, and are usually marketed

Table 12. Comparative overview of beeswax quality parameters according to FAO (2005), European legislation (2009/10/EC; EP, 2007) and International Honey Commission (Bogdanov, 2016a).

Parameter	FAO (2005)	2009/10/EC	EP 5.0 (2007) Yellow beeswax	EP 5.0 (2007) White beeswax	IHC (2016)
Water Content					<1%
Melting range (°C)	62–65	62–65	61–66	61–66	61–65
Specific gravity (D_{2020})		0,96	0.960	0.960	
Refractive index (75 °C)					1.4398–1.4451
Solubility	Insoluble in water; sparingly soluble in alcohol; very soluble in ether	Insoluble in water; sparingly soluble in alcohol; very soluble in chloroform and ether	Practically insoluble in water, partially soluble in hot alcohol (90% *V/V*) and completely soluble in fatty and essential oils.	Practically insoluble in water, partially soluble in hot alcohol (90% *V/V*) and completely soluble in fatty and essential oils.	
Acid value (mg KOH/g):	17–24	17–24	17–22	17–24	17–22
Saponification value (mg KOH/g)	87–104	87–104	87–102	87–104	87–102
Ester value (mg KOH/g)			70–80	70–80	70–90
Ester/acid ratio					3.3–4.3
Peroxide value (mM H_2O_2/kg):	<5	<5			
Glycerol and other polyols:	<0,5% (as glycerol)	<0,5% (as glycerol)	Absent	Absent	Absent
Carnuba wax	Passes test				
Ceresin, paraffins and other waxes:	Passes test	Absent	Absent	Absent	Absent
Fats, Japan wax, resin and soaps	Passes test	Absent	Absent	Absent	Absent
Arsenic		<3 mg/kg			
Lead	<2 mg/kg	<5 mg/kg			
Mercury		<1 mg/kg			

(imported) as category 3 material which includes ABPs that do not present a potential risk for the food chain as they must not contain residues of other (foreign) substances and environmental contaminants (Reg. (EC) No. 1069/2009, Reg. (EU) No 142/2011, USDA/APHIS regulations – most of other regulations are in compliance with mentioned EC legislation for export/import purposes). However, beeswax is frequently marketed as "safe" category 3 even when it contains substances of questionable origin and chemical background (such as most commonly used adulterants, paraffin and stearic acid/stearin, as well as pesticide residues), due to the lack of obligatory legal regulations controlling its authenticity (FFN, 2017; Svečnjak et al., 2018). This problem is not referring only to the aspects of uncontrolled contamination via comb foundation trade (deliberate addition of adulterants into comb foundations that are not subjected to authenticity testing prior to their placement on the market) but also to the fact that adulterants are being accumulated in the apiculture sector through the comb foundation production process as comb foundations re-enter beekeeping technology (Svečnjak et al., 2016). Consequently, adulterated beeswax may enter other industries, such as cosmetic, pharmacy and food industry. Furthermore, given that beeswax (honeycomb built upon comb foundation) comes into contact with honey during the honey production process, food safety is another concerning aspect of beeswax adulteration. The negative aspects and potential risks of beeswax adulteration for animal and public health were recently brought to the attention of the European Commission (EC) by the EU Food Fraud Network (FFN, 2017).

Nowadays, there are more than 15 different natural (petroleum-derived, mineral, animal, and plant waxes) and industrial waxes used as beeswax adulterants worldwide (Bogdanov, 2004b, 2016a). Among them, paraffin waxes represent the greatest problem due to their wide availability and low price. Additionally, physico-chemical properties of paraffin (it is a chemically inert, almost odourless, and white or colourless substance) makes it almost "ideal" for adulteration. Other adulterants, such as stearic acid, stearin, tallow, microcrystalline wax, and others, are observed sporadically. The presence of high amounts of paraffin ($>$ 50%; up to 93%), as well as the sporadic appearance of stearic acid and/or stearin in comb foundations collected from the market has been reported in several recent studies (Maia et al., 2013; Serra Bonvehí & Orantes Bermejo, 2012; Svečnjak, Baranović et al., 2015, 2016, 2018; Waś et al., 2016). The negative effects of beeswax adulterated with stearin on the brood development have also been recently reported (Reybroeck & Van Nevel, 2018).

The disconcerting situation on the comb foundation market indicated by the results obtained in these studies implies an urgent need for routine beeswax authenticity control. This kind of outcome is a consequence of a larger-scale problem: general deficit of beeswax and "chronic" accumulation and circulation of paraffin (sporadically, other adulterants as well) in the comb foundation production process. The lack of regulations and standardized analytical methods for beeswax authenticity testing definitely contribute to this issue (Svečnjak, Baranović et al., 2015; Waś et al., 2015, 2016). We therefore provide a detailed description of the methods for beeswax adulteration detection using classical physico-chemical parameters, sensory analysis and spectroscopic techniques (GC-MS and FTIR-ATR).

6.2.2. Beeswax authentication by classical analytical methods (physico-chemical parameters)

Determination of physico-chemical parameters describing both physical and chemical properties of different organic substances have been widely used to establish the reference values defining quality (authenticity) criteria for particular substance, including beeswax. Over the course of the last six decades, considerable research has been invested in the attempt to build a reliable reference physico-chemical model defining pure beeswax (Bogdanov, 2016a; Tulloch, 1973, 1980; White, Riethof, & Kushnir, 1960).

Among the list of analytical methods and physico-chemical parameters defining beeswax quality criteria finally proposed by IHC based on pharmaceutical regulations (Bogdanov, 2016a; Table 11), the melting point, acid value, saponification value, ester value and ester/acid ratio, are the most commonly used to evaluate the beeswax quality and to detect possible adulteration. Nevertheless, the use of these parameters for detection of beeswax adulteration has some drawbacks in detecting the common adulterants, (paraffin, tallow, stearic acid and carnauba wax); as presented in Table 13, the minimum amount of mentioned adulterants that can be detected by corresponding methods is relatively high (high detection thresholds: 5–50%, depending on the type of adulterant).

Although the majority of proposed analytical range values characterizing pure beeswax are generally accepted and widely utilized, corresponding methods have not yet been officially employed for general beeswax authentication and quality control due to unresolved legislation issues mentioned in previous section. Another possible reason why these methods are not implemented in sectors other than pharmacy and food industry might be related to analytical deviations (values of acid, saponification, and ester value, as well as ester/acid ratio outside the proposed ranges) observed and reported in numerous studies in the past and current literature (Bennett, 1944; Bernal et al., 2005; Maia & Nunes, 2013; Serra Bonvehí, 1990; Svečnjak, Baranović et al., 2015; Tulloch, 1973). An overview of the ranges of physico-chemical parameters reported in the literature for authentic beeswax are summarized in Table 14 (Bennett, 1944; Bernal et al., 2005; Maia & Nunes, 2013; Nunes and Maia, 2017, unpublished data; Poncini, Poncini, & Prakash, 1993; Puleo & Rit, 1992; Serra

Table 13. Minimum adulteration percentages detected in beeswax by the measurement of reference physico-chemical parameters (Bernal et al., 2005).

Parameter	Paraffin (54–74 °C)	Stearic acid	Tallow	Carnauba wax
Melting point	30–50%	30%	40%	5%
Acid value	10%	2%	10%	20%
Saponification value	10%	3%	15%	[a]
Ester value	5%	5%	10%	[a]
Ester/acid ratio	10%	15%	10%	40%
Iodine value	15%	15%	15%	[a]

[a]Not useful for beeswax adulteration adulteration.

Bonvehí, 1990; Serra Bonvehí & Orantes Bernejo, 2012; Svečnjak, Baranović et al., 2015; Tulloch, 1973; Tulloch & Hoffman, 1972). The major factor affecting the analytical range values (primarily saponification value, and consequently, ester value and ester/acid ratio) is an exposure of beeswax to high heat treatment (Tulloch, 1973) which represents an integral part of the comb foundation production process given that beeswax used for comb foundation production is commonly subjected to different high heat treatments (121–140 °C) necessary to kill the spores of the heat-resistant *Paenibacillus larvae*. Thus, anomalous range values can be partially explained by the heat treatment applied (>100 °C) during beeswax recycling and processing (Bogdanov, 2016a; Svečnjak, Baranović et al., 2015; Tulloch, 1973): However, this does not explain the anomalous range values reported for virgin beeswax samples collected directly from the beehives (Bernal et al., 2005; Maia & Nunes, 2013; Svečnjak, Baranović et al., 2015). Certain deviations may also arise from a different geographical origin of the beeswax (Beverly et al., 1995), but the overall analytical deviation effects are not yet fully explained. Mentioned deviations of the range values proposed as beeswax quality standards do not affect standard methods for determining the respective parameters, but should be considered when performing physico-chemical tests (especially on comb foundations) for authentication purposes.

Along with proposed analytical measurands mentioned above, other physico-chemical parameters have been utilized sporadically for the purpose of beeswax authentication, namely, density, peroxide value (Bernal et al., 2005; Bogdanov, 2004a, 2004b), ash content and iodine number (Bernal et al., 2005; Puleo & Rit, 1992; Serra Bonvehí, 1990).

The methods for determination of all physico-chemical parameters listed above, as well as the challenges related to their use in beeswax adulteration detection, are described in the following sections (with an exception of ash content determination which has already been presented in Section 5.4.1).

6.2.2.1. Drop point. The drop point of beeswax is the temperature at which a drop of beeswax grease is extruded from the bottom of a special cup under the conditions of this test. The range of values defined by the European Pharmacopoeia and EU legislation is 61–66 °C.

It should be noted that determination of drop point and melting point (described in the following section) are not reliable analytical methods for beeswax adulteration detection if adulterants of the same or similar melting point as beeswax (60–65 °C) were used (often in the case of beeswax adulteration with paraffin wax).

An apparatus for the determination of drop point presented in Figure 36 (dimensions in mm) consists of 2 metal sheaths (A) and (B) screwed together. Sheath (A) is fixed to a mercury thermometer. A metal cup (F) is loosely fixed to the lower part of sheath (B) by means of 2 tightening bands (E). Fixed supports (D) 2 mm long determine the exact position of the cup in addition to which they are used to centre the thermometer. A hole (C) pierced in the wall of sheath (B) is used to balance the pressure. The draining surface of the cup must be flat and the edges of the outflow orifice must be at right angles to it. The lower part of the mercury thermometer has the form and size shown in the figure; it covers a range from 0 to 110 °C and on its scale a distance of 1 mm represents a difference of 1 °C. The mercury reservoir of the thermometer has a diameter of 3.5 ± 0.2 mm and a height of 6.0 ± 0.3 mm. The

Table 14. Ranges of the physico-chemical parameters reported in the literature for authentic beeswax.

Parameter	Melting point	Acid value	Saponification value	Ester value	Ester/acid ratio	Peroxide value	Iodine number
Bennett (1944)	61–65	16–23	85–101	72–79	3.6–4.3		4.0–12.0
Tulloch and Hoffman (1972)	63.4–65.1	17.4–21.8		70.3–75.4	3.38–4.12		
Tulloch (1973)		19.1		73.5	3.84		
Serra Bonvehí (1990)	61.9–64.1	17.4–19.8	90.1–90.8	70.3–79.0	3.54–4.34		9.6–17.3
Puleo and Rit (1992)	61–65	17–24	87–104	70–80			7–12
Bernal et al. (2005)	64–66	17.1–21.9	82.8–147.1	62.7–74.8	3.09–7.08	<0.01	7.6–13.1
Serra Bonvehí and Orantes Bermejo (2012)	61.9–64.1		90.1–98.3				
Maia and Nunes (2013)	63.0–67.3	14.4–23.0	65.5–124.2				
Svečnjak, Baranović et al. (2015)	60–65	20.7–30.2	57.5–134.0	31.1–112.2	1.18–5.14		
Nunes and Maia (2017, unpublished data)				46.4–103.3	2.3–5.3	0–19.9	5.9–14.3

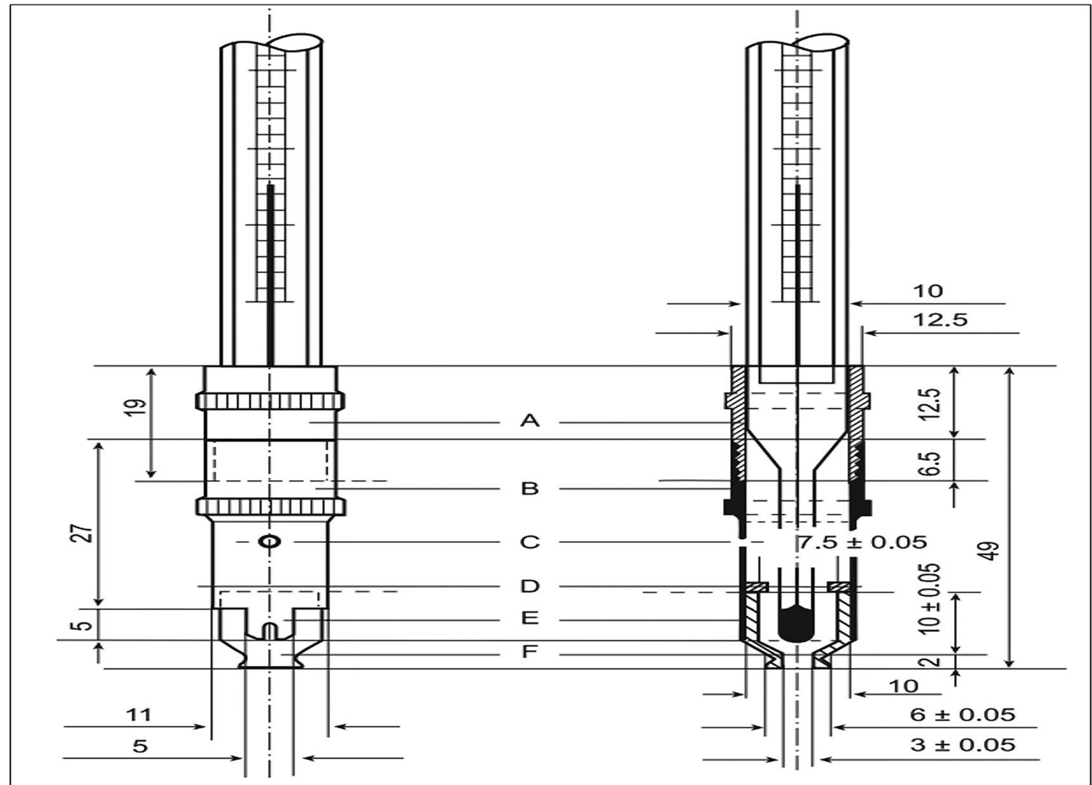


Figure 36. Apparatus for the determination of beeswax drop point (dimensions in mm); metal sheath fixed to a mercury thermometer (A), lower part of sheath (B), hole pierced in the wall of sheath (C), fixed supports (D), 2 tightening bands (E), metal cup fixed to the lower part of the sheath (F).

apparatus is placed in the axis of a tube about 200 mm long and with an external diameter of about 40 mm. It is fixed to the test-tube by means of a stopper through which the thermometer passes, and is provided with a side groove. The opening of the cup is placed about 15 mm from the bottom of the test-tube

Equipment required:

- Water bath with controlled temperature
- Drop point apparatus
- Thermometer
- 1L beaker
- Heat and stirring plate
- Stirrer

Procedure:

1. Melt the beeswax (10 g) by heating on a water-bath
2. Pour onto a glass plate.
3. Allow cooling to a semi-solid mass.
4. Fill the metal cup (Figure 36) by inserting the wider end into the beeswax.
5. Repeat the procedure until beeswax extrudes from the narrow opening.
6. Remove the excess with a spatula.
7. Insert the thermometer immediately.
8. Remove the beeswax displaced.
9. Allow to stand at room temperature for at least 12 h before determining the drop point.
10. Place the apparatus in the water-bath (the whole device is immersed in a beaker with a capacity of about 1 L) filled with water.
    The bottom of the test-tube is placed about 25 mm from the bottom of the beaker. The water level reaches the upper part of sheath (A). A stirrer is used to ensure that the temperature of the water remains uniform.
11. Heat the water-bath.
12. When the temperature is at about 10 °C below the presumed drop point, adjust the heating rate to about 1 °C/min.
13. Note the temperature at the fall of the first drop.
14. Carry out at least 3 determinations, each time with a fresh beeswax sample. The difference between the readings must not exceed 3 °C. The mean of three readings is the drop point of the beeswax sample.

6.2.2.2. Melting point. This index is determined following the "capillary tube method" as described by Bernal et al. (2005).

Equipment required:

- Water bath with controlled temperature
- 10 cm long 62 mm internal diameter thin-wall hollow capillary tube

Procedure:

1. Melt beeswax (see Section 2.2)
2. Insert melted beeswax in a 10 cm long 62 mm internal diameter thin-wall hollow capillary tube, up to about 1 cm.
3. Allow sample to solidify.
4. Keep at RT for 24 h once solidified.
5. Put the capillary tube containing beeswax into a water bath.
6. Slowly warm the water bath: 1–2 °C/min
   The temperature is checked with a thermometer whose bulb had to be as close as possible to the capillary tube with beeswax.
7. The melting temperature is that at which the beeswax is completely molten: the beeswax liquid is entirely transparent without turbidity.
8. Repeat steps 1–7 twice so that the analysis is performed in triplicate.

Alternatively, the capillary tube-based instruments for automatic determination of melting point (or slip melting point – SMP) employing simple and fast protocols based on microprocessor-controlled temperature ramping up system and in some models, a built-in digital camera that allows the observation of beeswax melting, can be used (for example, MP 55 Melting Point System, Mettler Toledo).

6.2.2.3. Acid value. The acid value is defined as the amount of KOH in milligrams needed to neutralize 1 g of beeswax. This method is intended to give a measure of free fatty acids present in beeswax, although the presence of other interfering acid substances can affect this relation.

The acid value determination is one of the most efficient and simple methods for detecting beeswax adulteration with paraffin and stearic acid. Acid value decreases with the addition of paraffin (Bernal et al., 2005; Maia & Nunes, 2013; Svečnjak, Baranović et al., 2015), and increases with the addition of stearic acid (Bernal et al., 2005) (Figures 37 and 38, respectively).

Equipment required:

- Balance with a 0.1 mg precision
- 250 mL conical flask
- Reflux condenser
- Glass beads
- Electric plate
- Burette

Materials and reagents required:

- Xylene p.a.
- Ethanol p.a.
- Phenolphthalein p.a.
- KOH p.a. or available standardized 0.5 M KOH solution

Procedure:

1. Weigh 2 g of beeswax in a 250 mL conical flask fitted with a reflux condenser (***m***, g).
2. Add 40 mL of xylene.
3. Add a few glass beads.
4. Heat on an electric plate at 60–70 °C until the material is completely dissolved.
5. Add 20 mL of ethanol.
6. Add 0.5 mL of phenolphthalein solution (1% phenolphthalein in 95% ethanol).
7. Titrate the hot solution with a standardized c.a. 0.5 M potassium hydroxide (***C***, mol/L) in ethanol until the change in colour of the indicator (appearance of a persistent red colour for at least 10 s), (***v1***, mL).
8. Repeat steps 2–7 to create the reference (blank) solution without the addition of sample, (***v2***, mL).
9. Calculate the acid value using the formula:

$$\text{Acid value} = \frac{(v1 - v2) \times C \times 56.1}{m}$$

6.2.2.4. Saponification value. The saponification value is defined as the amount of KOH in milligrams needed to

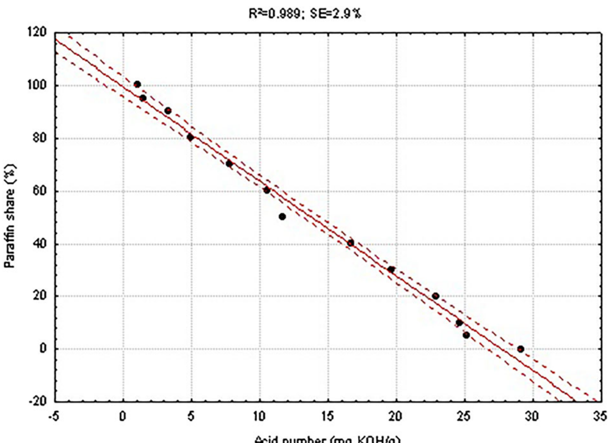


Figure 37. Effect of different levels of beeswax contamination with paraffin on the acid value of beeswax (Svečnjak, Baranović et al., 2015).

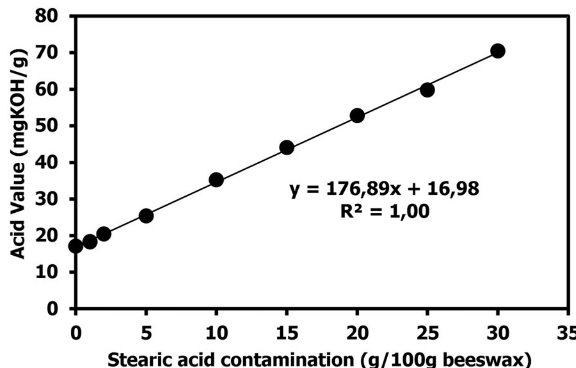


Figure 38. Effect of different levels of beeswax contamination with stearic acid on the acid value of beeswax (Nunes & Maia, 2017, unpublished data).

neutralize 1 g of beeswax after saponification, i.e., alkaline hydrolysis. This method is intended to give a measure of the total fatty acids present in beeswax, either free or esterified, although the presence of other interfering acid substances can affect this relation (any acid or ester present but not associated directly with the sample being analyzed, including adulterant-originated compounds).

Determination of saponification value (and consequently, ester value and ester/acid ratio) is not a reliable for detecting adulterants in comb foundations due to analytical anomalies that may be caused by high heat treatment applied during the comb foundation production process. Also, it is a time-consuming analysis, not suitable for routine testing.

Equipment required:

- Balance with a 0.1 mg precision
- 250 mL conical flask
- Reflux condenser
- Glass beads
- Electric plate
- Burette

Materials and reagents required:

- Xylene p.a.
- Ethanol p.a.
- Phenolphthalein p.a.
- KOH p.a or available standardised 0.5 M KOH solution
- HCl p.a. or available standardised 0.5 M HCl solution

Procedure:

1. Weigh 2 g of beeswax in a 250 mL conical flask fitted with a reflux condenser (***m***, g).
2. Add 30 mL of a mixture of ethanol:xylene (1:1 v/v)
3. Add a few glass beads.
4. Heat until the material is dissolved.
5. Add 25.00 mL of standardized c.a. 0.5 M potassium hydroxide (***C***, mol/L)
6. Heat under reflux during 3 h.
7. Titrate the hot solution immediately with a standardized solution of c.a. 0.5 M of HCl, using 1 mL of phenolphthalein solution (1% phenolphthalein in 95% ethanol) as indicator (***v3***, mL).
8. Reheat the solution to boiling several times (usually three to four times) during the titration to dissolve the material.
9. Repeat steps 2–8 to create the reference (blank) solution without the addition of sample, (***v4***, mL).
10. Calculate the saponification value using the formula:

$$\text{Saponification value} = \frac{(v4 - v3) \times C \times 56.1}{m}$$

6.2.2.5. Ester value.

$$\text{Ester value} = \text{saponification value} - \text{acid value}$$

The ester value is defined as the amount of KOH in milligrams needed to neutralize 1 g of ester linked acids of beeswax being calculated by subtracting the saponification value (Section 6.2.2.4) by the acid value (Section 6.2.2.3). This method is intended to give a measure of esterified fatty acids in beeswax.

6.2.2.6. Iodine number. The iodine number is defined as the amount in grams of iodine absorbed by 100 g of lipids. The iodine value gives an indication of the average degree of unsaturation, i.e., the number of double bonds of a lipid. There are various methods available in the literature for determining the iodine number of a lipid; the two more common methods are those of Wijs and Hanus. The method mostly used for determination of the iodine number of beeswax is the Hanus method.

The iodine number can also be advantageously used to detect adulteration with paraffin, tallow, and stearic acid (Bernal et al., 2005). An example for iodine number increase in case of adulteration with tallow, and its decrease in case of paraffin adulteration is presented in Figures 39 and 40, respectively.

Equipment required:

- Balance with a 0.1 mg precision
- 250 mL conical flask with stopper
- Burette

Materials and reagents required:

- Chloroform p.a.
- Iodine p.a.
- Glacial acetic acid p.a.
- Bromine water p.a.
- Potassium iodide p.a.
- Sodium thiosulfate p.a or available standardised 0.1 N sodium thiosulfate solution
- Starch

Procedure:

1. Prepare Hanus solution:
   a. Dissolve 18.2 g of iodine in 1 L of glacial acetic acid
   b. Add 3 mL of bromine water for doubling the halogen content.
   Alternatively, there are commercially available Hanus solutions from various distributors.
2. Weigh the appropriate amount of beeswax (***m***, g) in a 250 mL conical flask.

   The amount of material used in iodine value determination is dependent on its expected iodine value

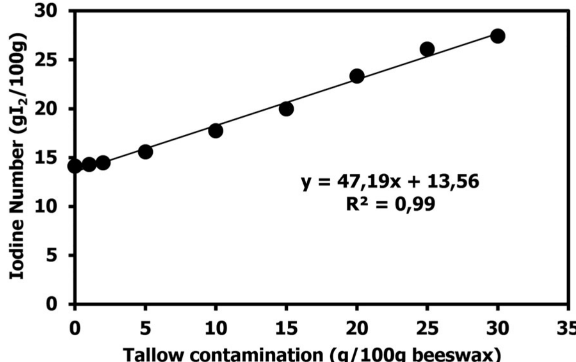


Figure 39. Effect of different levels of beeswax contamination with tallow on the beeswax iodine number (Nunes & Maia, 2017, unpublished data).

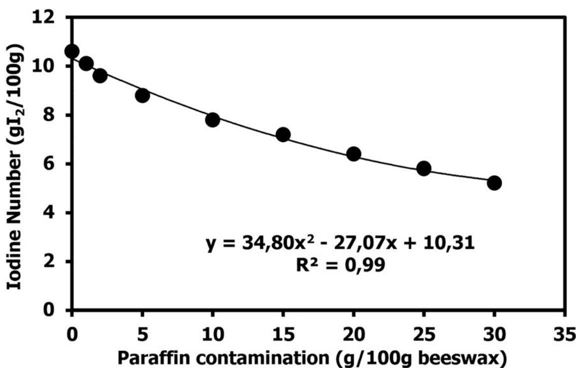


Figure 40. Effect of different levels of beeswax contamination with paraffin on the beeswax iodine number (Nunes & Maia, 2017, unpublished data).

and should be determined by a previous titration of the sample before carrying out the analytical determination, as presented in Table 15.

3. Add 10 mL of chloroform.
4. Add 25mL of Hanus solution by draining in a definite time (the time of draining should be kept constant between analysis).
5. Close the flask with a glass stopper.
6. Mix thoroughly.
7. Allow standing in the dark for 30 min with occasional shaking.
8. Add 10 mL of 15% potassium iodide solution.
9. Shake thoroughly.
10. Add 100 mL of freshly boiled and cooled distilled water, washing down any free iodine on the stopper.
11. Titrate against standardized 0.1 N sodium thiosulfate (***N***, equivalent L^{-1}) solution until the yellow solution turns almost colourless.
12. Add 2–3 drops of starch solution (1%); a deep blue colour must be formed.
13. Shake the stoppered flask vigorously (so that any iodine remaining in the chloroform is taken up by the potassium iodide solution).
14. Continue with titration until the blue colour disappears (***v5***, mL).

Table 15. Amount of sample to be weighed in the iodine number determination as a function of the expected iodine number.

Expected iodine number	Mass of sample to be used (g)
0–30	0.8000
30–50	0.5000
50–100	0.2500
100–150	0.1700
150–200	0.1200

15. Repeat steps 3 to 14 to create the reference (blank) solution without the addition of sample, (***v6***, mL).
16. Calculate the iodine number using the formula:

$$\text{Iodine number} = \frac{(v6 - v5) \times N \times 12.69}{m}$$

6.2.2.7. Peroxide value. The peroxide value of a sample gives a measure of the amount of hydroperoxides present, being a measure of the oxidation of a sample, expressed commonly as milliequivalents of peroxide per kilogram of sample (AOAC 965.33, 2000).

The method has two main sources of errors, the oxidation of the reagents by air, accelerated by light, and the re-absorption of the liberated iodine by the material, which can reduce the peroxide value obtained and these sources of errors should be taken into account when interpreting the results.

Determination of peroxide value is commonly used for beeswax purity testing in the frame of chemical and technical assessment of beeswax as food additive (FAO, 2005; JECFA, 2005).

Equipment required:

- Balance with a 0.1 mg precision
- 250 mL conical flask with stopper
- Burette

Materials and reagents required:

- Chloroform p.a.
- Glacial acetic acid p.a.
- Potassium iodide p.a.
- Sodium thiosulfate p.a or available standardised 0.1 N sodium thiosulfate solution
- Starch

Procedure:

1. Transfer approximately 5 g of the sample (***m***, g) into a 250 mL Erlenmeyer flask with glass stopper.
2. Add 30 mL of the glacial acetic acid: chloroform 3:2 (v/v).
3. Swirl to dissolve.
4. Add 0.5 mL of saturated potassium iodide solution, freshly prepared (dissolve excess KI in freshly boiled water, excess solid must remain, store in the dark).

5. Shake thoroughly for 60 ± 1 s while reaction is occurring.
6. Add 30 mL of water.
7. Shake vigorously.
8. Slowly titrate with 0.1 N sodium thiosulfate solution (***N***, equivalent L^{-1}) with vigorous shaking until the solution presents a pale yellow colour.
9. Add 0.5 mL of 1% aqueous starch solution and continue titration with vigorous shaking to release all iodine from the organic chloroform layer, until the colour disappears. (***v7***, mL).
10. If less than 0.5 mL of the 0.1 N sodium thiosulfate solution is used, repeat the determination using the standardized 0.01 N sodium thiosulfate solution (step 8).
11. Repeat steps 2–10 to create the reference (blank) solution without the addition of sample (the volume used should be less than 0.1 mL of the 0.1 N thiosulfate solution) (***v8***, mL).
12. Calculate the peroxide value using the formula:

$$\text{Peroxide value} = \frac{(v7 - v8) \times N}{m} \times 1000$$

6.2.2.8. Detection of carnauba wax.

Equipment required:

- Balance with a 0.1 mg precision
- Test tubes
- Water bath with controlled temperature
- Beaker
- Light microscope

Materials and reagents required:

- *n*-butanol p.a.

Procedure:

1. Weigh 100 mg of the sample into a test tube.
2. Add 20 mL of *n*-butanol.
3. Heat the test tube in a boiling water bath.
4. Shake the mixture gently until the sample dissolves completely.
5. Transfer the test tube to a beaker of water at 60 °C.
6. Allow the water to cool to room temperature.
7. A loose mass of fine, needlelike crystals separates from clear mother liquor. Under the microscope, the crystals appear as loose needles or stellate clusters, and no amorphous masses are observed, indicating the absence of carnauba wax.

6.2.2.9. Detection of ceresin, paraffin and other waxes.

Equipment required:

- Balance with a 0.1 mg precision
- Round-bottomed flask
- Reflux condenser
- Thermometer
- Water bath with controlled temperature

Materials and reagents required:

- KOH p.a.
- Ethanol p.a.

Procedure:

1. Weigh 3 g of material in a round-bottomed flask.
2. Add 30 mL of a 40 g/L solution of KOH in aldehyde-free ethanol.
3. Boil gently during 2 h under reflux by attaching a condenser.
4. Remove the condenser and immediately insert a thermometer.
5. Place the round-bottomed flask in a water-bath at 80 °C.
6. Allow cooling by swirling the solution continuously.
7. For pure beeswax no precipitate is formed up to 65 °C, although the solution may be slightly opalescent. If above 65 °C there is a formation of precipitates, this indicates the presence of ceresin, paraffin and other waxes. At 59 °C, the solution of pure beeswax is cloudy.

6.2.2.10. Detection of glycerol and other polyols.

Equipment required:

- Balance with a 0.1 mg precision
- Round-bottomed flask
- Reflux condenser
- G4 glass filter
- Test tubes
- Thermometer
- Water bath with controlled temperature

Materials and reagents required:

- KOH p.a.
- Ethanol p.a.
- Sulfuric acid p.a.
- Glycerol p.a.
- Sodium periodate p.a.
- Fuchsin p.a.
- Sodium sulphite p.a.
- HCl p.a.
- Activated charcoal

Procedure:

1. Add 10 mL of ethanolic potassium hydroxide solution (0.5 N) to 0.20 g of beeswax.
2. Heat on a water-bath under a reflux condenser for 30 min.
3. Add 50 mL of dilute sulphuric acid (98 g/L of H_2SO_4)

4. Cool.
5. Filter through a G4 glass filter.
6. Rinse the flask and the filter with dilute sulphuric acid.
7. Combine the filtrate and washes.
8. Dilute to 100.0 mL with dilute sulphuric acid.
9. Prepare a 10 mg/L glycerol standard in diluted sulphuric acid (98 g/L of H_2SO_4).
10. Place 1.0 mL of the test solution in a test-tube.
11. Place 1 mL of the glycerol standard in another test tube.
12. Add 0.5 mL of a 10.7 g/L solution of sodium periodate to each tube
13. Mix thoroughly.
14. Allow to stand for 5 min.
15. Prepare decolorized fuchsin solution by dissolving 0.1 g of basic fuchsin in 60 mL of water.
    a. Allow the fuchsin solution to stand protected from light for at least 12 h.
    b. Add a solution of 1 g of anhydrous sodium sulfite (reagent grade) in 10 mL of water.
    c. Add 2 mL of hydrochloric acid slowly and with continuous shaking of the solution.
    d. Dilute to 100 mL with water.
    e. Decolorize with activated charcoal.
    f. Filter through a G4 glass filter.
    g. If the solution becomes cloudy, filter again before use.
    h. If by standing the solution becomes violet, decolorize again by adding activated charcoal.
    i. Store protected from light.
16. Add 1.0 mL of decolorized fuchsin solution to each tube.
17. Mix thoroughly (any precipitate disappears).
18. Place the tube in a beaker containing water at 40 °C.
19. During cooling observe for 10–15 min.
20. The violet blue colour developed by the pure beeswax containing solution should not be more intense than that of the glycerol standard prepared and analyzed simultaneously [using 1.0 mL of a 0.001% (w/v) solution of glycerol in dilute sulphuric acid].

6.2.2.11. Advantages and disadvantages of physico-chemical methods.

Advantages:

- Inexpensive equipment, material and reagents.
- Easy to implement.
- No need for advanced chemical analysis training.
- Existence of reference values for pure beeswax for the majority of physico-chemical parameters (though analytical anomalies that may occur should be taken into consideration).

Disadvantages:

- Questionable reliability of certain physico-chemical methods for beeswax adulteration detection (determination of melting point, saponification and ester value, and ester/acid ratio) in relation to the type of beeswax specimen or adulterant type.
- High detection thresholds: 5–50% (depending on the type of adulterant and parameter used for its detection).
- Methods require high amounts of the sample for analysis (compared to GC-MS and FTIR-ATR method).
- Analyses are destructive.
- Provide no chemical information about the beeswax constituents.
- Performance of more than one analysis is required to determine the presence of adulterants.
- For quantification purposes (and sometimes reliability), the beeswax adulteration should be confirmed by more reliable analytical tools - spectroscopic techniques (GC-MS or FTIR-ATR).

6.2.2.12. Conclusion. Considering all the challenges associated with the use of physico-chemical methods for beeswax adulteration detection, we recommend the following approach to be applied when assessing the authenticity of beeswax using physico-chemical parameters:

- Always combine a set of several physico-chemical measurements (at least 3; an exclusive measurement of only one physico-chemical parameter should not be used for detecting adulterates in beeswax; the most reliable combination of physico - chemical measurements to discriminate between pure and adulterated beeswaxes is determination of acid value, iodine number and peroxide value (Bernal et al., 2005).
- For reliable confirmation of adulterants presence (detection of in average <5–15% of adulterants) and quantification of adulterants in beeswax, physico-chemical measurements should be complemented with spectroscopic analysis (GC-MS and/or FTIR-ATR technique described below in Sections 6.2.4 and 6.2.5, respectively).

6.2.3. Sensory analysis of beeswax

Determination of the sensory characteristics of beeswax is not sufficient evidence of adulteration, but in some cases can give hints on possible adulteration. In this way, this kind of analysis can be important to beekeepers because they are fast and cheap and can be a useful as a preliminary analysis of the beeswax quality in industry. The negative control for sensory assessment is authentic beeswax (collected in accordance with guidelines described in Section 2.1.1).

To obtain the beeswax sample from comb wax, comb foundation or wax blocks for sensory assessment, follow the procedure described in Section 2.1. Beeswax melting and storage should be performed as described in Sections 2.2 and 2.3, respectively.

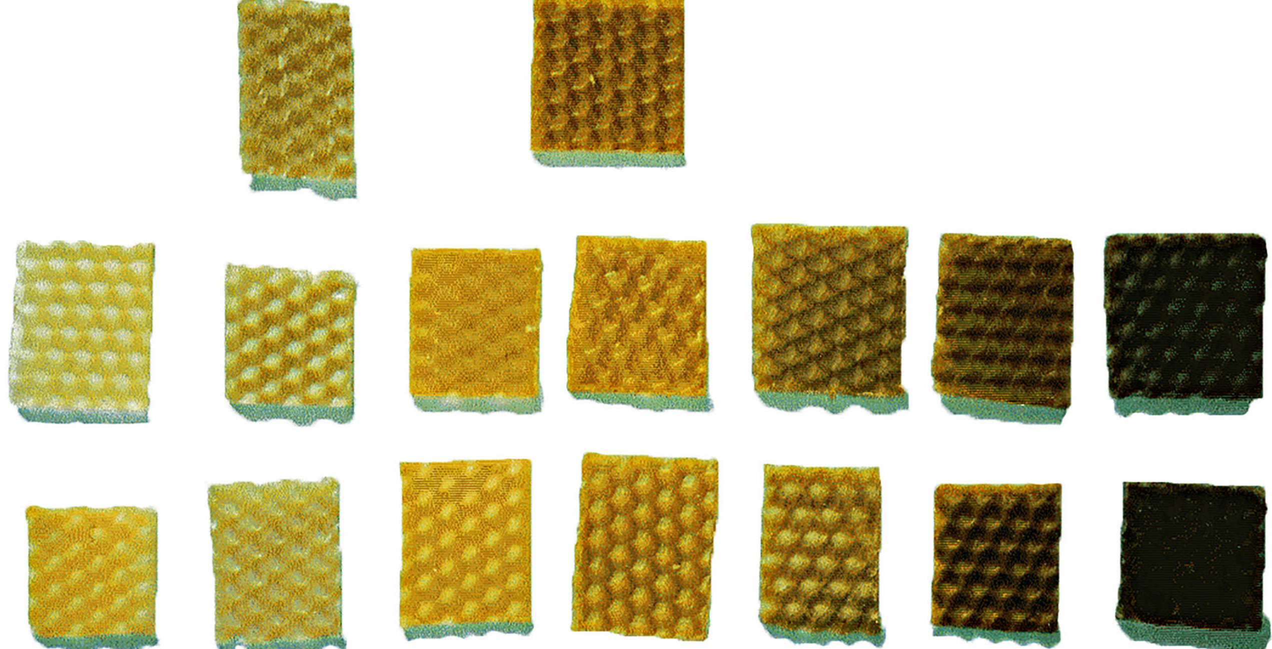


Figure 41. Diversity of colours in unadulterated comb foundations due to mixing of different wax blocks and absence of wax bleaching. Photo: Maia M.

6.2.3.1. Organoleptic characteristics.

6.2.3.1.1. Colour of beeswax. The colour of the freshly produced beeswax is white, later it turns to yellow. This yellow colour originates from propolis and pollen colorants (Bogdanov, 2004a). The colour of the beeswax is an important quality parameter (point of view of refiners and industry), but should not be taken as a criterion for establishing wax purity (Barros, Nunes, & Maia, 2009). Figure 41 shows comb foundation of naturally different colours (without adulterants).

In the industry, the colour of beeswax can be altered by bleaching or by the addition of colorants (Bianchi, 1990). Bleaching is often done with chemicals. The use of these chemicals can be problematic from an environmental and toxicological point of view. Beeswax can be brightened simply by exposing it to the sun, or by chemicals using the following methods.

1. Use 2–3 g of oxalic acid per kg wax and 1 L of water. Add acid to water and not *vice versa* (Bogdanov, 2016a).
2. Use 1 mL concentrated of sulphuric acid to 1 L of water per kg wax.
3. Add concentrated hydrogen peroxide solution (about 35% in basic *milieu*) to hot wax (100 °C). There is no point in using more than the amount of hydrogen peroxide suggested, and any excess may cause too much frothing and a boiling over of wax (Brown, 2009). An excess of hydrogen peroxide can cause problems in the manufacture of creams and ointments.

6.2.3.1.2. Aroma and taste of beeswax. The odour is very important to characterize the beeswax sensory profile and/or to indicate an addition of certain adulterants, such as oils, tallow, paraffin or resins. Beeswax has a characteristic odour, originating from the bees, propolis and honey (Bogdanov, 2004b). Odour is best assessed by placing the wax sample in a test tube (Bianchi, 1990) and should be pleasant and honey-like (Bogdanov, 2016a). Some factors can contribute to the change of aroma of wax, like the use of combs containing fermented honey and the use of some solvents (Bogdanov, 2004b). The taste of beeswax is normally pleasant and is not specific – any unpleasant taste is a sign of quality deterioration due to foreign matter (Bogdanov, 2016a). See Table 16 to compare the odour and taste of pure beeswax and adulterated beeswax. It should be noted that it is not recommendable to assess the taste of beeswax in case of suspicious beeswax samples given the unknown chemical background of various foreign substances that might be used for beeswax adulteration.

6.2.3.1.3. Consistency of beeswax. Consistency level is an important test to indicate the presence of some adulterants in beeswax (Bianchi, 1990). Consistency test should be performed as follows:

1. Weigh 1 g of beeswax and melt in a test tube.
2. Put into a watch glass and leave 24 h at room temperature.
3. After this period, take the sample between index finger and thumb and make a ball.
4. See Table 16 for differences between pure beeswax and mixtures of beeswax with different adulterants.

Table 16. Differences between pure beeswax and beeswax mixture with some adulterants.

Substance	Odour/taste	Consistency
Beeswax	Should have a honey-like smell	The mass of beeswax is slightly sticky to fingers and has a homogeneous, transparent and dull appearance
Paraffin	It also has a peculiar odour of petroleum (the more intense the smell, the greater the percentage of paraffin)	Adulteration with paraffin is recognised by the appearance of an intense brightness in the moulded beeswax sample between the fingers, and become slippery between the fingers
Tallow	Fat-like odour	Elastic, easily mouldable
Stearin/stearic acid	Hard to characterize specific odour/not distinctive	Like porcelain bright, with visible crystalline appearance, undoes easily between fingers

6.2.3.2. Qualitative methods for rapid detection of beeswax adulteration. The qualitative tests are quick and interesting because they can indicate, with some assurance, the level of adulteration, especially with regard to paraffin (Barros et al., 2009). The description of these tests is modified from Bianchi (1990).

6.2.3.2.1. Detection of paraffin.

Procedure:

1. The alcohol solution of 12% KOH should be prepared on the same day.
2. Weigh 5 g of beeswax in 250 mL beaker.
3. Add 25 mL of 12% KOH alcohol solution.
4. Heat to a pasty consistency (at ∼ 70–80 °C).
5. Quickly dissolve with 20 mL of hot glycerine.
6. Add hot distilled water.
7. In the presence of paraffin or ceresin, a milky liquid is produced.
8. Can be converted into a precipitate if there are high amounts of paraffin.

It is possible to designate wax samples as adulterated with paraffin in excess of 15%. We also found that in case of adulteration with 10% of paraffin, there was a slight increase in the opacity of the solution. However, we think it may not be convincing enough to confirm that a wax sample is adulterated, because this type of analysis is highly dependent on the operator's observation. For samples with high percentages of paraffin (> 20%), this methodology is quite persuasive, independently of the operator, because the milky appearance of the liquid is very typical (Figure 42). Results of this test are related with acid value, and these two methods are complementary to each other for the detection of paraffin. The qualitative method for rapid detection of paraffin generates less than 5% false positives; the absence of paraffin was confirmed by FTIR-ATR (Maia & Nunes, 2017, unpublished data).

6.2.3.2.2. Detection of tallow.

Procedure:

1. In a beaker (50 mL), bring to the boil 1 g of beeswax with 10 mL of sodium carbonate solution at anhydride 30%, until its dilution.
2. Leave it to cool at room temperature.
3. Allow standing for another 3 min.
4. Carefully pour the liquid into the test tube.
5. Add 5 mL of water.
6. In the presence of fat, a milky liquid rapidly form some lumps that rise to the surface after ±10 min (Figure 43).

6.2.3.2.3. Detection of stearin.

Procedure:

1. Heat 1 g of beeswax in a beaker (50 mL) until it is melted.
2. Add 7 mL of ethyl alcohol and over 3 mL of water until its dissolution.
3. Cool to 20 °C.
4. Filter into a test tube (filter used: Whatman 114).
5. Add 4 mL of distilled water to the filtrate.
6. In the presence of stearin, a milky white precipitate is observed (Figure 44).

6.2.3.2.4. Detection of resin.

Procedure:

1. Heat 1 g of beeswax and 4 mL of concentrated nitric acid (65%) in a beaker (50 mL) until it is melted.
2. Boil for 1 min.
3. Leave it to cool at room temperature.
4. Dilute with 4 mL of water.
5. Rapidly neutralize with with few drops of ammonia.
6. In the presence of resins, reddish vapours are formed during the heating of the blend and the final solution is a reddish-brown color.

6.2.3.2.5. Detection of starch.

Procedure:

1. Place 1g beeswax in a 50 mL beaker.
2. Heat (at 62–65 °C, until melting starts)
3. Add 12 mL of essence of turpentine until its dissolution.
4. Add an aqueous solution of iodine if a white deposit was formed.
5. If the deposit becomes blue, the sample contained starch or flour.

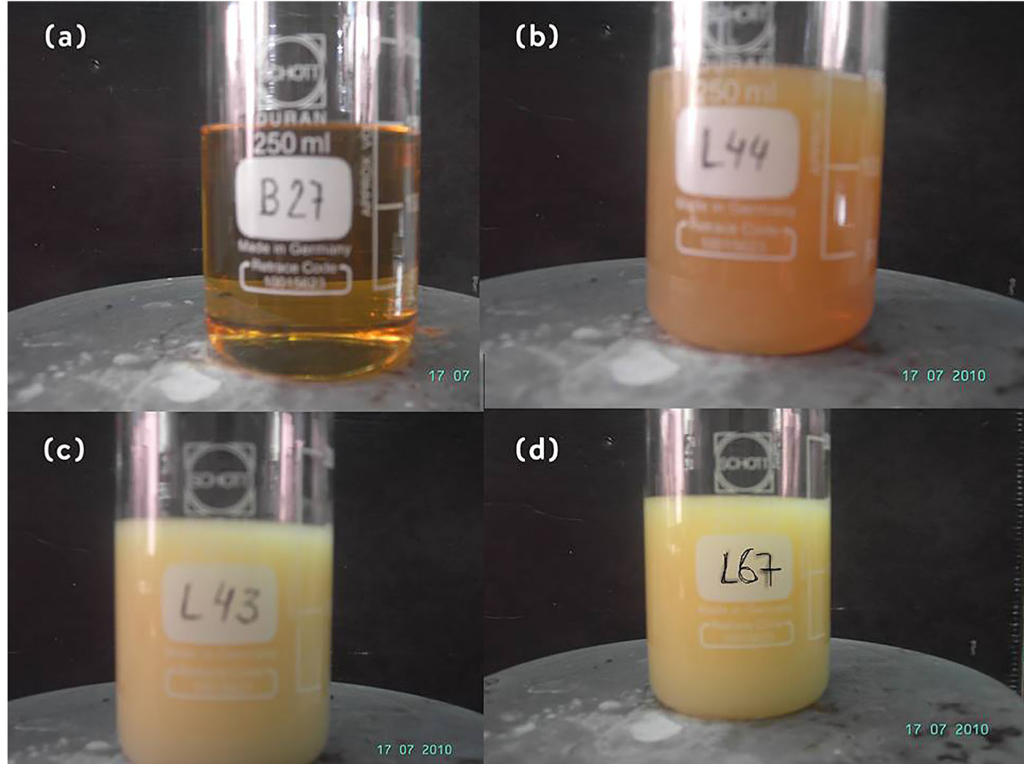


Figure 42. Detection of paraffin by qualitative methods: (a) sample without paraffin; (b) sample containing 15% of paraffin; (c) sample containing 20% of paraffin; (d) sample containing 30% of paraffin.

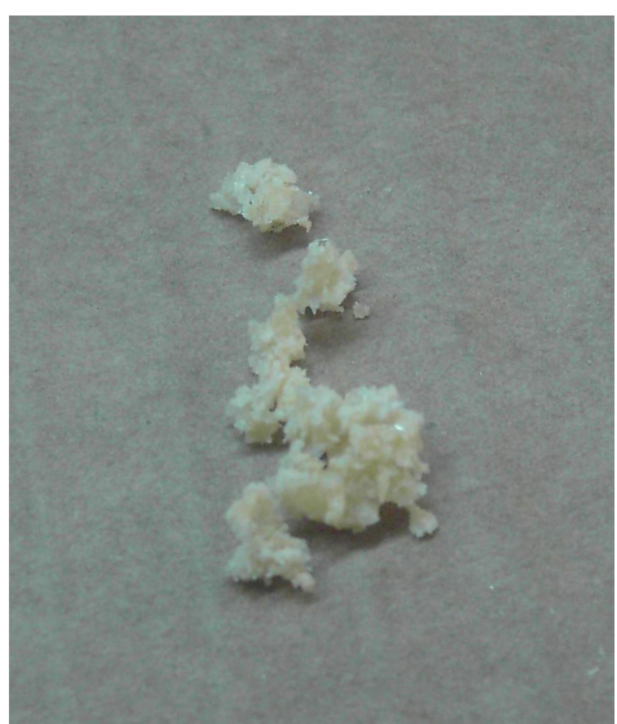


Figure 43. Formation of lumps characteristic for tallow adulteration.

6.2.3.2.6. Detection of minerals.

Procedure:

1. Heat 1 g of beeswax in a beaker (50 mL) until it is melted.
2. Add 12 mL of chloroform until dilution of the beeswax dilution.
3. In the presence of minerals, an insoluble residue is formed.

6.2.3.3. Advantages and disadvantages of sensory analysis.

Advantages:

- Cheap and fast methods.
- Can be related to other analytical parameters (physico-chemical, spectroscopic) and complement the confirmation of adulteration.
- Low percentage of false positives.

Disadvantages:

- Sensory analysis comprises both objective and subjective evaluation
- Subjective measures
- High detection limit for some adulterants (paraffin: 10–15%).
- For some parameters, the detection of adulteration may depend on the operator experience.

6.2.4. Adulteration detection by GC-MS technique

The GC-MS method elaborated by Waś et al. (2014a) for the determination of beeswax hydrocarbons is also suitable for the detection of beeswax adulteration with hydrocarbons of foreign origin, e.g., paraffin or ceresin

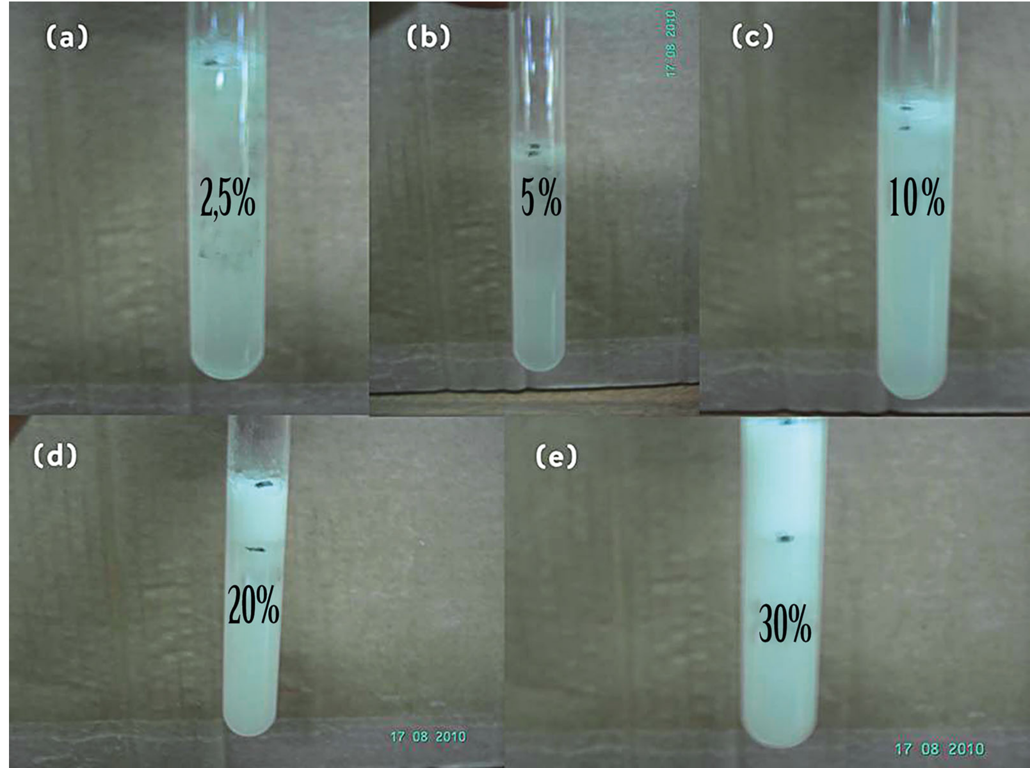


Figure 44. Adulteration with stearin: (a) sample containing 2.5% of stearin; (b) sample containing 5% of stearin; (c) sample containing 10% of stearin; (d) sample containing 20% of stearin; (e) sample containing 30% of stearin.

(Waś et al., 2015, 2016). The method is schematically illustrated in Figure 45.

The GC-MS method which is described in details in Section 5.2.1 focuses on identification of hydrocarbons (alkanes, alkenes, and dienes) and quantitative analysis of n-alkanes. These compounds naturally occur in beeswax, but they can also come from adulteration with substances such as paraffin or ceresin. The routine analysis of detection of beeswax adulteration with hydrocarbons of foreign origin can be carried out rapidly by comparing the chromatograms (fingerprints) of hydrocarbons in pure and adulterated beeswax as is presented in Figure 46. The proposed method also enables the quantitative analysis of n-alkanes, which could be used for estimation a degree of beeswax adulteration with paraffin. The minimum estimated percent of paraffin detectable using the GC-MS technique is 3%. According to the criteria established by Waś et al. (2016) the adulteration of beeswax with paraffin is indicated by the presence of hydrocarbons containing over 35 atoms of carbon in the molecule, and by the higher contents of individual n-alkanes ($C_{20}H_{42}$–$C_{35}H_{72}$) as well as a higher content for the total of these compounds, in comparison to the maximum contents determined in beeswax (Waś et al., 2014b). Similar criteria, but with slightly different critical values for the contents of beeswax hydrocarbons, were recommended by other authors (Jiménez et al., 2007).

6.2.4.1. Detection of beeswax adulteration with foreign hydrocarbons. The analytical procedure for detection of beeswax adulteration with hydrocarbons of foreign origin (substances such as paraffin or ceresin) is the same as procedure for determination of hydrocarbons in pure beeswax. This procedure is described step by step in Section 5.2.1 (along with the list of equipment and materials/reagents required):

1. Preparation of standard solution (5.2.1.1).
2. Beeswax sample preparation (5.2.1.2).
3. Conditions of GC-MS analysis (5.2.1.3).
4. Qualitative analysis of beeswax hydrocarbons (5.2.1.4).
5. Quantitative analysis of n-alkanes in beeswax (5.2.1.5).

6.2.4.2. Quality assessment of beeswax using GC-MS. In assessing the quality of commercial beeswax, the following criteria indicating its adulteration with hydrocarbons of foreign origin (e.g., paraffin) should be applied:

1. Presence of hydrocarbons containing over 35 carbon atoms in the molecule.
2. Higher contents of individual n-alkanes ($C_{20}H_{42}$–$C_{35}H_{72}$).
3. Higher content for the total of n-alkanes in comparison to the maximum values determined in pure beeswax.

In the routine preliminary quality control of beeswax is recommend the comparative analysis of the hydrocarbon chromatograms, an example of which is given in

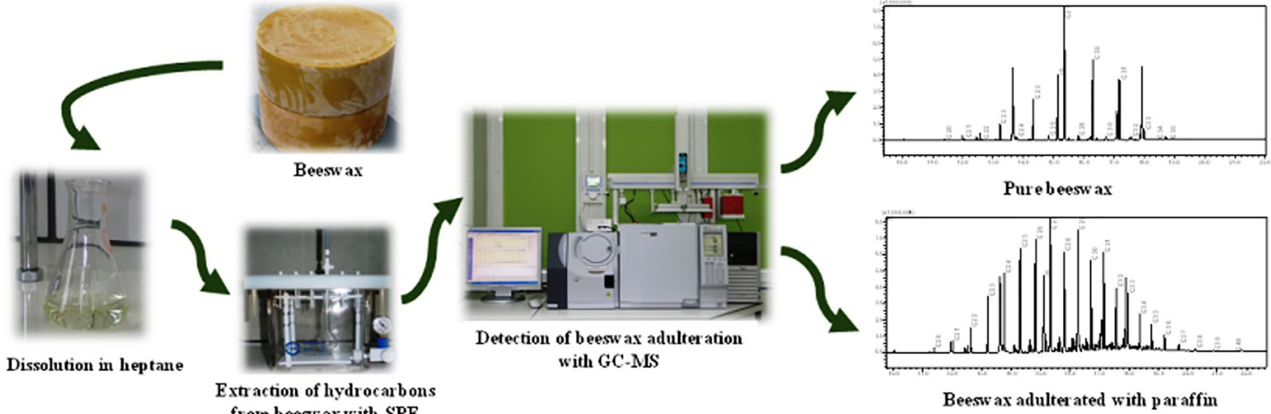


Figure 45. General procedure of the detection of beeswax adulteration by GC-MS.

Figure 46. For the compaerative analysis of chromatograms the same amounts of beeswax samples should be taken. The GC-MS chromatogram of hydrocarbons presented in Figure 46(a) is proposed as standard fingerprint of beeswax originate from *A. mellifera*. By comparing of the chromatograms of hydrocarbons in pure beeswax (Figure 46(a)) and adulterated with paraffin (Figure 46(b,c)), it can be easily noted that the intensity of the peaks of individual alkanes in beeswax increases when the paraffin is added. The increase of the peak intensities is particularly visible for the alkanes with even numbers of carbon atoms in the molecule ($C_{24}H_{50}$, $C_{26}H_{54}$, $C_{28}H_{58}$, $C_{30}H_{62}$, $C_{32}H_{66}$, $C_{34}H_{70}$), even with only a 3% addition of paraffin (Figure 46(b)). Moreover, the addition of substances such as paraffin or ceresin to beeswax resulted in the occurrence of alkanes containing over 35 atoms of carbon in the molecule (Figure 46(b,c)), which are not detected in pure beeswax (Figure 46(a)).

In the next step, it is advisable to confirm the qualitative results based on chromatograms by quantitative analysis performed using the internal standard method (described in Section 5.2.1.5). Then, n-alkane contents determined in unknown sample must be compared with the maximum contents determined for pure beeswax, proposed as the concentration – guide values to distinguish between pure and adulterated beeswax (Waś et al., 2014b) shown in Table 17. According to these requirements, the maximum concentrations accepted for pure beeswax amounted to 11.7 g/100 g (for total n-alkanes $C_{20}H_{42}$–$C_{35}H_{72}$) and 1.0 g/100 g (for total even-numbered n-alkanes from $C_{20}H_{42}$ to $C_{34}H_{70}$).

The most frequently used adulterant is paraffin. However, various types of paraffin available on the market differ not only in physico-chemical properties (such as melting point), but also in the hydrocarbon composition. The results of studies investigating different types of paraffin and their mixtures with beeswax were presented by Waś et al. (2015, 2016). In all investigated types of paraffin, the homologous series of n-alkanes much longer than those in beeswax were found. Also the amounts of n-alkanes were different depending on the type of paraffin. These results might be helpful and useful in detection of beeswax adulteration with paraffin. Depending on the type and amount of paraffin used for beeswax adulteration, chromatograms and determined contents of hydrocarbons in beeswax adulterated with paraffin may be different. Nonetheless, the same criteria and rules defined in this paragraph for assaying the quality of unknown beeswax sample should be applied.

6.2.4.3. Advantages and disadvantages of GC-MS method for beeswax adulteration detection.

Advantages:

- more precise, accurate and selective in comparisons to classical physico-chemical methods
- efficient in detection of beeswax adulteration with hydrocarbons of alien origin e.g., paraffin, ceresin (allows detection of about 3% of paraffin added to beeswax)
- preliminary purification of a sample with SPE, eliminates the overlapping of peaks and prevents inaccurate results of the quantitative analysis
- Suitable for a routine control of beeswax carried out rapidly by comparing the chromatograms (fingerprints); possible without SPE procedure.

Disadvantages:

- allows only the detection of adulteration with hydrocarbons of alien origin (paraffin, ceresin)
- expensive due to a very specialized equipment

6.2.5. Detection of beeswax adulteration by FTIR-ATR spectroscopy

Definitions, acronyms (in addition to those listed in Section 5.3):

- RS: reference (calibration) standard/s
- ABM: adulterant–beeswax mixtures
- *R*: coefficient of correlation

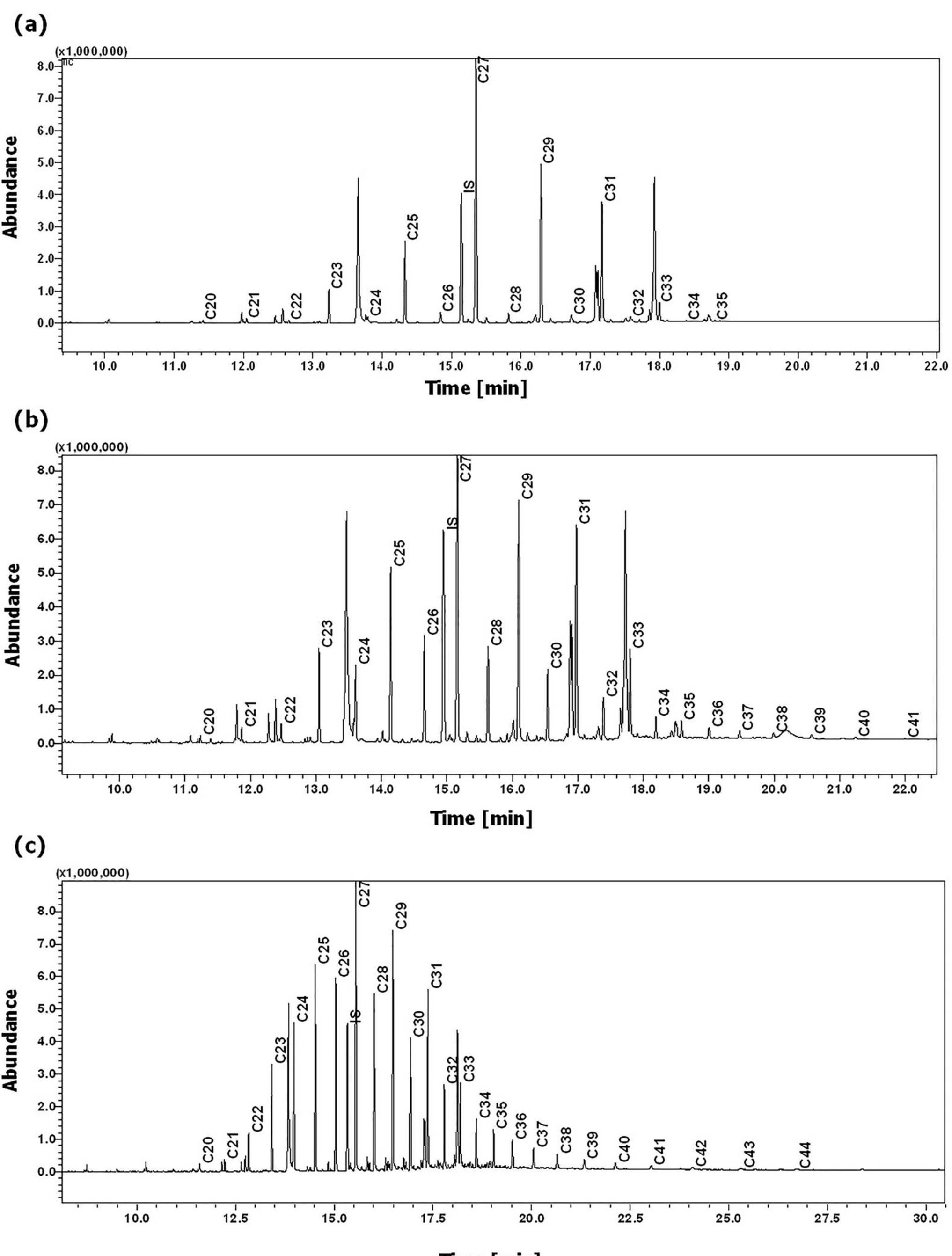


Figure 46. GC-MS chromatogram of hydrocarbons in beeswax: (a) pure beeswax originated from *A. mellifera*, (b) beeswax with 3% addition of paraffin, (c) beeswax with 30% addition of paraffin; C20–C44 – n-alkanes with the formula $C_{20}H_{42}$–$C_{44}H_{90}$; IS – internal standard ($C_{30}H_{62}$).

- R^2: coefficient of determination
- SE: standard error
- c_v: coefficient of variation
- RSD: relative standard deviation

FTIR spectroscopy is generally well-recognized as very reliable or promising method in analysis of various food systems (such as honey, milk, olive oil, juice, etc.) for authentication purposes (Casale & Simonetti, 2014; Cozzolino, Corbella, & Smyth, 2011; Karoui, Downey, & Blecker, 2010; Rios-Corripio, Rojas-López, & Delgado-Macuil, 2012), and even more demanding systems such as those involved in forensic sciences (Yu & Butler, 2015). However, it was not used for the authentication of beeswax until the second decade of this century. In 2013, Maia et al. demonstrated a feasibility study of FTIR-ATR for detection of beeswax adulterants with good detection limits (≤5%) of the four most commonly used beeswax adulterants: paraffin, microcrystalline wax, tallow, and stearic acid. Shortly after, Svečnjak, Baranović et al. (2015) established an analytical procedure for routine detection of beeswax adulteration by

Table 17. Contents of hydrocarbons (min-max) proposed by different authors as concentration guide-values to distinguish between pure and adulterated beeswax originated from *A. mellifera*.

Compound	Jiménez et al. (2007)[a]	Waś et al. (2016)[b]
Heptadecane	0.03–0.22	Not detected
Nonadecane	0.08–0.64	Not detected
Eicosane	0.03–0.13	0.01–0.06
Heneicosane	0.19–0.61	0.03–0.10
Docosane	0.02–0.13	0.02–0.09
Tricosene	0.03–0.18	Not determined quantitatively
Tricosane	0.69–1.33	0.12–0.68
Tetracosane	0.04–0.18	0.03–0.13
Pentacosene	0.05–0.18	Not determined quantitatively
Pentacosane	1.26–1.78	0.42–1.47
Hexacosane	0.18–0.35	0.06–0.22
Heptacosene	0.05–0.12	Not determined quantitatively
Heptacosene isomer	0.09–0.21	Not determined quantitatively
Heptacosane	2.55–3.20	2.44–4.40
Octacosane	0.14–0.37	0.06–0.19
Nonacosene	0.06–0.52	Not determined quantitatively
Nonacosane	1.87–2.68	1.68–2.73
Triacontene	0.02–0.07	Not detected
Triacontene isomer	0.02–0.05	Not detected
Triacontane	0.11–0.31	0.05–0.19
Hentriacontadiene	0.01–0.08	Not determined quantitatively
Hentriacontene	0.61–0.89	Not determined quantitatively
Hentriacontene isomer	1.05–1.58	Not determined quantitatively
Hentriacontane	1.62–2.45	1.53–2.64
Dotriacontene	0.06–0.12	Not detected
Dotriacontane	0.04–0.14	0.01–0.12
Tritriacontadiene	0.01–0.08	Not determined quantitatively
Tritriacontene	1.19–1.82	Not determined quantitatively
Tritriacontene isomer	0.06–0.38	Not detected
Tritriacontane	0.34–0.72	0.31–0.76
Tetratriacontane	Not detected	<0.025[c]–0.03
Pentatriacontene	0.03–0.16	Not determined quantitatively
Pentatriacontene isomer	0.02–0.08	Not determined quantitatively
Pentatriacontane	0.01–0.09	<0.025[c]–0.03
Total		8.27–11.66

[a]GC-FID method, concentration in wt%, related to the internal standard (octadecyl octadecanoate).
[b]GC-MS method, concentration in g/100 g, internal standard method with using standard mixture of n-alkanes ($C_{20}H_{42}$-$C_{40}H_{82}$) and squalane ($C_{30}H_{62}$) used as IS.
[c]Limit of determination.

generating the calibration standards and curves for detection of various adulterants.

Considering the advantages of this spectroscopic technique and observations reported in studies mentioned above, we here present a simple and reliable FTIR-ATR method that enables distinguishing between authentic and adulterated beeswax samples, including the procedure for the quantification of adulterants in contaminated beeswax samples [method by Svečnjak, Baranović et al. (2015) is complemented and slightly modified for this purpose]. The general list of equipment and materials required is given in Section 5.3.

6.2.5.1. Generating IR spectral database of reference samples. Prior to infrared analysis aiming to detect the presence of adulterants in beeswax, it is necessary to conduct detailed IR characterization of beeswax and adulterant samples (as described in Section 5.3). To detect and quantify foreign substances in beeswax, a reference IR spectral database of authentic (genuine) beeswax, adulterants (as much different types as possible), and their mixtures should be generated. This requires preparation of in-house quality control materials, as described in the following subsections. Generating IR spectral database of the reference samples involves several steps described in detail bellow:

1. Sampling of the reference specimens (genuine beeswax, adulterants) – see Section 6.2.5.1.1.
2. Preparation of adulterant–beeswax mixtures/reference (calibration) standards – see Section 6.2.5.1.2.
3. Acquisition of FTIR-ATR spectra of prepared samples – see Section 6.2.5.1.3.

6.2.5.1.1. Sampling of reference specimens.

6.2.5.1.1.1. Genuine beeswax – sampling and storage conditions. To obtain the reference IR spectra of genuine beeswax, virgin beeswax samples (wild-built combs) should be collected directly from the hives maintained

in controlled conditions, as described in Section 2.1.1. Sampling, melting and storage of comb wax and other types of beeswax specimens is performed as described in Section 2.

It is recommendable to collect as much wild-built combs as possible (>10) from different hives to determine minor chemical variations naturally occurring among different beeswaxes in particular geographical region (this is important to reduce the level of uncertainty when evaluating test samples, as explained in Section 6.2.5.2.3.3.).

6.2.5.1.1.2. Adulterants – purchasing and storage conditions. Adulterants of interest can be purchased on the national or international market depending on their availability in different countries. Only certified products purchased from the commercial suppliers (companies and/or specialized shops) that guarantee product's authenticity should be used for generating the IR spectral database of the reference adulterant specimens.

For example, crude paraffin wax with melting point 56 °C (*paraffinum solidum*, Ph. Eur. 7.8) and stearic acid (*acidum stearicum*, Ph. Eur. 8.1) can be purchased from the local pharmaceutical supplier. Different types of high melting point paraffin waxes, commonly used in the beekeeping technology for hive waxing, can also be purchased for this purpose if they have a product specification. Animal fats are available at different market places, but it is preferable to obtain beef and/or mutton tallow from the local butcher shop.

The majority of wax-based adulterants can be stored in original packaging with lids and stored according to the manufacturer's instructions or as described in Section 2. However, due to the lower stability (biological degradation tendency) of particular animal-derived specimens, such as beef or mutton tallow, these must be melted and filtered before storing. Melting and filtering are necessary to remove needless tissues (venation) that are naturally present in animal fat. Commercial paper filters or canvas filters can be used for this purpose. Afterwards, samples should be stored in an airtight glass containers at +4 °C to prevent oxidation and decomposition.

6.2.5.1.2. Preparation of adulterant–beeswax mixtures (reference standards). To obtain reference samples exhibiting IR spectral features specific for particular level of adulteration, and ensure precise prediction and quantification of the same levels in test samples, mixtures of genuine beeswax containing different proportions of particular adulterant (adulterant–beeswax mixtures – ABM) should be prepared as follows (as an example, we present a preparation description for 5 g mixtures):

1. Weigh the material (beeswax and adulterant) for the preparation of ABM containing different proportions of adulterant (w/w) in accordance with the recipe given in Table 18 (recipe for 5 g ABM; higher amounts can also be prepared if required).
   Note: prepared mixtures are to be used for the establishment of calibration (standard) curves exhibiting a relationship between the FTIR instrument response and the known concentrations of the analyte (adulterant). It is therefore of crucial importance to conduct an accurate weighing a precise analytical balance (precision: ±0.001 g).
2. Place the weighed specimens in small glass thermostable storage containers.
3. Place the containers in a temperature chamber for 3 h at 90 °C for melting and homogenisation (stir the mixtures gently several times during this period for better homogenization).
   Note: complete set of material prepared must be subjected to the same temperature treatment (including pure beeswax and pure adulterant, i.e., mixtures 1 and 13 from Table 18). 90 °C temperature treatment must be applied to ensure melting of adulterants with higher melting point (such as carnauba wax: 83 °C, some paraffin waxes: >75 °C).
4. Re-solidify the mixtures by allowing to cool to room temperature (without any additional cooling treatment aiming to decrease the temperature rapidly) (Figure 47).
   Note: some adulterants have a tendency to precipitate after melting (tallow for instance) – stir those mixtures continuously to the solid state to avoid a formation of separated layers.
5. Store the mixtures (with a lid on) in a dark place at room temperature, as described in Section 2.3 on beeswax storage.

The source of the material used to prepare the RS requires careful consideration regarding its authenticity. For example, more than 20 wild-built combs collected from different hives were used as beeswax material for the preparation of mixtures presented here, while adulterants were purchased as specified in Section 6.2.5.1.1.2. Furthermore, make sure a sufficient quantity of material for the preparation of the whole set of mixtures (13) is provided.

Along with the preparation of mixtures by following usual 10% increasing sequence of adulterant addition (10, 20, 30, …, 90%), we propose a preparation of two additional mixtures containing 5% and 95% of adulterant to obtain more precise results based on additional marginal range values. This can be useful in case of "borderline" test samples (5%) or almost pure adulterants (95%). Also, mixtures can be prepared to cover a wider range of adulteration level (5, 10, 15, 20, 25%, … sequence) and decrease the calibration error. Nonetheless, we here describe the methodology using adulterant proportions (Table 18) that provide simple application and reliable detection of adulterants with good detection limits (≤3%).

Table 18. Recipe for the preparation of 5 g adulterant–beeswax mixtures (w/w).

Mixture	Adulterant:beeswax ratio (%)	Adulterant (g)	Beeswax (g)
1	0:100 (pure beeswax)	–	5
2	5:95	0.25	4.75
3	10:90	0.5	4.5
4	20:80	1	4
5	30:70	1.5	3.5
6	40:60	2	3
7	50:50	2.5	2.5
8	60:40	3	2
9	70:30	3.5	1.5
10	80:20	4	1
11	90:10	4.5	0.5
12	95:5	4.75	0.25
13	100:0 (pure adulterant)	5	–
Total		32.5	32.5

6.2.5.2. Acquisition of IR spectra of reference standards. The IR spectra of all sample types (beeswax, adulterants, ABM) should be recorded in accordance with the procedure described in Section 5.3.2.2. Given the solid state of the RS after melting and re-solidification, as well as their storage in containers, placing the samples on the ATR crystal is best done by using a laboratory scoop or scraper (this helps to tranfer small aliquots that will melt on the crystal prior to spectral analysis). In case of recording the spectra of samples with naturally higher melting point, the temperature of the ATR crystal should be increased to 80–90 °C during the spectra acquisition.

6.2.5.3. Detection of adulterants using IR spectral data. Detection of adulterants based on IR spectral data involves several basic steps:

I. Visual exploration of spectral similarities (and dissimilarities) between beeswax and adulterants and identification of adulterant-specific spectral region(s) of interest in ABM (see Section 6.2.5.3.1 for details).

   Visual exploration represents the first step towards selecting a suitable spectral region of interest indicative for adulteration detection (further used for calibration purposes). Each adulterant type exhibits different spectral features as both individual sample and when mixed with beeswax. We describe examples of spectral alteration based on four adulterant types and respective adulterant–beeswax mixtures (RS):

   1. paraffin wax–beeswax mixtures (petroleum-derived adulterant)
   2. tallow–beeswax mixtures (adulterant of animal origin)
   3. stearic acid–beeswax mixtures (synthetic adulterant)
   4. carnauba wax–beeswax mixtures (adulterant of plant origin)

II. Construction of a calibration curve using the spectral data of RS (see Section 6.2.5.3.2 for details).

   Construction of a calibration curve using the spectral data of pre-analyzed reference (calibration) standards is conducted by following several steps:

   1. Estimate the coefficient of correlation (R) to determine the strength and direction of a relationship between the instrument response and known adulterant proportions in the indicative spectral regions identified in RS (see Section 6.2.5.3.2.1 for details).
   2. Select the indicative spectral region (targeted peak areas) showing the best correlation effects.
   3. Generate (plot) a calibration curve based on the best correlation results

      The spectral regions with reference peaks exhibiting the best correlation parameters (R $\geq$ 0.998; SE $<$ 0.05%), are used for the construction of a calibration curve and further quantification of adulterants in test samples (see Section 6.2.5.3.2.2 for details).
   4. Carry out statistical (linear regression) analysis to estimate the prediction strength and

Figure 47. Containers with prepared adulterant (paraffin/*paraffinum solidum* – PS)–beeswax mixtures after melting. Photo: Svečnjak L.

prediction error of selected calibration model for adulteration level detection (determination of the coefficient of determination – R2, and standard error – SE). This is essential for the final interpretation of results which includes both the predicted value (% of adulterant in beeswax) and measurement uncertainty in terms of prediction accuracy (see Section 6.2.5.3.2.3 for details).

III. Detection of adulterants in test samples (see Section 6.2.5.3.3 for details).
The quantification of adulterants in the test samples includes the following steps:
1. Acquire the spectrum of test sample under the same measurement conditions as RS.
2. Estimate the measurement uncertainty associated with the instrument and the sample being analyzed (see Sections 6.2.5.3.3.1 and 6.2.5.3.3.2 for details).
3. Determine (quantify) the exact amount of adulterant in test sample (see Section 6.2.5.3.3.3 for details).

6.2.5.3.1. Identification of adulterant-specific spectral regions of interest in adulterant–beeswax mixtures. As mentioned, each adulterant type exhibits different spectral features (Figure 48) as both individual sample and when mixed with beeswax. Thus, IR spectra of prepared ABM should reveal a unique trend of spectral alterations (increasing and/or decreasing absorption intensities) which follow the increasing amounts of adulterant added to beeswax. These alterations represent spectral regions of interest that are further used for calibration purposes.

Comparative spectral features of beeswax versus selected adulterants (paraffin, beef tallow, stearic acid, and carnauba wax) are described and presented below in the following sections along with IR spectra of the reference standards (ABM) and identification of spectral regions indicative for adulteration.

6.2.5.3.1.1. Paraffin–beeswax mixtures. In comparison to the beeswax IR spectrum, a typical FTIR-ATR spectrum of *paraffin* is characterised by a simple molecular structure related to hydrocarbon absorption bands (at 2921, 2852, 1464, and 720 cm^{-1}), as shown in Figure 48. The main spectral differences between pure beeswax and paraffin (as well as other adulterants) are observed in the fingerprint region. The analyte signals related to the ester and free fatty acids vibrations (at 1738, 1714, and 1172 cm^{-1}) are not present in the IR spectrum of paraffin, and therefore, represent valuable and indicative spectroscopic data for detection of beeswax adulteration.

When prepared and recorded in a satisfactory manner as described above, IR spectra of *paraffin–beeswax mixtures* should reveal a clear linear decrease in the absorption intensities of the lipid components following the increasing percentages of the paraffin (Figure 49). These effects are observed in the spectral region between 1760 and 1700 cm^{-1} (esters and free fatty acids with absorption maximums at 1738 cm^{-1} and 1714 cm^{-1}, respectively), and between 1260 and 1100 cm^{-1} (esters with absorption maximum at 1172 cm^{-1}).

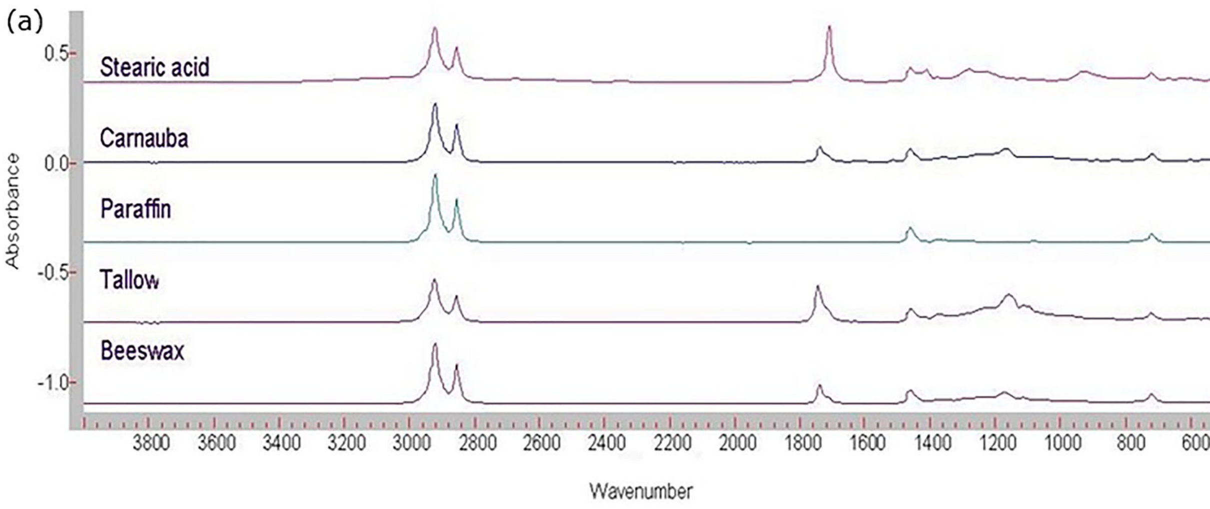
Paraffin waxes with different melting point exhibit the same IR spectra (Figure 50). Thus, all types of paraffin waxes can be used for calibration; there are no effects on the reliability of results.

6.2.5.3.1.2. Tallow–beeswax mixtures. Contrary to the lack of particular lipid-based signals in the fingerprint region of a paraffin spectrum, a characteristic IR spectrum of *beef tallow* is dominated by the strong intensive absorption bends that correspond to the esters vibrations, occurring at 1744 and 1160 cm^{-1} (Figure 48). These signals are considerably stronger and characterised by the absorption maxima shifts in comparison to beeswax signals.

Tallow-beeswax mixtures also exhibits linear behaviour trend, but in the opposite direction in comparison to paraffin; IR absorption intensities (most prominent in the spectral ranges between 1770–1710 cm^{-1} and 1260–1070 cm^{-1}) increased linearly for the increasing percentages of the tallow added to beeswax (Figure 51). This increasing trend is withal accompanied by the characteristic absorption maximum shifts in comparison to beeswax signals (from 1738 to 1744 cm^{-1}, and from 1172 to 1160 cm^{-1}). Different types of tallow might give spectra with slightly different signal properties, especially in the region 1770–1710 cm^{-1} due to a different composition of free fatty acids. However, these spectral data are equally valid and reliable for the purposes of further calibration.

6.2.5.3.1.3. Stearic acid–beeswax mixtures. Stearic acid exhibits the most distinctive IR spectrum, with numerous absorption bands that are not observable in the IR spectrum of beeswax, more specifically, absorptions at 1710, 1412, 1281, and 929 cm^{-1} (Figure 48).

Stearic acid–beeswax mixtures exhibit unique and complex spectral features with numerous regions that are indicative for adulteration (Figure 52). The appearance of a strong band at 1710 cm^{-1} in the IR spectrum of stearic acid can be attributed to the $C=O$ stretching vibrations of saturated aliphatic carboxylic acids as dimer given that these bands characteristically appear in the region between 1730 and 1700 cm^{-1}. Such spectral effect can be explained by the dimer association of stearic acid in different solutions and mixtures; the crystallization process of stearic acid in various media may lead to different polymorphic forms depending on the different crystallization parameters (Garti, Sato, Schlichter, & Wellner, 1986). Pielichowska et al. (2008) reported the similar appearance of a band at 1710 cm^{-1} in stearic acid, belonging to $C=O$ stretching in dimers in liquid state. The fingerprint region contains a series of bands that are very useful for detection of stearic



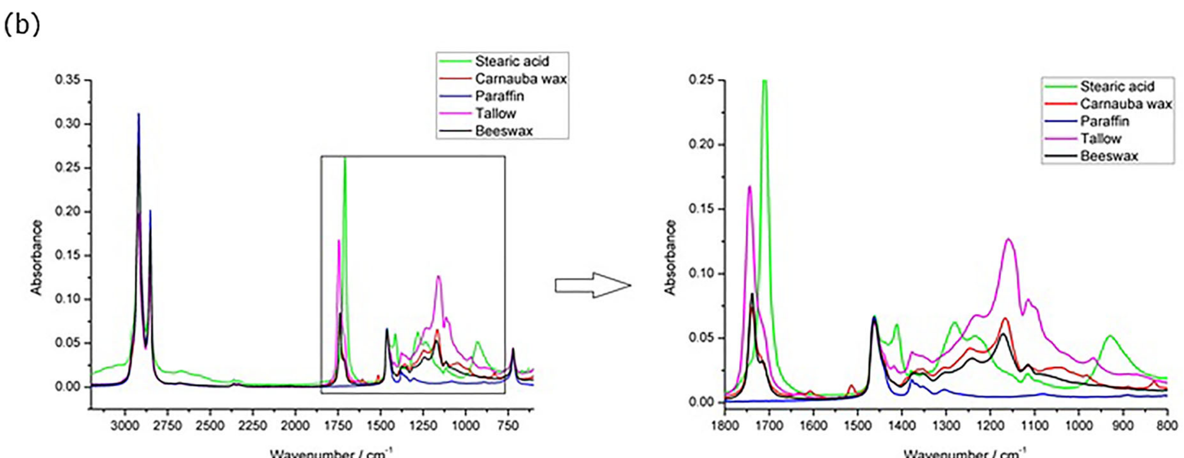


Figure 48. Comparative spectral features of beeswax and four commonly used adulterants (paraffin, tallow, stearic acid, and carnauba wax): (a) individual spectra and, (b) combined spectra representation (modified from Svečnjak, Baranović et al., 2015).

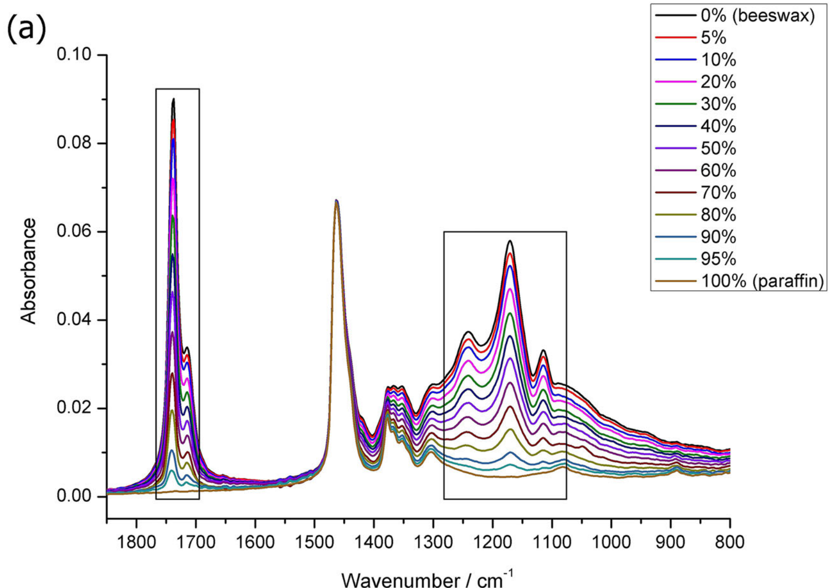
acid in beeswax; the most indicative are vibrations associated with carboxylic acids observed at 1281 cm^{-1} (C–O stretching vibrations) and 929 cm^{-1} (O–H bending vibration) (Pielichowska et al., 2008; Socrates, 2001).

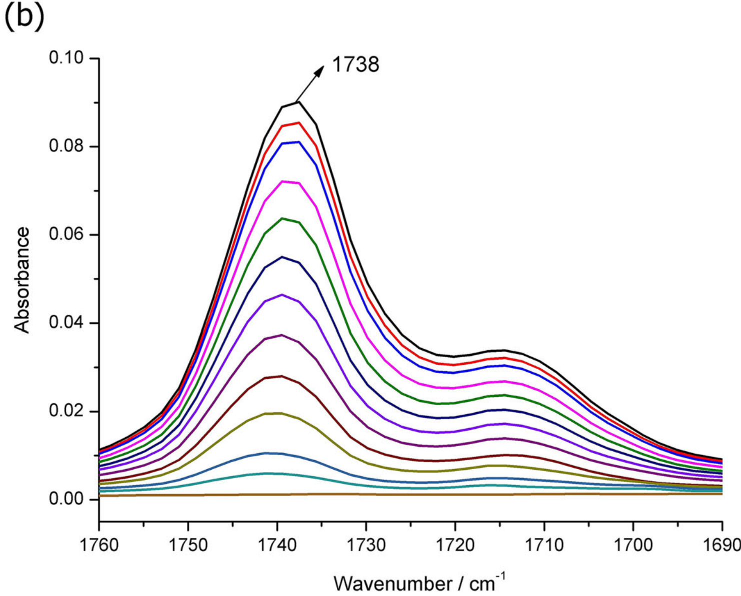
6.2.5.3.1.4. Carnauba wax–beeswax mixtures. Unlike other adulterants that show a unique spectral fingerprint which can easily be distinguished from beeswax, the *carnauba wax* spectrum is very similar to the beeswax spectrum with only minor spectral differences (Figure 48), which makes a detection of carnauba wax the most challenging. Differences can be observed in the region where variations among different genuine beeswax samples also appear (1090–1015 cm^{-1} in particular). Therefore, these spectral regions should not be used for further calibration modelling. However, specific small-intensity absorption bands that are observable at 1633, 1607, 1514, and 830 cm^{-1}, specify IR spectrum of carnauba wax and carnauba wax–beeswax mixtures (Figure 53). An integrated visual identity of these absorptions should be considered when detecting the presence of carnauba wax in beeswax. However, these small bands do not necessarily show an equal linear increasing trend following increasing amonts of carnauba wax in carnauba-beeswax mixtures. In case of the example presented here, linear trend is well represented for absorbance at 1514 cm^{-1}, while other two peaks show a desultory in linearity (Figure 53). The same goes for the region from 840 to 820 cm^{-1}.

The indicative regions (and corresponding distinctive absorption bands/peak areas) determined for a particular adulterant of interest are further used in the calibration process, starting with an evaluation of a correlation coefficient between IR measurements (absorbance intensities) and known (real) proportions of adulterant (described below in the following subsections).

6.2.5.3.2. Construction of a calibration curve using the spectral data of reference standards.

6.2.5.3.2.1. Estimation of the correlation coefficient (R). The correlation coefficient (*R*) is one of the statistical parameters commonly used in analytical measurements prior





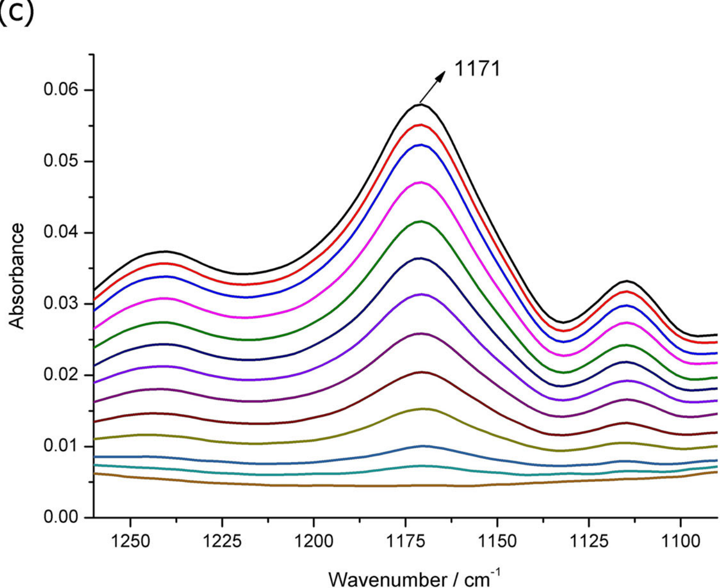


Figure 49. IR spectra of beeswax, paraffin, and paraffin–beeswax mixtures (calibration standards) containing different proportions of paraffin; an emphasis on the spectral regions indicative for paraffin detection: (a) fingerprint region – 1800–800 cm^{-1}; (b) spectral region from 1760 to 1700 cm^{-1}; (c) spectral region from 1250 to 1100 cm^{-1} (modified from Svečnjak, Baranović et al., 2015).

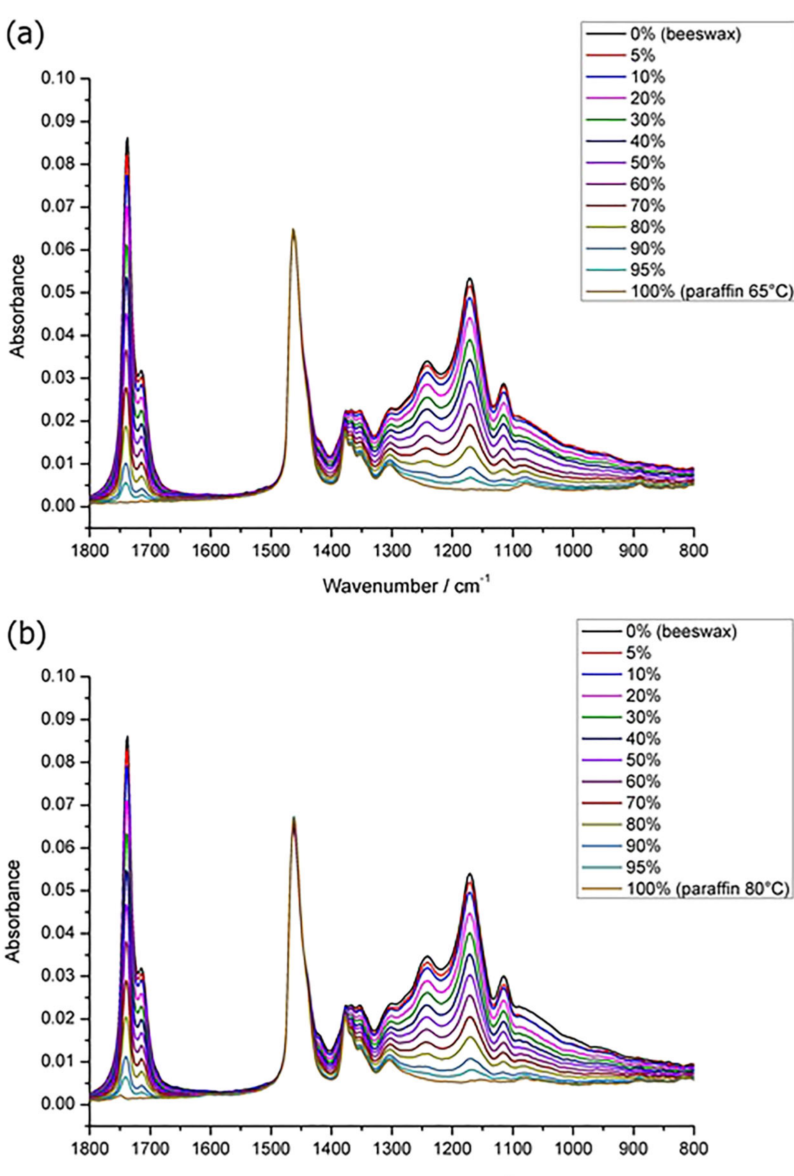


Figure 50. IR spectra of paraffins with different melting point: (a) 65 °C; (b) 80 °C.

to setting up a calibration curve. The correlation coefficient represents the strength and direction of a relationship (degree of correlation) between the FTIR instrument response and the known (real) concentrations of the analyte (amount of adulterant).

The overall correlation results related to the target peaks of indicative spectral regions for four different adulterant (paraffin, beef tallow, stearic acid, and carnauba wax)–beeswax mixtures are given in Table 19. The results representing the best correlation between the instrument response and known adulterant proportions in the adulterant–beeswax RS are shown in Figures 54–57, for paraffin, tallow, stearic acid, and carnauba wax, respectively. In these figures we show the validation plots representing the instrument response for each adulterant–beeswax RS (mixtures containing 0–100% of adulterant; marked as 1–13 point in the plots), i.e., its indicative spectral regions. We recommend these spectral regions (absorbances at selected reference peak areas) to be used for further calibration process for corresponding adulterants (recommended peak areas are also highlighted with asterisks in Table 19). Also, we recommend estimating and selecting the best *R* value for other adulterants (not presented here) in like manner. It should be noted that stearic and palmitic acid, as well as commercially available stearin (often present on the market as a mixture of stearic and palmitic acid) exhibit almost equal IR spectral features and therefore, the same indicative spectral regions as those recommended for stearic acid, can be used to detect palmitic acid and stearin in beeswax. The same goes for commercial stearic acid which is also often available on the market as a mixture of stearic and palmitic acids.

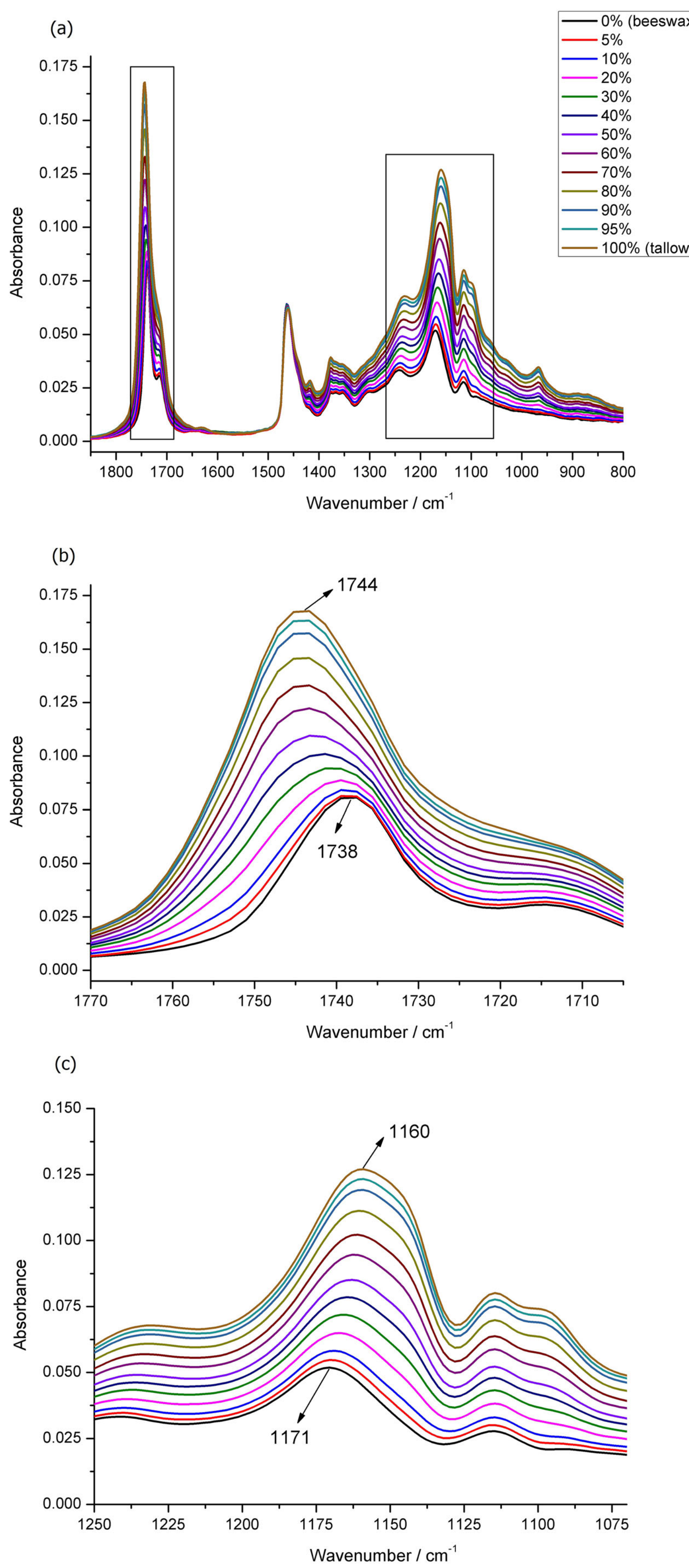


Figure 51. IR spectra of beeswax, tallow, and tallow–beeswax mixtures (calibration standards) containing different proportions of tallow; an emphasis on the spectral regions indicative for tallow detection: (a) fingerprint region – 1800–800 cm^{-1}; (b) spectral region from 1770 to 1710 cm^{-1}; (c) spectral region from 1250 to 1075 cm^{-1} (modified from Svečnjak, Baranović et al., 2015).

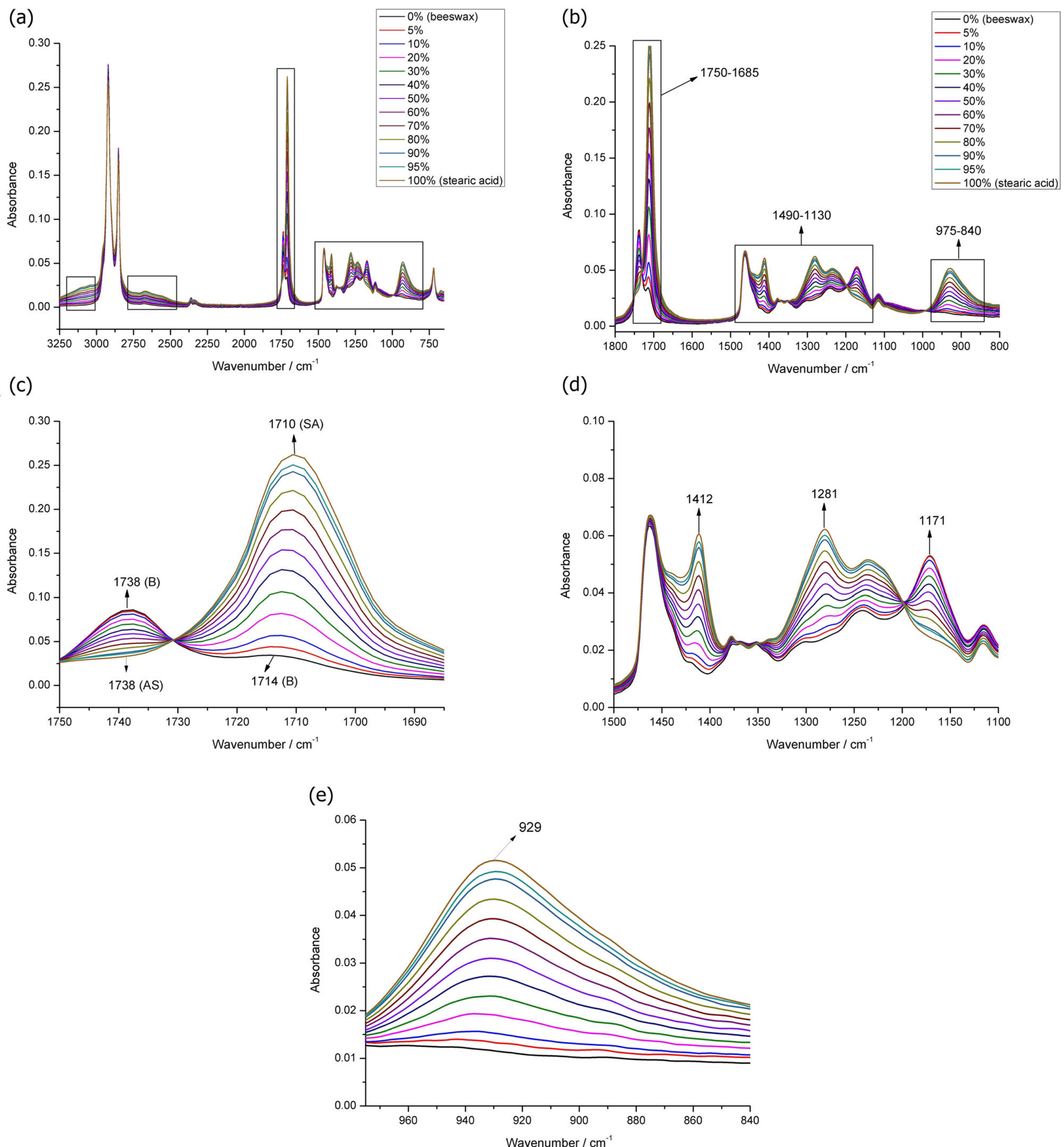
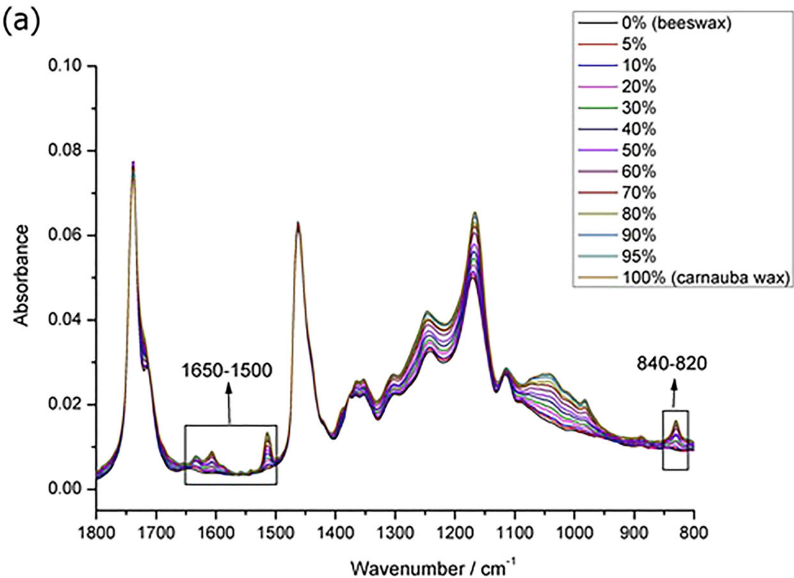


Figure 52. IR spectra of beeswax, stearic acid, and stearic acid–beeswax mixtures (calibration standards) containing different proportions of stearic acid; an emphasis on the spectral regions indicative for stearic acid detection: (a) whole spectrum – 3250–700 cm^{-1}; (b) fingerprint region – 1800–800 cm^{-1}; (c) spectral region from 1750 to 1690 cm^{-1}; (d) spectral region from 1500 to 1100 cm^{-1}; (e) spectral region from 970 to 840 cm^{-1} (Svečnjak, 2018, unpublished data).

The correlation assessment presented here was performed using the Resolutions Pro version 5.3.0 software package (Agilent Technologies). Commonly used statistical packages (such as SPSS, SAS, R, Statistica-StatSoft line of softwares, etc.), as well as those specialized for the spectral data manipulation (such as Origin) can also be used for this purpose as they provide simple instructions for calculating the coefficient of correlation (R). R value can also be determined using Microsoft Excel spreadsheet. For more details, see Section 7 in the *BEEBOOK* article on statistics (Pirk et al., 2013).

6.2.5.3.2.2. Generating a calibration curve. The spectral regions with reference peak areas exhibiting the best correlation parameters ($R \geq 0.998$) are used for the construction of a calibration curve that will be further utilized for the quantification of adulterants in test samples. Most of modern FTIR spectrometers have



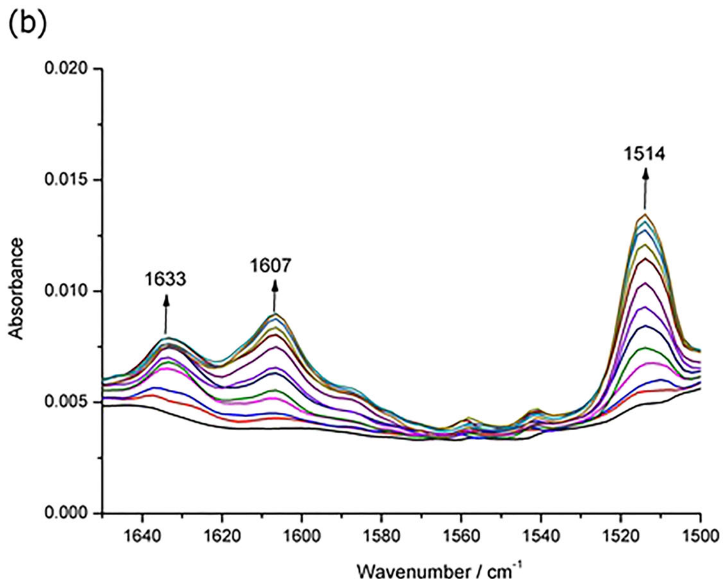


Figure 53. IR spectra of beeswax, carnauba wax, and carnauba wax–beeswax mixtures (calibration standards) containing different proportions of carnauba wax; an emphasis on the spectral regions indicative for carnauba wax detection: (a) fingerprint region – 1800–800 cm^{-1}; (b) spectral region from 1650 to 1500 cm^{-1} (Svečnjak, 2017, unpublished data).

accompanying software that enables default quantification of the analyte of interest after recording the spectra of the reference (calibration) standards. To detect and quantify the analyte of interest (adulterant) in the samples of unknown chemical background (test samples), the calibration data (targeted peak areas of reference/calibration standards showing the best *R* value) must be previously stored as special (calibration) file formats (e.g., as BSQ file format in case of Agilent/Cary 660 FTIR spectrometer and accompanying software Resolutions Pro). Afterwards, the quantitative prediction of adulterant amount in test samples (based on calibration curve – known adulterant amounts in RS) is performed by a simple click on respective quantification toolbar button.

If there is no specialzed software for default quantification available, other statistical packages (such as those listed in Section 6.2.5.3.2.1) can be used to perform a factor-based Partial Least Squares Regression (PLSR) method for determining the adulteration level based on ABM (calibration set) and validation (test) set of samples (see Svečnjak, Baranović et al., 2015 for details).

6.2.5.3.2.3. Estimation of the prediction performance parameters. Interpretation of the results and complete analytical report on the amount of adulterant in beeswax test samples should include both the best estimate/predicted value (% of adulterant in beeswax based on generated calibration curves) and level of measurement uncertainty in terms of prediction accuracy (±%). Therefore, after estimating the *R* values, the spectral regions with reference peaks exhibiting the best correlation parameters (used for calibration purposes) are further subjected to linear regression modelling.

Table 19. Determination of a correlation coefficient conducted on the target peaks of indicative spectral regions of four different adulterant (paraffin, beef tallow, stearic acid and carnauba wax)–beeswax mixtures (Svečnjak, Baranović et al., 2015; Svečnjak, 2017, unpublished data).

Reference standard (adulterant–beeswax)	Reference peak (cm^{-1})	Peak area (cm^{-1})	Predominant beeswax compound associated to reference peak	Correlation coefficient (R)
Paraffin	1738	1750–1727	Monoesters	0.9999[a]
	1171	1195–1147	Esters of aliphatic acids	0.9998[b]
Tallow	1738	1753–1724	Monoesters	0.9969
	1171	1195–1148	Esters of aliphatic acids	0.9995[a]
Stearic acid	1738	1747–1730	Monoesters	0.9971
	1710	1721–1707	Free fatty acids	0.9982
	1412	1423–1400	Esters (shoulder)	0.9989
	1281	1308–1253	Free fatty acids	0.9996[a]
	929	978–880	None	0.9983
Carnauba wax	1633	1638–1628	None	0.2609
	1607	1610–1603	None	0.9859
	1514	1523–1506	None	0.9995[a]
	830	740–820	None	0.9889

[a]The highest R values determined for each adulterant (used for further calibration process).
[b]Reference peak 1171 cm^{-1} can also be used for further calibration due to almost equally good correlation results as for 1738 cm^{-1}.

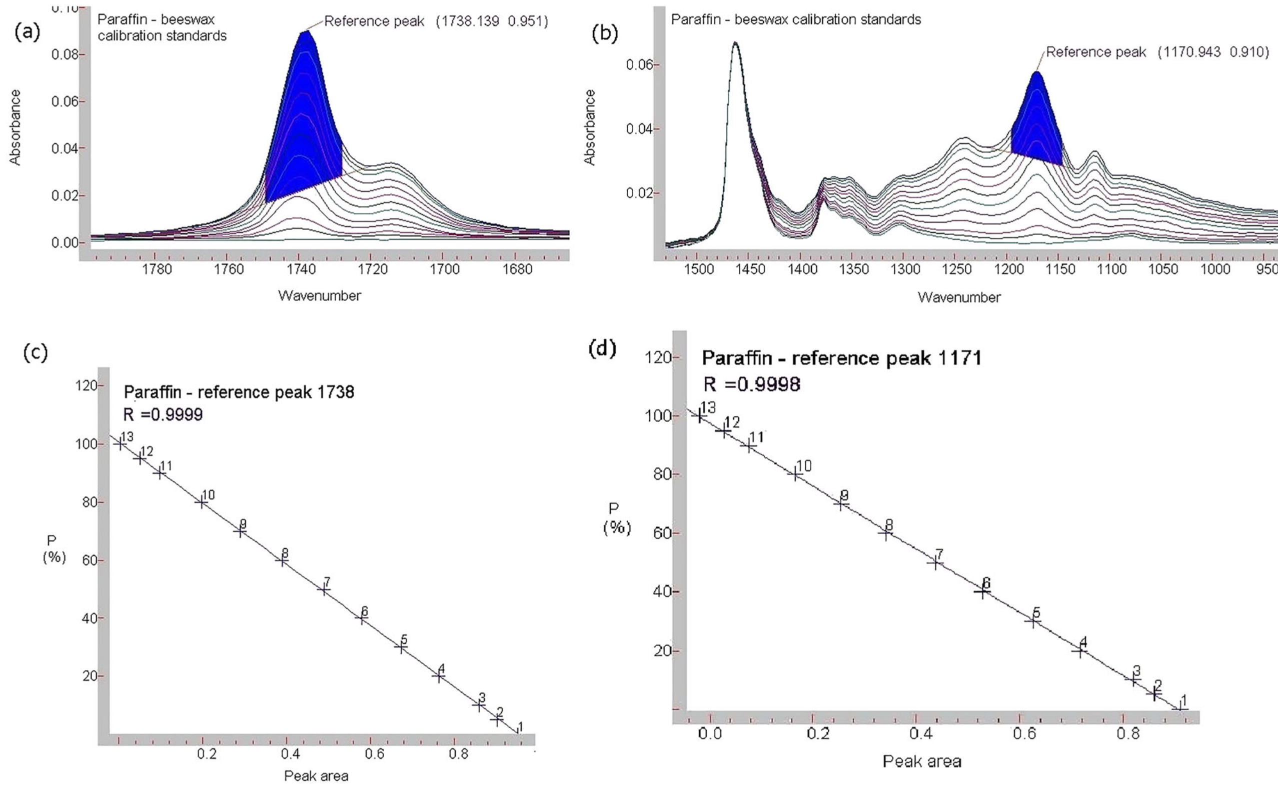
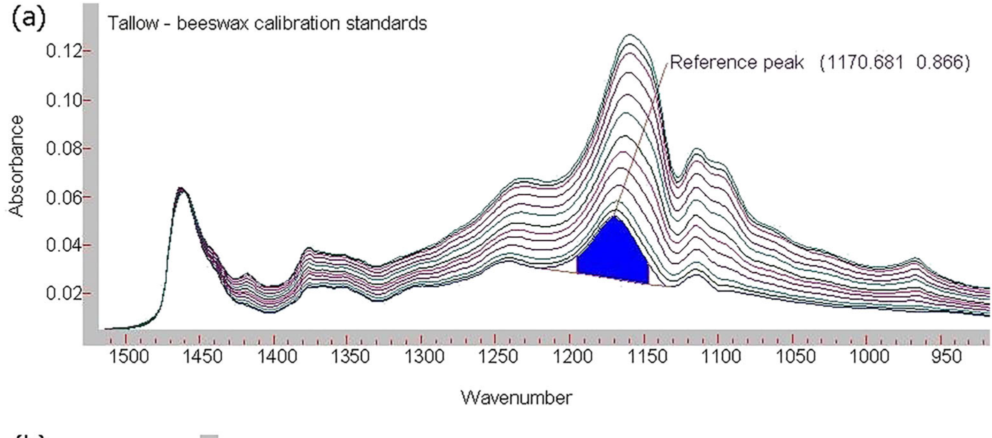


Figure 54. Reference peak areas: (a) absorbance at 1738 cm^{-1}, and (b) absorbance at 1171 cm^{-1} exhibiting (c, d) the best results of correlation between the instrument response and known paraffin proportions in the paraffin–beeswax reference standards (Svečnjak, 2017, unpublished data).

The aim of linear regression is to estimate the prediction strength and prediction error of selected calibration curve for adulteration level detection, and this is achieved by calculating the coefficient of determination (R^2) and standard error (SE), respectively. R^2 can be considered here as adjusted R, while SE estimates the measurement uncertainty in terms of accuracy (closeness of agreement between measured adulterant share values and true adulterant share values). An example of prediction performance parameters of the calibration curve constructed for determination of stearic acid share in beeswax based on stearic acid-beeswax mixtures is shown in Figure 58). For reliable quantification of adulterants in beeswax, it is best to hold to the following performance parameters: $R^2 \geq 0.997$, SE $< 0.05\%$.

The statistical analysis presented here (Figure 58) was performed using the statistical package Origin



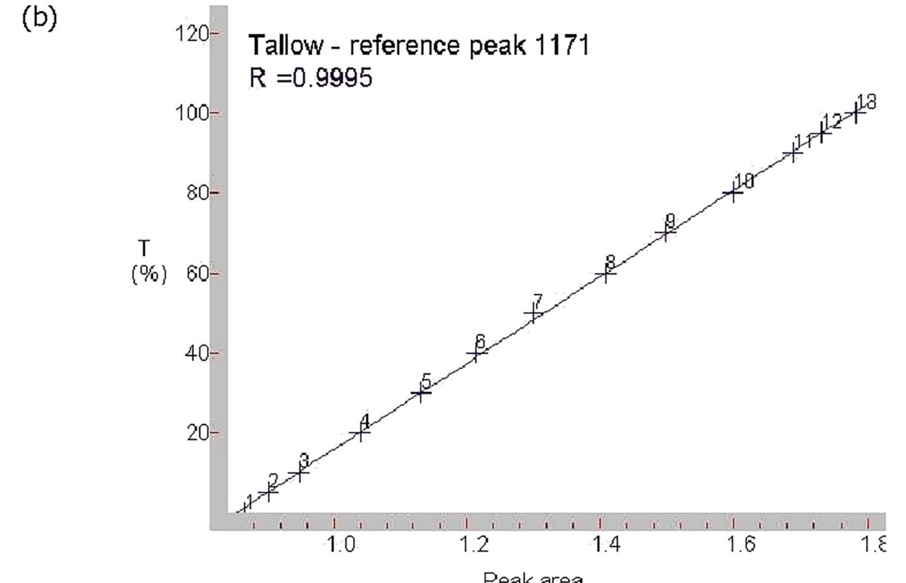


Figure 55. (a) Reference peak area (absorbance at 1171 cm^{-1}), (b) exhibiting the best results of correlation between the instrument response and known tallow proportions in the tallow–beeswax reference standards (Svečnjak, 2017, unpublished data).

version 8.1 (OriginLab Corporation), but other statistical packages may also be used (examples of respective packages are provided in Section 6.2.5.3.2.1). For general guidelines on linear regression models, see Sections 5 and 5.2 in the *BEEBOOK* paper on statistics (Pirk et al., 2013).

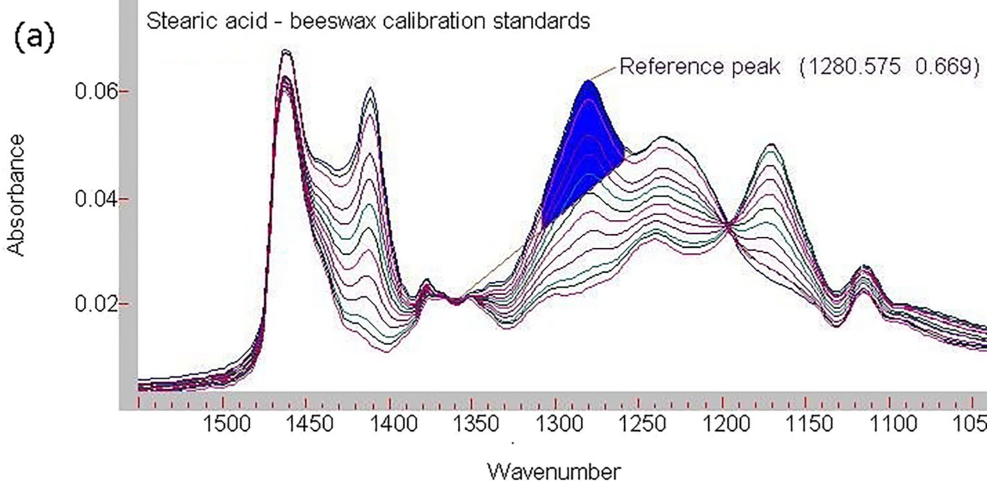
6.2.5.3.3. Detection of adulterants in test samples. In practice, the test samples are usually comb foundations of unknown chemical background (origin) collected from the market, and they often contain adulterants.

Quantification of the analytes of interest (adulterants) in test samples is performed by comparing the unknown samples to a set of reference/calibration standards of known adulteration level (based on the generated calibration curve). After calibration curves are constructed and evaluated to be satisfactory ($R^2 \geq 0.997$, SE $< 0.05\%$), they can be used to estimate the proportion (%) of the targeted analyte (adulterant) in test samples. This requires each test sample to be analyzed (two replicate spectra) under the same measurement conditions as RS (see Section 6.2.5.2), and the result (predicted value/best estimate) is obtained based on the generated calibration curve, as described in Section 6.2.5.3.2.2. However, the complete result on the proportion of adulterants in test samples should include an estimate of the level of confidence associated with the estimated value, which is commonly represented as:

$$\text{adulterant share} = \text{predicted value} \pm \text{measurement uncertainty } \%$$

Therefore, prior to determining the adulteration level in test samples, it is necessary to estimate several aspects of measurement uncertainty. Along with the measurement uncertainty (in terms of prediction accuracy) determined by means of SE (see previous section), the measurement uncertainty associated with the measuring instrument and the sample being analyzed should also be estimated.

6.2.5.3.3.1. Measurement uncertainty arising from the sample. When comparing minor spectral variations identified in 20 different genuine *A. mellifera* beeswax samples (presented in Section 5.3.3.1; Figure 33) with absorption changes observed in paraffin–beeswax mixtures, it is



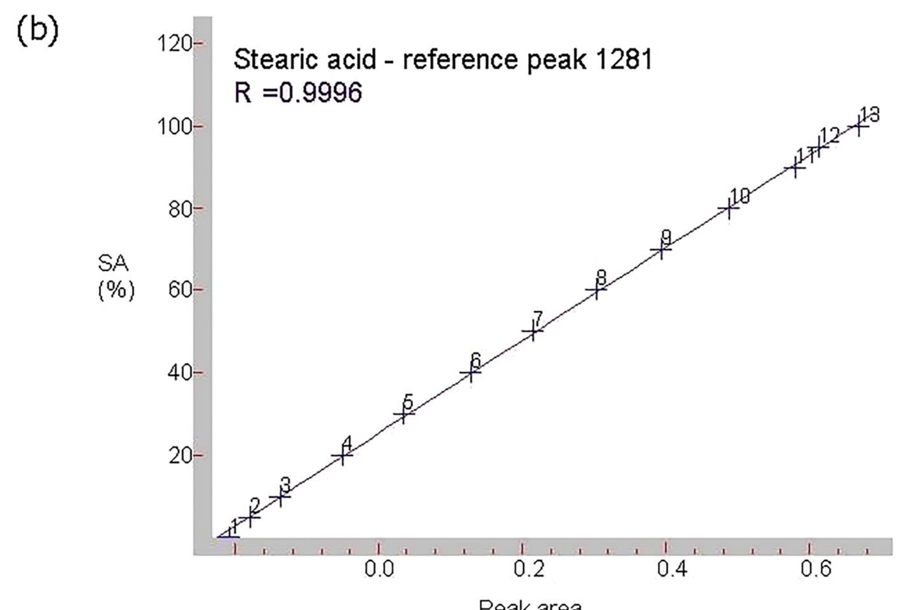
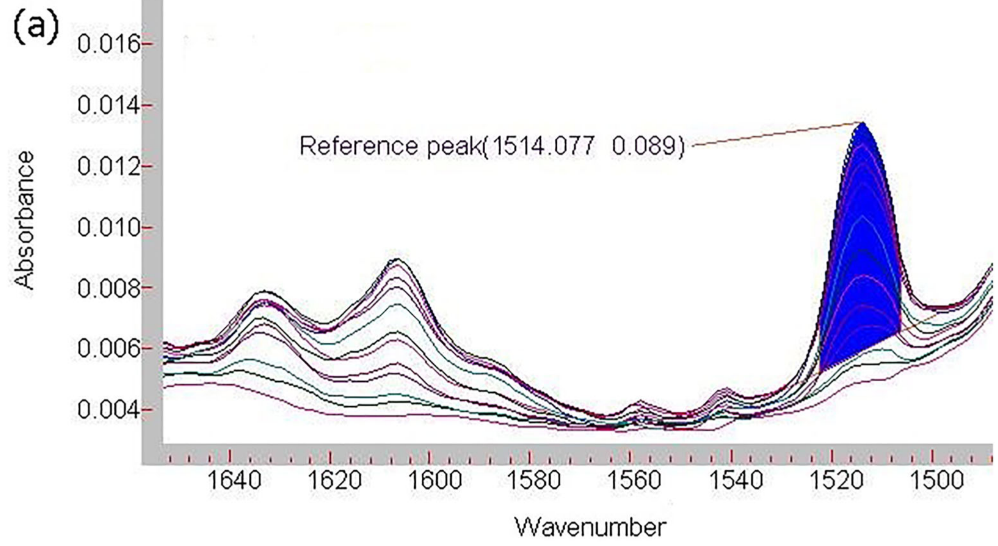


Figure 56. (a) Reference peak area (absorbance at 1281 cm^{-1}), (b) exhibiting the best results of correlation between the instrument response and known stearic acid proportions in the stearic acid–beeswax reference standards (Svečnjak, 2017, unpublished data).

obvious that the most intensive spectral variations determined in different beeswaxes (observed between 1090 and 1015 cm^{-1}) do not intercept those crucial for detection of beeswax adulteration with paraffin (absorbance at 1738 and 1171 cm^{-1}; Figure 50). However, these minor differences may slightly affect the results for test samples. When detecting paraffin in beeswax, we detect the components whose share decreases by addition of paraffin. This reflects in the lower absorbance intensities of bands associated with esters and fatty acids. Thus, it can be concluded that paraffin in some respects "dilutes" the beeswax. Minor spectral variations that occur among different comb wax samples may also reflect slightly lower absorbance intensities of mentioned absorption bands throughout spectrum (Figures 33 and 59). To avoid uncertainty and potential declaration of false negative results for test samples (especially in cases of "borderline" test samples containing $\leq$5% of paraffin), and determine the exact amount of paraffin in beeswax, it is necessary to quantify spectral variations occurring in different genuine beeswax samples, at a spectral region that overlaps with the paraffin calibration pattern (with maximum absorbance at 1738 cm^{-1}, or 1171 cm^{-1} if used for calibration). This is achieved by calculating the coefficient of variation (c_v), also known as relative standard deviation (RSD). c_v should be determined using the absorbance at 1738 cm^{-1} (or 1171 cm^{-1}). Using an example of spectra obtained from 20 different beeswax samples presented in Section 5.3.3.1 (Figure 33), we present a calculation of the following: mean absorbance at 1738 cm^{-1} (μ_A= 0.5665), with standard deviation σ= 0.0197. The coefficient of variation c_v, defined as:

$$c_v = \frac{\sigma}{\mu_A}$$

is 0.0348 (3.48%), which means that 3.48% of variation



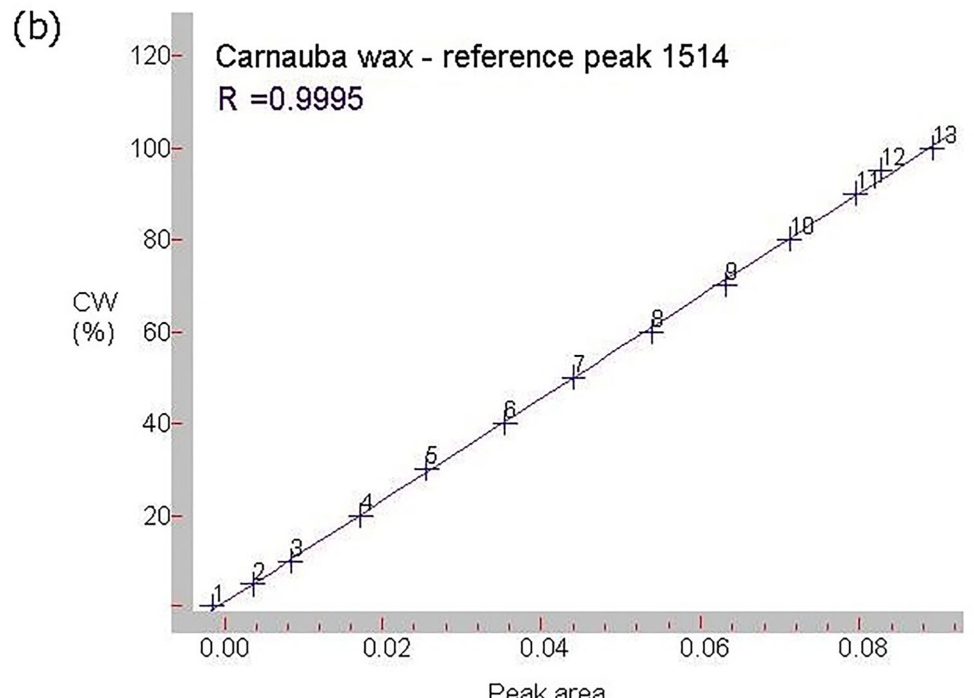


Figure 57. (a) Reference peak area (absorbance at 1514 cm^{-1}), (b) exhibiting the best results of correlation between the instrument response and known carnauba wax proportions in the carnauba wax–beeswax reference standards (Svečnjak, 2017, unpublished data).

can be assigned to spectral alterations naturally occurring in authentic beeswax samples (Figure 59).

Obtained c_v value should be considered in the final result.

The uncertainty test procedure related to the natural minor variations occurring in different beeswax samples (c_v – sample) should be applied when detecting adulterants whose calibration curves involve reference peaks associated with the compounds present in the beeswax (e.g., it should be applied in the case of paraffin, tallow, and stearic acid detection, while can be omitted in the case of carnauba wax; see Table 19. for details).

6.2.5.3.3.2. Instrument related measurement uncertainty. Measurement uncertainties can also come from the measuring instrument (FTIR spectrometer) so the precision (repeatability) of a measurement system has to be assessed. This is achieved by determining the c_v value based on ten-fold measurement of different aliquots of the same beeswax sample, following the calculation steps described in previous section. For comparison, c_v value (repeatability) determined by ten-fold measurement of different aliquots of genuine beeswax by using the maximum absorbance (at 2921 cm^{-1}) is 0.3% (generally, it should not exceed 0.5%).

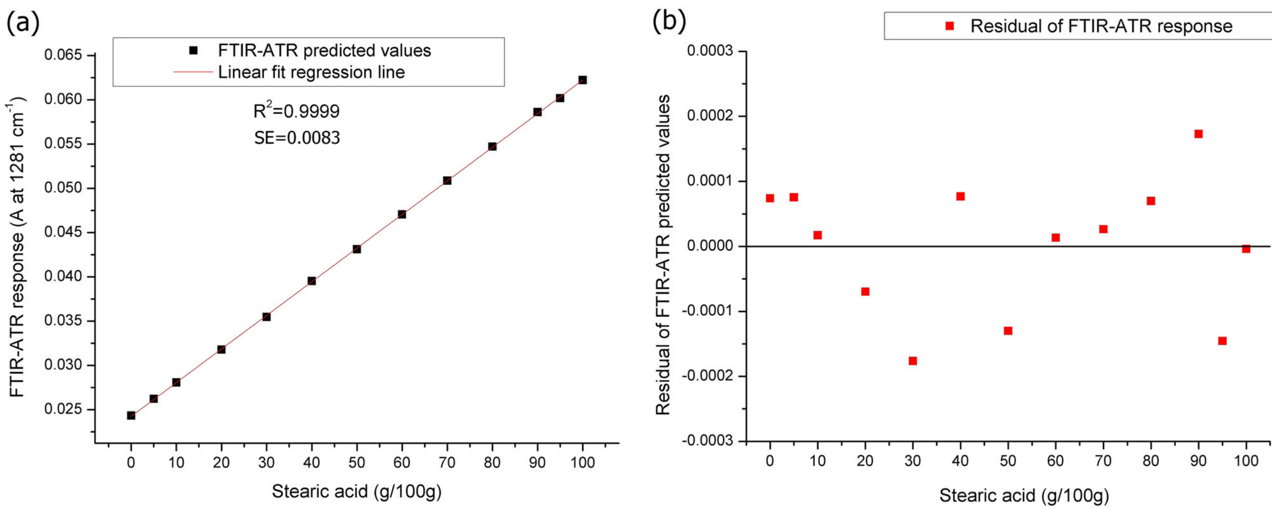


Figure 58. (a) Prediction performance parameters of the calibration curve constructed for determination of the stearic acid share in beeswax: a scatter plot of instrument response data (FTIR-ATR predicted values) versus real stearic acid share values; (b) residuals of FTIR-ATR prediction (Svečnjak, 2018, unpublished data).

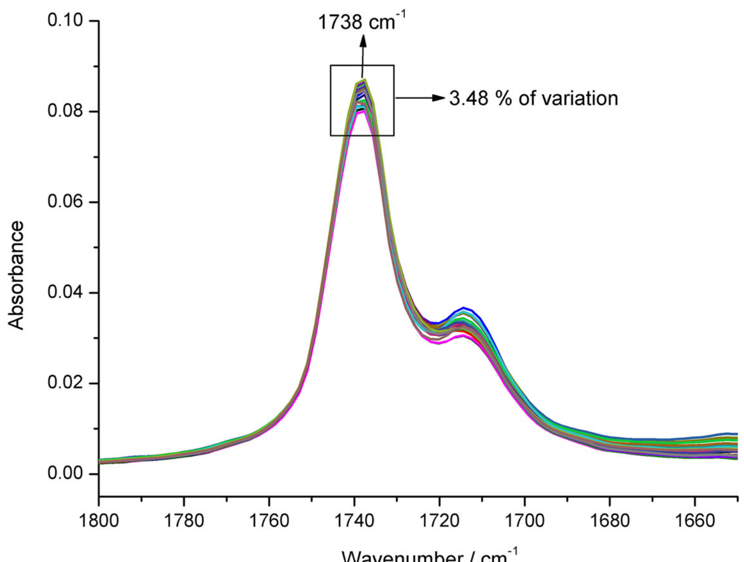


Figure 59. IR spectra and spectral variations (c_v = 3.48%) between different beeswax samples ($n = 20$) originating from different *A. mellifera* colonies observed at 1798 cm^{-1} (Svečnjak, 2017, unpublished data).

6.2.5.3.3.3. Interpretation of results. For a proper interpretation of the results, the complete measurement result on the adulterant share in the test sample should account for all measurement uncertainties determined in above sections, i.e., in Section 6.2.5.3.2.3 (SE/prediction accuracy), Section 6.2.5.3.3.1 (c_v – sample/natural variations in genuine beeswax), and Section 6.2.5.3.3.2 (c_v – repeatability/measurement precision). Thus, if for example 19.3% of paraffin has been detected in the test sample, the final result should be presented as follows:

$$\text{paraffin share} = 19.3 \pm 3.8\%$$

where the final uncertainty value of 3.8% is consisted of 0.023% (SE determined for paraffin), 3.48% (c_v – sample), and 0.3% (c_v – repeatability).

6.2.5.4. Advantages and disadvantages of FTIR-ATR for beeswax adulteration detection. Compared to other analytical tools used for detection of adulterants in beeswax described above, FTIR-ATR analytical approach is characterized by the following:

Advantages:

- Numerous instrumental and measurement advantages as presented in 5.3.4. 'Advantages and disadvantages of FTIR-ATR measurements' (summarized: fast, cheap, reliable - good accuracy and precision, reagent-free, and easy-to-use method).
- Suitable for qualitative and quantitativedetection of all adulterant types.

- High temperature treatment commonly used in comb foundation production process (~125 °C) has no influence on the spectral values.

Disadvantages:

- The method relies entirely on the in-house preparation of reference standards, i.e., adulterant–beeswaxmixtures (time-consuming at initial stage).
- Relatively high price of FTIR spectrometer coupled with ATR accessory (but cheaper compared to GC-MS and IRMS instrumentation).
- Hard to distinguish microcrystalline wax from paraffin wax, and stearic acid from palmitic acid and stearin (although irrelevant from the viewpoint of authenticity given that mentioned substances represent foreign matters that should not be present in beeswax).

6.2.6. Investigating brood survival on adulterated comb foundations

Field studies on the acceptance or rejection of adulterated comb foundation by the honey bee colony are scarce (Castro, Medici, Sarlo, & Eguaras, 2010; Darchen & Lavie, 1958; Medici, Castro, Sarlo, Marioli, & Eguaras, 2012; Ribeiro da Silva, Ribeiro, Toledo, & Toledo, 2002; Semkiw & Skubida, 2013; Toledo, 1991). As reported in several studies (Maia et al., 2013; Serra Bonvehí & Orantes Bermejo, 2012; Svečnjak, Baranović et al., 2015), comb foundation without any adulterants are rare and most contain 5–20% of paraffin. Foundations with significantly higher percentages, i.e., > 50% of adulterants, have occasionally been found on the European market. These results indicate the variability of beeswax quality, probably due to various factors, such as the recycling of old combs and accumulation of residual amounts of paraffin (Maia & Nunes, 2013; Svečnjak, Baranović et al., 2015; Svečnjak, Prđun et al., 2015), the use of 10% of paraffin during the comb foundation production process to improve the mold of beeswax (Castro et al., 2010), mixing of beeswax from different countries, and also, the different types of paraffin wax and other substances used for adulteration (Serra Bonvehí & Orantes Bermejo, 2012).

This market situation implies a need for implementing bioassays to understand the consequences of particular biological factors on honey bee colonies (Section 6.2.6.2), as well as economic losses for the beekeepers (see Section 6.2.6.3). However, it is necessary to homogenize productive, environmental, management and technical factors that may influence the behaviour of honey bee colonies against pure and adulterated comb foundations. In Section 6.2.6.1, we present several points that justify the necessity of the bioassays having the same conditions of the factors indicated above.

6.2.6.1. General factors important for bioassay standardization.

1. For general guidelines to equalize colony size see Section 3.1 or 3.2 (setting up experimental colonies of uniform strength) in the *BEEBOOK* paper on *A. mellifera* strength parameters (Delaplane, van der Steen, & Guzman, 2013)
2. The races of honey bees: different races can produce different quantity of beeswax in equal conditions (Hepburn, 1986; Toledo, 1991).
3. Age of a queen: colonies with new queen can have more capacity to produce more beeswax (Hepburn et al., 2014).
4. Age of honey bee and physiological state of the colony: pre-swarming colony have more potential to construct comb wax (Hepburn, 1986), and queen loss and swarming tend to be the most disruptive factors to colony populations (Delaplane et al., 2013).
5. Temperature values (mean, maximum and minimum) and humidity (%): these factors can also influence the construction of the comb (Szabo, 1977).
6. Nectar/pollen/beebread availability: the production of beeswax varies with the quantity and quality of food (Pratt, 2004) and inadequate pollen nutrition can interfere with normal wax gland development (Hepburn, 1986).
7. Condition of the combs: honey bee colonies with more honey reserves produce more wax than colonies with scarce reserves, even during the nectar flow period (Pratt, 2004). If possible, use manual or electronic hive scales for weighing full hives and to compare the development between colonies. See Section 4.1. "Research methods at the colony level" in the *BEEBOOK* paper on miscellaneous methods for *A. mellifera* research (Human et al., 2013).
8. Type of artificial diet (sugar and protein): normally, beekeepers stimulate honey bee colonies with sugar syrup at a ratio of 1:1 (by weight). In case of proteins, it is better to use a commercial product because botanical origin of pollen can differ in nutritional value between different geographical regions.
9. Sanitary management (type of acaricide). Varroa control treatment conducted before bioassay is important to avoid the death of brood. Some organic acaricides can interfere with brood development (Rademacher & Harz, 2006).
10. Type of beehive or nucleus: different volume of the hive and colony size can have some interactions.
11. Quality of adulterants (especially paraffin with different melting point): the beeswax industry uses paraffin with different melting points taking into account two points of view: paraffin with low melting point (<58 °C) because it is cheaper, and paraffin with higher melting points (60–65 °C) because of the similarity to the melting point of the beeswax. The adulteration impact on the bee colonies may depend on

the type of the paraffin used, especially in relation to its melting point (Semkiw & Skubida, 2013). For instance, the use of low melting point paraffin wax can result in comb deformations and/or collapses.

12. Thickness of the comb foundation: the thickness of the comb foundation can influence the comb construction (Toledo, 1991): (a) if the foundation is thicker, the honey bees spend more time working on building the combs; (b) larger areas of honeycomb are constructed on the comb foundation with the finest thickness.
13. Beeswax origin: it is important that beeswax has the same origin since different concentration of residues and adulterants can significantly influence test results, especially if the wax comes from different suppliers (Büchler et al., 2013).

6.2.6.2. Bioassay: brood survival versus beeswax adulteration. For biological factors, it is important to consider the dynamics of the honey bee colonies because some studies showed that the addition of adulterants may decrease the area built on the comb foundation, reduce the queen acceptance for oviposition, cause the appearance of irregular brood patterns, and may cause other negative effects (Castro et al., 2010; Darchen & Lavie, 1958; Medici et al., 2012; Semkiw & Skubida, 2013; Toledo, 1991). The brood survival on adulterated comb foundation experiment should be conducted as follows:

1. Use pure comb foundation (made of genuine beeswax) and comb foundation adulterated with paraffin (or other adulterant of interest)
2. Adulteration levels should be similar to the situation on the beeswax market (the adulterant amounts can range from 5 to >90%).
3. Adulterated beeswax can be obtained from a stock proven (or suspected) to be adulterated to a certain percentage or adulteration can be performed by the investigator. Adulterated wax foundations can be obtained as follows:
   a. Choose the adulterant of interest (note that the most commonly used/available solid paraffin wax has melting point ranging from 56 to 58 °C; if different melting point want to be tested, they can be found in specialized shops).
   b. Melt the beeswax and paraffin wax (or other adulterant) in the same container. Take into account the proportions of each to obtain the levels of adulteration desired, and the total quantity required (see Section 2.2. for details on melting for general guidelines).
   c. When the wax mixture melts, place a smooth wet table board in cold water.
   d. Dip the board in the wax mixture 2–3 times to obtain a sheet of wax.
   e. Allow to cool.
   f. Remove the smooth wax sheet.
   g. For each sheet obtained always wet the board to facilitate separation
   h. Place the wax sheet in press machine.(alternatively, put the melted wax in the portable beeswax machine).
4. Insert experimental frames in the hives: one frame with comb foundation made of pure beeswax and another frame with a sheet containing certain percentage of paraffin (or other adulterant) in each beehive. Repeat this operation on 3 hives (minimum) (Figure 60).
5. To avoid any effect of the frame position, two experimental frames are placed in contact with the brood nest in each hive; one on the left and the other on the right side of the brood.
6. To equalize colony size see above Section 6.2.6.1, step 1.
7. The measurements of the brood survival can be carried out in two ways:
   a. Compare the survival of brood in each hive and each experimental frame, the one with particular percentage of adulterant, and with pure beeswax (see Section 4.1. "Measuring colony strength at end of experiment" in the BEEBOOK paper on *A. mellifera* strength parameters' (Delaplane et al., 2013). Survival of brood can be quantified by first selecting a relatively contiguous patch of brood in the late larval/capped stage, and overlaying on the patch a 10-cm horizontal transect and a 10 cm vertical transect intersecting at the centre (Delaplane et al., 2013).
   b. Methodology by Medici et al. (2012) can also be applied: count the number of worker honey bee cells with eggs (day 0) over a rectangular area of 7 x 6 cm^2 marked on each wax section on both sides. This is repeated at intervals of ten days (day 10), counting the number of pupae. The brood survival rate in each treatment is estimated by the following formula:

$$\text{Brood survival} = \left(\frac{\text{N of pupae}}{\text{N of eggs}}\right) \times 100$$

8. The experiment should last at least 7 weeks to cover two generations.

6.2.6.3. Bioassay: colony development versus beeswax adulteration. This methodology has the objective of verifying the economic impact of adulteration on the beekeeping by assessing the colonies that develop on pure compared to adulterated combs. The rejection or bad acceptance of comb foundation by the honey bees is frequently observed by the beekeepers, and it is cosely related to the poor quality of commercial comb foundations. Comb foundation adulteration may cause an economic loss in the beekeeping because it delays the development of the honey bee colonies. This usually result in the delay in placing the honey supers (negative

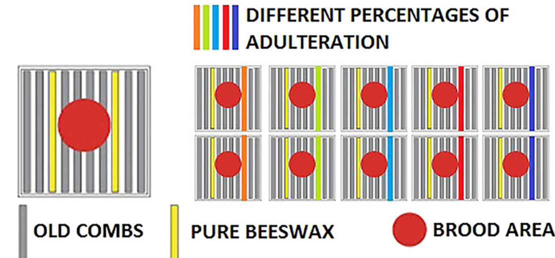


Figure 60. Bioassay for assessing biological factors.

impact on honey production) and compromises the production of swarms. Also, given that good apicultural practices recommend replacing at least 2–3 old combs per colony with comb foundations each year (Bogdanov et al., 2009), it is important that the honeybees are given wax that they can use as fast as possible to build their combs (Johansson & Johansson, 1978):

Bioassay tests for economic factors should be performed as follows:

1. Use pure comb foundation and comb foundation adulterated with paraffin or other adulterant of interest (see step 1. and 2. in Section 6.2.6.2).
2. Place comb foundation with the same percentage of paraffin (or other adulterant) in the beehive. This option consists of performing a method as close as possible to the operations in normal bee management (usually beekeepers acquire beeswax at the same supplier).
3. Use three test frames to replace frames with old combs.
4. Put the frames between the last brood comb and the first comb with honey and pollen (Figure 61).
5. To transfer a nucleus (five frames) or a package of bees to a beehive (10 frames), put five frames with bees, open and closed brood and food reserves (pollen and honey) into a beehive (Figure 62).
6. Put marks (numbers or letters) or spray the top of the experimental frames (pure and adulterated comb foundation) with different colours.
7. Before use in experiment, weigh experimental frames (Figure 63).
8. Use 1 L of sugar syrup at a ratio of 1:1 (by weight) to stimulate colonies in the first day. Remember: this bioassay is performed when there is a nectar flow and it is not economical to use more syrup. Feed with more syrup during the experiment period only in case of unfavourable climatic conditions (prolonged rain, the wind that dries the production of nectar during the flowering season, etc.).
9. Check the development of the colonies weekly. Use manual or electronic hive scales to weigh full hives (see Section 4.1 "Research methods at the colony level" in the *BEEBOOK* article Miscellaneous standard methods for *Apis mellifera* research' (Human et al., 2013).

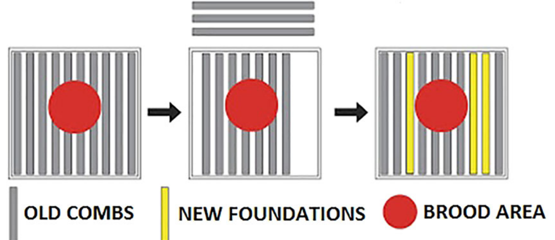


Figure 61. Replacement of the old combs (generally three frames).

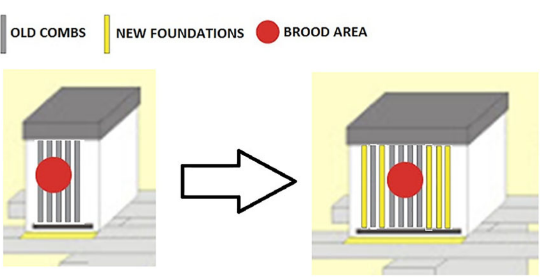


Figure 62. Transferring of a nucleus (five frames) or a package of bees to a beehive (ten frames).

10. Each time a beehive is checked, weigh the experimental frames (pure and adulterated comb foundation) with the same scale.
11. Compare the weigh between colonies and each experimental frame (built upon pure and adulterated comb foundation) (Figure 63). In this case, the weight includes the production of wax, the storage of honey, the honey bees and the brood.
12. Exchange the fully drawn frames from the middle (near the frames with brood) with the initially or partially built frames from outside positions (near nectar and pollen frames) during the weekly visits (step 9), immediately after weighing the combs. This operation is only performed if this is required (if the comb foundations are pulled out with wax).
Caution: do not insert the comb foundation into the hive without pulling in the middle of brood to avoid development of some brood diseases. The placement of experimental frames between the brood area can divide the colony; consequently, a part of the brood may not receive enough heat and some brood diseases, such as fouldbrood and chalkbrood, may develop.
13. A comb is considered built when the cells are more than 0.5 cm deep (Szabo, 1977) and when honey bees have constructed cells on more than 80% of the comb foundation area.
14. The duration of the study is 3 weeks (sufficient time for the colony to pull the pure wax out). However, this time period need to be adjusted according to climatic conditions and various nectar flow. It is important to compare the development of the colonies with different ratios of adulterated wax in the same environmental conditions, and at the same time.

Figure 63. Weighing of the experimental frames in the field.

6.2.6.4. Advantages and disadvantages.

Advantages:

- Better understanding of the consequences of beeswax adulterants on biological and economic factors in beekeeping technology.
- Potential for conducting complementary studies related to the adulterants (especially paraffin wax) that act as diluents of residual contaminants present in comb foundations; for instance, for verifying diluent action of the paraffin with the acaricides and consequent contamination of honey and brood (Medici et al., 2012; Wilmart et al., 2016).

Disadvantages:

- Possible need for a considerable number of colonies to carry out this kind of study.
- For some parameters, there may be difficulties in the measurement procedure, such as the evolution of the colony weight and relating economic factors (it might be necessary to purchase scales and instruments that may not be economically viable).

6.3. Standard methods for detection of pesticide residues in beeswax

Definitions, acronyms:

- MRL: maximum residue limits
- GC: gas chromatography
- ECD: electron capture detector
- NPD: nitrogen-phosphorus detector
- FPD: flame photometric detector
- MS: mass spectrometry
- LC: liquid chromatography
- PES: polyethersulfone
- DE: diatomaceous earth
- OCLLE: on-column liquid-liquid extraction using diatomaceous earth
- SPE: solid-phase extraction
- QuEChERS: quick easy cheap effective rugged safe
- PSA: primary secondary amine
- CDL: curved desolvation line
- ESI: electrospray ionisation
- LOD: limits of detection
- LOQ: limits of quantification

6.3.1. Residues in beeswax

Data on beeswax contamination by pesticides are less available and mostly come from nationwide surveys, rather than from regular inspections, simply because beeswax is not directly consumed by humans. Therefore, an official maximum residue limits (MRLs) are not set. On the other hand, beeswax is not only recycled almost continuously in the form of comb foundation, but it is also processed for pharmaceutical purposes or for cosmetics industries, and in the food industry as a glazing agent, for the surface treatment of certain fruits and as a carrier for flavour and colours (EFSA, 2007).

Beeswax is often contaminated by persistent lipophilic acaricides (Bogdanov, 2006; Jiménez et al., 2005). Moreover, many substances can easily migrate from

wax to honey (Tremolada et al., 2004; Wallner, 1999), hence pesticide residues even at trace levels are problematic. The contamination level of residues in beeswax has to be controlled and the accumulation process driven by wax recycling should be limited.

Gas chromatography (GC), initially in combination with selective detectors (electron capture detector (ECD), nitrogen-phosphorus detector (NPD), flame photometric detector (FPD) and subsequently in combination with mass spectrometry (MS) analyser, has been the main instrumental technique used in the analysis of pesticide residues since the 70s. GC and liquid chromatography (LC) coupled with MS detection are currently the most widely used techniques for multi-residue analysis of pesticides in bee products (Wiest et al., 2011). The choice of the chromatographic technique depends mainly on the physicochemical characteristics of the pesticides of interest. Volatile, semi-volatile and thermally stable compounds can be determined by GC technique, while non-volatile and/or thermally unstable compounds are usually determined by LC technique (Kujawski et al., 2014). The number of active ingredients that are determined by LC has increased significantly in the last years, since the pesticides used today are more polar, thermally unstable or not easily vaporizable, which makes them more suitable for this technique (Albero, Sanchez-Brunete, & Tadeo, 2004; Wille et al., 2011).

Over last recent years many studies have been focused in the multiple determination of pesticides in bee matrices covering insecticides, acaricides, fungicides and herbicides which can be divided into distinct chemical classes and mechanism of action such as organophosphates, organochlorurates, carbamates, phenylpyrazoles, pyrethroids, neonicotinoids, spynosins, benzoylureas and more. The next sections will describe the most used methods for wax extraction and purification for the subsequent analysis with LC-MS (see Section 6.3.2.1) and GC-MS techniques (see Section 6.3.3.1), and related chromatographic and MS conditions (see Sections 6.3.2.2 and 6.3.3.2).

6.3.2. Trace-level determination of pesticides in beeswax by LC-MS

6.3.2.1. Extraction and purification. The extraction of pesticide residues and the partial or total removal of the interfering substances are two critical issues in the analysis of complex matrices like beeswax. These operations affect performance parameters of sensitivity, selectivity and trueness of the method.

There is no official method for sample preparation for pesticide detection in beeswax. There are many methods that can be used and they are usually chosen based on the materials and equipment present in the laboratory. The extraction and clean up protocols more used can be distinguished into three types: on-column liquid-liquid extraction using diatomaceous earth, solid phase extraction (SPE) and modified QuEChERS (Quick Easy Cheap Effective Rugged Safe) protocol. The QuEChERS technique is the most commonly used method as it is able to determine a broad spectrum of analytes and it is certainly faster than using diatomaceous earth (OCLLE) or other SPE cartridges, despite a lower sample purification.

To get the best sample homogeneity of wax and improve the extraction of residues is always recommended to:

1. Reduce the wax in small pieces.
2. Place them in the milling chamber.
3. Freeze the wax pouring liquid nitrogen in the milling chamber.
4. Grind it finely with the analytical mill.

6.3.2.1.1. OCLLE protocol. (Nguyen et al., 2009):

1. Weigh 2 g of homogenized wax.
2. Add 15 mL of n-hexane mixture/isopropanol (8:2 v/v).
3. Heat to 50 °C for 3 min until wax is dissolved.
4. Add 10 mL of water.
5. Centrifuge for 5 min at 50 °C and 700 rpm.
6. Transfer the aqueous phase in diatomaceous earths cartridge.
7. Elute the analytes after 15 min with 20 mL of acetone, collecting the extract into a glass flask.
8. Dry on the rotary evaporator.
9. Reconstitute with 1 mL of acetonitrile/water (50:50 v/v).
10. Filter (polyethersulfone (PES) syringe filter, diameter 13 mm, pore size 0.45 µm).
11. Transfer into vial.

6.3.2.1.2. SPE protocols. (Yáñez, Bernal, Nozal, Martín, & Bernal, 2013):

1. Weigh 0.5 g of homogenized wax.
2. Add 1.5 mL of water.
3. Add 2.5 mL of ethanol.
4. Stir for 1 min.
5. Centrifuge for 20 min.
6. Condition the ChemElut SPE cartridge with 1 mL NaCl 20%.
7. Transfer the supernatant solution in the cartridge.
8. After 15 min, twice elute the analytes with 10 mL of n-hexane-dichloromethane (50:50 v/v).
9. Dry at 30 °C under gentle stream of nitrogen.
10. Reconstitute with 200 µL of acetonitrile-water (50:50 v/v).
11. Filter and transfer into vial.

SPE protocol (Wallner, 1993):

1. Weigh 0.2 g of homogenized wax.
2. Add 6 mL of hexane.

3. Heat to 70 °C.
4. Centrifuge at −10 °C for 5 min.
5. Transfer the supernatant solution in a glass tube.
6. Repeat the steps 2, 3, 4 and 5 with 3 mL hexane two times.
7. Condition a 500 mg Florisil column with 6 mL of hexane.
8. Run the collected solution through the column.
9. Elute with 1.5 mL of acetone/hexane (1:1).
10. Transfer 0.5 mL into vial.

6.3.2.1.3. Modified QuEChERS protocol. (Herrera Lòpez, Lozano, Sosa, Hernando, & Fernàndez-Alba, 2016) according to the European EN 15662 (CEN 2008) method:

1. Weigh 10 g of homogenized sample.
2. Add 10 mL of acetonitrile.
3. Stir for 1 min.
4. Add the premixed extraction salts, consisting of 4 g of Magnesium sulphate anhydrous, Sodium chloride 1 g, 1 g of Trisodium citrate dihydrate and 0.5 g of Disodium hydrogencitrate sesquihydrate.
5. Stir for another min.
6. Centrifuge for 5 min at 4000 rpm.
7. Transfer 6 mL of the liquid phase in a polypropylene tube.
8. Add 150 mg of Primary Secondary Amine (PSA) and 900 mg of magnesium sulphate anhydrous.
9. Stir for 1 min.
10. Centrifuge for 2 min at 6000 rpm.
11. Transfer 1 mL of extract in a test tube with 220 µL of acetonitrile.
12. Vortex and transfer into vial.

6.3.2.2. LC-MS detection. This subsection presents a short review of parameters relative to LC-MS detection of pesticides in beeswax performed by different studies (summarized in Table 20). The used parameters depend on the equipment and instrumentation available to the laboratory, and the investigated analytes. The expexted result consists on a chromatogram with acceptable peaks shape and resolution.

Chromatographic separation of pesticides was more often achieved by reversed-phase chromatography that includes any chromatographic method that uses a hydrophobic stationary phase, and the most used column is the octadecyl carbon chain (C18)-bonded silica, such as:

- Polaris C18-A HPLC column (3 µm, 2.0 × 150 mm) (Pirard et al., 2007).
- Agilent Zorbax SB-C18 column (3.5 µm, 2.1 x 150 mm) (Mullin et al., 2010).
- Fused-core type column Kinetex C18 (2.6 µm, 4.6 x 150 mm) (Yáñez et al., 2013, 2014).
- Halo C18, Eksigent column (2.7 µm, 0.5 x 50 mm) (Herrera Lòpez et al., 2016).

Kinetex Phenyl-Hexyl column (2.6 µm, 2.1 x 100 mm) (Jabot et al., 2015) as it displays a higher affinity for aromatic and moderately polar analytes, and RP-Amide Ascentis Express column (2.7 µm, 2.1 x 100 mm) (Porrini et al., 2016) that provides enhanced selectivity for samples containing acidic and basic compounds that can act as a hydrogen-bond donor, have also been used.

Chromatographic conditions:

- Column oven temperatures settings were quite variable depending on the research study, ranging from 25 to 60 °C. In detail, 25 °C (Yáñez et al., 2014), 30 °C (Herrera Lòpez et al., 2016), 35 °C (Yáñez et al., 2013), 40 °C (Pirard et al., 2007; Porrini et al., 2016), and 60 °C (Jabot et al., 2015) were the different temperatures used.
- Chromatographic mobile phases consisted of an acidified aqueous solution (mobile phase A) and an organic phase (mobile phase B) as acetonitrile or methanol, acidified or not. In detail, different combinations have been used:
    - (A) water and (B) acetonitrile, both acidified with 0.1% of acetic acid (Pirard et al., 2007) or with 0.1% formic acid (Herrera Lòpez et al., 2016).
    - (A) 0.1% formic acid in water and (B) acetonitrile (Yáñez et al., 2013, 2014).
    - (A) 0.04 mM ammonium acetate in water with 0.01% formic acid and (B) methanol (Jabot et al., 2015).
    - (A) 5 mM ammonium formiate in water with 0.1% formic acid and (B) 5 mM ammonium formiate in methanol with 0.1% formic acid (Porrini et al., 2016).
    - Only in one case an isocratic elution mode was described (Yáñez et al., 2014), but usually different gradient modes were performed. The flow rate varied from 0.4 to 0.5 mL/min.

Electrospray ionisation mass spectrometry (ESI-MS) is the elective technique. ESI uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. In positive ion mode, as performed for pesticide detection in beeswax, the analyte is sprayed at low pH to encourage positive ion formation.

The instrumental parameters vary in the different papers:

- Jabot et al. (2015): capillary voltage 3200 V, desolvation temperature 450 °C, source temperature 150 °C and nitrogen desolvation and nebulizer gas flows 900 and 150 L/h, respectively.
- Pirard et al. (2007): capillary and cone voltages 3 kV and 35 V, respectively, temperature source 125 °C, desolvatation temperature 250 °C. Nitrogen used as cone and desolvating gas at a flow rate of 100 and 680L/h, respectively.

Table 20. Summary of LC coupled to MS methods for detection of pesticides in beeswax.

Number of chemicals detected	Pesticides extraction	Instrument	Chromatographic column	Mobile phases	LOQ	Validation	Advantages/ disadvantages	BeeBook section number	References
120	QuEChERS method	Microflow-LC-ESI-MS/MS	Halo C18 (50 × 0.5 mm × 2.7 µm)	(A) Water (B) ACN both with 0.1% formic acid	5 µg/kg (for most compounds)	Yes	Well described method parameters	6.3.2.1.3., 6.3.2.2.	Herrera Lòpez et al. (2016)
128	QuEChERS method	LC-ESI-MS	RP-Amide Ascentis Express (100 × 2.1 mm × 2.7µm)	(A) Water (B) ACN both with 0.1% formic acid and 5 mmol ammonium formiate	n.r. (5 µg/kg LOD)	n.r.	LOQ not available	6.3.2.2.	Porrini et al. (2016)
13	On-column liquid–liquid extraction (OCLLE) using diatomaceous earth	LC-ESI-MS/MS	Kinetex phenyl-hexyl (100 × 2.1 mm × 2.6 µm)	(A) 0.1% formic acid and 0.04 mmol ammonium acetate in water (B) MeOH	1–40 µg/kg	Yes	Few chemicals detected	6.3.2.2.	Jabot et al. (2015)
300	QuEChERS method	LC-ESI-MS/MS	n.r.	n.r.	10 µg/kg (for most compounds)	n.r.	Lacking critical information		Ravoet et al. (2015)
99	Solid phase extraction	LC-ESI-MS/MS	n.r.	n.r.	100 µg/kg (for most compounds)	n.r.	Lacking critical information	6.3.2.2.	Simon-Delso et al. (2015)
2	Solid-liquid extraction	LC-ESI-MS	Kinetex C18 (150 × 4.6 mm × 2.6 µm)	(A) Water with 0.1% formic acid (B) ACN	0.4–0.7 µg/kg	Yes	Few chemicals detected	6.3.2.2.	Yáñez et al. (2014)
7	OCLLE using diatomaceous earth	LC-ESI-MS	Kinetex C18 (150 × 4.6 mm × 2.6µm)	(A) Water (B) ACN both with 0.1% formic acid	1.5–7 µg/kg	Yes	Few chemicals detected	6.3.2.1.2., 6.3.2.2.	Yáñez et al. (2013)
200	QuEChERS method	LC-ESI-MS/MS	Agilent Zorbax SB-C18 column (150 × 2.1 mm × 3.5 µm)	n.r.	n.r. (0.1–50 ng/kg LOD)	n.r.	Chemicals detected by LC and GC techniques, not mentioned which and how many compounds for each technique.	6.3.2.2.	Mullin et al. (2010)
1	OCLLE using diatomaceous earth	LC-ESI-MS/MS	n.r.	n.r.	0.5 µg/kg	n.r.	Few chemicals detected	6.3.2.1.1., 6.3.2.2.	Nguyen et al. (2009)
19	OCLLE using diatomaceous earth	LC-ESI-MS/MS	Polaris C18 (150 × 2.0 mm × 3 µm)	(A) Water (B) ACN both with 0.1% acetic acid	0.0002–1.232 µg/kg (CCβ)	Yes	Few chemicals detected	6.3.2.2.	Pirard et al. (2007)

ACN, acetonitrile; MeOH, methanol; n.r., not reported.

- Porrini et al. (2016): ESI interface voltage 1.5 kV, curved desolvation line (CDL) temperature 250 °C, block heater temperature 200 °C. Nitrogen was the nebulising gas with a flow rate of 1.5 L/min.
- Yáñez et al. (2013, 2014): fragmentor voltage 140 V, drying gas (N_2) flow 9 L/min, drying gas (N_2) temperature 340° C, nebulizer gas (N_2) pressure 40 or 60 psi, capillary voltage 2500 or 3000 V.

Limits of detection (LOD) of the methods ranged from 0.05 to 50 ng/kg and limits of quantification (LOQ) from 0.5 to 70 μg/kg, strictly depending on the analyte and on the studies considered, as summarised in Table 20.

6.3.3. Chromatographic multiresidue analysis by GC-MS

In the scientific literature concerning determination of pesticides in wax by GC-MS, prevails the analyses of residues of active ingredients linked to pharmacological treatments in the control of *Varroa* mite (miticides). Few publications describe analysis methods for pesticides determination in wax (Bogdanov, Kilchenmann, & Imdorf, 1998; Boi et al., 2016; Lodesani, Costa, Serra, Colombo, & Sabatini, 2008; Mullin et al., 2010; Wallner, 1993, 1999).

The next sections will describe the most used methods for wax extraction and purification for the subsequent analysis with GC-MS (see Section 6.3.3.1) and related chromatographic and MS conditions (see Section 6.3.3.2).

6.3.3.1. Extraction and purification. Similarly to what has already been written for the phases of extraction and purification in LC-MS paragraph (see Section 6.3.2.1), also in GC analysis these phases assume a relevant importance, due to the chemical complexity of the wax.

To obtain the best homogeneity of the sample is recommendable to freeze the wax with liquid nitrogen and then to grind it finely (as described in Section 6.3.2.1.).

The extraction and purification protocols can be distinguished into three types according to the use of diatomaceous earth (DE), SPE or QuEChERS method.

6.3.3.1.1. DE protocol. (Boi et al., 2016):

1. Weigh 3 g of wax in a beaker.
2. Melt at 80 °C in oven.
3. Add 5 mL of hexane.
4. Add 15 g of diatomaceous earth.
5. Vortex the powdered mixture.
6. Fill an empty 80 mL cartridge with the resulting powdered mixture.
7. Elute the sample with approximately 120 mL of acetonitrile.
8. Freeze the extract at -18 °C, when the elution is complete.
9. Dry the extract in rotary evaporator at low pressure with water bath at 55 °C.
10. Dissolve the residue with 3 mL of 2,2,4-trimethylpentane.
11. Filter (PES syringe filter, 13 mm, 0.45 μm) the sample in vials for GC-MS analysis.

Advantages:

- performance of good extractions and purifications

Disadvantages:

- requires large volumes of solvent and large freezers

6.3.3.1.2. SPE protocol. (Bogdanov et al., 1998):

1. Weigh 1 g of wax into a centrifugation tube.
2. Add 10 mL hexane.
3. Extract in ultrasonic bath for 45 min.
4. Cool the n-hexane solution in a freezer for 1.5 h.
5. Centrifuge for 18 min at 10,000 rpm at −5 °C.
6. Concentrate the supernatant to a volume of 1 mL using a Rotor evaporator.
7. Transfer to a new centrifuge tube.
8. Repeat twice freezing and centrifugation.
9. Load the final supernatant (1 mL) into a Florisil (60/100 mesh) column (1.5 g Florisil + 0.5 g anhydrous Na_2SO_4 in a 6 mm i.d. glass column).
10. Add 20 mL of n-hexane.
11. Discard this fraction.
12. Add 10 mL of n-hexane/acetone (1:1).
13. Concentrate this fraction by Rotor evaporator.
14. Dry under a gentle nitrogen stream.
15. Re-suspend purified extract in 0.5 mL of n-hexane for GC-MS quantification.

Advantages:

- performance of good extractions and purifications

Disadvantages:

- time-consuming analysis

6.3.3.1.3. QuEChERS protocol. (Mullin et al., 2010):

1. Weigh 3 g of wax into a 50 mL centrifuge tube.
2. Add 100 μL of process control spiking solution.
3. Prepare an adequate volume of extraction solution by mixing water:acetonitrile:glacial acetic acid, 44:55:1 (v/v).
4. Add 27 mL of extraction solution.
5. Melt the sample at 80 °C in water bath.
6. Cool the sample to room temperature.

7. Add the premixed extraction salts, consisting of 6 g of $MgSO_4$ and 1.5 g of sodium acetate.
8. Seal tube.
9. Shake vigorously for 1 min.
10. Centrifuge for 1 min.
11. Add to a dual layer SPE cartridge (containing 250 mg graphitized carbon black and 500 mg PSA) about 80 mg of anhydrous magnesium sulfate.
12. Condition cartridge by adding 4.0 mL of acetone/toluene (7:3, v:v).
13. Elute solvent to waste, using a positive pressure or vacuum manifold.
14. Add 2 mL of supernatant obtained after centrifugation (step 10), to the top of the cartridge.
15. Elute cartridge using 3–4 mL of acetone/toluene (7:3, v:v) into a 15 mL graduate glass centrifuge tube.
16. Dry eluate to a final volume of 0.4 mL in evaporator at 50 °C.
17. Sample is ready for GC-MS analysis.

Advantages:

- quick and simple technique

Disadvantages:

- technique does not provide an excellent purification of the analytes

6.3.3.2. GC-MS detection. Most of the scientific literature (Boi et al., 2016; Bonzini et al., 2011; Serra Bonvehí & Orantes Bermejo, 2010) agrees that a DB5 MS column 30 m × 0.25 mm × 0.25 μm is the best choice for the chromatographic separation of pesticides by GC.

Instrumental parameters settings:

- Injection volume: 1 μL.
- Injector: splitless mode (60 s).
- Injector temperature: 280 °C.
- Carrier gas: helium.
- Flow rate: 1 mL min^{-1}.
- Temperature program:
  - 60 °C for 2min.
  - 20 °C min^{-1} to 150 °C.
  - 4 °C min^{-1} to 260 °C.
  - 15 °C min^{-1} to 320 °C.
  - 320 °C for 7 min.
- Transfer line temperature: 325 °C.
- Electron ionization: 70 eV.
- Source temperature: 230 °C.
- Quadrupole temperature 150 °C.
- Scan range: 50–600 mass unit.

The limits of detection (LOD) and quantification (LOQ) vary greatly in relation to the monitored active ingredient (see Table 21 for some examples, the conditions they were acquired in and the advantages and disadvantages of each).

6.3.4. Other methods for pesticides detection in beeswax

Other techniques different from LC or GC-MS are currently not used commonly for the detection of pesticides in beeswax, except for the pesticides group of acaricides, mostly used to treat honey bee colonies to control *Varroa* mite infestation.

The methods for acaricide detection in beeswax are mainly based on the determination by GC with different detectors (apart from MS analyser, described in Section 6.3.3) such as flame photometric detector (FPD), ion trap detector (ITD), flame ionization detector (FID) and electron capture detector (ECD), but only allow for the detection of a restricted number of analytes.

Only a few researchers used LC, and then only for the determination of a limited number of active principles, mainly acaricides. A widely used LC detection is the fluorescence detector but its use is restricted to analytes that are fluorescent or are converted to fluorescent derivatives.

Given the limited number of analytes and the relatively high limits of detection, these methods are to be considered of secondary importance compared to multiresidual studies previously described in Sections 6.3.2., 6.3.3., and 6.3.4., and these have been summarized in Table 22. They are outdated and of very limited interest and can therefore be considered as historical literature, not recommended for future studies.

7. Standard methods for detection of pathogens in beeswax

Beeswax is an important bee product which returns partly into the hive in the form of comb foundation (Máchová, 1993). Consequently, investigation methods applicable to beeswax herein described aim at detecting and quantifying honey bee pathogens and consequently guaranteeing sterility or at least the absence of specific pathogens from comb foundation. When beeswax is processed for producing foundations, cleaning and disinfection could necessitate chemicals, e.g., 0.5% H_2SO_4, and a temperature of 80–90 °C for 30 min. This procedure is sufficient to kill vegetative stages of *Paenibacillus larvae* (Rose, 1969), but does not destroy *P. larvae* spores; their heat resistance can even be increased at these temperatures (Gerhardt & Marquis, 1989). A temperature of 121 °C at a pressure of 101.325 kilopascal (kPa) for 20–30 min to remove *P. larvae* spores from beeswax is recommended (Hornitzky & Wills, 1983; Plessis, Du-Rensbrug, & Van-Johannsmeier, 1985; Shimanuki, Herbert, & Knox, 1984). Temperature not combined with pressure could also be effective but longer application is required (Savov & Arsenov, 1963). Methods for isolation of *P. larvae* spores in beeswax had been already elaborated in the 70s by Kostecki and

Table 21. Summary of GC coupled to MS methods for detection of pesticides in beeswax.

Number of chemicals detected	Pesticides extraction	Chromatographic column	LOD	Validation	Advantages/disadvantages of the method	Beebook section number	References
5	DE	J&W DB 5MS 30 m, 0.25 mm, 0.25 μm	1 μg/kg	Yes	Few active ingredients	6.3.3.1.1.	Boi et al. (2016)
200[a]	QuEChERS method	J&W DB 5MS 30 m, 0.25 mm, 2 μm	0.1–50 μg/kg	n.r.	Number of chemicals detected by GC not indicated	6.3.3.1.3.	Mullin et al. (2010)
5	SPE	HP 5 30 m, 0.25 mm, 0.25 μm	n.r.	n.r.	Few active ingredients	6.3.3.2.	Bonzini et al. (2011)
14	SPE	J&W DB-17MS 30 m, 0.25 mm, 0.25 μm	7–38 μg/kg	Yes	Few active ingredients	6.3.3.2.	Serra Bonvehí and Orantes Bermejo (2010)

n.r., not reported.
[a]Used in combination with LC-MSMS technique.

Orlowski (1975), Kostecki and Jelinski (1977) and then by Hansen and Rasmussen (1991). More recently wax (hive) debris have been investigated as indicator of the possible presence of spores of *P. larvae* and to predict the onset of AFB in the colony in the next season (Bzdil, 2007; Forsgren & Lauden, 2014; Ryba, Titera, Haklova, & Stopka, 2009; Titěra & Haklová, 2003). Beeswax is the most relevant component of hive debris and it is where pathogens can accumulate. Other than *P. larvae* here we report on the protocols to determine other pathogens from beeswax and wax (hive) debris, i.e., *Melissococcus plutonius, Ascosphaera apis*, *Nosema* spp. and small hive beetle (SHB). Furthermore, the reader is referred to *COLOSS BEEBOOK Volume II* for more general information on the different pathogens of honey bees.

7.1. Early detection of American foulbrood by beeswax analysis

AFB is a devastating brood disease of the honey bee caused by the spore-forming, Gram-positive rod-shaped bacterium *P. larvae* (de Graaf et al., 2013; Hansen & Brodsgaard, 1999). AFB is one of the bee diseases listed in the OIE (Office International des Epizooties – the World Organization for Animal Health) Terrestrial Animal Health Code (OIE, 2018a) and internationally recognised diagnosis protocols are available in the OIE Manual for diagnostic tests and vaccines for terrestrial animals (OIE, 2018b). The possible role of beeswax in the spread of pathogens through trade in honey bees and their products has been considered by Mutinelli (2011). An AFB infected dead honey bee larva transforms in a dark scale adherent to the cell wall that contains up to 2.5 billion spores of *P. larvae* (Figure 64) and can contaminate beeswax. Methods of detecting *P. larvae* in beeswax have implications in both sterility of foundations and prognostic value for the development of the disease.

We provide these methods in a simple and easy-to-be-applied form to facilitate their implementation in the laboratory. However, reference to original papers is provided. Furthermore, readers are addressed to COLOSS *BEEBOOK Volume II Standard methods for American foulbrood research* (de Graaf et al., 2013) that covers a broad set of technical information on *P. larvae*.

7.1.1. Determination of P. larvae *spores in beeswax*

7.1.1.1. Choice of solvent. Wax samples should be dissolved in Tween 80 that is today considered the most appropriate solvent (Bzidil, 2007) (Section 7.1.1.2.2). An historical overview of different solvents used to extract *P. larvae* spores from beeswax and wax (hive) debris is presented in Table 23.

All the solvents should be stored in a flammable cabinet and used under chemical safety hood using personal protective equipment, i.e., laboratory suit, gloves and googles.

Table 22. List of publications concerning the research of pesticides in beeswax using techniques other than liquid and gas chromatography coupled with mass spectrometry.

Analytes	Technique	LOD (μg/kg)	Reference
Malathion, coumaphos	GC-FPD	–	Thrasyvoulou and Pappas (1988)
Amitraz	GC-ITD	1	Leníček et al. (2006)
Para-dichlorobenzene	GC-FID	700	Bogdanov et al. (2004)
Amitraz, fluvalinate, bromopropylate	GC-ECD	1, 1, 50	Lodesani et al. (1992)
Bromopropylate, coumaphos, flumethrin, fluvalinate	GC-ECD	100–300	Bogdanov et al. (1998) and Bogdanov, Kilchenmann, and Bütikofer (2003)
Fluvalinate	GC-ECD	100	Tsigouri, Menkissoglu-Spiroudi, Thrasyvoulou, and Diamantidis (2000)
Amitraz and metabolites (DPMF, DMA)	GC-ECD	80	Jiménez, Bernal, del Nozal, and Alonso (2004b)
Lindane, chlorpyriphos, z-chlorfenvinphos, endosulfan A and B, 4,4′-DDE, 4,4′-TDE, acrinathrine, bromopropylate, tetradifon, coumaphos, fluvalinate	GC-ECD	5–40	Jiménez, Bernal, del Nozal, and Alonso (2004a)
Benomyl, carbendazim	LC-FL	10–50	Bernal et al. (1997)
Coumaphos	LC-FL	2	Van Buren, Marien, Velthuis, and Oudejans (1992)
Amitraz	LC-UV	–	Korta et al. (2001)
Fluvalinate, coumaphos, bromopropylate and 4,4′-dibromobenzophenone	LC-DAD	2–100	Adamczyk, Làzaro, Pérez-Arquillué, and Herrera (2007)

FPD, Flame Photometric Detector; ITD, Ion Trap Detector; FID, Flame Ionization Detector; ECD, Electron Capture Detector; FL, Fluorimetric Detector; UV, Ultra-Violet detector; DAD, Diode Array Detector.

The following characteristics are examined visually providing direct information about the solvent efficacy:

- solubility of wax samples.
- formation of mist or sediment with solvent.

In the latter, mist should be removed by replicated dissolving and centrifugation until beeswax is completely dissolved.

According to the results obtained by Bzdil (2007), the Tween method is statistically significantly more effective in detecting *P. larvae* spores than toluene. This might be explained by high aggressiveness of toluene that might kill a part of spore-forming microorganisms in one of the sporulation stages. It is also possible that toluene residues on culture media plates negatively influence the germination and growth of microorganisms.

The viable *P. larvae* spores trapped in beeswax and extracted by solvent is determined by sample plating on MYPGP medium.

7.1.1.2. Method of isolation of P. larvae *spores from beeswax and wax (hive) debris.*

Definitions, acronyms:

- Spiked sample: sample to be examined spiked with spores of *P. larvae*.
- Positive control: reference strain of *P. larvae*, ATCC 9545.
- Negative control: matrix without spores.
- H_2O_2: hydrogen peroxide

Equipment required:

- Bunsen or Laminar flow cabinet
- Sterile 50 mL tubes
- Sterile Pasteur pipette
- 50–200 μL micropipette
- 200μL sterile tips
- Sterile disposable loops
- Bench centrifuge
- Disposable L-shaped spatula
- Microscopic slides
- Incubator +37 °C
- Dry heat oven 90 °C
- Freezer -18 °C
- Refrigerator +2 °C/+8 °C
- Jar for microaerobic conditions.
- Water bath +50 °C/+80 °C
- Microscope with objective 100x

Reagents required:

- Sterile distilled water stored at RT
- MYPGP agar stored at 5 ± 2 °C:
    - 10 g Mueller-Hinton broth (Oxoid CM0405)
    - 15 g yeast extract
    - 3 g K_2HPO_4
    - 1 g Na-pyruvate
    - 20 g agar
    - Autoclave at 121 °C/15 min.
    - Add 20 mL 10% glucose (autoclaved separately).
    - Cool the media to 50 °C and add the antibiotics to a final concentration of 20 μg/mL for nalidixic acid and 10 μg/mL for pipemidic acid:

Figure 64. Comb with typical signs of AFB: dark scales adhering to the wall of the cells; perforated caps with brown dense material draining from the cells. Once dried this material becomes scale.

- Nalidixic acid stock solution (1 mg/mL) is prepared by dissolving 0.1 g in 2 mL of 1 M NaOH and diluting to 100 mL with 0.01 M phosphate buffer (pH 7.2).
- Pipemidic acid stock solution (2 mg/mL) is prepared by dissolving 0.2 g in 2 mL of 1 M NaOH and then diluting to 100 mL with 0.01 M phosphate buffer (pH 7.2).
- Both antibiotic solutions are filter sterilized.

The medium is poured (20 mL) into sterile Petri dishes and plates are dried before use (15 min).

- Campygen (Oxoid) gas pack for generating microaerobic conditions stored at RT
- 3% oxigen peroxide solution stored at 5 ± 2 °C
- Tween 80 solution stored at RT
- Reagents for GRAM staining (Hucker's technique modified): safranine, cristal violet and Lugol's solution stored at RT
- Saline solution stored at RT
- Positive control stored at -20 °C
- Negative control stored at RT

7.1.1.2.1. Spiked beeswax preparation to be used as Paenibacillus larvae *positive control.*

1. Weigh 1 g of beeswax.
2. Cut in small pieces in a 50 mL tube.
3. Add an AFB scale (Figure 64), previously tested positive for *P. larvae* (see 7.1.1.2.5.).

7.1.1.2.2. Culture.

1. Weigh 1 g of beeswax or wax debris.
2. Cut in small pieces (<3 mm) in a 50 mL tube.
3. Add 8.5 mL sterile distilled water.
4. Add 0.5 mL Tween 80 previously heated for 30 min in water bath at 70 ± 2 °C to reduce its viscosity.
5. Put the sample for 30 min in water bath at 70 ± 2 °C.
6. Stir in longitudinal direction every 10 min (if beeswax is not properly melt or the beeswax size is >3 mm, put in water bath for additionally 30 min). The result is a grey-brown pulp.
7. Cool at RT for 2–4 h to clearly separate liquid/solid phase.
8. Transfer 2–5 mL of liquid in a sterile tube (15 mL).
9. Add the same volume of sterile distilled water.
10. Stir for 5 min.
11. Put in incubator at 90 ± 2 °C for 10 min.
12. Cool at RT.
13. Smear 200 μL of the suspension on MYPGP agar using a spatula.
14. Incubate at +37 ± 1 °C in a jar with CampyGen OXOID gas pack for 5–8 days.
15. After 5–8 days, evaluate the presence of colonies compatible with *P. larvae*.
16. Small, regular, rough, flat or convex, whitish to beige colonies should be considered suspect.
17. Colony morphology is not conclusive but might serve to select the bacterial colonies for further identification through catalase test or Gram staining. Molecular methods can also be applied for identification and characterisation (see COLOSS *BEEBOOK VOLUME II*).

7.1.1.2.3. Catalase test.

1. Transfer a colony to a clean microscope slide.
2. Add a drop of 3% hydrogen peroxide on the colony.
3. Observe the drop with the naked eye.
4. *P. larvae* colonies do not produce bubbly foam or only delayed and weak (Haynes, 1972).

7.1.1.2.4. Gram staining (OIE, *2018b*).

1. Flood (cover completely) slide of a heat fixed smear with crystal violet.
2. Leave for about 60 s.

Table 23. Solvents used to extract *P. larvae* spores from beeswax and hive debris.

References	Rose (1969) and Wilson (1972)	Máchová (1993)	Ritter and Metzinger (1998) and Ritter (2003)	Titěra and Haklová (2003)	Bzdil (2007)	Ryba et al. (2009)	Forsgren and Laugen (2014)
Matrix/solvents	Beeswax	Beeswax	Beeswax	Hive debris	Beeswax/ hive debris	Hive debris	Hive debris
Benzene	Was found to be the best	Was found to be the best	–	–	–	–	–
Ether also named diethyl ether	–	Mixed with wax, combined to form a sediment which did not disappear even after repeated centrifugation. After plating, a wax crust was formed in which the spores did not germinate. There was a reduction in the number of spores in replicated dissolution, sedimentation and decantation in the lower part of the jelly supernatant.	Only this solvent was used and found appropriate.	–	–	–	–
Chloroform	–	As above	–	–	–	–	–
Toluene	–	An excessive growth of additional microflora in the Petri dishes was observed in an experiment with toluene.	–	Only this solvent was used and found appropriate.	–	Toluene (common in cultivation tests) gave consistent results.	–
Tween 80	–	–	–	–	Tween method is statistically significantly more effective in detecting *P. larvae* spores than the Toluene method (Titěra & Haklová (2003)).	According to Bzdil (2007).	According to Bzdil (2007).
Isopropanol and ethanol	–	–	–	–	–	They showed the average value of efficiency.	–
Water + Tween 80	–	–	–	–	–	Extraction into water with Tween 80 did not show any differences compared with water alone.	–
Water	–	–	–	–	–	Showed the highest efficiency.	–

3. Wash the slide for 5 s with water. The smear should appear blue-violet when observed with the naked eye.
4. Flood the slide with the iodine solution.
5. Leave for about 60 s.
6. Rinse the slide with water for 5 s and immediately proceed. At this point, the smear should still be blue-violet.
   Note: The next step involves addition of the decolouriser, ethanol. This step is somewhat subjective because using too much decolouriser could result in a false Gram (–) result. Likewise, not using enough decolouriser may yield a false Gram (+) result.
7. To be safe, add the ethanol drop-wise until the blue-violet colour is no longer emitted from the specimen.
8. Rinse with water for 5 s.
9. Flood the slide with the counter-stain safranin.
10. Let this stand for about 60 s to allow the bacteria to incorporate the dye.
11. Rinse with water for 5 s to remove any excess of dye.
12. Blot the slide gently with bibulous paper or allow it to air dry before viewing it under the microscope (Figure 65).

7.1.1.2.5. Bacterial DNA extraction from wax (hive) debris.

1. Weight 1 g of debris.
2. Mix 1 g of debris with 5 mL of sterile, distilled water.
3. Shake at RT for 1 h.
4. Immediately subject 100 µL of the suspension to DNA extraction using the QIAamp® genomic DNA isolation mini kit for Gram-positive bacteria (Qiagen) (Ryba et al., 2009).
5. Elute the DNA with 100 µL elution buffer and store at -20 °C until further use.

7.1.1.2.6. Quantitative real-time PCR for P. larvae *16s RNA sequence.* The quantitative real-time PCR assay is modified from Martinez, Simon, Gonzalez, and Conget (2010):

1. The reaction contained 10 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad), 0.4 µM each of primers PL2-Fw and PL2-Rev, 2 µL of template.
2. The final reaction volume should be adjusted to 20 µL with nuclease free water.
3. A negative control containing water instead of DNA template is included in each run.
4. All real time qPCR reactions (standards, unknown samples and controls) are performed in duplicate in neighbouring wells on the sample plate.
5. The amplification and data acquisition is carried out using a CFX Connect® (Bio-Rad) real-time PCR machine under the following cycling condition.
6. Enzyme activation step, 98 °C for 2 min, PCR cycling (40 cycles of 98 °C for 5 s (denaturation), 58 °C for 5 s (annealing/extension) and data collection.
7. The identity of the amplified product is confirmed using a melting curve analysis, by raising the temperature from 55 to 95 °C in 0.5 °C increments with a hold of 1 s at each increment.
8. The assay specificity and the presence/absence of non-specific amplification products is determined through the melting temperature(s) (T_m) of the amplified product(s).
9. Each set of PCR assays included serial dilutions of DNA extracted from known concentrations of *P. larvae* spores (total microscopic count) as external standards, for relating the qPCR data to spore counts (Forsgren, Stevanovic, & Fries, 2008).
10. Standard curves are prepared by using serial dilutions of target DNA ranging from 10^2 to 10^8 as quantification standards in every run.
11. The quantification data are normalized to tissue or debris weight and converted to bacterial cells per bee or bacterial cells per gram debris.

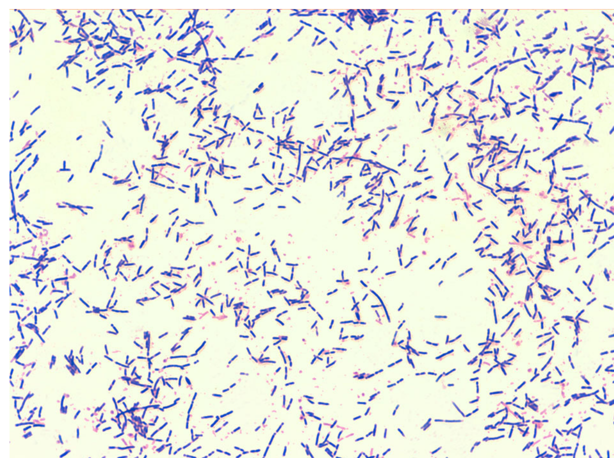


Figure 65. Gram + staining of *P. larvae*. Gram staining, 1000×, immersion oil.

This PCR method on debris samples revealed the highest bacterial levels if compared to honey bee samples (Forsgren & Laugen, 2014). Eventually, sub-clinical levels of *P. larvae* can be easily detected by this PCR in hive debris irrespective of disease symptoms.

7.2. Detection of other pathogens/pests in beeswax and wax (hive) debris

7.2.1. European foulbrood

There is no reference about investigation of *Melissococcus plutonius*, the causative agent of European foulbrood, in beeswax except for McKee, Djordjevic, Goodman, and Hornitzky (2003) who developed a hemi-nested PCR to improve the detection of *Melissococcus plutonius* in honey bees and their products starting from a previous attempt by Djordjevic, Noone, Smith, and Hornitzky (1998). Briefly, to obtain brood comb cell washes from colonies to be screened for the presence of *M. plutonius*:

1. Soak a sterile swab in sterile distilled water.
2. Rotate it in a single comb cell containing a freshly laid egg.

3. Agitate the swab in 1 mL of sterile distilled water for 30 s.

The application of this hemi-nested PCR to brood comb cell washes could be useful to provide information about the possible presence of *M. plutonius* on beeswax (McKee et al., 2003).

Further information on *M. plutonius* is available in COLOSS *BEEBOOK* volume II Standard methods for European foulbrood research (Forsgren, Budge, Charrière, & Hornitzky, 2013).

The lack of references about the detection of *M. plutonius* in beeswax is likely related to its non-spore forming nature. Hence, it is not considered relevant as beeswax contaminant.

7.2.2. *Ascosphaera apis*

Beeswax, provisions, brood and excreta are rich in nutrients and serve for fungal growth when sufficient moisture is available. Fungi associated with honey bee hives (including yeasts) may be parasites, commensals or mutualistics. *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive and Spiltoir is a pathogen that affects honey bee brood and causes chalkbrood (Johnson et al., 2005). This species occurs widely in temperate regions, being the most widespread infectious fungal disease (Gilliam, Taber, Lorenz, & Prest, 1988). Due to the intimate contact between larvae and comb cells (beeswax) it is reasonable to hypothesize that fungal spores are embedded in beeswax. Foundations artificially contaminated with ground sporulated mummies (mummy is the name and typical clinical presentation of infected larvae covered by *A. apis* iphae and/or mycelium) were able to infect honey bee larvae (Flores, Spivak, & Gutierrez, 2005).

Here we present the protocol to analyse the fungal community from brood combs (beeswax) and wax (hive) debris according to Rivas and Bettucci (2007).

7.2.2.1. *Protocol for determination of A. apis in comb wax and wax (hive) debris.*

1. Place brood comb and bottom board debris on Petri dishes containing MEA 20% sucrose.
   Malt extract agar (for 1 L of MEA + 20% sucrose):
   - Glucose 20 g,
   - Malt extract 20 g,
   - Sucrose 200 g,
   - Peptone 1 g, Agar 20 g,
   - Distilled water1 L.
     Preparation: dissolve ingredients and sterilize at 121 °C for 15 min.
     Final pH: 5.0–5.5.
     Store at 4 °C.
2. Incubate at 25 °C until the fungal colonies develop.
3. Identify grown colonies based on COLOSS *BEEBOOK Volume II* (Jensen Bruun et al., 2013).

Detailed information on further methods to study fungal disease of honey bee is available in COLOSS *BEEBOOK Volume II Standard methods for fungal brood disease research* (Jensen Bruun et al., 2013).

7.2.3. Nosema *spp.*

An extensive literature review exists on *Nosema* spp. in COLOSS *BEEBOOK Volume II Standard methods for Nosema research* (Fries et al., 2013). However, detection of *Nosema* spores in beeswax is a rarely studied subject. The nature of beeswax could make retrieving of spores difficult and during wax separation spore viability and genomic DNA could be affected. Different methods may be suitable for spore separation from wax, spore purification and DNA extraction. Here, we present a rapid method developed for a successful separation of *Nosema* spores from beeswax (Muz, Özdemir, & Mutinelli, 2017).

7.2.3.1. *Beeswax melting and spore extraction by water.*

1. Heat 100 mL of distilled water to 81 °C on a digital hot-plate with magnetic stirrer device combined with an encapsulated magnetic stir bar to mix the solution in a borosilicate quality glass chamber.
2. Gently add 10 g of the sampled beeswax to heated water.
3. Close the glass cover well for 30 min.
4. Cool the mixture beeswax-water very slowly at a rate of 1 °C/2 min.
   This facilitates the separation of *Nosema* spores from beeswax.
5. Stop magnetic stirring.
6. Remove the magnetic stir when temperature reaches 66 °C.
7. Let the temperature of the hot-plate decrease at a rate of 0.5 °C/2 min until 60 °C.
8. Gently aspirate the water in a centrifuge tube.
9. Centrifuge at 14,000 rpm for 3 min.
10. Gently discard the supernatant and get *Nosema* spore pellet ready for DNA extraction (Fries et al., 2013).

If spore purification is needed, this can be done by Percoll® centrifugation (Pertoft, Laurent, Laas, & Kagedal, 1978).

7.2.3.2. *Beeswax melting and spore extraction by organic solvents.*

If the expected spore number is very low, different chemicals like dichloromethane, acetonitrile, acetone, ethyl acetate, hexane, toluene and xylene should replace water:

1. Use a microtube heating block.
2. Add 0.25 g of beeswax to 1 mL of any of the solvents above to the microtube.
3. Close microtube.
4. Melt at 81 °C in a fume hood.

5. Gently vortex the tube for 5 min.
6. Incubate for 30 min.
7. Cool heating block until 60 °C.
8. Put a drop of solvent containing dissolved beeswax and spores on a microscopy slide.
9. Cover the slide with a cover slip.
10. Check under light microscope (400x) for *Nosema* spore.
11. If the drop contains spores, quickly transfer the drop to a glass tube.
12. Keep the tube open and wait until the solvent evaporates completely in fume hood.
13. Add 1 mL purified water into the glass tube
14. Vortex gently.

The beeswax separated and purified *Nosema* spore suspension is ready to use in any commercial gDNA extraction kit or any traditional method like cetyl trimethylammonium bromide (CTAB), phenol-chloroform or DNAzol methods. See also Fries et al. (2013) for further study methods.

Laboratory trials demonstrated that acetonitrile, acetone, ethyl acetate and xylene caused a reduction in the total number of spores of 34, 25, 19 and 10% respectively and their use is then discouraged. Toluene and hexane had instead no noteworthy effect on the total number of *Nosema* spores recovered (Muz et al., 2017).

7.2.4. Small hive beetle. The small hive beetle (SHB), *Aethina tumida*, is a parasite and scavenger of honey bee colonies (Neumann, Pettis, & Schäfer, 2016). It has recently become an invasive exotic species creating the need for an efficient and reliable detection method. Ward et al. (2007) developed a method to screen wax (hive) debris for the presence of SHB using real-time. This method (modified) was included in COLOSS *BEEBOOK volume II Standard methods for small hive beetle research*, and recommended to screen imported hives for SHB (Neumann et al., 2013).

7.2.4.1. Protocol for determination of A. tumida in wax (hive) debris in the context of monitoring programs. Hive debris were collected from 398 colonies in a monitoring study on SHB carried out during 2010 and 2011 in Spain (Cepero, Higes, Martínez-Salvador, Meana, & Martín-Hernández, 2014). Following the protocol of mentioned study, hive debris should be collected and processed as follows:

1. Collect the debris of the bottom of six hives randomly selected (8.5 g per the six colonies as average) in a plastic jar (125 mL).
2. To prepare the pooled samples, introduce 5 g from each sample of debris into a double bag strainer (Seward, BA6040).
3. Add 18 mL of milliQ water (MQW).
4. Crush in a Stomacher 80 (Biomaster) for 120 s.
5. Add 9 mL of MQW.
6. Homogenise the suspension again for 60 s.
7. Collect the macerate obtained in a 50 mL tube.
8. Centrifuge at 1751xg for 10 m.
9. Discard the supernatant.
10. Add 1 mL of MQW to the pellet.
11. Re-suspend an aliquot of 50 mg (on average) from the pellet in 3 mL of MQW and homogenise.
12. Add 400 µl to a 96-well plate and process for DNA extraction as described by Botías et al. (2012); include some wells with water alone as negative controls.
13. Freeze the plates at −20 °C until use.

Real-time PCR should be performed in a LightCycler 480 (Roche) using the LightCycler 480 Probes Master mix (Roche, 04887301001), and PCR is carried out as described by Ward et al. (2007).

7.3. Conclusions

We have listed the available protocols to determine pest and pathogens analysing beeswax and wax (hive) debris (of which beeswax is the most relevant component). Available literature is most concentrated on protocols to extract *P. larvae* spores from beeswax and more recently from wax (hive) debris based on the relevance and the impact this bacterium has on the apiculture industry. According to the results presented, the Tween method (Bzdil, 2007) (Section 7.1.1.2.2.) appeared statistically significantly more effective in detecting *P. larvae* spores than the toluene method (Titěra & Haklová, 2003) as well as the others listed in Table 23. In addition, Ryba et al. (2009) and Forsgren and Laugen (2014) successfully used the Bzdil's method on hive debris. Hive debris gained importance during the last decade since they have a predictive value on the possible development of a disease condition of the colony, particularly for AFB. Protocols to identify this bacterium are well established (de Graaf et al., 2013; OIE, 2018b), but as for other matrices and pathogens, it is important to make available fast and sensitive methods of detection from beeswax and wax (hive) debris based on PCR that do not require time-consuming culture methods. Protocols to determine other pathogens from beeswax and wax (hive) debris, i.e., *A. apis*, *Nosema* sp. and SHB have also been provided based on PCR at least for the latter two.

8. Overview of other methods and perspectives

There are numerous methods covering different aspects of beeswax research that are not presented here in detail as it was not possible to encompass such an extensive beeswax research area, i.e., to standardize all methods available in the literature. However, these methods should also be mentioned here in terms of

their perspectives for standardization in the future. We highlight the following ones:

- Methods for beeswax synthesis and secretion research covering investigation of wax gland complex (Hepburn et al., 1991).
- Investigation of biodegradability of beeswax by adapted CO_2 evolution test (Hanstveit, 1992).
- Methods for investigating cell cappings (Goetz & Koeniger, 1992; Hepburn, 1998).
- Method for research on mechanical properties of beeswax, such as electromechanical test system methodology (Buchwald et al., 2006, 2009), investigation of beeswax viscoelasticity and resistance to deformation (Shellhammer, Rumsey, & Krochta, 1997), determination of fracture toughness (Kurstjens, Hepburn, Schoening, & Davidson, 1985), investigation of tensile properties (Zhang, Duan, Karihaloo, & Wang, 2010), honeycomb vibratory signals tested by laser - Doppler vibrometers (Sandeman et al., 1996; Tautz & Lindauer, 1997; Tautz et al., 2001), research on crystal texture and alterations (Davidson & Hepburn, 1986; Hepburn & Kurstjens, 1988; Kurstjens et al., 1985; Kurstjens, Mcclain, & Hepburn, 1990), and optical and X ray diffraction studies (Mellema, 2009; Nikolova, Panchev, Kovacheva, & Pashova, 2009).
- Methods for investigating wax role in nestmate recognition (Breed, Garry et al., 1995, Breed, Page et al., 1995, Breed et al., 1998; Breed, 1998; D'ettorre et al., 2006).
- Molecular structure analysis of beeswax by nuclear magnetic resonance (solid-state ^{13}C NMR) (Kameda, 2004).
- Methods for investigating cell structure: polyester resin cast method described by Pirk et al. (2004), and silicone-based moulding rubber method for investigation of cell bases described by Hepburn et al. (2007).
- Determination of honeycomb order parameters from image analysis (Kaatz et al., 2008).
- Investigation of ultrasonic acoustic resonances exploited by the bees during comb construction, and accuracy of the hexagonal symmetry (Kadmon, Ishay, & Bergman, 2009).
- Investigation of comb integrity by environmental scanning electron microscope (ESEM) (Zhang, Duan et al., 2010).
- Thermographic analysis of comb and hexagonal cell building (Bauer & Bienefeld, 2013).
- Methods for honey bee silk research employing various molecular, spectroscopic and chromatographic analytical tools (Campbell et al., 2014; Sutherland et al., 2007, 2011, 2014; Walker, Warden, Trueman, Weisman, & Sutherland, 2013;; Weisman et al., 2010; Wittmer et al., 2011), and ESEM (Zhang, Si, Duan, & Wang, 2010).
- Method for research on thermal properties of beeswax based on classical melting point determination, determination of the heat of fusion, differential scanning calorimetry (DSC), and thermal conductivity determination (Buchwald et al., 2005; Buchwald, Breed, & Greenberg, 2008; Timbers & Gochnauer, 1982; Timbers, Robertson, & Gochnauer, 1977).

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