



Standard methods for *Apis mellifera* honey research

Ligia Bicudo de Almeida-Muradian, Ortrud Monika Barth, Vincent Dietemann, Michael Eyer, Alex da Silva de Freitas, Anne-Claire Martel, Gian Luigi Marcazzan, Carla Marina Marchese, Carla Mucignat-Caretta, Ana Pascual-Maté, Wim Reybroeck, M. Teresa Sancho & José Augusto Gasparotto Sattler

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REVIEW ARTICLE

Standard methods for *Apis mellifera* honey research

Ligia Bicudo de Almeida-Muradian^{a,*} , Ortrud Monika Barth^b , Vincent Dietemann^c , Michael Eyer^c , Alex da Silva de Freitas^d , Anne-Claire Martel^e , Gian Luigi Marcazzan^f , Carla Marina Marchese^g , Carla Mucignat-Caretta^h , Ana Pascual-Matè^{i,j}, Wim Reybroeck^k , M. Teresa Sanchoⁱ  and José Augusto Gasparotto Sattler^a 

^aPharmaceutical Science School, University of São Paulo, São Paulo, Brazil; ^bInstituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil; ^cAgroscope, Swiss Bee Research Centre, Bern, Switzerland; ^dInstituto de Geociências, Universidade Federal Fluminense, Niterói, Brazil; ^eSophia Antipolis Laboratory, Honeybee Pathology Unit, ANSES, Sophia, France; ^fCREA – Council for Agricultural Research and Economics, Research Centre for Agriculture and Environment, Bologna, Italy; ^gRed Bee Honey, American Honey Tasting Society, Weston, FL, USA; ^hDepartment of Molecular Medicine, University of Padova, Padua, Italy; ⁱDepartamento de Biotecnología y Ciencia de los Alimentos, Nutrición y Bromatología, Universidad de Burgos, Burgos, Spain; ^jFacultad de Ciencias de la Salud, Universidad Isabel I, Spain; ^kResearch Institute for Agriculture, Fisheries and Food; Technology and Food Science Unit, Melle, Belgium

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Honey is an important food for man and has been used as a natural sweetener since ancient times. It is a viscous and aromatic product made by honey bees using the nectar of flowers or honeydew. Honey is composed of a complex mixture of carbohydrates and other substances such as organic acids, amino acids, proteins, minerals, vitamins, lipids, aroma compounds, flavonoids, pigments, waxes, pollen grains, several enzymes, and other phytochemicals. This chapter presents some properties of *Apis mellifera* honey as well as the main methods of honey analysis. All methods are based on specialized literature, including the Codex Alimentarius, AOAC, and publications of the International Honey Commission. Herein, we describe methods related to honey authenticity, botanical origin, geographical origin, physicochemical analysis, radioentomology, pesticide and antibiotic contamination, chemotherapeutics, and sensory analysis. All methods are described in a step-by-step model in order to facilitate their use.

La miel es un alimento importante para el hombre y se ha utilizado como edulcorante natural desde la antigüedad. Es un producto viscoso y aromático hecho por las abejas de la miel usando el néctar de las flores o el rocío de miel. La miel está compuesta por una mezcla compleja de carbohidratos y otras sustancias como ácidos orgánicos, aminoácidos, proteínas, minerales, vitaminas, lípidos, compuestos aromáticos, flavonoides, pigmentos, ceras, granos de polen, varias enzimas y otros fitoquímicos. En este capítulo se presentan algunas propiedades de la miel de *Apis mellifera*, así como los principales métodos de análisis de la miel. Todos los métodos se basan en literatura especializada, incluido el Codex Alimentarius, AOAC y publicaciones de la Comisión Internacional de la Miel. A continuación, describimos métodos relacionados con la autenticidad de la miel, el origen botánico, el origen geográfico, el análisis fisicoquímico, la radioentomología, la contaminación por pesticidas y antibióticos, la quimioterapia y el análisis sensorial. Todos los métodos se describen en un modelo paso a paso para facilitar su uso.

蜂蜜是人类重要的食品, 自古以来就被用作天然甜味剂。它是蜜蜂使用花蜜或蜜露酿造而成的粘性芳香产品。蜂蜜由碳水化合物和其他物质(例如有机酸、氨基酸、蛋白质、矿物质、维生素、脂质、香气化合物、类黄酮、色素、蜡、花粉粒、几种酶和其他植物化学物质)组成的复杂混合物。本章介绍西方蜜蜂蜂蜜的一些特性以及主要分析方法。所有方法均基于专业文献, 包括食品法典, AOAC和国际蜂蜜委员会的出版物。在此, 我们描述与蜂蜜真实性、蜜源植物、地理来源、理化分析、放射昆虫学、农药和抗生素污染、化学治疗和感官分析有关的方法。为了便于使用, 所有方法都采用分步描述的方式加以介绍

Keywords: *Apis mellifera*; honey; physicochemical analysis; honey authenticity; botanical and geographical origin; diagnostic radioentomology; pesticides; sensory; antibiotics and chemotherapeutics

*Corresponding author. Email: ligiabi@usp.br

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1. Introduction

The honey chapter is divided into seven parts describing the main properties of honey as well as their methods of analysis. Each method is described step-by-step. The authors come from many countries such as Belgium, Brazil, France, Italy, Spain, and Switzerland, with many of the authors being members of the International Honey Commission. Honey is the most important product from the apiary and is consumed globally, making it economically important (Almeida-Muradian et al., 2012). As the only natural sweetener, honey historically was an important food for man. Honey was mentioned in many ancient cultures (the holy books of ancient India (the Vedas), book of songs Shi Jing (written in the sixth century BC in ancient China), the Holy Bible, just to name a few), and depicted in many wall drawings in ancient Egypt, Rome (mentioned by the writers Vergil, Varro, and Plinius) and also in medieval high cultures. In ancient Greece, the honey bee, a sacred symbol of Artemis, was an important design on Ephesian coins for almost six centuries (Bogdanov, 2011).

Honey is a viscous and aromatic product, widely appreciated by man and prepared by honey bees (Hymenoptera: Apidae, *Apis* spp.) from the nectar of flowers or honeydew, the latter being produced through an intermediary, generally an aphid (Ouchemoukh et al., 2007; Almeida-Muradian et al., 2013; Ferreira et al., 2009). This natural product is generally composed of a complex mixture of carbohydrates and other less frequent substances, such as organic acids, amino acids, proteins, minerals, vitamins, lipids, aroma compounds, flavonoids, vitamins, pigments, waxes, pollen grains, several enzymes, and other phytochemicals (Almeida-Muradian et al., 2013; Almeida-Muradian & Penteadó, 2015; Gomes et al., 2010; Lazarevic et al., 2010).

All methods related to honey research cited herein are based on the specialized literature, and the authors recommend reading the primary references such as Codex Alimentarius, Association of Official Agricultural Chemists (AOAC), and International Honey Commission publications. Herein, we include the main methods related to research with honey, from the hive to the final product consumed.

2. Honey authenticity—botanical, geographical, and entomological origin

Pollen analysis is the initial technique used to determine the botanical origin of the honey produced by bees. This knowledge enables one to characterize the honey producing area by means of pollen grain identification. *Apis* and *Meliponini* bees show different preferences when foraging plant products.

The management of honey harvesting must follow quality procedures, aiming not only at its efficient collection, but mainly at maintaining its original

characteristics and the quality of the final product. The beekeeper, when handling the harvest, must wear appropriate clothing for beekeeping. It is recommended not to collect honey on rainy days or with high relative humidity, as it could lead to an increase in the moisture content of the product. Harvesting should take place selectively during the opening of the beehive. The beekeeper must inspect each frame, giving priority to removing only the frames that have at least 90% of their operculated storage cells, this being indicative of the maturity of the honey in relation to the percentage of moisture. In order to guarantee the quality of the final product, during the extraction of honey, all the equipment and utensils used in the various stages of handling must be specific to this activity, and there is no room for adaptation. In the case of equipment and utensils that will have direct contact with the product, all must be stainless steel, specific for food products.

Pollen analysis is the initial technique used to determine the botanical origin of the honey produced by bees. This knowledge enables one to characterize the honey producing area by means of pollen grain identification. *Apis* and *Meliponini* bees show different preferences when foraging plant products. For palynological analysis, honey samples of *Apis* have to be received in glass vials and kept at room temperature. In case of stingless bees, the honey has to be kept in a refrigerator at about 8°C to avoid fermentation.

For more details in sampling, please see “Sampling” sections.

2.1. Botanical origin

Pollen grains and other structured elements that compose honey sediments are indicative of the botanical origin (Barth, 1989; Vit et al., 2018). A complete palynological analysis reveals the real provenance of the raw material. Pollen grains are introduced into honey by different means (Barth, 1989). Besides the nectariferous plants, that secrete nectar in addition to producing pollen, the polliniferous plants mainly produce pollen grains and have low concentrations of nectar (Barth, 1989).

2.1.1. Pollen grains

The first universal technique aiming at the recognition and evaluation of the pollen in honey was elaborated by the French, Swiss, and Dutch specialists on honey analysis. This started with Zander's work in 1935, which was published by Louveaux et al. (1970a, 1970b) and updated by Louveaux et al. (1978). The latter includes many standard methods, including the acetolysis method. A more recent publication on pollen analysis was published in von der Ohe et al. (2004).

These methodologies are used today. For the first technique, the pollen grain content is not removed, making it difficult to observe some of the structures. However, more of the elements occurring in honey sediments are preserved. The acetolysis technique

enables a better visualization of pollen grain structures when only the outer pollen grain wall (exine) is preserved, but there is a significant loss of non-resistant elements such as oils, fungi hyphae, yeast, insect fragments, and organic matter (Haidamus et al. 2019). A detailed analysis of the technique established by Louveaux et al. (1978) was presented also by von der Ohe et al. (2004) considering quantitative and qualitative pollen counting.

2.1.2. Other structured elements

Honey sediments may contain several types of particles besides pollen grains (Barth, 1989; Zander & Maurizio, 1975). These structured elements, not nectar indicative, show what happens during honey sampling and storage, and reflect honey quality. Some adulterants include a variety of fungal spores, yeasts, colorless molds and air dispersed spores, green algae, bacteria, insect bristles and scales, and pathogenic mites, as well as starch and synthetic fibers.

2.2. Methodology to prepare honey sediment following Louveaux et al. (1970a)

- (1) Weigh 10 g of honey into a beaker.
- (2) Add 20 mL of distilled water and dissolve well by stirring (10–15 min).
- (3) If the honey is crystallized, heat the mixture for a few seconds in a microwave oven to complete the dissolution process of the sugar crystals.
- (4) Divide the solution eventually between two conical centrifuge tubes of 15 mL capacity.
- (5) Centrifuge at least 252 RCF (relative centrifugal force or G) that, according to a centrifuge model, may comprise on average 1500 rpm (revolutions per minute), for 15 min.
- (6) Discard the supernatant and rapidly invert the tubes for approximately 30 s before returning to normal position.
- (7) Add 10 mL of distilled water to the pellet.
- (8) Centrifuge at 1500 rpm for 15 min.
- (9) Discard the supernatant and rapidly invert the centrifuge tubes for approximately 30 s before returning to normal position.
- (10) Add 5 mL of glycerin water (solution I: 1 with distilled water and glycerin) to the pellet.
- (11) Wait for 30 min and then centrifuge at least 1500 rpm for 15 min.
- (12) Discard the supernatant and rapidly invert the tube onto absorbent paper (never return to the normal position) to allow all the liquid to drain.
- (13) Prepare two microscope slides.
- (14) Put a little piece of glycerin jelly (0.5 mm^3) onto a needle tip and collect part of the sediment into the centrifuge tube.
- (15) Place the sediment on the center of the microscope slide, heat gently on a hot plate, only to dissolve the glycerin jelly.
- (16) Cover with a coverslip, and seal with paraffin.

- (17) The pollen grain identification is based on the reference pollen slides library from regional/country vegetation and with the aid of reference catalogs (e.g., Roubik & Moreno, 1991).
- (18) After the identification and palynological analysis, the prepared slides were stored in a pollen library.

2.3. The acetolysis technique

Acetolysis (Erdtman, 1952) is an artificial fossilization method to prepare pollen grains in order to get the best transparency of the grain envelope.

- (1) Weigh 10 g of honey into a beaker.
- (2) Add 20 mL of distilled water and dissolve well by stirring (10–15 min).
- (3) If the honey is crystallized, heat the mixture for a few seconds in a microwave oven to complete the dissolution process of the sugar crystals.
- (4) Divide the solution evenly between two centrifuge tubes of 15 mL capacity.
- (5) Centrifuge at least 1500 rpm for 15 min.
- (6) Discard the supernatant and rapidly invert the tubes for approximately 30 s before returning to normal position.
- (7) Add 5 mL of acetic acid (32%), centrifuge at least 1500 rpm for 15 min and discard the supernatant.
- (8) Add the acetolysis mixture (4.5 mL of acetic anhydride (98%) and 0.5 mL of sulfuric acid (99%)).
- (9) Heat in a water bath just to 80°C for 3 min.
- (10) Centrifuge at least 1500 rpm for 15 min.
- (11) Discard the supernatant and add 20 mL of distilled water.
- (12) Wash twice.
- (13) Discard the supernatant and rapidly invert the tubes for approximately 30 s before returning to normal position.
- (14) Add 5 mL of glycerin water (solution I: 1 with distilled water and glycerin) to the pellet. Wait for 30 min.
- (15) Centrifuge at least 1500 rpm for 15 min.
- (16) Discard the supernatant and rapidly invert the tube onto absorbent paper (never return to the normal position) to allow all the liquid to drain.
- (17) Prepare two microscope slides.
- (18) Put a little piece of glycerin jelly (0.5 mm^3) onto a needle tip and collect part of the sediment in the centrifuge tube.
- (19) Place the sediment on the center of the microscope slide, heat gently on a hot plate, only to dissolve the glycerin jelly.
- (20) Cover with a coverslip, and seal with paraffin.

2.4. Additional techniques

A number of other techniques to prepare honey samples were purposed and published. In general, they are complex to execute and require a broad assortment of equipment.

Table 1. Evaluation of a *Croton* monofloral honey sample, a strong nectariferous and subrepresented plant taxon.

Total number of pollen grains counted	962	= 100% of the pollen
Total of anemophilous pollen counted	842	= 87.5% of the pollen
Total of polliniferous pollen counted	52	= 5.4% of the pollen
Total of nectariferous pollen counted (=100%)	68	= 7.1% of the pollen
<i>Croton</i>	18	= 26.5% of nectariferous pollen, a subrepresented taxon
<i>Solanum</i>	18	= 26.5% of nectariferous pollen
<i>Myrcia</i>	10	= 1.0% of nectariferous pollen
Final evaluation: Monofloral honey of <i>Croton</i> (Euphorbiaceae).		

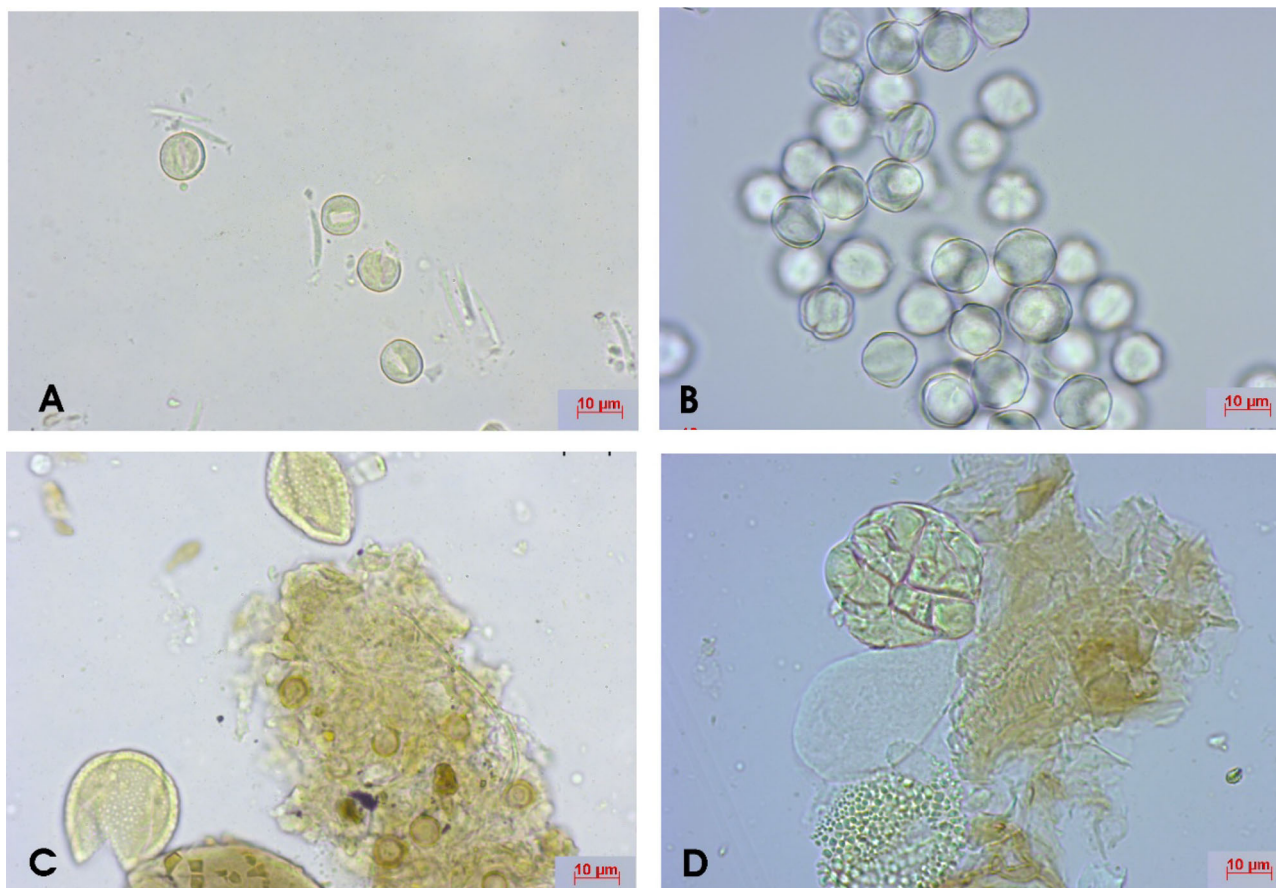


Figure 1. (A) Anemophilous pollen of *Piper*; (B) Polliniferous pollen of Melastomataceae; (C) *Citrus* monofloral honey with honeydew contribution (organic material and spores); (D) Group of algae cells and a broken pollen grain of *Croton* of a honeydew honey.

Filtration (Lutier & Vaissière, 1993), ethanol dilution (Bryant & Jones, 2001), mid-infrared spectroscopy (Ruoff et al., 2006), multivariate analysis Principal Component Analysis (PCA) (Aronne & de Micco, 2010), ultraviolet spectroscopy (Roshan et al., 2013), isotopes (Wu et al., 2015), and pollen grain DNA metabarcoding (Hawkins et al., 2015) have all been used to determine honey origin.

2.5. Honey sample evaluation

Since the standard work of Louveaux et al. (1978), the qualitative and quantitative evaluation of honey samples (Maurizio, 1977) has been a subject of many investigations until the data assembly in von der Ohe et al. (2004). The reliability of pollen analysis in honey samples was discussed in detail by Behm et al. (1996). Pollen coefficients were discussed by the *R*-values of honey pollen coefficients by Bryant and Jones (2001).

2.5.1. Super and subrepresentation of pollen types

For each region of honey production, there are plants that are significant nectar or/and pollen producers. The pollen grains present in the honey sediments can reveal the bee foraging behavior. In general, the number of pollen grains present on one microscope slide provides information to use to assign a frequency class, indicating if the pollen types observed in the honey samples are predominant (PP), accessory (AP), important minor pollen (IMP), or minor pollen (MP) (Louveaux et al., 1970a, 1970b).

Few pollen grains found in a honey sample suggests that the bees visited plant species of a low pollen production, but that have a high nectar availability like *Citrus* spp. and *Croton* sp. (Table 1, Figure 1). A high quantity of pollen grains from polliniferous plants (e.g., Poaceae and Melastomataceae) can indicate a super

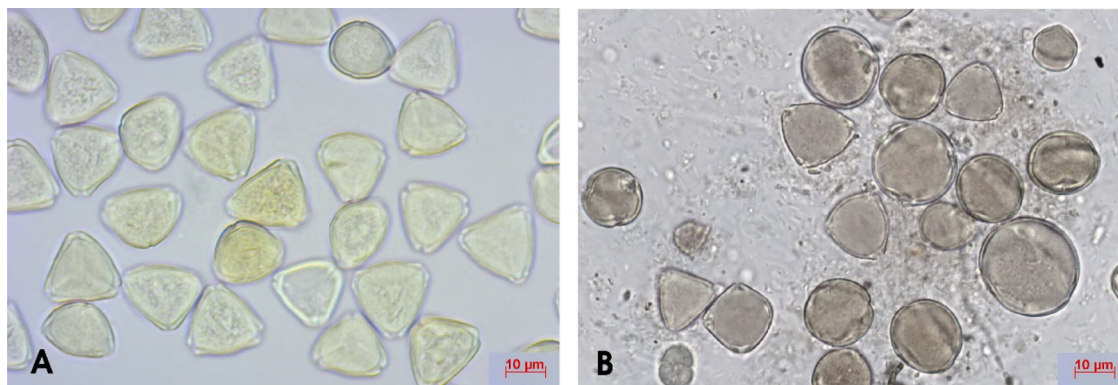


Figure 2. (A) Monofloral *Myrcia* honey. (B) Heterofloral honey.

representation since these species produce a lot of pollen but little or no nectar (Louveaux et al., 1978).

2.5.2. Monofloral and heterofloral honeys

- (1) Monofloral/unifloral honeys contain predominantly pollen grains from an unique plant species ($\geq 45\%$ of all nectariferous pollen grains counted), denominated a (“predominant pollen type,” PP)
- (2) Bifloral honeys contain pollen grains from two plant species with a frequency of 15–45% per nectariferous species (“accessory pollen types,” AP)
- (3) Heterofloral/plurifloral honeys contain pollen grains from three or more nectariferous plant species with frequencies in the 3–15% (“important minor pollen types,” IMP) or $< 3\%$ (“minor pollen types,” MP) (Barth, 1989; Louveaux et al., 1978) (Figure 2).

2.5.3. Honeydew

Bees can use the excreta of aphids (Aphidae) that feed on plant xylem to create honeydew. This bee product can be difficult to classify and presents a high electrical conductivity and mineral salt content. Generally, pollen grain occurrence is low and a moderate number of fungal spores can be observed (Barth, 1971; Maurizio, 1959) (Figure 1).

2.6. Geographical origin

Pollen grain analysis allows one to determine the nectar origin of honey. The assembly of plant species identified by pollen analysis provides information about honey’s local or regional origin. A reference pollen slide collection (pollen library) must be available in order to compare the morphology of pollen grains obtained directly from flowers with the ones obtained in the honey sediment collection. The scope of this collection depends upon the study area and the radius of action of the bees. Plant assemblies recognized in the honey sediment by means of pollen grain morphology inform about the nectariferous and non-nectariferous, local and regional potential of honey production (Aronne & de Micco,

2010; Consonni & Cagliani, 2008; Persano Oddo & Bogdanov, 2004; Salgado-Laurenti et al., 2017).

Unfortunately, some honeys do not contain much pollen. In these cases, the honey origin is difficult to determine. It may derive from strong nectar secreting plant species such as those from the Asclepiadaceae. Sometimes, certain flowers secrete abundant nectar that will drip from the flower inflorescences, making it unlikely to contain pollen. This happens with *Dombeya* (Sterculiaceae, a shrub) and with some plants that grow in very humid environments.

2.7. Entomological origin

For purposes of this manuscript, we are discussing honey produced by bees of the family Apidae, genus *Apis*, species *A. mellifera*. There are several subspecies/races of *A. mellifera* occurring in different parts of the world (Carreck et al., 2013; Meixner et al., 2013). Humans have cultivated this bee since antiquity given that it has provided a source of sugar. There are other types of honey producing bees. Collectively called stingless bees (also Apidae), these bees occur mainly in the tribes Meliponini and Trigonini. Bees from both tribes produce a different type of honey than that produced by *A. mellifera*.

Stingless bee honey contains less sugar and more water than the honey produced by *Apis* spp. These bees are very important pollinators in their native habitats, being responsible for maintenance of the local vegetation, mainly the forests, and for seed production. There are circa 500–800 species occurring exclusively in the tropical and sub-tropical Neotropical regions. *Apis* spp. and *Meliponini* spp. forage on some of the same plants, but many different plants as well. Species specific preferences for various forage resources depend upon the bee species’ affinity, as well as the flowering vegetation strata and environmental conditions (Barth et al., 2013).

3. Standard honey physicochemical analysis for nutritional composition, chemical composition, and antioxidant activity

In this section, we present the main physicochemical analyses for nutritional composition, chemical

composition, and antioxidant activity of honey in step-by-step format. Review articles on the composition and properties of honey from *A. mellifera* colonies are present in the literature (De-Melo et al., 2017; Pascual-Maté et al., 2018).

We recommend reading of the primary reference on this topic: Codex Standard for Honey (2001).

3.1. Water content determination (moisture)

Water content of honey is related to the botanical and geographical origin of the nectar, edaphic and climatic conditions, season of harvesting, manipulation by beekeepers, processing/storage conditions, etc. (De-Melo et al., 2017). It is an important physicochemical parameter for honey shelf life (Bogdanov, 2011) and it normally ranges between 13% and 25% (Uran et al., 2017), with the average being about 17% (Fallico et al., 2009). Honeys with high moisture content (>18%) have a higher probability of fermenting upon storage (Bogdanov, 2011) while those with a low moisture content (<15%) are likely to granulate.

The Council Directive 2001/110/EC (European Commission, 2001) as well as the Brazilian legislation (Ministério da Agricultura Pecuária e Abastecimento, 2000) recommend the limit of 20% for moisture in honey samples (Thrasyloulou et al., 2018).

The standard method for honey moisture determination, indicated by Codex Standard for Honey (2001), uses the refractometric method which is simple and reproducible. It is possible to use an Abbé refractometer or digital refractometers for this purpose (AOAC, 1992; Bogdanov, 2009, 2011). The refractometric method is described here as a reference method (AOAC, 1990; Bogdanov, 2009).

3.1.1. Principle

Water content or moisture determined by the refractometric method is based on the fact that the refractive index increases with solid content. In honey, the refraction index can be converted in moisture content using the Chataway Table (Bogdanov et al., 1997).

3.1.2. Apparatus and reagents

- Water bath or incubator
- Honey sample
- Refractometer

3.1.3. Procedure

3.1.3.1. Samples and standards preparation and determination

- (1) If honey is granulated, place it in an airtight closed flask and dissolve crystals in a water bath or incubator at 50 °C.
- (2) Cool the solution to room temperature and stir.

- (3) Ensure that the prism of the refractometer is clean and dry.
- (4) Cover the surface of the prism from the refractometer with the sample and read the refractive index.
- (5) Read the corresponding moisture content from the Chataway Table (Table 2), making the temperature correction, if necessary.

Other methods can be used to determine water content of honey including Karl Fischer method and nonstandardized method Fourier transform infrared spectroscopy (FTIR) (Almeida-Muradian et al., 2013, 2014; Almeida-Muradian & Gallmann, 2012; Bogdanov, 2009).

3.2. Sugar content determination (HPLC method)

Honey is mainly composed of sugars (60–85% of the total volume). Monosaccharides (fructose and glucose) represent about 75% of the sugars found in honey. Disaccharides (sucrose, maltose, turanose, isomaltose, maltulose, trehalose, nigerose, and kojibiose) compose about 10–15% of the sugars. The rest is composed of a small amount of other sugars, including trisaccharides (mainly maltotriose and melezitose).

Due to the wide variety of botanical sources, the sugar profile presents great variation; however, it has been demonstrated that among the same species of plants, the profile of sugars and other physicochemical components can be used as fingerprint for the identification of the botanical origin of monofloral honeys (El Sohaimy et al., 2015; Ruoff et al., 2005, 2006).

According to the publication of the Harmonized Methods of the International Honey Commission (2009), the methodologies for sugar determination are grouped as specific and nonspecific. Liquid chromatography - refractive index detector (specific methodology) and a methodology proposed by Lane-Eynon (nonspecific methodology) are among the most used methodologies for the quantification of sugars presented in honeys (Bogdanov, 2009).

There are other methods described in the literature for the determination of sugars. These include (1) enzymatic determination (Gómez-Díaz et al., 2012; Huidobro & Simal, 1984), (2) high performance liquid chromatography with pulsed amperometric detector and anion exchange column (HPLC-PAD) (Bogdanov, 2009; Cano et al., 2006; Nascimento et al., 2018; Ouchemoukh et al., 2010), (3) ultra-performance LC with and evaporative light scattering detector (UPLC-ELSD) (Zhou et al., 2014), (4) gas chromatography (GC) (Bogdanov, 2009; Ruiz-Matute et al., 2007), (5) capillary electrophoresis (CE) (Dominguez et al., 2016), (6) electrochemical determination (EL Alami EL Hassani et al., 2018), (7) FTIR (Almeida-Muradian et al., 2012; Almeida-Muradian, Sousa, et al., 2014; Almeida-Muradian, Stramm, et al., 2014; Anjos et al., 2015), and (8) Raman spectroscopy (RAMAN) (Özbalci et al., 2013). High-Performance Liquid Chromatography with Refractive Index Detection (HPLC-RI) (Bogdanov, 2009) is described herein.

Table 2. Chataway table—refractive index and moisture content (%) 20 °C. (source: International Honey Commission, 2009).

Water content (g/100 g)	Refractive index (20 °C)	Water content (g/100 g)	Refractive index (20 °C)
13.0	1.5044	19.0	1.4890
13.2	1.5038	19.2	1.4885
13.4	1.5033	19.4	1.4880
13.6	1.5028	19.6	1.4875
13.8	1.5023	19.8	1.4870
14.0	1.5018	20.0	1.4865
14.2	1.5012	20.2	1.4860
14.4	1.5007	20.4	1.4855
14.6	1.5002	20.6	1.4850
14.8	1.4997	20.8	1.4845
15.0	1.4992	21.0	1.4840
15.2	1.4987	21.2	1.4835
15.4	1.4982	21.4	1.4830
15.6	1.4976	21.6	1.4825
15.8	1.4971	21.8	1.4820
16.0	1.4966	22.0	1.4815
16.2	1.4961	22.2	1.4810
16.4	1.4956	22.4	1.4805
16.6	1.4951	22.6	1.4800
16.8	1.4946	22.8	1.4795
17.0	1.4940	23.0	1.4790
17.2	1.4935	23.2	1.4785
17.4	1.4930	23.4	1.4780
17.6	1.4925	23.6	1.4775
17.8	1.4920	23.8	1.4770
18.0	1.4915	24.0	1.4765
18.2	1.4910	24.2	1.4760
18.4	1.4905	24.4	1.4755
18.6	1.4900	24.6	1.4750
18.8	1.4895	24.8	1.4745
		25.0	1.4740

3.2.1. Apparatus and reagents

Apparatus:

- HPLC system consisting of pump, sample applicator, temperature-regulated RI-detector (30 °C), and temperature-regulated column oven (30 °C).
- Ultrasonic bath
- Micro-membrane 0.45 µm
- Volumetric flask (100 mL)
- Syringe (1 mL)
- Beaker (50 and 100 mL)
- Pipette (25 mL)
- Sample vials (1.5 mL)

Reagents:

- Water (HPLC grade)
- Methanol (HPLC grade)
- Acetonitrile (HPLC grade)
- Analytical stainless-steel column, containing amine-modified silica gel with 5 µm particle size, 250 mm in length; 4.6 mm in diameter.

3.2.2. Procedure

3.2.2.1. Sample preparation and determination

- (1) Weigh 5 g of honey into a beaker and dissolve in 40 mL distilled water.

- (2) Pipette 25 mL of methanol into a 100 mL volumetric flask and transfer the honey solution quantitatively to the flask.
- (3) Complete the volumetric flask with water until 100 mL.
- (4) Filter the solution through a 0.45 µm membrane and collect the filtered solution into sample vials.
- (5) Store for four weeks in the refrigerator (4 °C) or for six months at freezer (−18 °C).

3.2.2.2. Chromatographic conditions.

- Flow rate: 1.3 mL/min.
- Mobile phase: acetonitrile: water (80:20, v/v).
- Column and detector temperature: 30 °C.
- Sample volume: 10 µL.

3.2.2.3. Calculation and expression of results. Honey sugars can be identified and quantified by comparison of their retention times and the peak areas with those of the standard sugars (external standard method).

The mass percentage of the sugars (*W*) is calculated according to the following formula:

$$W = A_1 \times V_1 \times m_1 \times 100 / A_2 \times V_2 \times m_0$$

W = Mass percentage of sugars (g /100 g).

*A*₁ = Peak areas or peak heights of the given sugar compound in the sample solution (expressed as units of area, length, or integration).

A_2 = Peak areas or peak heights of the given sugar compound in the standard solution (expressed as units of area, length, or integration).

V_1 = Total volume of the sample solution (mL).

V_2 = Total volume of the standard solution (mL).

m_1 = Mass amount of the sugar in grams in the total volume of the standard (V_2).

m_0 = sample weight (g)

3.3. Reducing sugars

Apparent reducing sugars and apparent sucrose content of honey are most commonly determined for honey quality control purposes. The Lane and Eynon method (Codex Alimentarius Standard, 1969) is used for the determination of sugars. Apparent reducing sugars are defined as those sugars that reduce a Fehling's reagent under the conditions specified (Bogdanov et al., 1997; Bogdanov & Martin, 2002; Bogdanov, 2009; Granato & Nunes, 2016).

3.3.1. Apparatus and reagents

Apparatus:

- Burette (25 mL)
- Volumetric pipette (2 mL)
- Volumetric flasks (25 and 100 mL)
- Erlenmeyer flasks (250 mL)
- Beakers (50 and 100 mL)
- Pipette (25 mL)
- Heating plate

Reagents:

- Copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)
- Potassium sodium tartrate tetrahydrate ($\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4\text{H}_2\text{O}$)
- Sodium hydroxide (NaOH)
- Glucose

3.3.2. Procedure

3.3.2.1. Solutions preparation.

Fehling's solution A:

- (1) Weigh 69.28 g of copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in a beaker and solubilize by stirring with distilled water.
- (2) Transfer to a 1 L volumetric flask.

Fehling's solution B:

- (1) Weigh 346 g of sodium and potassium double tartrate ($\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4\text{H}_2\text{O}$) in 300 mL of distilled water.
- (2) Separately, dissolve 100 g of sodium hydroxide (NaOH) into 200 mL of distilled water in a plastic beaker and ice bath in the hood.

- (3) Combine the solutions of sodium and potassium tartrate and sodium hydroxide in a 1 L volumetric flask.
- (4) Homogenize by stirring.
- (5) Raise the volume of the flask to full with distilled water.

3.3.2.2. Standardization of Fehling solutions. It is necessary to standardize Fehling solutions to obtain the correction factor.

- (1) Prepare a 0.5% (w/v) glucose solution.
- (2) Weigh 0.5 g of glucose in a beaker, add distilled water, dissolve, and transfer to a 100 mL flask.
- (3) Fill the burette with the glucose solution and holder as described (in section 3.3.2.4).
- (4) For each standardization, make a minimum of four titrations. Apply the average of these values in the formula to obtain the correction factor (F) of the solutions of Fehling:

$$F = (\% \text{ of glucose}) \times (\text{average of volumes spent}) \times 0.01$$

The correction factor corresponds to the amount of sugar required to reduce 10 mL of the Fehling solutions.

3.3.2.3. Preparation of the sample.

- (1) Dissolve 5.0 g of honey in distilled water up to 25 mL in a volumetric flask (Solution 1:5).
- (2) From this homogenized solution, transfer 2 mL to a 100 mL volumetric flask.
- (3) Make up to volume with distilled water and homogenize (final dilution 1:250). The initial diluted solution 1:5 should be reserved for the analysis of apparent sucrose.

3.3.2.4. Titration. The titration is done with a 25 mL burette containing the diluted honey (solution 1:5) and an Erlenmeyer flask with 5 mL of Fehling A solution, 5 mL of Fehling B, and 40 mL of distilled water.

- (1) Heat the Erlenmeyer to a hot plate, using a tripod and a screen.
- (2) Heat the solution with the heating plate until boil, then the titration is initiated, releasing in one go 5 mL of the sugar solution of the burette. With the restart of the boiling, the Fehling solution becomes reddish, but still with a lot of blue color (Cu^{2+} ions).
- (3) The titration should be restarted, this time dropwise, under stirring and the color modification being observed. The reaction is over when the solution, against a fluorescent light, does not present any shade or blue reflection, being colored an intense red. Titration should not exceed 3 min.

3.3.2.5. Calculation.

The calculation of the percentage of reducing sugars is given by the following formula:

$$\%AR = \frac{(\text{dilution of honey solution} = 250) \times F \times 100}{\text{average of the volumes spent in the titration}}$$

AR = reducing sugars

F = correction factor obtained from the standardization of Fehling's solutions.

3.4. Apparent sucrose

Apparent sucrose content is determined indirectly by calculating difference in total reducing sugar before and after inversion of sugars in honey. The determination of apparent sucrose requires the use of acid hydrolysis to break the glycoside bonds of the disaccharides, thus releasing reducing sugars such as glucose and fructose (Bogdanov et al., 1997; Granato & Nunes, 2016).

3.4.1. Apparatus and reagents

Apparatus:

- 100 mL glass beaker
- 100 and 500 mL volumetric flask
- 1 and 2 mL volumetric pipettes
- 25 mL burette
- 250 mL Erlenmeyer flask
- Hot plate

Reagents:

- Concentrated hydrochloric acid (HCl)
- Fehling A solution
- Fehling B solution
- Sodium hydroxide (NaOH)

3.4.2. Procedure

3.4.2.1. Sodium hydroxide solution (5 mol/L)

- (1) Weigh 100 g of NaOH and dissolve in distilled water.
- (2) Increase the volume in a volumetric flask to 500 mL with distilled water.
- (3) Condition the solution in a plastic container.

3.4.2.2. Sample preparation

- (1) From the 1:5 diluted honey solution prepared in the reducing sugars analysis, transfer 2 mL to a 100 mL glass beaker and add 40 mL of distilled water and 1 mL of concentrated HCl.
- (2) This solution must be boiled, cooled, neutralized by adding 5 mol/L NaOH until a pH of 7 ± 0.2 .
- (3) Add distilled water up to 100 mL in a volumetric flask and make the titration according to the same procedure as "Reducing sugars" (3.3.2.4.). The result is expressed as percentage of total sugars (% AT):

$$\%AT = \frac{(\text{dilution of honey solution} = 250) \times F \times 100}{\text{average of the volumes spent in the titration}}$$

AT = total sugars

F = correction factor obtained from the standardization of Fehling's solutions

Heating to boiling under strongly acid conditions ensures that the sucrose is hydrolyzed; having as products the separate molecules of glucose and fructose. The molecular mass of sucrose (MM = 342 mol/L) is 95% of the molecular mass of glucose and fructose together (MM = 360 mol/L), taking into account that 5% of the mass refers to water (MM = 18 mol/L) which was used in the hydrolysis. Thus, the percentage of apparent sucrose in a sample is calculated using the following formula:

$$\% \text{ apparent sucrose} = (\% \text{ total sugars} - \% \text{ reducing sugars}) \times 0.95$$

3.5. Proteins (proline)

The presence of protein in honey is low and varies depending on the species of honey bee (*A. mellifera* 0.2–1.6% and *A. cerana* 0.1–3.3%) (Lee et al., 1998) and the contact of these with flower pollen, honeydew, and nectar (Davies, 1975).

In the last decades, the use of more sensitive and robust methodologies has enabled the identification of a larger quantity of proteins and amino acids in honey samples from several countries (De-Melo et al., 2017). Among the currently used methodologies, the most preferred is HPLC (Hermosín et al., 2003) associated with identification by mass spectrometry (Chua et al., 2013).

The classical method to determine protein uses the total nitrogen content that is usually calculated by multiplying the total nitrogen content from the Kjeldahl method by a factor of 6.25 (AOAC, 1990). However, about 40–80% of the total honey's nitrogen comes from the protein fraction and most of the remainder resides in the free amino acids (Chua et al., 2013).

According to the published update "Harmonized Methods of the International Honey Commission" in 2009, the proline content is recommended for quality control (being considered a criterion of honey ripeness) and it can be an indicator of sugar adulteration (Bogdanov, 2009). Since most of the amino acids are present in trace quantities, only proline content is included for the quality parameters of honey. No other methods for protein quantification are included in the methods referenced above or in the Codex Standard for Honey (2001).

3.5.1. Principle

Proline and ninhydrin form a colored complex. After adding 2-propanol, color extinction of the sample solution and a reference solution at a wavelength maximum is determined. The proline content is determined from the ratio of both and it is expressed in mg/kg. The method is based on the original method of Ough (1969).

3.5.2. Apparatus and reagents

Apparatus:

- Spectrophotometer measuring in the range of 500–520 nm
- Cells 1 cm
- 20 mL tubes with screw cap or stopper
- 100 mL measuring flask and 100 mL beaker
- Water bath

Reagents:

- Distilled Water
- Formic acid (H.COOH), 98–100%
- Solution of 3% ninhydrin in ethylene glycol monomethyl ether (methyl-cellosolve)
- Proline reference solution: Prepare an aqueous proline stock solution containing 40 mg proline to 50 mL distilled water (volumetric flask). Dilute 1 mL to 25 mL (volumetric flask) with distilled water (solution containing 0.8 mg/25 mL)
- 2-propanol solution, 50% by volume in water

Proline reference solution.

- (1) Prepare an aqueous proline stock solution containing 40 mg proline to 50 mL distilled water.
- (2) Dilute 1 mL to 25 mL with distilled water (solution containing 0.8 mg/25 mL).
- (3) Prepare a 2-propanol solution, 50% by volume in water.

3.5.3. Procedure

3.5.3.1. Preparation of the sample solution.

- (1) Weigh to the nearest milligram about 5 g honey into a beaker and dissolve in 50 mL distilled water.
- (2) After dissolving, transfer all the solution to a 100 mL volumetric flask.
- (3) Complete the volume with distilled water and shake well.

3.5.3.2. Determination. Note that the coefficient of extinction is not constant. Therefore, for each series of measurements, the average of the extinction coefficient of the proline standard solution must be determined at least in triplicate.

- (1) Pipette 0.5 mL of the sample solution in one tube, 0.5 mL of distilled water (blank test) into a second tube, and 0.5 mL of proline standard solution into each of three additional tubes.
- (2) Add 1 mL of formic acid and 1 mL of ninhydrin solution to each tube.
- (3) Cap the tubes carefully and shake vigorously using a vortex machine for 15 min.

- (4) Place tubes in a boiling water bath for 15 min, immersing the tubes below the level of the solution.
- (5) Transfer to a water bath at 70 °C for 10 min.
- (6) Add 5 mL of the 2-propanol-water-solution to each tube and cap immediately.
- (7) Leave to cool and determine the absorbance using a spectrophotometer for 45 min after removing from the 70 °C water bath at 510 nm, using 1 cm cells.

Note: Adherence to the above times is critical.

3.5.3.3. Calculation and expression of results. Proline in mg/kg honey at one decimal place is calculated according to following equation:

$$\text{Proline (mg/kg)} = \frac{E_s}{E_a} \times \frac{E_1}{E_2} \times 80$$

E_s = Absorbance of the sample solution

E_a = Absorbance of the proline standard solution (average of two readings)

E_1 = mg proline taken for the standard solution

E_2 = Weight of honey in grams

80 = Dilution factor

In Germany, a honey with < 180 mg proline/kg honey is considered either unripe or adulterated (Bogdanov, 2009).

3.6. Vitamins (HPLC)

The presence of vitamins in honey is quite variable and is related to the botanical origin of the honey. Water soluble vitamins (vitamins C and B-complex) occur in higher levels in honey than do lipid-soluble vitamins (A, D, E, and K) due to the low presence of lipids in honey. In general, this foodstuff cannot be considered as an important source of vitamins due to the fact that they are in very low quantities. As mentioned by León-Ruiz et al. (2013), the identification of vitamins in different honeys allows their characterization to botanical type and is a valuable approach of honey quality control, but no official methods have been described (De-Melo et al., 2017).

There are few studies that identified and quantified vitamins in honey. Some authors such as Ciulu et al. (2011) and León-Ruiz et al. (2013), published works aiming to investigate the presence of vitamins in honey using HPLC. The method outlined by León-Ruiz et al. (2013) is described herein.

3.6.1. Apparatus and reagents

Apparatus:

- HPLC system consisting of pump, sample applicator, UV-vis variable wavelength detector
- Micro-membrane 0.45 μm
- Volumetric flask (25 mL)
- Syringe (1 mL)

Table 3. Elution program in the RP-HPLC gradient elution of water-soluble vitamins in honey.

Time (min)	Solvent A (TFA aqueous solution, 0.025%, v/v) (%)	Solvent B (acetonitrile) (%)
0 ^a	100	0
5 ^a	100	0
11 ^a	75	25
11 ^b	75	25
19 ^b	55	45
20 ^b	60	40
22 ^b	100	0

Note: flow rate: 1.0 mL/min; a—Operative wavelength: 254 nm; b—Operative wavelength: 210 nm.

- Beaker (50 and 100 mL)
- Pipette (25 mL)
- Sample vials (1.5 mL)

Reagents:

- Trifluoroacetic acid (0.025%, v/v) (HPLC grade)
- Acetonitrile (HPLC grade)
- Analytical column (C18 column, 250 mm × 4.6 mm, 5 μm particle size).

3.6.2. Procedure

3.6.2.1. Sample preparation.

- (1) Weigh 10 g of honey into a 50 mL beaker and dissolve in 10 mL of ultra-pure water.
- (2) Add 1 mL of NaOH 2M to complete solubilization.
- (3) Add 12.5 mL of phosphate buffer 1M (pH = 5.5).
- (4) Raise the volume to 25 mL using ultra-pure water.
- (5) Filter the solution through a 0.45 μm membrane and collect in sample vials.
- (6) Store in the refrigerator if it is necessary.

3.6.2.2. Standards preparation. The stock standard solution is prepared by weighing in a 100 mL volumetric flask: 10.0 mg of vitamin B2; 25.0 mg of vitamin B5; and 10.0 mg of vitamin B9.

- (1) Add 40 mL of ultra-pure water.
- (2) Add 4 mL of NaOH to complete dissolution.
- (3) Add 50 mL of phosphate buffer 1M (pH = 5.5); 10.0 mg of vitamin B3; then 10.0 mg of vitamin C.
- (4) Fill the flask to 100 mL using distilled water.
- (5) Store the standard solution in the dark at 4 °C.
- (6) Prepare the solution fresh daily as needed.

3.6.2.3. Chromatographic analysis. The vitamins are identified and quantified by comparison of the retention times and the peak area of the pure standards. The elution program in the RP-HPLC gradient elution of water-soluble vitamins in honey is described in Table 3.

3.7. Minerals

The mineral content of honey samples has been receiving significant attention from many scientists globally. In

recent years, the mineral profile of honey became an important indicator of quality control and environmental contamination by agrochemicals (Almeida-Silva et al., 2011; Kacaniová et al., 2009). In the same way, some authors proposed that mineral profile can be used as an authentication analysis for botanical identification or designation of geographical origin (Anklam, 1998; Louppis et al., 2017).

Many factors can contribute to the mineral composition of honey, including the botanical origin of nectar, the harvesting treatment and material of storage, seasonal climatic variations, and geographical origin (Almeida-Silva et al., 2011; Anklam, 1998). Other minerals in honey may derive from those present in the air, water, and soil. Furthermore, bees can be exposed to minerals while visiting flowers, contacting branches and leaves, drinking water from pools, and while flying (Kacaniová et al., 2009).

The mineral content in honey ranges from 0.04% in light honeys to 0.2% in dark honeys (Alqarni et al., 2014). Potassium is the most abundant mineral in honey, composing generally up to 1/3 of the total mineral content (Alqarni et al., 2014; Yücel & Sultanoglu, 2013). In smaller quantities, honey also contains sodium, iron, copper, silicon, manganese, calcium, and magnesium. Macro elements (such as potassium, calcium, and sodium) and trace minerals (such as iron, copper, zinc, and manganese) perform a fundamental function in biological systems: maintaining normal physiological responses, inducing the overall metabolism, influencing the circulatory system and reproduction, and acting as catalysts in various biochemical reactions (Alqarni et al., 2014; da Silva et al., 2016).

Honey should be free from heavy metals in amounts that may represent a hazard to human health. However, maximum residue levels of these potentially toxic elements in honey have not been established. The World Health Organization (WHO, 1996), has proposed acceptable levels of 15 μg/kg for arsenic, 25 μg/kg for lead, 5 μg/kg for mercury, and 7 μg/kg for cadmium.

As described by Bogdanov et al. (2007), the characterization of trace elements by inductively coupled sector-field plasma mass spectrometry (ICP-SFMS) is significantly improved by virtue of enhanced sensitivity and separation of polyatomic interferences in a high resolution mode. In contrast, the option of inductively coupled plasma with mass spectroscopy detector (ICP-

Table 4. Instrumental working parameters for mineral analysis of honey.

Torch injector	Quartz
Spray chamber	Peltier cooled cyclonic
Sample uptake	0.3 rps (rounds per second)
Nebulizer type	MicroMist
Interface	Pt-cones
RF power	1550W
Ar gas flow rate (L/min)	Plasma 15; auxiliary 0.9
Nebulizer pump	0.1 rps
He gas flow rate	0.03 mL/min
Ion lenses model	X-lens
Lens voltage	10.7 V
Omega bias	-90V
Omega lens	10.2 V
Acquisition mode	Spectrum
Peak Pattern	1 point
Integration time	2000 ms
Replicate	3
Sweeps/replicate	100
Tune mode	No gas: 0 s; 0.1 s
(Stabilization time; Integ. Time/mass)	He: 5 s; 0.5 s HEHe: 5 s; 1 s
ICP-MS (standard mode)	No gas: Be ⁹ , Na ²³ , Mg ²⁴ , Al ²⁹ , K ³⁹ , As ⁷⁵ , Mo ⁹⁵ , Ag ¹⁰⁷ , Sb ¹²¹ , Ba ¹³⁷ , Tl ²⁰⁵ , Pb ²⁰⁸ , U ²³⁸ He mode: Ca ⁴³ , V ⁵¹ , Cr ⁵² , Mn ⁵⁵ , Co ⁵⁹ , Ni ⁶⁰ , Cu ⁶³ , Zn ⁶⁶ , Cd ¹¹¹ HEHe: Se ⁷⁸ , Fe ⁵⁶
Internal standards	²⁰⁹ Bi, ¹¹⁵ In, ⁴⁵ Sc

MS) is widely used by researchers and yields accurate results. Bilandžić et al. (2017) published a work with mineral composition of 24 honey samples using an ICP-MS system with a great percentual recovery in the determinations. This work shows detailed parameters of analysis validation and we chose this method as the standard analysis for determining mineral content.

3.7.1. Apparatus and reagents

Apparatus:

- High-pressure microwave oven
- Inductively coupled plasma instrument with mass detector
- Teflon dishes
- Volumetric flasks (10 and 50 mL)

Reagents:

- Nitric acid (HNO₃)
- Certified standards consisting of Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Se, Sb, Th, U, V, and Zn
- Internal Standard Multi-Element Mix consisting of Li, Sc, Ga, Y, In, Tb, and Bi
- Ultrapure water

3.7.2. Procedure

3.7.2.1. Sample extraction

- (1) Weigh 0.5 g of honey samples into a Teflon dish.
- (2) Add 3 mL ultra-pure water.
- (3) Add 2.5 mL HNO₃ (65%).

- (4) Perform a wet digestion of the solution using a microwave oven set at a digestion program consisting of three potency steps: first step at 500 W for 2.5 min, second step at 1000 W for 20 min, and the third step at 1200 W for 30 min.
- (5) Following the wet digestion, cool the samples to room temperature.
- (6) Transfer the digested clear solution to a 50 mL volumetric flask and fill it using ultra-pure water.
- (7) All solutions should be spiked with the internal standard to a final concentration of 10 µg/L.

3.7.2.2. Calculation and expression of results.

- (1) Perform a quantitative analysis using the calibration curve method.
- (2) Calibration curves should be constructed with a minimum of five concentrations of standards per element.
- (3) The limits of detection (LODs) should be calculated as three times the standard deviation of 10 consecutive measurements of the reagent blank, multiplied by the dilution factor used for sample preparation (Table 4).

3.8. Calories

Honey calories can be determined indirectly using calculation criteria according to the Brazilian Food Composition Table (Food Research Center, 2017) and (WHO/FAO, 2002) which uses the Atwater general factors system.

The calculation is done by multiplying the content of proteins and available carbohydrates by the factors described as follows:

- Proteins: 17.0 kJ/g or 4.0 kcal/g
- Available carbohydrates: 16.0 kJ/g or 4.0 kcal/g

Observation: In the case of lipids, dietary fiber, and alcohol, it is zero for honey.

3.9. Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is a compound produced by sugar degradation (dehydration of hexoses in an acidic medium) (Bogdanov, 2011). HMF is a freshness parameter of honey; and in fresh honeys, it is absent or occurs in trace amounts. High values of HMF are naturally present in honeys from areas with warm climates (Sodré et al., 2011).

HMF is enhanced with honey processing and heat treatment. Adulteration with commercial sugars and long storage can also enhance HMF (Bogdanov, 2011).

There is more than one method found in literature to determine HMF content (HPLC and spectrophotometric method). We have chosen to summarize the spectrophotometric method of Bogdanov et al. (1997).

3.9.1. Principle

The spectrophotometric method is based on the principle that HMF absorbs at 284 nm.

3.9.2. Apparatus and reagents

Apparatus:

- Spectrophotometer able to measure 284 and 336 nm
- Quartz cells 1 cm (two cells)
- Vortex mixer
- Beaker (50 mL)
- Volumetric flask (50 mL)
- Filter paper
- Pipettes (0.5 and 5 mL)
- Test tubes (18 x 150 mm)
- Carrez solution I
- Carrez solution II
- Sodium bisulphite solution 0.2%
- Sample solution (initial honey solution = 5.0 mL)

Reagents:

- Distilled water.
- Carrez solution I
- Carrez solution II
- Sodium bisulphite solution 0.2%
- Sample solution (initial honey solution = 5.0 mL)

3.9.3. Procedure

The HMF spectrophotometric procedure can be found in AOAC (1990) and Bogdanov et al. (1997).

3.9.3.1. Samples, standards preparation and determination

Carrez I Solution—Dissolve 15 g of ferrocyanide in water and fill to 100 mL in volumetric flask.

Carrez II Solution—Dissolve 30 g of zinc acetate in water and fill to 100 mL in volumetric flask.

Sodium Bisulfite Solution 0.2%—Dissolve 0.20 g of sodium bisulfite in water and dilute to 100 mL in volumetric flask.

- (1) Weigh approximately 5 g of honey into a 50 mL beaker.
- (2) Dissolve the sample in approximately 25 mL of distilled water and transfer quantitatively into a 50 mL volumetric flask.
- (3) Add 0.5 mL of Carrez solution I to the 50 mL volumetric flask and mix with a vortex mixer.
- (4) Add 0.5 mL of Carrez solution II to the 50 mL volumetric flask, mix with a vortex mixer, and fill to 50 mL total volume with distilled water.
- (5) Filter the liquid through filter paper (rejecting the first 10 mL of the filtrate).
- (6) Pipette 5.0 mL of the filtered liquid into each of two test tubes (18 x 150 mm).
- (7) Add 5.0 mL of distilled water to one of the test tubes and mix with the vortex (sample solution).
- (8) Add 5.0 mL of sodium bisulphite solution 0.2% to the second test tube and mix with a vortex (reference solution).
- (9) To the sample solution (initial honey solution = 5.0 mL), add 5.0 mL of distilled water.
- (10) To the reference solution (initial honey solution = 5.0 mL), add 5.0 mL of 0.2% sodium bisulphite solution.
- (11) Determine the absorbance of the sample solution against the reference solution at 284 and 336 nm in 10 mm quartz cells within 1 h.
- (12) If the absorbance at 284 nm exceeds a value of about 0.6, dilute the sample solution with distilled water and the reference solution with sodium bisulphite solution (as described before) in order to obtain a sample absorbance low enough for accuracy.

3.9.3.2. Calculations. Results are calculated using the formula:

$$\text{HMF in mg/kg} = (A_{284} - A_{336}) \times 149.7 \times 5 \times D/W$$

Where:

A_{284} = absorbance at 284 nm;

A_{336} = absorbance at 336 nm;

$$149.7 = \frac{1.26 \times 1000 \times 1000}{16830 \times 10 \times 5} = \text{Constant}$$

126 = molecular weight of HMF

16,830 = molar absorptivity ϵ of HMF at $\lambda = 284$ nm

1000 = conversion g into mg

10 = conversion 5 into 50 mL

1000 = conversion g of honey into kg

5 = theoretical nominal sample weight

D = dilution factor (in case dilution is necessary)
 W = Weight in g of the honey sample
 Express the results in mg/kg.

3.10. Ashes

Ashes as a quality parameter is reported for Brazilian regulation, but this method will probably be replaced by the conductivity measurement. Ash content is related to honey origin (e.g., blossom honeys have lower ash content compared with honeydew honey) (Bogdanov, 2009). The procedure for ash determination is described in Bogdanov (2009).

3.10.1. Principle

Samples are ashed using a temperature $< 660^\circ\text{C}$ and the residue is weighted.

3.10.2. Apparatus

- Crucible
- Electric furnace (can run a temperature of 600°C)
- Desiccator with dry agent
- Analytical balance

3.10.3. Procedure

3.10.3.1. Samples, standards preparation and determination

- (1) Prepare the ash dish by heating it in the electrical furnace (ashing temperature 600°C at least 1 h).
- (2) Cool the ash dish in a desiccator to room temperature and weigh to 0.001 g (m_2).
- (3) Weigh 5–10 g of the honey sample to the nearest 0.001 g into an ash dish that has been previously prepared in (1).
- (4) Cool the ash dish in the desiccator (2 h) and weigh.
- (5) Continue the ashing procedure (steps 4 and 5) until a constant weight is reached (m_1).

3.10.3.2. Calculations. Make the calculation using the following formula:

$$W_A = \frac{(m_1 - m_2)100}{m_0}$$

Where:

W_A is expressed in g/100 g
 m_0 = weight of honey
 m_1 = weight of dish + ash
 m_2 = weight of dish

3.11. Free acidity

Free acidity is determined using the method of recommended by the AOAC (1990). The International Honey Commission has proposed 50 milliequivalents as the maximum permitted acidity in honey (Bogdanov, 2009). Furthermore, the Council Directive 2001/110/EC (European Commission, 2001) mentions free acidity as a

measured honey quality. Finally, the Brazilian regulation established the maximum of 50 milliequivalents/kg of honey.

3.11.1. Principle

A honey sample is dissolved in distilled water and free acidity is measured by titration with 0.1 M sodium hydroxide solution at pH 8.0.

3.11.2. Apparatus and reagents

Apparatus:

- pH meter
- Magnetic stirrer
- Burette or automatic titrator
- Beaker (250 mL)

Reagents:

- Distilled water (carbon dioxide free)
- Buffer solutions (pH 4.0 and 9.0)
- 0.1 M sodium hydroxide solution

3.11.3. Procedure

The procedure for free acidity is found in Bogdanov (2009).

3.11.3.1. Samples and standards preparation.

- (1) The pH meter should be calibrated.
- (2) Dissolve 10 g of the honey sample in 75 mL of carbon dioxide-free water in a 250 mL beaker.
- (3) Stir with the magnetic stirrer.
- (4) Immerse the pH electrodes in the solution and record the pH.
- (5) Titrate with 0.1 M NaOH to a pH of 8.30 (complete the titration within 2 min).
- (6) Record the reading to the nearest 0.2 mL when using a 10 mL burette and to 0.01 mL if the automatic titrator has enough precision.

3.11.3.2. Calculations. Free acidity is calculated using the formula:

$$\begin{aligned} \text{Free acidity (milliequivalents or millimoles acid/kg honey)} \\ = \text{mL of 0.1 M NaOH} \times 10 \end{aligned}$$

3.12. Insoluble solids

Honey insoluble solids include pollen, honey-comb debris, bees and filth particles, and is considered a criterion of honey cleanness. It is possible to determine insoluble solid content by filtering honey diluted with distilled water at 80°C in porous plate crucibles (Bogdanov, 2009; Bogdanov et al., 1997; Codex, 2001).

3.12.1 Apparatus and reagents

- Drying oven at 105°C
- Drying oven

- Pump vacuum
- Thermometer
- Desiccator with silica gel
- Metal spatula
- Beakers (50 and 250 mL)
- Porous crucible # 3
- 1000 mL kitassato flask

3.12.2 Procedure

- (1) Weigh about 20 g of honey into a 250 mL Beaker.
- (2) Dilute with hot distilled water (80 °C) until the sample is dissolved.
- (3) Transfer to a porous crucible # 3 coupled in the kitassato flask (the crucible must be pre-dried at 105 °C for 12 h, cooled in desiccator and weighed).
- (4) Filter under vacuum and wash the honey sample in the crucible with distilled water at 80 °C until the volume of each filtrate reaches 1 L.
- (5) Dry the crucible again at 105 °C for 12 h.
- (6) Cool the crucible in a desiccator and weigh it.

3.12.3 Calculation

The percentage of insoluble solids is calculated by the following ratio:

$$\text{Original sample mass} = 100\% \text{ Mass of solids} \\ \text{(filtered and dried crucible} \\ \text{– dried crucible)} = x\%$$

3.13. Diastase activity

Diastases (α - and β -amylases) are enzymes naturally present in honey. Diastase content depends on the floral and geographical origins of the honey. Their function is to digest the starch molecule in a mixture of maltose (disaccharide) and maltotriose (trisaccharide). Diastase is sensitive to heat (thermolabile) and consequently are able to indicate overheating of the product and the degree of preservation (Ahmed, 2013; da Silva et al., 2016; Granato & Nunes, 2016).

For this analysis, the method recommended by The International Honey Commission (2009) and Codex Alimentarius (2001) is based on the “diastase activity” that corresponds to the activity of the enzyme present in 1 g of honey, which can hydrolyze 0.01 g of starch in 1 h at 40 °C, expressed as the diastase number in Göthe units (Bogdanov et al., 1997). In this step-by-step method, the modifications proposed by Santos et al. (2003) were included to give improvements in the solutions preparation and spectrophotometer procedures.

3.13.1. Apparatus and reagents

Apparatus:

- UV/VIS Spectrophotometer
- Bucket 1 cm

- Analytical balance
- pH meter
- Thermometer
- Stopwatch
- Water bath
- 250 mL Erlenmeyer flask
- Volumetric flasks of 50, 100, and 500 mL
- 1 L dark volumetric flask
- 50 mL Beaker
- 100 mL test tube
- 20 mL volumetric pipettes
- Micropipette with a variable volume of 100–1000 μ L

Reagents:

- Sodium acetate (CH_3COONa)
- Glacial acetic acid (CH_3COOH)
- Sodium hydroxide (NaOH)
- Soluble starch
- Anhydrous sodium chloride
- Twice sublimated Iodine
- Potassium iodide (KI)
- Destilated water

3.13.2. Standard preparation

Acetate buffer solution 0.1 mol/L pH 5.3:

- (1) Pipette 0.57 mL acetic acid into a 50 mL volumetric flask and fill with distilled water (Solution A).
- (2) Weigh 1.64 g of sodium acetate and dissolve it into 80 mL of distilled water. Adjust the volume to 100 mL in a volumetric flask (Solution B).
- (3) Mix 10.5 mL of Solution A with 39.5 mL of Solution B.
- (4) Check the pH value using the pH meter and adjust, if necessary, to 5.3 with sodium acetate if the pH value is less than 5.3 or with acetic acid if the pH value is above 5.3.
- (5) Transfer the solution to a 100 mL volumetric flask and fill with distilled water. Keep refrigerated.

Sodium chloride solution 0.1 mol/L:

- (1) Weigh 0.585 g of sodium chloride into a 50 mL Beaker.
- (2) Dissolve in distilled water.
- (3) Transfer to a 100 mL volumetric flask and fill up to volume with distilled water.

Iodine solution 0.02 mol/L:

- (1) Weigh 4 g of KI into a 100 mL test tube.
- (2) Transfer with about 40 mL of distilled water to a dark volumetric flask, glass stopper, with a capacity of 1 L.
- (3) Weigh 2.54 g of twice sublimated iodine and transfer to the KI-containing flask. Fill up to volume with distilled water.

- (4) Shake until complete dissolution of iodine.
- (5) Transfer to a 500 mL amber milled flask.
- (6) Store the solution at room temperature with no light.

Starch solution 1% (m/v):

- (1) Weigh 1.0 g of soluble starch into a 250 mL Erlenmeyer flask.
- (2) Mix with 70 mL of distilled water.
- (3) Heat to boiling under constant stirring and keep for 3 min. Transfer the final volume of 100 mL in a volumetric flask and cool down to room temperature in running water. The solution should be prepared at the time of use, do not store to avoid contamination.

Sodium hydroxide solution 0.1 mol/L:

- (1) Weigh 2.0 g of NaOH and dissolve in distilled water by filling the volume in a volumetric flask to 500 mL.
- (2) Store the solution in a plastic container.

3.13.3. Experimental procedure

- (1) Weigh 5.0 g of honey into flask.
- (2) Add 20 mL of distilled water to the flask.
- (3) Correct the pH of this solution to a value of 5.3 by adding 0.1 mol/L NaOH until the correct pH is obtained.
- (4) Fill the flask to 50 mL with distilled water.
- (5) Reaction system: Add 5 mL of the honey solution to a test tube.
- (6) Add 500 μ L of the acetate buffer 0.1 mol/L pH 5.3 to the tube and mix. It is essential that the honey solution is buffered before contact with sodium chloride, because at pH below 4, diastase activity is inhibited.
- (7) Add 500 μ L of the sodium chloride solution 0.1 mol/L to the tube.
- (8) Add 150 mL of the solution of 0.02 mol/L iodine and 9.6 mL distilled water to the tube and mix.
- (9) Keep the tube and its contents in a water bath at 40 °C \pm 1 °C.
- (10) Add 250 μ L of the 1% starch solution (m/v).
- (11) Start the stopwatch, shaking the solution until complete homogenization occurs.
- (12) Transfer a part of the volume of the reaction system to complete a 1 cm cell and measure the absorbance of the solution in the spectrophotometer at 660 nm against a water blank. This first reading is the initial absorbance value (Ab_{si}). The tube with the solution should remain in the water bath at 40 °C.
- (13) Make periodic readings of absorbance, always returning the tube to the water bath when not being read, until a value between 0.24 and 0.20 is reached.

- (14) When this value is reached, stop the timer and record the elapsed time value.
- (15) The last recorded absorbance value is considered the final absorbance (Ab_{sf}).
- (16) Calculate the diastase index using the following formula:

$$\text{Diastase index} = \frac{(\text{Abs}_i - \text{Abs}_f) \times 0.3}{T(\text{h}) \times V \times 0.016}$$

0.3 = absorbance constant = 0.3 mg⁻¹ (previously determined by honey-free assay, given by method)

T (h) = time (in hours) between Ab_{si} and Ab_{sf} measurements

V = volume of the 10% honey solution in the test tube (mL)

0.016 = total volume in liters of the solution in the test tube (16 mL)

3.14. Electrical conductivity

This method is easy, quick, and involves inexpensive instrumentation. Electrical conductivity determination depends on the ash and acid contents of honey (e.g., the higher ash and acids, the higher the result for electrical conductivity). This parameter has been used as criterion of the botanical origin of honey (Bogdanov, 2009).

According to the European regulation (European Commission, 2001), electrical conductivity of blossom honey must be < 0.8 mS/cm of EC, while electrical conductivity of honeydew honey and chestnut honey must be > 0.8 mS/cm. Exceptions are honeys from *Arbutus*, *Banksia*, *Erica*, *Leptospermum*, *Melaleuca*, *Eucalyptus*, *Tilia*, and blends (De-Melo et al., 2017).

3.14.1. Principle

Electrical conductivity uses the measurement of the electrical resistance.

3.14.2. Apparatus and reagents

- Conductimeter lower range 10⁻⁷ S.
- Conductivity cell (platinized double electrode—immersion electrode)
- Thermometer
- Water bath
- Volumetric flasks (100 mL and 1000 mL)
- Beakers
- Distilled water
- Potassium chloride

3.14.3. Procedure

The standard method for measuring honey electrical conductivity was outlined by Bogdanov (2009).

3.14.3.1. Samples and standards preparation.

- (1) If the cell constant of the conductivity cell is not known, transfer 40 mL of the potassium chloride solution (0.1 M) to a beaker.

- (2) *Potassium chloride solution (0.1 M) preparation*: dissolve 7.4557 g of potassium chloride (KCl), dried at 130 °C, in freshly distilled water and fill up to 1000 mL volumetric flask. Prepare fresh on the day of use.
- (3) Connect the conductivity cell to the conductivity meter.
- (4) Rinse the cell thoroughly with the potassium chloride solution and immerse the cell in the solution, together with a thermometer.
- (5) Read the electrical conductance of this solution in mS after the temperature has equilibrated to 20 °C.
- (6) Calculate the cell constant K , using the formula: $K = 11.691 \times 1/G$, where: K = cell constant cm^{-1} ; G = Electrical conductance (mS), measured with the conductivity cell; 11.691 = the sum of the mean value of the electrical conductivity of freshly distilled water in $\text{mS}\cdot\text{cm}^{-1}$ and the electrical conductivity of a 0.1 M potassium chloride solution, at 20 °C.
- (7) Rinse the electrode with distilled water after the determination of the cell constant.
- (8) Dissolve an amount of honey, equivalent to 20 g of dry matter of honey, in distilled water.

3.14.3.2. Determination.

- (1) Transfer 20 mL of the solution created in section "Samples and standards preparation" to a 100 mL volumetric flask and fill up to 100 mL with distilled water.
- (2) Pour 40 mL of the sample solution into a beaker and place the beaker in a thermostated water bath at 20 °C.
- (3) Rinse the conductivity cell thoroughly with the remaining part of the sample solution (Step 1).
- (4) Immerse the conductivity cell in the sample solution and read the conductance in mS after temperature equilibrium has been reached.
- (5) If the determination is carried out at a different temperature, because of a lack of thermostated cell, a correction factor can be used for calculating the value at 20 °C. For temperatures above 20 °C, subtract 3.2% of the value per °C. For temperatures below 20 °C, add 3.2% of the value per °C.

3.14.3.3. Calculations. Calculate the electrical conductivity of the honey solution, using the following formula:

$$S_H = K \times G$$

Where: S_H = electrical conductivity of the honey solution in $\text{mS}\cdot\text{cm}^{-1}$; K = cell constant in cm^{-1} ; G = conductance in mS. Express the result to the nearest 0.01 $\text{mS}\cdot\text{cm}^{-1}$.

Also, FTIR could be used in order to determine honey electrical conductivity (Almeida-Muradian et al., 2012, 2013).

3.15. Trolox equivalent antioxidant capacity (TEAC)

Determining the scavenging ability of honey to the radical cation of ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)], using trolox, a water-soluble vitamin E analog, as the standard for the calibration curve (Miller et al., 1993; Re et al., 1999; Sancho et al., 2016) is the method we propose for measuring antioxidant capacity. The described method is a modification of Re et al. (1999) procedure, optimized by Sancho et al. (2016):

3.15.1. Apparatus and reagents

- Spectrophotometer (Visible), to measure absorbance (A) at 734 nm
- Glass or Plastic cells 1 cm
- Ultrasonic bath
- Shaker
- Stopwatch
- 100 mL volumetric flask
- 100 mL beakers
- Pipettes
- Distilled water
- ABTS
- $\text{K}_2\text{S}_2\text{O}_8$
- Trolox

3.15.2. Solutions

3.15.2.1. Preparing the solutions.

- (1) 7mM ABTS aqueous solution: Dissolve 0.3841 g 2,20 – azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS) in distilled H_2O , stir and dilute to 100 mL with distilled H_2O .
- (2) 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ aqueous solution: Dissolve 0.0662 g potassium persulfate (di-potassium peroxodisulfate) in distilled H_2O , stir, and dilute to 100 mL with distilled H_2O .
- (3) 5mM trolox solution: Dissolve 0.1251 g (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) into a mixture of absolute ethanol-distilled H_2O (1:1). The use of an ultrasonic bath for 5 min aids dissolution. Dilute to 100 mL with distilled H_2O .

3.15.2.2. Generation and dilution of ABTS radical cation (ABTS^{*+})

- (1) Undiluted ABTS^{*+} solution: mix 7mM ABTS aqueous solution and 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ aqueous solution in equal amounts (1:1, v/v) and allow them to react in the dark for 16 h at room temperature. Undiluted ABTS^{*+} solution is stable two days, stored in the dark at room temperature.

- (2) Working ABTS^{•+} solution: Dilute the undiluted ABTS^{•+} solution with distilled water to obtain an absorbance between 0.70 and 0.80 at 734 nm. The working ABTS^{•+} solution must be prepared fresh daily.

3.15.2.3. Samples and standards preparation.

Sample preparation (H-sample):

- (1) Weigh 10 g honey into a beaker.
- (2) Dissolve in distilled water.
- (3) Transfer to a 100 mL volumetric flask.
- (4) Further dilute to 100 mL with distilled water.

solution (H-sample), referred to section "Determination" (4).

Linear regression: a standard trolox calibration curve is drawn by representing trolox concentrations (mM) on the x-axis, and % inhibition on the y-axis.

$$\% \text{ inhibition} = ax + b$$

x = concentration trolox (mM)

a = slope

b = intercept

TEAC value of the honey sample ($\mu\text{mol trolox/g honey}$): TEAC antioxidant activity of honey is calculated as follows:

$$\text{TEAC } (\mu\text{mol trolox/g honey}) = \frac{(\% \text{ inhibition H-samples} - b)}{a} \times \frac{100}{\text{honey weight (g)}}$$

Trolox calibration curve (standard):

- (1) In six volumetric flasks, dilute the 5 mM trolox solution with distilled water to obtain the following concentrations: 0.25 mM, 0.50 mM, 0.75 mM, 1.00 mM, 1.25 mM, and 1.50 mM.

3.15.2.4. Determination.

- (1) Using a pipette, transfer 990 μL of the diluted ABTS^{•+} working solution (see "Generation and dilution of ABTS radical cation (ABTS^{•+})" section) into each cell. One cell corresponds to distilled water (no antioxidant), six cells correspond to the calibration curve (with trolox standard solutions of section "Samples and standards preparation"), and another cell corresponds to the sample (of section "Samples and standards preparation").
- (2) Start the reaction by adding 10 μL of distilled water (DW), trolox standard solution (STANDARD), or sample solution (H-sample) to the corresponding cell.
- (3) Mix immediately by turning the cell upside down. Determine the absorbance (A_0).
- (4) Determine the absorbance (A) after 6 min (A_6) at 734 nm in the cell, against a blank of distilled water.

This procedure is performed in triplicate.

3.15.2.5. Calculations.

Percentage of inhibition:

$$\% \text{ inhibition} = \frac{(A_0 - A_6) \times 100}{A_0}$$

A_0 is the absorbance at initial time of distilled water (DW), standard solution (STANDARD), or sample solution (H-sample), referred to section "Determination" (3).

A_6 is the absorbance measured at 6 min of distilled water (DW), standard solution (STANDARD), or sample

a = slope

b = intercept

Where 100 (of the formula) = 1000 ($\mu\text{mol/mmol}$) * 100 (mL honey solution)/1000 (mL/L).

3.16. Color of honey by the CIELab system

The colorimeter measures the reflected color of the honey samples, both liquid and crystallized, of the whole visible spectrum in a wavelength interval between 380 and 740 nm. Other methods for measuring a honey sample's color require honey dilution before measuring its color by spectrophotometry at a given wavelength. Or, it is necessary to dissolve any crystals in the honey to obtain a completely liquid honey that is transparent. Both procedures modify the honey color. The advantage of the colorimeter method is that it is the only instrument that gives the samples' color the way the human eye does, whether the sample is liquid or crystallized. Moreover, honey remains suitable for use in other analysis (Sancho et al., 2016).

3.16.1. Apparatus

- $L^*a^*b^*$ Colorimeter glass
- Ultrasonic bath

3.16.2. Procedure

- (1) Fill the colorimeter glass completely with honey. Bubbles in the honey can interfere with honey color. They can be removed from the honey if it is placed in an ultrasonic bath at room temperature for a few minutes before color determination (avoiding honey heating).
- (2) Determine the color parameters L^* (lightness, 100 for white and 0 for black), and the chromaticity coordinates a^* (positive values for redness and

negative values for greenness) and b^* (positive values for yellowness and negative values for blueness), using illuminant D65, 10° observation angle and 45°/0° geometry illumination.

- (3) Measure the color in triplicate (Sancho et al., 2016).
- (4) Other interesting values for honey comparison or characterization, calculated from a^* and b^* , are the coordinates C^* (chroma, saturation, vividness, or purity of a color) and h^* (hue angle or tone) (Tuberoso et al., 2014).

$$C^* = \sqrt{a^{*2} + b^{*2}}$$

$$h^* = \arctg b^*/a^*$$

4. Investigating honey production with diagnostic radioentomology

Diagnostic radioentomology is a technique based on the measurement of density from component materials using X-rays. The density measured by computer tomography (CT) scanning reflects the degree to which the energy of the X-ray beam is reduced when penetrating a certain material. This technique allows for the quantification and visualization of differences in density with minimal disturbance of the colony since the hive does not need to be opened to introduce light into the dark nest and since no sample need be collected. Thus, it becomes possible to investigate, noninvasively, phenomena that are affected by other common research methods that require destructive sampling.

Honey originates from nectar and honeydew and undergoes a process of concentration (i.e., dehydration). The increase in sugar concentration affects honey density. This can be tracked using radioentomology.

Despite the importance of honey for beekeeping, the processes that lead to its production within the honey bee nest have rarely been investigated (Eyer, Greco, et al., 2016; Eyer, Neumann, et al., 2016). Diagnostic radioentomology was recently used to shed light on nectar storage strategies of bees by allowing for the measure of sugar concentration of the content of a large number of storage cells (Eyer et al., 2016; Greco et al., 2013). By following the evolution of the sugar concentration in individual cells over time, it is also possible to monitor several stages of honey production by the workers, from initial deposition of nectar or honeydew in cells to the capping of mature honey (Eyer et al., 2016).

This technique could be used further to determine the influence of environmental (e.g., high or low nectar flow, diverse or monotonous nectar flow, varying weather conditions) and internal factors (e.g., hive design, air circulation, colony demography) on honey ripening and storage. A better knowledge of the factors affecting honey production processes by honey bee workers could not only lead to a better knowledge of how the honey bee superorganism manages its food stock but

could also lead to the improvement of beekeeping management practices by favoring a rapid ripening of honey or by increasing productivity. A further application of this technique is to track the deposition and spread of substances applied into hives (Rademacher et al., 2013) that might contaminate honey stores. The concentration of crop content of live, yet motionless honey bees can also be measured with this technique. Rapidly moving individuals will appear blurred in the scan and thus hinder precise measurements. In the following sections, we describe how to perform measurements of sugar concentration of the content of carbohydrate reserves stored in wax combs with a CT scanner.

4.1. Experimental conditions and material required to conduct diagnostic radioentomology trials

4.1.1. Hive to scanner size ratio

The most commonly available CT-scanners are those used for human or veterinary medicine. Their dimensions allow for scanning hives of most standard sizes without honey supers. Larger scanners exist for veterinary applications and can be used to study hives with supers. When high resolution is required, it can be obtained by reducing the size of the experimental hive (e.g., using Miniplus (R) hives, 30 × 30 × 34 cm). The focalization of the X-ray beam on a smaller area increases scan resolution. Micro-CT scanners are also available to study small items (e.g., individual motionless workers, a few cells) with high resolution.

4.1.2. Material to be scanned

Combs built from foundation sheets in moveable frames have a regular and planar shape, which facilitates scanning and data analysis. However, diagnostic radioentomology can be used to study honey production in combs of any shape (e.g., in wild nests in logs) thanks to 3D reconstruction tools. Depending on the research question, combs already containing stores can be used as starting material. When the starting point of the study is an empty cell, a drawn empty comb can be inserted in the test colony. Single combs without workers, workers alone or entire hives with workers can be scanned. Single combs can be gathered in an empty hive or super box and all scanned at once to decrease the number of scans required (Figure 3).

When hives containing honey bees are to be scanned, safety of scanning facility staff and of bystanders needs to be considered. Hive entrances should be closed early on the day of scanning before foragers start their activity and tight hives should be used to prevent honey bees from escaping. Ventilation (e.g., through a screened bottom) should nevertheless be possible for the workers to be able to adjust hive temperature. Combs and hives have to be labeled in a way that they can be recognized on the scan images. Notches cut in the wood or thin metallic letters or numbers can be

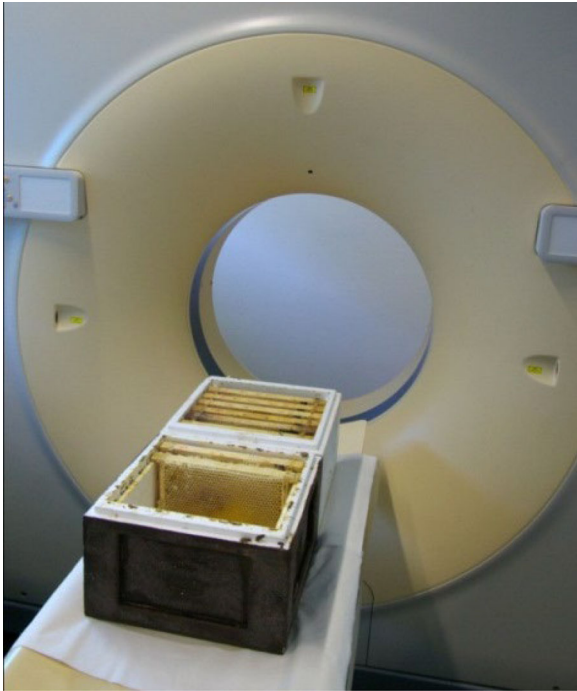


Figure 3. Experimental combs are placed in hive bodies for a CT scan.

used. Metallic parts like nails and wires deflect the X-ray beam and produce glares that make data analysis difficult. It is thus necessary to remove all metallic parts from the material to be scanned or to use plastic or polystyrol hive material.

4.1.3. Monitoring the storage of artificial diets

Nectar and honeydew collected by foragers are highly diverse in their composition. Consequently, it can be useful to use artificial diets to simplify the study of honey production. The storing of artificial diets (e.g., commercial feeding solutions) can also be the purpose of the experiment. Commercial feeding solutions or a solution of controlled sugar concentration can be provided to the colonies in feeders within the hive or outside of the hive at a close distance, which reflects a more natural foraging situation.

To prevent the foragers from collecting other sources of carbohydrates than those experimentally provided, test colonies can be placed in tunnel cages (see Medrzycki et al. (2013), section “Scanning settings”). When tunnel cages are not available, field colonies can be used during a period without natural nectar flow. To confirm absence of incoming nectar, the concentration of returning foragers’ crop content can be measured at regular interval using the following method:

- (1) Collect returning foragers that have no pollen baskets on their legs at the hive entrance with the help of forceps.

- (2) Gently squeeze their abdomens between your fingers to force them to regurgitate a small droplet of crop content.
- (3) Place this droplet onto a refractometer adapted to the range of expected concentrations (see Human et al. (2013), section 4.7.3.1).
- (4) Read the sugar concentration through the refractometer.
- (5) Repeat this procedure daily for 10–50 foragers per test-colony.

If the concentration of crop content does not correspond to that of the diet provided, the foragers are collecting nectar from other sources. If this input cannot be accounted for in the analysis, the experiment should be performed when naturally available resources do not interfere with the aim of the study.

4.1.4. Studying the storage of natural diets

When the aim of the experiment is to study natural diets, field colonies with freely flying honey bees can be used. Choose a period with the nectar or honeydew flow of interest for your research question. The range of sugar concentrations collected by foragers can be verified by squeezing the abdomen of returning foragers (see section “Monitoring the storage of artificial diets”).

4.1.5. Labeling of the diet to increase contrast

When the storage of qualitatively different solutions of similar densities needs to be studied, it is possible to discriminate between these solutions by labeling one of them with a contrast agent (Eyer et al., 2016). One example of a suitable contrast agent is Visipaque, which is harmless to honey bee workers at a concentration of 10%. Visipaque (iodixanol Injection) is a contrast media solution, with multiple indications in human medicine (aortography, venography, urography, etc.) for use in X-ray scans. Other contrast agents may be used but their toxicity for honey bees need to be determined (Medrzycki et al. 2013).

If a contrast agent is used, whether it mixes with the solution or sediments in the cell needs to be determined prior to the experiment (Eyer et al., 2016). This verification is required to exclude possible bias from the contrast agent when monitoring density patterns or measuring density of cell or crop content.

4.1.6. Calibration

The density of cell content is measured in Hounsfield units. This unit is used as a proxy for sugar concentration. A regression of sugar concentrations on density of the artificial carbohydrate solutions or natural sources is thus required to convert Hounsfield units in % sugar concentration (Eyer et al., 2016). To

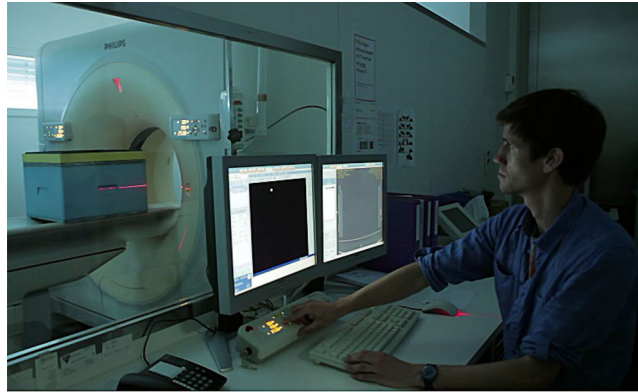


Figure 4. Movement of the scanner bed is programmed and the hive box is positioned using the scanner's laser beams.

generate a calibration curve, the following steps are conducted.

4.1.6.1. Artificial diet.

- (1) Prepare vials (20–50 mL) with carbohydrate solutions (mixing granulated sugar with water) covering the range of concentrations required for the experiment (e.g., 30%, 50%, 60%, 70%, and 80% sucrose).
- (2) Vortex to homogenize solution.
- (3) Scan the vials (see section “Computer tomography scanning” below).
- (4) Measure density of 10 randomly selected points in each vial (using the point measure tool of eFilm, for example) to consider putative density variations within the vial.
- (5) Average these measures.
- (6) Plot the averages for each vial with density measured on the x-axis and sugar concentration on the y-axis.
- (7) Add a linear trend line to the chart.
- (8) Obtain the equation describing the trend line in order to determine the slope (m) and the constant (t) of the calibration line.
- (9) Calculate the sugar concentration (y) by multiplying the density measured by the slope value and by adding the constant (t) ($y = (m * x) + t$). Repeat this calculation for each density value measured.

4.1.6.2. Natural diet.

- (1) Sample aliquot of nectar, cell, or crop content.
- (2) Place aliquot on a refractometer adapted to the expected sugar concentration (see Human et al. (2013), section 4.7.3.1).
- (3) Read the sugar concentration through the refractometer.
- (4) Repeat this procedure until a wide enough range of concentrations is obtained to perform calibration.
- (5) Perform steps (5–9) as indicated in “Artificial diet” section.

4.1.6.3. Labeled diet. A specific calibration curve has to be generated for the calculations of the sugar concentrations in labeled diets. Repeat steps described in Section “Monitoring

the storage of artificial diets” with labeled solutions of various sugar concentrations. Densities of labeled and nonlabeled diets should not overlap at any point of the concentration range.

4.2. Computer tomography scanning

4.2.1. Handling and transporting of the combs

Ideally, a portable CT-scanner should be used to avoid interfering with the biological processes in the colony. However, until the use of portable devices in the field becomes more practical and affordable and since the most frequently accessible devices are fixed, it is recommended to minimize transport of the test hives or combs by conducting the field component of the experiments near the CT-facility. In cases when transportation cannot be avoided, its effects can be minimized by careful handling, avoidance of shaking and shocking the hives or combs to be tested. If combs are scanned outside of the hive, not using smoke during comb collection will limit methodological bias by not provoking cell content take up by workers. Workers can be removed from the combs by gently brushing them off, taking care that the bristles do not contact the cell content. Shaking the combs to get rid of workers is not recommended due to possible effects on cell content, especially if storage of nectar of low sugar concentration is investigated. In the absence of honey bees, further biases such as cell content evaporation can be minimized by scanning the comb immediately after collection.

4.2.2. Scanning settings

The scans can be performed with a Philips Brilliance CT 16-slice apparatus (e.g., Philips Healthcare, 5680 DA Best, The Netherlands) using 120.0 kVp and 183.0 mA as settings. For other models, detailed device setting should be discussed with scanner operator according to the output required.

4.2.3. Performing a scan

Constant conditions during scanning should be maintained, holding the temperature in the scanning room at

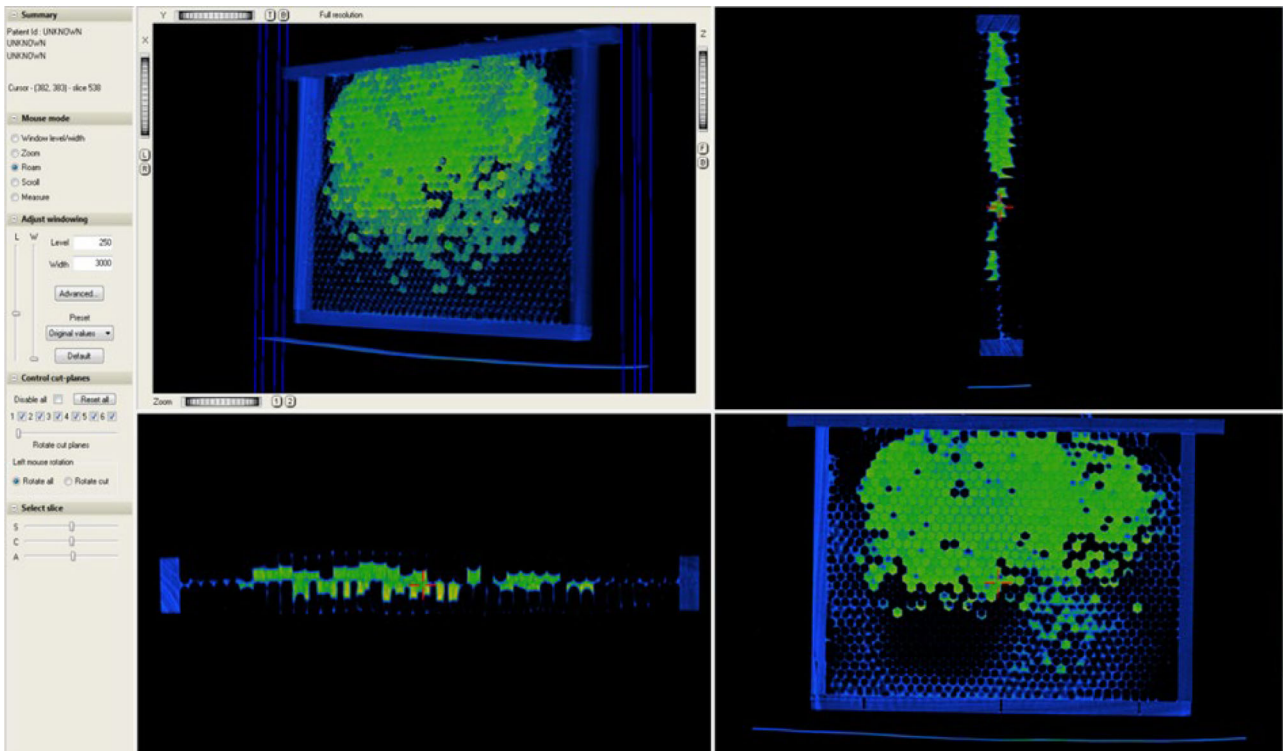


Figure 5. Screenshot of a 3D imaging software with 3D reconstruction panel (top left) and three section planes (sagittal: top right, transverse: bottom left, coronal bottom right).

18–20 °C. If required by the study question, scans can be repeated at defined time intervals. Adhere to safety measures as instructed by scanner operator to avoid the danger of exposure to X-rays.

- (1) Place the hive or combs to be scanned on the scanner bed.
- (2) Adjust bed movement range and position of material to be scanned using the device's positioning laser beams (Figure 4).
- (3) Place yourself in the designated, protected area; personal other than operators leave the room.
- (4) Press scan and survey the scan-procedure on the computer.
- (5) Wait until scan is complete (usually indicated by the extinction of an acoustic signal) before leaving the designated area.
- (6) Save the scan output to a specific location on a server or a hard drive.

4.3. Data analysis

4.3.1. Analysis of density patterns in individual cell

For the visualization of cell content density patterns, CT-images can be analyzed with specific 3D rendering software that permits the visualization of differences in density with a color gradient (Figure 5). This feature enables visual monitoring of density measured following this procedure:

- (1) Load file.
- (2) Choose parameter settings. Detailed device settings for the windowing feature should be discussed with the scanner operator according to the output required. A dark color represents low density content, whereas light color represents high density content. Image analysis software also has several coloration presets (e.g., Figure 5) that can be tried to render the best image output for the analysis. There is no empirical manner to determine the best color settings. The appearance of the images generated will determine which is the most informative.
- (3) Apply and record the chosen settings.
- (4) Inspect cells from sagittal, transverse, and coronal perspectives for specific cell content patterns (Figures 5 and 6).

4.3.2. Measuring density of cell content

Density, quantified in Hounsfield Units (HU), can be measured using the software eFilm, for example (30-day test-version is available under <https://estore.merge.com/na/index.aspx>). Density of individual cell content can be measured with the ellipse tool as described below. If required, more precise measurements of cell content can be obtained with the point measurement tool.

- (1) Load image file.
- (2) Navigate/Scroll vision plane to the cells of interest.

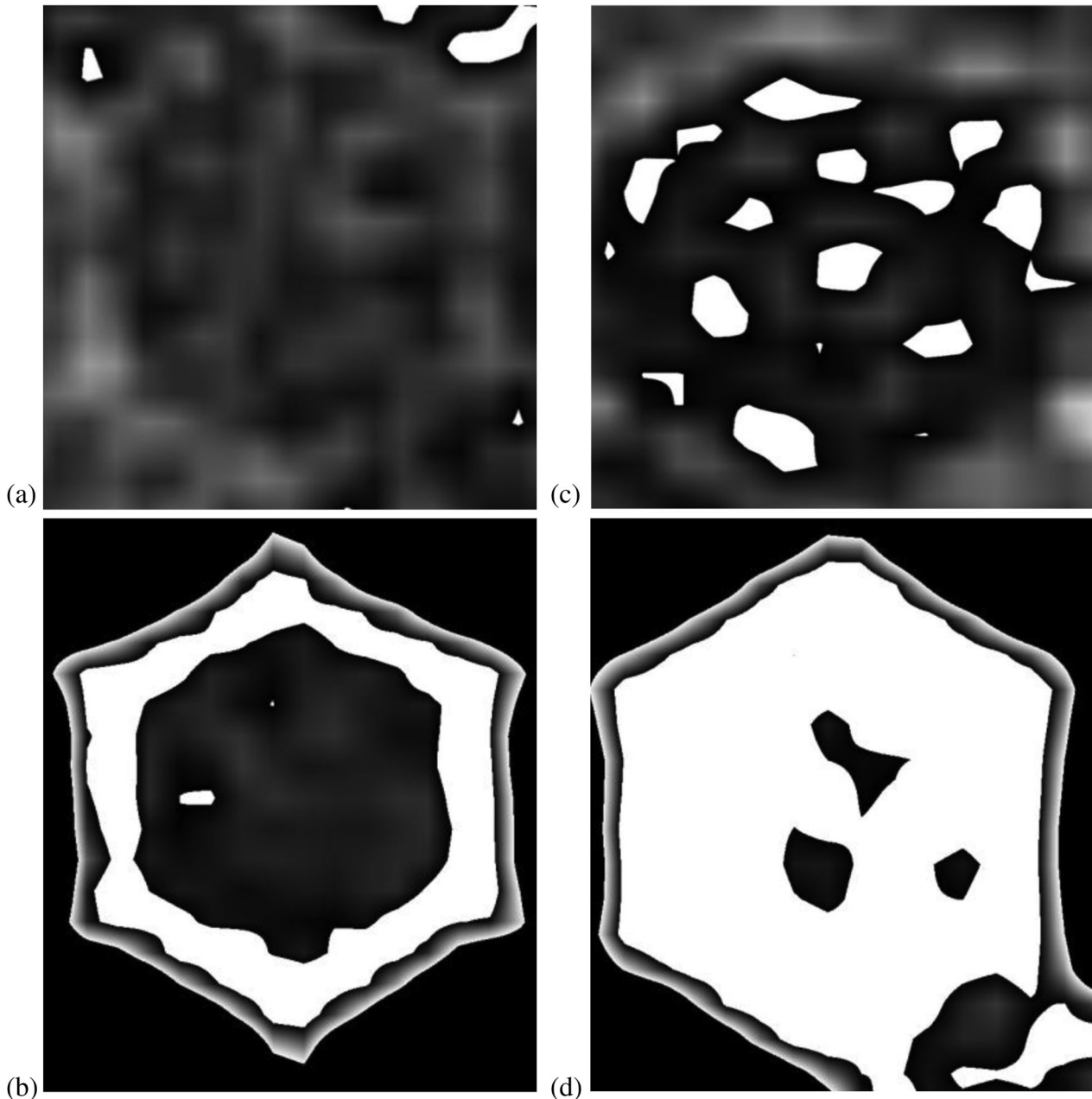


Figure 6: Density patterns in individual cells: (a) low density homogeneous pattern; (b) ring of high density content along the cell walls; (c) inhomogeneous pattern with high density speckles; (d) homogeneous high density cell content. Modified from images published in Eyer et al., 2016 under CC BY 4.0.

- (3) Use the ellipse tool to draw a circle over the content of the cell, three scanning frames away from its bottom end.
- (4) Repeat the measurement of the same cell, three frames below the surface of the cell content.

Steps 3 and 4 are necessary to take into account within cells variation of density (see Eyer et al., 2016). Scanning a few frames away from cell bottoms and openings helps avoiding the inclusion of air volumes or wax in the circles, which would strongly bias the measurement.

- (5) Average the values of these measurements.

4.3.3. Measuring cell filling status

The filling status of the cells can be estimated as the number of scanning frames for which the content fills the whole cell diameter.

- (1) Load image file.
- (2) Place vision plane at cell bottom.
- (3) Scroll through each frame with full content while counting.

4.3.4. Analyzing content of individual cells over time

- (1) Take a picture of each test comb or load the image file with eFilm.

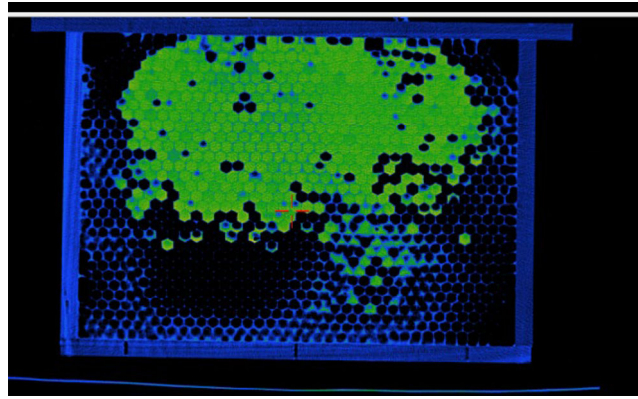


Figure 7. A coronal scan through a partly filled comb. Colors reflect differences in density, with black for low density to yellow for high density. Cell content appear in green-yellow shades, wax and wood in blue, air in black.

- (2) Select and mark 10 individual cells (on picture, screenshots (Figure 7), or printouts) for each test comb.
- (3) Define and use specific landmarks (e.g., particular shapes on the wax combs or marks on the frames that are not likely to change over time) on the image files/pictures to easily identify individual cells in subsequent scans.
- (4) Analyze content of individual cells with eFilm or other dedicated software on subsequent scan times. This allows the investigation of their filling and ripening dynamics over time.
- (8) Chose the fixed distance model with the mean distance between the centers of two neighboring cells. This distance can be measured with the line tool of eFilm.
- (9) Run the analysis and consider the I-index obtained: I-indices close to zero indicate random pattern, whereas positive indices indicate a tendency toward clustering. Negative indices indicate a tendency toward uniformity (Eyer et al., 2016). *P*-values indicate whether the distribution patterns are significant.

In case the whole process of honey production is to be monitored but not all cells are capped at the end of the experiment, the selection of cells measured can be done *a posteriori*, once cells have been capped.

4.3.5. Spatial analyses at comb level

The following method can be used to investigate spatial patterns in nectar processing (Eyer et al., 2016).

- (1) Select combs that contain a sufficient number of filled cells (e.g., Figure 7).
- (2) Select a single vertical scan frame perpendicular to the cells' long axes and parallel to the comb midrib for each comb. Choose this frame in order to maximize the number of cells showing content.
- (3) Measure the density of content of a predefined number of cell (e.g., using the ellipse or point tool of eFilm, see section "Measuring density of cell content").
- (4) Determine the projected X and Y coordinates of each cell (e.g., with the help of the probe tool of eFilm).
- (5) Enter coordinates in a spreadsheet in which the density values will be recorded.
- (6) Analyze spatial patterns (e.g., using the spatial autocorrelation Moran's I test statistic in Arc GIS 10.2).
- (7) Enter feature locations (projected coordinates) and attribute values (sugar concentration) to calculate the spatial autocorrelation (I index).

4.4. Pros and cons

Pros: The use of CT-scanning permits nondestructive observations and measurements within the dark hive. Repeating the observations and measurements in time is thus possible without disrupting the phenomenon under scrutiny. Three-dimension imaging is possible.

Cons: Different materials of similar density cannot be distinguished based on their Hounsfield value. Unless a portable device is available, the material to be scanned has to be transported to the scanning facility. Only snapshots and hence low frequency time lapse images can be captured, filming is not possible to observe the behavior of workers for example. Metallic parts create glare and need to be removed from the scanned material.

4.5. Perspectives

Diagnostic radioentomology is a powerful tool to monitor otherwise difficult to observe processes. However, this recently developed method could be further developed to increase its usefulness. Scans performed immediately after behavioral observations in hives with transparent sides can help relate density patterns with the behavior that generated them. Better labeling techniques (e.g., avoiding sedimentation of contrast agent) of cell content would provide further opportunities to monitor processes involved in nectar storage and honey production with an even higher resolution. Scanning of storage combs should also be performed more

frequently as done to date, aiming at investigating the processes of honey production with higher time resolution. Further, generating a database of densities of nectar and honey of specific origins (with various sugar compositions) or at different ripening stages could improve the sets of tools available and might relieve the need for calibration before each experiment. Scanning honey bee workers, to investigate crop content, for example, is limited by the relatively low scanning speed. This is especially the case with older devices and when workers are moving. However, the motors of new generations of scanners are spinning the X-ray and detection units at high speeds and will allow for the freezing of a greater proportion of the worker's movement. Other imaging techniques (e.g., radioactive labeling and laser scanning microscopy) could also be employed for studying honey production by workers.

5. Pesticide residues in honey

The term “pesticides” represents many different substances used in various crop protection products to treat plants against pests and in veterinary drugs used against animal pests/parasites (including those used in honey bee colonies to control bee pests/parasites), in/around structures to protect against structural pests, etc.

Pesticides applied on crops can contaminate plants, soil, water, and air, and honey bees may be exposed to them via contact with these matrices. The bees collect and transport contaminated products (nectar, pollen, and water) into the hive. There is also a risk of finding pesticide residues in bee products following treatment of the hive.

Honey samples can be screened for pesticide residues for food safety purposes (“Commission Regulation (EU) No 37/2010”, European Union Commission, 2009; “Regulation (EU) No 396/2005”, 2005). The European Union (EU) requires that honey be tested for pesticide residues within the framework of the monitoring program covered by the Council Directive 96/23/EC (European Communities, 1996). Maximum residue limits (MRLs) are defined for pesticides in honey (EU Pesticides Database) which mostly are between 0.01 and 0.05 mg/kg. For acaricides used in beekeeping, two MRLs were defined in the Commission Regulation (EU) No 37/2010 (2009) for amitraz (and its metabolite, 2,4-dimethylaniline) and coumaphos: 0.2 and 0.1 mg/kg, respectively.

5.1. Chemical families of pesticides

The most common use of pesticides is as plant protection products in agriculture. Pesticides are classified in three main groups according to the nature of the “pest” to be controlled: herbicides, fungicides, and insecticides (including acaricides). In each group, there are different chemical families of pesticides.

5.1.1. Herbicides

Herbicides are used to eliminate weeds which disturb the growth of the crops. They are widely used in agriculture to kill plants or to inhibit their growth or development. Residues are found in air, water, and on various plant parts. There are multiple chemical classes of herbicides: carbamates, triazines, triazoles, and ureas to name a few.

5.1.2. Fungicides

Fungicides are used to kill fungi in plants, stored products, or soil, or to inhibit their development. Fungicides can either be contact, translaminalar, or systemic. The main chemical families of fungicides used are benzimidazoles, dicarboximides, triazoles, chloronitriles, and carbamates.

5.1.3. Insecticides and acaricides

Insecticides and acaricides are used to kill or disrupt the growth/development of insects or mites. Insecticides are applied on crops to protect them against pests. They can be classified into two groups. (i) Systemic insecticides are incorporated into the tissues of treated plants. Insects ingest the insecticide while feeding on the plants. (ii) Contact insecticides are toxic by contact to insects. Acaricides are also used against honey bee parasites such as *Varroa* in hives (Dietemann et al., 2013). The main chemical insecticide families are organochlorines, organophosphorus, pyrethroids, neonicotinoids, carbamates, and phenylpyrazoles.

5.2. Analytical methods

For protecting the health of consumers, the analytical challenge is to achieve limits of quantification at or below the MRL specified for pesticides under EU or other similar legislation. The laboratories conducting the residue analyses usually develop and validate a multi-residue method. Within the framework of official controls on pesticide residues, laboratories follow the requirements specified in the guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed (“Document SANTE/11813/2017”, European Commission, 2018).

The development of residue analysis methods depends on the properties of both the matrices (honey, wax, pollen, etc.) and pesticides. Residue analysis involves several steps. There are many extraction and clean-up procedures used by different authors to determine the amount of pesticides in honey (Bargańska & Namieśnik, 2010). Solvent extraction and solid-phase extraction (SPE) are the techniques most commonly used for the extraction of pesticides from honey.

There are two general analytical approaches used to determine residues in food and environmental samples: (i) specific methods where a single pesticide and its metabolites are quantitatively determined in the sample, and (ii)

Table 5. Analysis of pesticide residues in honey using a liquid–liquid extraction.

Pesticide analyzed	Extraction protocol	References
Organochlorines (α -, β -, and γ -hexachlorocyclohexane (HCH), hexachlorobenzene (HCB), aldrin, p,p'-DDE, p,p'-DDD, o,p'-DDT and p,p'-DDT)	<ol style="list-style-type: none"> 1. Dissolve 5 g of honey with 50 mL 4% aqueous solution of sodium sulfate in a centrifuge tube. 2. Add 20 mL of ethyl acetate to the sample. Repeat the liquid-liquid extraction two times with 15 mL of ethyl acetate. 3. Centrifuge at 3000 rpm for 10 min if emulsion is formed. 4. Filtrate the organic phase through anhydrous sodium sulfate. 5. Evaporate the organic phase under a stream of nitrogen to 2.5 mL for analysis in graduated centrifuge tube. 6. Analyze by GC-ECD, and Gas Chromatography/Mass Spectrometry (GC-MS) for confirmatory analysis following authors' protocols. 	Blasco, Lino et al. (2004)
Coumaphos, bromopropylate, amitraz and tau-fluvalinate	<ol style="list-style-type: none"> 1. Mix 20 g of honey in an Ultra-Turrax blender with a mixture of <i>n</i>-hexane (60 mL), propanol-2 (30 mL) and 0.28% of ammonia. The pH of this mixture is 8. 2. Filter the solution through a filter paper. 3. Repeat steps 1 and 2 with the mixture of <i>n</i>-hexane (60 mL), propanol-2 (30 mL) and 0.28% of ammonia. 4. Rinse the Ultra-Turrax with 40 mL of <i>n</i>-hexane and filter this washing solution on the same filter paper as in step 2. 5. Transfer the combined extracts from steps 2, 3 and 4 to a separating funnel (500 mL). 6. Add 50 mL of distilled water and 0.28% of ammonia (pH 10). 7. Shake the separating funnel vigorously. 8. Allow the filtrate to separate into two phases. 9. Discard the aqueous phase (lower). 10. Repeat steps 6, 7, 8 and 9 twice. 11. Filter the <i>n</i>-hexane phase through a layer of anhydrous sodium sulfate placed in a funnel plugged with a filter paper. 12. Concentrate the extract by evaporation to dryness under reduced pressure in rotary evaporator using a 35–40 °C water bath. 13. Recover the residue obtained with 1 mL of acetone before analysis. 14. Analyze by HPLC-DAD following authors' protocol. 	Martel and Zeggane (2002)

multiresidue methods which are analytical methodologies for the simultaneous analysis of trace amounts of a large number of analytes. The number of pesticides tested in the sample can be limited in order to get reliable results, higher recoveries, and lower quantification limits.

Several analytical methods have been used to separate and detect pesticides in honey. Gas chromatography (GC) and liquid chromatography (LC) are used for the detection and quantification of pesticide residues (Souza Tette et al., 2016). The choice of the separation technique depends mostly on the characteristics of the pesticides of interest. The volatile, semi-volatile, and thermally stable compounds can be determined by GC, whereas nonvolatile and/or thermally unstable ones should be determined by LC.

At least three steps are required for the analysis of pesticides, among them extraction, separation, and detection. Each one of these steps will be described in the sections that follow.

5.2.1. Sampling

The samples to be analyzed must be representative of the entire honey batch in question. The different steps for the sampling of honey are as follow:

- (1) If the honey sample contains impurities (e.g., wax), the sample should be filtered through a stainless-steel sieve. If needed, the honey can be gently pressed through the sieve with a spatula.
- (2) If a honeycomb is sampled, the honeycomb is drained through a 0.5 mm sieve without heating in order to separate honey from the comb.
- (3) For crystallized honey, the sample is homogenized with a spatula and an analytical test portion is collected by coring of the honey.
- (4) Honey must be homogenized before analysis.
- (5) According to the protocol of sample preparation applied for extraction of pesticide residues in honey, collect 1–20 g honey into a centrifuge tube or a beaker for sample preparation.

5.2.2. General requirements for pesticide residue analyses

- (1) All analyses should include negative control honey (honey free from pesticide residues—the “blank”) and matrix calibration standards prepared by adding pesticides to blank honey before pretreatment of the samples.
- (2) The sample spiked with pesticides at the level corresponding to the limit of quantification (LOQ) for

Table 6. Analysis of pesticide residues in honey using a solid-phase extraction (SPE).

Pesticide analyzed	Extraction protocol	References
450 pesticides	<ol style="list-style-type: none"> 1. Dilute 15 g of the test sample in a 250 mL glass jar with 30 mL of water. 2. Shake for 15 min at 40 °C in a shaking water bath. 3. Add 10 mL of acetone to the jar. 4. Transfer the jar contents to a 250 mL separating funnel. 5. Rinse the jar with 40 mL of dichloromethane and transfer this rinse to the separating funnel for partitioning. 6. Shake the funnel eight times and pass the bottom layer through a funnel containing anhydrous sodium sulfate into a 200 mL pear-shaped flask. 7. Add 5 mL of acetone and 40 mL of dichloromethane into the separating funnel. 8. Repeat steps 6 and 7 twice. 9. Evaporate the organic phase to about 1 mL with a rotary evaporator at 40 °C for clean-up. 10. Add sodium sulfate into a graphitized carbon black cartridge to about 2 cm. 11. Connect the cartridge to the top of the aminopropyl cartridge in series. 12. Condition the cartridges with 4 mL acetonitrile/toluene 3:1 (v/v). 13. Add 1 mL of the sample. 14. Rinse the pear-shaped flask with 3 × 2 mL acetonitrile/toluene 3:1 (v/v) and decant it into the cartridges. 15. Elute the pesticides with 25 mL acetonitrile/toluene 3:1 (v/v). 16. Evaporate the eluate to about 0.5 mL using a rotary evaporator at 40 °C. 17. Analyze by GC-MS or LC-MS/MS following authors' protocols according to the group of pesticides. 	Pang et al. (2006)
Organochlorines (α -, β -, and γ -hexachlorocyclohexane (HCH), alachlor, heptachlor, aldrin, endosulfan II, 4,4'-DDE, dieldrin, endrin and 4,4'-DDD) and primiphos-ethyl (internal standard)	<ol style="list-style-type: none"> 1. Add 10 g of honey in a jar and heat the honey at 35 °C for 15 min. 2. Add 50 mL of distilled water to dissolve honey. 3. Extract with 3 × 30 mL portions of a binary mixture of petroleum ether/ethyl acetate 80:20 (v/v) in a separating funnel. 4. Dry the combined organic extract over anhydrous sodium sulfate. 5. Evaporate to 2 mL with rotary evaporator and transfer into a 5 mL glass tube concentrator. 6. Evaporate to dryness under a stream of nitrogen. 7. Dissolve the dried residue with 0.4 mL of <i>n</i>-hexane. 8. Condition the florisol cartridge with 10 mL of <i>n</i>-hexane. 9. Load the concentrated extract obtained in step 7 onto the cartridge. 10. Elute the pesticides with 25 mL of 20% (v/v) of diethyl ether in <i>n</i>-hexane. 11. Evaporate the eluate to dryness under a stream of nitrogen. 12. Dissolve the dried residue with 0.4 mL of <i>n</i>-hexane containing 1 µg/mL of internal standard. 13. Analyze by GC-MS/MS following authors' protocol. 	Tahboub et al. (2006)
15 organophosphorus (OP), 17 organochlorines (OC), 8 pyrethroids (PYR), 12 N-methyl-carbamate (NMC), bromopropylate and the internal standards: 4-bromo-3,5-dimethylphenyl-Nmethylcarbamate (4-Br-NMC), triphenylphosphate (TPP) and polychlorinated biphenyl (PCB) 209	<ol style="list-style-type: none"> 1. Dissolve 10 g of honey in a 60 mL glass tube with 10 mL of water and 10 mL of acetone. 2. Homogenize the mixture with Ultra Turrax for 2 min. 3. Wash the Ultra Turrax with about 2 mL of acetone and collect the washings into the glass tube. 4. Load the solution obtained in step 3 into an EXTrelut®NT 20 column. 5. Allow to drain for 10 min to obtain an even distribution into the filling material. 6. Elute pesticides with 5 × 20 mL of dichloromethane using the first aliquot to wash the glass tube. 7. Collect the eluate into a 150 mL Erlenmeyer flask. 8. Concentrate to nearly 1 mL with a rotary evaporator at 40 °C (reduced pressure) and by drying manually by rotating the flask. 9. Dissolve the residue in 2 mL of <i>n</i>-hexane. 10. Divide the sample solution obtained in step 9 in two portions. 11. Transfer one portion of the sample (1 mL) into a 25 mL Erlenmeyer flask and dry by manually rotating the flask. 12. Dissolve the residue in 0.5 mL water/acetonitrile 50:50 (v/v). 13. Add the appropriate amount of 4-Br-NMC as an internal standard. 14. Inject the sample into the LC/DD/FI (liquid chromatography-double derivatization coupled with spectrofluorimetric detector) for the determination of NMC pesticides following author's protocol. 15. Add the appropriate internal standards (PCB 209 and TPP) in the second portion of the original sample left in the Erlenmeyer flask (1 mL). 16. Inject this portion in GC-MSD and in GC-FPD following authors' protocols in order to analyze bromopropylate, OC, PYR and OP residues respectively. 	Amendola et al. (2010)

each target analyte must be prepared with other samples to control the sensitivity of the equipment.

- (3) A supplementary test (named “test sample”) is conducted to measure the recovery of all target analytes.
- (4) For each analytical sequence, the extracts are injected into the analytical instrument in the following order: blank solvent, negative control honey (blank sample), samples spiked with pesticides for calibration (the “matrix calibration standards”), blank sample, unknown samples (samples to quantify), test sample (to calculate the recovery for each pesticide) and, again, a spiked sample from the calibration to control any variation during the sequence and blank sample.
- (5) At the end of the sequence, the sample spiked at level I (corresponding to the LOQ) is injected to verify the ability of the equipment to detect the LOQ. Validation of the analytical method should be performed according to the Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed (Document SANTE/11813/2017, 2018).

5.2.3. Sample preparation (extraction and clean-up)

The purpose of the extraction is to extract the substance from the sample with minimum co-extractives matrix interferences. The choice of the solvent is important. Acetone, acetonitrile, ethyl acetate, and hexane are commonly used in this step. The analytes are extracted from the matrix and then, through a clean-up process, the co-extractives are removed.

The most frequent technique used for sample extraction is via the homogenization of the honey sample with an organic solvent or water followed by clean-up with a liquid–liquid extraction (LLE) or a solid phase extraction (SPE) on column. Relatively new techniques like the dispersive solid phase extraction (dSPE) and solid phase microextraction (SPME) are used. Main parameters of methodologies mentioned in the literature for the extraction of pesticides from honey are reported by Rial-Otero et al. (2007) and Souza Tette et al. (2016). Different techniques are presented below with corresponding protocols. The main criterion is to find one method that gives acceptable recoveries for all analytes with one protocol.

5.2.3.1. Liquid-liquid extraction (LLE). In multiresidue methods, an important step is the extraction procedure, especially for complex matrices such as honey, which contains high sugar content. LLE is the most common extraction and purification technique used in the determination of pesticides in honey (Souza Tette et al., 2016). However, LLE usually employs large sample sizes and toxic organic solvents. It is also characterized by the use of multiple sample handling steps, which makes

it susceptible to error and contamination. Furthermore, it usually enables the extraction of analytes belonging to only one chemical class. Despite the disadvantages described above, LLE continues to be used in the analysis of pesticides in honey. Bargańska and Namieśnik (2010) reviewed in their paper the techniques already used to extract pesticides from samples of honey. The general protocol involves dissolving honey in water and applying a LLE with various solvents (such as petroleum ether, hexane, dichloromethane, ethyl acetate or mixtures of petroleum ether/ethyl acetate (Tahboub et al., 2006), acetonitrile/acetone/ethyl acetate/dichloromethane (Rissato et al., 2007), acetone/dichloromethane (Pang et al., 2006), hexane/propanol-2 (Martel & Zeggane, 2002)) to extract pesticides according to the pesticide’s polarity. The experimental procedures are given in Table 5.

The extracts obtained before analysis by chromatography are clean but these methods use a considerable amount of solvent. Furthermore, these methods are limited to the extraction of a few pesticides due to their different solubility.

5.2.3.2. Solid phase extraction (SPE) and dispersive solid phase extraction (dSPE). Sometimes, after a LLE, a clean-up step on a SPE cartridge with different adsorbents may be necessary before quantification of pesticide residues in honey. Protocols using SPE are given in Table 6 for the main classes of pesticides.

The SPE technique (Bargańska & Namieśnik, 2010; Rial-Otero et al., 2007; Singh et al., 2014; Souza Tette et al., 2016) combines in a single step the extraction and the clean-up procedures based on the separation of LC, where the solubility and functional group interactions of sample, solvent, and sorbent are optimized to affect retention and elution. In SPE, the sample is passed through a cartridge or a packed column filled with a solid sorbent where the pesticides are adsorbed and then eluted with an organic solvent. Moderately polar to polar analytes are extracted from nonpolar solvents on polar sorbents. Nonpolar to moderately polar analytes are extracted from polar solvents on nonpolar sorbents. Most multiresidue methods include a clean-up step using adsorption columns on polar sorbents (florisil, alumina, silica gel) for normal-phase SPE and on nonpolar sorbent like C18 for reversed-phase SPE. The reversed-phase C18 cartridge is by far the most common choice used by researchers for the extraction of insecticides, acaricides, fungicides, herbicides, organochlorines, and organophosphorus pesticides from honey (Rial-Otero et al., 2007). As florisil retains some lipids preferentially (25 g florisil with 3% water retains 1 g of fat), it is particularly well suited for the clean-up of fatty foods. When a florisil column is eluted with solvent mixtures of low polarity, nonpolar residues are recovered almost quantitatively (Singh et al., 2014). Florisil sorbent has been used for

pyrethroids, organochlorines and organophosphorus pesticides. Lores et al. (1987) used a silica gel clean-up method for organophosphorus pesticide analysis.

Other SPE clean-up approaches include the combination of GCB (graphitized carbon black) and PSA (primary secondary amine) columns, the combination of C18, GCB and aminopropyl, and the combination of GCB, PSA, and SAX (strong anion-exchange sorbent) columns. GCB is such a sorbent, being nonspecific and generally of hydrophobic nature. Contrary to sorbents based on SiO₂, these may be used without the pH of the treated solutions being considered. Because of difficulties with elution of certain planar or aromatic pesticides from GCB, only PSA is used for very efficient clean-up of acetonitrile extracts. The dSPE with PSA is effectively used to remove many polar matrix components, such as organic acids, certain polar pigments, and sugars. Thus, the PSA clean-up method is selected as the most efficient for cleaning honey samples.

The choice of one sorbent or another depends on the analyte polarity and on the possible co-extracted interferences. Sample pH can be critical to obtain high yields of pesticide retention in the sorbent. Thus, in some cases, sample pH modification can be necessary in order to stabilize the pesticides and increase their absorption in the solid phase. Once the pesticides have been retained in the SPE cartridges, they are then eluted with an organic solvent such as acetone, dichloromethane, ethyl acetate, hexane, methanol, tetrahydrofuran or mixtures of hexane/ethyl acetate, hexane/dichloromethane, and methanol/water or methanol/ethyl acetate/dichloromethane.

There is also a method based on SPE, the on-column liquid-liquid extraction (OCLLE) or liquid-liquid extraction on a solid support (SLE), a technique based on classical LLE principle, but assisted by inert solid support (Pirard et al., 2007). This inert matrix consists of diatomaceous earth, well-known for its high porosity, its high dispersing capacities and its high capacity for aqueous adsorption. Pesticides in honey were studied by Amendola et al. (2010) using EXtrelut®NT 20 columns packed with a specially processed wide pore kieselguhr with a high pore volume as support for the repartition process. Honey is dissolved in a mixture of water and acetone and is loaded into an EXtrelut®NT 20. The majority of the co-extractive compounds are retained on the adsorbent material of the column, while the pesticides are eluted by dichloromethane (Amendola et al., 2010). Other columns can be used such as ChemElut 5 mL cartridges (Kujawski et al., 2014).

5.2.3.3. Quick Easy Cheap Effective Rugged and Safe (QuEChERS). The QuEChERS method consists in salting-out LLE using acetonitrile, MgSO₄, and NaCl salts and a dSPE step based on primary and secondary amine bonded silica (PSA) to remove co-extractive impurities

(U.S. EPA, 2013; Bargańska & Namieśnik, 2010). The QuEChERS method is particularly applied for the determination of polar, middle polar, and nonpolar pesticide residues in various matrices. This method is combined with sensitive analytical techniques such as LC-MS/MS and GC-MS/MS. Tomasini et al. (2012) demonstrated that the matrix effect depends on the floral origin of honey samples and that quantification by the standard addition method in blank matrix is needed.

With this approach, the sample should be >75% water. Then, an initial dissolution of the honey sample is required. Acetonitrile is used as the water-miscible extraction solvent and the separation phase is achieved by the addition of MgSO₄. The heat produced by the water binding process promotes extraction to acetonitrile. The addition of NaCl also increases the extraction efficiency (Kujawski et al., 2014). The supernatant is further extracted and cleaned using a dSPE technique. The dSPE centrifuge tube format (available in 2 mL and 15 mL sizes) contains magnesium sulfate (to remove residual water) and PSA sorbent (to remove sugars and fatty acids). These tubes are available with or without GCB (to remove pigments and sterols) and/or C18-EC (endcapped) packing (to remove nonpolar interferences such as lipids). Blasco et al. (2011) showed that the QuEChERS method presented the highest recoveries (mean recovery 91.67%) followed by the SPE (mean recovery 90.25%) whereas the solid-phase microextraction (SPME) showed the lowest recovery (mean recovery of 49.75%) for the pesticides studied. Thus, the QuEChERS method was the most adapted method with around 58% of recoveries >90%.

The QuEChERS multiresidue procedure replaces many complicated analytical steps commonly employed in traditional methods with easier ones. The QuEChERS method has been the most commonly used method for the analysis of pesticides in honey. However, one limitation is that the sample should be >75% water; thus, an initial dissolution of the honey sample is required, which leads to lower concentration of the sample compared to other sample preparation techniques. In order to overcome this limitation, Wiest et al. (2011) added a sample concentration step by evaporation which was satisfactory for extraction of organohalogenes, organophosphorus, pyrethroids, and insect growth regulators in honey. According to these authors, evaporation may be necessary when the MRL is lower than the method LOQ. A general protocol is given in the Table 7.

5.2.3.4. Solid phase microextraction (SPME). SPME is a rapid and simple procedure of extraction that can be easily automated and does not need an organic solvent. This technique consists of two separate steps: an extraction step and a desorption step. Both steps must be optimized for the procedure to be successful (Singh et al., 2014).

Table 7. Analysis of pesticide residues in honey using the QuEChERS methodology.

Pesticide analyzed	Extraction protocol	References
12 organophosphorus and carbamates insecticides (bromophos-ethyl, chlorpyrifos-methyl, chlorpyrifos-ethyl, diazinon, fenoxycarb, fonofos, phenthoate, phosalone, pirimiphos-methyl, profenofos, pyrazophos and temephos)	<ol style="list-style-type: none"> 1. Weigh 1.5 g of honey into a 50 mL polypropylene centrifuge tube. 2. Add 3 mL of hot water and vortex until dissolution. 3. Add 3 mL of acetonitrile to the sample and shake the tube vigorously by hand for 30 s. 4. Pour the sample and extract into the appropriate tube containing 6 g of MgSO₄ and 1.5 g of NaCl. 5. Shake the tube vigorously by hand for 1 min (avoiding formation of oversized MgSO₄ agglomerates). 6. Centrifuge the tube at 3000 rpm for 2 min. 7. Transfer 1 mL of acetonitrile extract (upper layer) to the dispersive-SPE tube containing 150 mg anhydrous of MgSO₄ and 50 mg of PSA. 8. Vortex the dSPE tube for 30 s and centrifuge at 3000 rpm for 2 min. 9. Transfer 0.5 mL of the final extract into the labeled autosampler vial. 10. Analyze by LC-MS/MS following authors' protocol. 	Blasco et al. (2011)
80 environmental contaminants	<ol style="list-style-type: none"> 1. Weigh 5 g of honey in a 50 mL centrifuge tube. 2. Add 10 mL of water in the centrifuge tube. 3. Shake the tube to dissolve honey. 4. When the mixture is homogeneous, add 10 mL of acetonitrile (ACN), 4 g of anhydrous MgSO₄, 1 g of sodium chloride, 1 g of sodium citrate dihydrate and 500 mg of disodium citrate sesquihydrate. 5. Shake the tube immediately by hand. 6. Vortex one minute and then centrifuge for 2 min at 5000 rpm. 7. Transfer 6 mL of supernatant in a pre-prepared 15 mL PSA tube (900 mg of anhydrous MgSO₄, 150 mg of PSA bonded silica). 8. Then, shake the tube immediately by hand. 9. Vortex 10 s and centrifuge for 2 min at 5000 rpm. 10. Evaporate 4 mL of the extract in a 10 mL glass cone-ended centrifuge tube until 50 µL are left. 11. Kept the remaining extract at -18 °C until analysis by LC-MS/MS or GC-MS following authors' protocols according to the group of pesticides. 	Wiest et al. (2011)
Chlorothalonil, heptachlor, captan, α-endosulfan, β-endosulfan, endosulfan sulfate, and dieldrin	<ol style="list-style-type: none"> 1. Place 10 g of honey sample into a polypropylene tube (50 mL) of conical base. 2. Homogenize with 10 mL of high purity water. 3. Add 15 mL of 1% acetic acid in ethyl acetate extraction solvent, also containing 6 g of MgSO₄ and 1.5 g of CH₃COONa anhydrous to the tube. 4. Shake by hand vigorously for 1 min. 5. Centrifuge at 5000 rpm for 5 min. 6. Transfer an aliquot of 1 mL of the supernatant to a 2 mL polypropylene tube containing 50 mg of PSA and 150 mg of MgSO₄. 7. Shake by hand vigorously for 30 s and centrifuge at 5000 rpm for 5 min. 8. Put 500 µL of the extract obtained in step 7 into the 1.5 mL vial and complete with 500 µL of ethyl acetate. 	Vilca et al. (2012)

(Continued)

Table 7. (Continued).

Pesticide analyzed	Extraction protocol	References
200 pesticides	<ol style="list-style-type: none"> 9. Analyze by GC-μECD following authors' protocol. 1. Weigh 5 g of the honey sample into a 50 mL polypropylene centrifuge tube. 2. Add 10 mL of deionized water. 3. Vortex and incubate in a water bath at 40 °C until complete homogeneity is obtained. 4. Add 10 mL of acetonitrile into the tube. 5. Shake the content for 1 min using a mechanical shaker. 6. Add the QuEChERS salt kit (containing 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate and 0.50 g of sodium hydrogen citrate sesquihydrate) and immediately shake for further 1 min. 7. Centrifuge at 15,000 rpm at 4–8 °C for 5 min. 8. Transfer the whole acetonitrile fraction into a 15 mL dSPE polypropylene tube (containing 150 mg of anhydrous magnesium sulfate and 25 mg of PSA). 9. Shake the tube for 1 min and centrifuge for 2 min at 15,000 rpm using a cooling centrifuge. 10. Transfer 2 mL of the supernatant into 50 mL round bottom glass flask. 11. Evaporate under vacuum at 40 °C till complete dryness. 12. Reconstitute the residue into 2 mL of hexane/acetone 9:1 (v/v). 13. Ultra-sonicate and filter the sample through a disposable 0.45 μm PTFE membrane filter into an amber glass vial. 14. Analyze by GC-MS/MS following the authors' protocol. 	Shendy et al. (2016)

A fused silica fiber coated with a polymeric film is immersed into an aqueous sample for a given amount of time. The analyte enrichment is by partitioning between the polymer and the aqueous phase according to their distribution constant. Factors influencing the extraction step include fiber type, extraction time, ionic strength, sample pH, extraction temperature, and sample agitation. The pesticides are adsorbed into the stationary phase and later thermally desorbed into the injection port of a gas chromatograph (Rial-Otero et al., 2007). Variables affecting the desorption step include temperature, desorption time, focusing oven temperature, the solvent used, and its volume. Most pesticides have been extracted with polydimethylsiloxane (PDMS, 100 μ m) fiber.

The PDMS has the following advantages: (i) enhanced reproducibility, (ii) lower detection limits, (iii) extended linearity, (iv) improved correlation coefficients, (v) low extraction time, and (vi) better chromatograms. The use of a sol-gel CROWN ETHER® fiber (40 μ m) was also proposed by Yu et al. (2004) to remove 11 organophosphorus pesticides from honey with good relative recoveries (74–105%) and low detection limits (<0.001 mg/kg). Blasco et al. (2011) employed silica fibers coating with 50 μ m carbowax/template resins

(CW/TPR) to analyze fenoxycarb, penthoate, temephos, fonofos, diazinon, pyrazophos, phosalone, profenofos, pirimiphos-ethyl, bromophos-ethyl, chlorpyrifos-methyl, and chlorpyrifos-ethyl in honey. The SPME is accurate as a monitoring method for the extraction of the selected pesticides from honey but cannot be implemented as currently applied as a quantification method due to its low recovery for pyrazophos, chlorpyrifos-methyl, temephos, and bromophos-ethyl. The application of the internal standard should be considered.

5.2.3.5. Stir bar sorptive extraction (SBSE). SBSE is a technique theoretically similar to SPME. It has been used with success for the extraction of organic compounds from aqueous food, biological, and environmental samples (Rial-Otero et al., 2007).

For SBSE, a stir bar is coated with a sorbent and immersed in the sample to extract the analyte from solution. The sample is stirred for a given time until the analyte reaches equilibrium between the polymer and the aqueous phase according to their distribution constant. Then, the analytes are desorbed by high temperatures into the injector port of the GC or by liquid removal for liquid chromatography-tandem mass spectrometry (LC-MS) analysis. Blasco et al. (2004) have

Table 8. Analysis of pesticide residues in honey using the SBSE protocol (Blasco et al., 2004).

Pesticide analyzed	Extraction protocol
6 organophosphorus insecticides (chlorpyrifos-methyl, diazinon, fonofos, phenthoate, phosalone, and pirimiphos-ethyl)	<ol style="list-style-type: none"> 1. Place 2.5 g of honey into a 50 mL glass beaker. 2. Dilute 1/10 ratio with water and homogenize over 15 min using a magnetic stirring bar coated with PDMS. 3. Carry out the sorption for 120 min while stirring at 900 rpm. 4. Remove the stir bar from the aqueous sample with tweezers. 5. Perform the desorption of the analytes into 2 mL vial filled with 1 mL of methanol. 6. Perform the desorption of the pesticides by agitating for 15 min. 7. Inject 5 μL of this extract into the LC-MS system following the authors' protocol.

applied this technique for the extraction of six organophosphorus pesticides from honey. These authors also compared the use of SBSE with SPME and concluded that although linearity and precision obtained by both techniques are similar, SBSE is more accurate and sensitive and the effect of honey matrix in the quantification is lower than that of SPME.

The most important advantages of SBSE are the same as those for SPME; however, higher recoveries are obtained with SBSE (Blasco et al., 2004) because of the thicker polydimethylsiloxane (PDMS) coating. The procedure is presented in Table 8. Nevertheless, recoveries are less than those obtained using other techniques (LLE, SPE, QuEChERS).

5.2.3.6. Dispersive liquid-liquid microextraction (DLLME). In 2006, the use of DLLME was developed in the field of separation science for the preconcentration of organic and inorganic analytes from aqueous matrices. The basic principle of DLLME is the dispersion of an extraction solvent (immiscible with water) and a disperser solvent (miscible in water and extraction solvent) in an aqueous solution that provides a large contact area between the aqueous phase and the extraction solvent. In DLLME, extraction and dispersive solvents are simultaneously and rapidly injected into the aqueous sample using a syringe. The main advantages of DLLME over conventional techniques are simplicity of operation, rapidity, low cost, easy handling, low consumption of organic solvents, high recovery, high factor enrichment, and compatibility with chromatographic techniques such as liquid chromatography (LC) and gas chromatography (GC). However, the QuEChERS method is demonstrated to be more robust and more suitable for the determination of pesticides in complex samples (Tomasini et al., 2011). A simple DLLME protocol for the determination of 15 organochlorine pesticides residues in honey is proposed by Zacharis et al. (2012) and is described in Table 9. The final DLLME protocol involves the addition of 750 μ L acetonitrile (disperser) and 50 μ L chloroform (extraction solvent) into a 5 mL aqueous honey solution followed by centrifugation. The sedimented organic phase (chloroform) is analyzed directly by GC-IT/MS (gas chromatography-ion

trap mass spectrometry) or evaporated and reconstituted in acetonitrile prior to the GC-ECD analysis.

5.2.4. Chromatographic detection

The quantification is performed by gas or liquid chromatography (Bargańska & Namieśnik, 2010; Rial-Otero et al., 2007) according to the characteristic of the analyte: (i) Gas chromatography (GC) with different detectors: electron capture (ECD), nitrogen-phosphorus (NPD) and mass spectrometry (GC-MS or GC-MS/MS); (ii) liquid chromatography coupled with mass spectrometry (LC-MS/MS).

Traditionally nonpolar and middle polar pesticides are analyzed with GC. This is the case for organochlorines, pyrethroids, and organophosphorus pesticides. With the emergence of new pesticides (e.g., neonicotinoids) and due to their physicochemical properties, the LC is used.

For the multiresidue analysis of pesticides, the most convenient detector would be a mass spectrometer (MS/MS, MS/TOF) coupled with either GC or LC, depending on the type of pesticides of interest. Volatile, semi-volatile, and thermally stable ones can be determined by GC, whereas nonvolatile and/or thermally unstable ones should be determined by LC. When there is a positive result, it has to be confirmed using mass spectrometry coupled with chromatography (GC-MS/MS and LC-MS/MS). Mass spectrometry coupled to a chromatographic separation method is a very powerful combination for identification of an analyte in the sample extract.

The choice of the GC column is a very important task in pesticide analysis. The stationary phase should be selected as a function of the polarity of the pesticides. Nonpolar columns (5% phenyl, 95% dimethylpolysiloxane) are the most commonly used for pesticide analysis in honey (e.g., DB-5, HP-5MS, and DB-XLB). Furthermore, other column parameters such as length, inner diameter, or film thickness can be optimized as a function of the number of pesticides to be determined simultaneously. Usually, the parameters of the column are: 30 m \times 0.25 mm \times 0.25 μ m (length \times inner diameter \times film thickness). In LC, a C18 column (e.g., 4.6 and 2.1 mm i.d.) is almost consensus for the separation of pesticides.

Table 9. Analysis of pesticide residues in honey using the DLLME protocol.

Pesticide analyzed	Extraction protocol	References
15 pesticides (etridiazole, chloroneb, propachlor, trifluralin, hexachlorobenzene, chlorothalonil, cyanazine, chlorpyrifos, DCPA, trans-chlordane, cis-chlordane, trans-nonachlor, chlorobenzilate, cis-permethrin, trans-permethrin)	<ol style="list-style-type: none"> 1. Prepare a solution of honey at 50 g/L (dissolve 10 g of honey in a flask with 200 mL of water). 2. Leave the sample to equilibrate for at least for 15 min prior to performing the DLLME extraction. 3. Transfer an aliquot of 5 mL of the diluted sample into a 10 mL screw cap glass tube with conical bottom. 4. Inject rapidly into the sample solution a mixture of 750 μL acetonitrile (dispenser) and 50 μL chloroform. 5. Gently shake the mixture by hand for 1 min. 6. Extract the pesticides and the ISTD (1-bromo decahexane) from the aqueous matrix/phase into the fine chloroform microdroplets. 7. Centrifuge the mixture for 3 min at 2500 rpm for phase separation. 8. Remove the sedimented chloroform volume using a microsyringe. 9. Transfer the extract into an autosampler vial with 50 μL insert and 2 μL of the organic solvent; directly inject this into the GC-IT/MS (GC with ion-trap mass spectrometer detector) following the authors' protocol. 10. For GC-ECD analysis, evaporate 20 μL of chloroform extract (obtained in step 8) to dryness by a gentle stream of nitrogen. 11. Reconstitute the sample by the same volume (20 μL) of acetonitrile. 	Zacharis et al. (2012)
Aldrin, endrin, lindane, 2,4'-DDT, 2,4'-DDD, 2,4'-DDE, 4,4'-DDT, 4,4'-DDE and α -endosulfan	<ol style="list-style-type: none"> 1. Dissolve in a centrifuge tube 0.5 g of a homogenized honey sample with 3 mL of ultrapure water. 2. Prepare and inject rapidly a mixture of 450 μL acetone (dispenser solvent) and 100 μL chloroform (extract) into the sample to obtain an emulsion. 3. After 20 s (including 5 s of shaking), centrifuge the sample (5 min, 4000 rpm). A two-phase solution is obtained. 4. The resulting volume of sediment phase is 80 μL. During the extraction, a precipitate formed between chloroform and aqueous phase. 5. Collect the chloroform phase at the bottom of the conical vial with a microlitre syringe. 6. Analyze by GC-MS (2 μL of sample are injected) following the authors' protocol. 	Kujawski et al. (2012)

6. Antibiotics and chemotherapeutics in honey

Honey is generally considered as a natural, healthy, and residue-free product. However, in the early 2000s, some imported honeys were often contaminated with antimicrobial residues, even with residues of chloramphenicol, a forbidden substance (Reybroeck, 2018). Importers of honey started with regular screening of honey for residues and food authorities increased the number of honey samples at border inspection posts and in national monitoring plans. In the beginning, the monitoring was focused on streptomycin, chloramphenicol, tetracyclines, and sulfonamides. Later, the scope was enlarged to include other compounds. Presently, honey is monitored for a large list of antibiotics and chemotherapeutics of interest in apiculture: tetracyclines (oxytetracycline), aminoglycosides (streptomycin), sulfonamides (sulfamethazine, sulfathiazole, sulfadiazine,

sulfamethoxazole, sulfamerazine, sulfadimethoxine), macrolides (tylosin, erythromycin), lincosamides (lincomycin), amphenicols (chloramphenicol), nitrofurans (furazolidone, furaltadone, nitrofurazone), nitroimidazoles (metronidazole), fluoroquinolones (enrofloxacin (ciprofloxacin), norfloxacin), and fumagillin (Reybroeck et al., 2012).

In general, residues of antimicrobials in honey originate from apicultural use since bee diseases caused by microorganisms such as American (*Paenibacillus larvae*, de Graaf et al. 2013) and European foulbrood (*Melissococcus plutonius*, Forsgren et al. 2013) can be cured by anti-infectious agents. Also nosemosis, caused by spores of the fungi or fungi-related *Nosema apis* or *N. ceranae* (Fischer & Palmer, 2005; Fries et al. 2013) is sometimes treated with antimicrobials like sulfonamides.

High levels (mg/kg or ppm) of antimicrobial residues of veterinary drugs applied to hives can be found in honey, especially the first week after dosing (Reybroeck et al., 2012). Afterward, the concentration of residues in honey decreases via a dilution effect by incoming nectar and consumption of contaminated honey by the bees. Some compounds (oxytetracycline, tylosin, furazolidone) are also degraded by metabolization. In contrast to other food producing animals, honey bees do not metabolize the drugs so residues could remain in the honey for more than a year (Reybroeck et al., 2012), see Table 10.

There are some cases reported of residues of antimicrobials in the honey due to agricultural practices, beekeeping practices, and environmental or fraud issues (Reybroeck, 2014): contaminated nectar from fruit trees treated with streptomycin against fireblight, natural production of streptomycin by certain *Streptomyces* bacteria, robbery by bees of contaminated honey, feeding bees contaminated honey, mixing clean honey with honey containing residues, the collection by bees of medicated drinking water from farms or surface water from fields where antibiotic-containing manure has been spread, contamination of nectar with sulfanilamide as a degradation product of the herbicide asulam, migration of residues from polluted wax foundation, semicarbazide formed from azodicarbonamide (ADC: a blowing agent used in the manufacturing of plastic gaskets in metal lids), and finally semicarbazide in heather honey formed from elevated arginine levels.

For residue analysis in honey, it is worth noting that some pharmacologically active compounds metabolize or degrade in honey. Thus, it is important to look for the suitable marker residue. For example, honey should be screened for both tylosin A and desmicosin (tylosin B) (Thompson et al., 2007). An overview of the most suitable marker residues for some antimicrobials of interest in beekeeping is given in Table 11. Reybroeck (2018) published a review about residues of antibiotics and chemotherapeutics in honey. This review can be a basis to decide which compounds to analyze in honey.

6.1. Legislation regarding residues of veterinary drugs in honey

MRLs were established for residues of veterinary medicinal products in foodstuffs of animal origin in order to protect public health. No MRLs have been established so far for antibiotics and sulfonamides in honey ("Commission Regulation (EU) No 37/2010 of 22 December 2009," 2009). This leads to the interpretation that the use of antibiotics in beekeeping is not permitted in the EU. However, based on the "cascade" system which is open to all animal species (including honey bees), antibiotics can be used for the treatment of bee diseases (Anonymous, 2007b) on condition that the active substance concerned is registered as an allowed substance in

Table 1 in the Annex of Commission Regulation (EU) No 37/2010, and a withholding period has been specified by the prescribing veterinarian. In this period, no honey could be harvested for human consumption. In reality, a very long withdrawal period needs to be considered.

Some EU Member States (Belgium, France) applied action limits, recommended target concentrations, nonconformity, or tolerance levels for antimicrobial residues in honey (Reybroeck et al., 2012). Presently, in application of the Commission Implementing Regulation (EU) 2018/470 for honey produced within the EU, the MRL to be considered for control purpose shall be the lowest of all the MRLs established for other target tissues in any animal species. However, some Member States do not allow treatment with antibiotics and chemotherapeutics under "cascade" in beekeeping which is making international honey trade more complex.

For certain prohibited or unauthorized analytes in food of animal origin, the regulatory limit is the Minimum Required Performance Limit (MRPL) or the Reference Point for Action (RPA). MRPLs were fixed to harmonize the level of control of those substances and to ensure the same level of consumer protection in the Community. So far in honey, a MRPL of 0.3 µg/kg was set for chloramphenicol (European Communities Commission, 2003), while the MRPL of 1 µg/kg for nitrofurans (furazolidone, furaltadone, nitrofurantoin, and nitrofurazone) metabolites in poultry meat and aquaculture products generally is considered as also applicable in honey (SANCO, 2004).

For the importation of products of animal origin from third countries, the MRPLs should be employed where they exist as RPAs to ensure a harmonized implementation of Council Directive 97/78/EC (Commission, 2005).

In a guidance paper of the Community Reference Laboratories (CRLs), recommended concentrations for testing were suggested for the harmonization of the performance of analytical methods for national residue control plans for substances without MRLs (Anonymous, 2007a). An overview of European regulatory limits and recommended concentrations for testing in honey for residues of antibiotics and chemotherapeutics of interest for use in beekeeping is given in Table 11.

In the USA, there are no authorized residue limits for antibiotics in honey despite the authorized use of certain antibiotic drugs (oxytetracycline, tylosin, and lincomycin) in beekeeping (Administration, 2017).

In Europe, for national residue monitoring plans, groups of substances that need to be monitored in honey are indicated ("Council Directive 96/23/EC of 29 April 1996," 1996). For honey, it concerns group B1 (antibacterial substances, including sulfonamides and quinolones), B2c (other veterinary drugs - carbamates and pyrethroids), B3a and B3b (organochlorine and organophosphorus compounds), and B3c (chemical elements).

Table 10. Classification of some veterinary drugs based on their stability in honey.

Compounds that do not degrade in honey	Compounds metabolizing or degrading in honey
Streptomycin	Tetracyclines: epimerization to 4-epimers
Sulfa drugs	Tylosin: degradation to desmycosin (tylosin B)
Lincomycin	Furazolidone: metabolization to AOZ*
Chloramphenicol	

*Note: AOZ: 3-amino-2-oxazolidone.

Despite not being indicated, honey should also be monitored (“essential”) for prohibited substances (group A6) such as chloramphenicol, nitrofurans, and nitroimidazoles (Anonymous, 2017).

Sampling rules are given in Annex IV of the Council Directive 96/23/EC. The same Directive specifies the frequencies and level of sampling while the Commission Decision 97/747/EC (European Communities Commission, 1997) provides levels and frequencies of sampling. The number of honey samples to be taken each year must equal 10 per 300 t of the annual production for the first 3,000 t of production, and one sample for each additional 300 t. Rules for official sampling procedures and the official treatment of samples until they reach the laboratory responsible for analysis are given in Commission Decision 98/179/EC (Commission, 1998).

6.2. Determination of residues of antibiotics and chemotherapeutics in honey

6.2.1. Sampling

The analyzed honey samples should be representative of the honey lot.

- (1) Crystallized honey should be homogenized before starting the analysis. This can be done by placing the sample in a water bath at maximum 40 °C until the honey is fully liquified and all of the sugar crystals are dissolved.
- (2) The honey should be homogenized before starting the analysis by stirring thoroughly (at least 2 min).
- (3) In the event the honey contains extraneous matter, the honey should be strained through a stainless-steel sieve with a mesh diameter of 0.5 mm.
- (4) If needed, the honey could be gently pressed through the sieve with a spatula.
- (5) Comb honey needs to be uncapped and drained through a 0.5 mm sieve without heating in order to separate honey from the comb.

6.2.2. Sample pretreatment

Some honeys require a special sample pretreatment. In honey, sulfonamides tend to bind sugars via the formation of N-glycosidic bonds through their aniline group (Sheth et al., 1990). Therefore, the sulfonamides need to be released from the sugar concentrates by

hydrolysis using strong acids (Schwaiger & Schuch, 2000) prior to analysis. Otherwise, it is possible that the sulfonamides are missed or underestimated.

6.2.3. General remarks for honey testing on antimicrobial residues

It is necessary to integrate at least one negative and one positive control sample in each test run. The negative honey is honey free from antimicrobial residues and, by preference, a mixture of the different types of honey (origin, color, and texture) all tested as negative in prior analysis. The positive control sample is prepared by contaminating blank honey with standard material. If commercial kits are used, follow the kit instructions set by the kit manufacturer and validate the method internally before using the method in routine testing. In Europe, validation should be performed according to Commission Decision 2002/657/EC (European Union Commission, 2002). For screening methods, the guidelines published by the Community Reference Laboratories for Residues could also be taken into account (Anonymous, 2010).

6.2.4. Microbiological screening tests

Microbiological screening is not often used for screening honey for antibiotic residues, this due to the very low detection capabilities ($CC\beta$) that need to be reached because of the issue of zero-tolerance for antimicrobial residues in honey applied in many countries. The high sugar content in this special matrix makes the use of microbial inhibitor tests also less evident. A broad-spectrum detection of antimicrobials in honey by Eclipse 50 (ZEULAB, Zaragoza, Spain) and PremiTest (R-Biopharm AG, Darmstadt, Germany) was suggested by Gaudin et al. (2013). In their validation, a high false positive rate of 5 and 14%, respectively, was observed. This study also showed that the detection capabilities are not in line with the action or reporting limits or recommended concentrations for testing for different compounds (e.g., streptomycin) applied in some European countries. The extraction and test procedure for the Eclipse 50 and PremiTest for honey testing are given in Tables 12 and 13, respectively.

Noncommercial microbiological methods, two for the detection of tetracyclines and one for the detection of tylosin, were published for honey based on the use of *Bacillus cereus* (Gordon, 1989), *Bacillus subtilis*

Table 11. Overview of European regulatory limits in honey and recommended concentrations for testing for residues of anti-infectious agents of interest for use in beekeeping.

Group	Substance	Marker residue	MRL (in µg/kg)	Recommended concentration for testing (in µg/kg)
Amphenicols	chloramphenicol	chloramphenicol	— ^a	0.3, MRPL
Nitrofurans	group (furazolidone, ...)	AOZ, AHD, SEM and AMOZ	— ^a	1, MRPL
Nitro-imidazoles	ronidazole	hydroxy-metabolites	— ^a	3
	dimetridazole	hydroxy-metabolites	— ^a	3
	metronidazole	hydroxy-metabolites	— ^a	3
Tetracyclines	tetracyclines	sum of parent drug and its 4-epimer	— ^b , 100 ^c	20
Sulfonamides (all substances belonging to the sulfonamide group)	sulfonamides	the combined total residues of all substances within the sulfonamide group	— ^b , 100 ^c	50
Aminoglycosides	streptomycin	streptomycin	— ^b , 200 ^c	40
Macrolides	erythromycin	erythromycin A	— ^b , 40 ^c	20
	tylosin	tylosin A	— ^b , 50 ^c	20

Note: MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 of the European Parliament and of the Council (The European Parliament & the Council of European Union, 2009) and Commission Regulation (EU) No 37/2010 and amendments as of August 1, 2019 (Commission, 2010); MRPL: Minimum Required Performance Limit, Commission Decision 2003/181/EC (European Communities Commission, 2003); Recommended concentration for testing: CRL guidance paper (Anonymous, 2007a); AOZ: 3-amino-2-oxazolidone; AHD: 1-aminohydantoin; SEM: semicarbazide; AMOZ: 3-amino-5-morpholinomethyl-2-oxazolidone; a: prohibited substance, Table 2 in Annex of Commission Regulation (EU) No 37/2010 (Commission, 2010); b: no MRL fixed in honey; c: MRL based on application of the Commission Implementing Regulation (EU) 2018/470 for honey produced within the EU.

Table 12. Extraction and test protocol for the Eclipse 50 for honey (Gaudin et al., 2013).

Extraction protocol	
1.	Weigh 2 g of honey in a 15 mL centrifuge tube.
2.	Add 5 mL of acetonitrile/acetone (v/v, 70/30).
3.	Mix for 30–40 s using a vortex.
4.	Incubate at 62.5 ± 2.5 °C for 5 min \pm 30 s to dissolve honey completely.
5.	Centrifuge at 4000 rpm for 15 min.
6.	Transfer the supernatant to a clean tube.
7.	Evaporate under a nitrogen stream at 55 °C for 15–20 min.
8.	Dissolve the residue in 250 µL of UHT consumption milk using a vortex for 1 min.
Test protocol	
1.	Transfer 50 µL of extract into an Eclipse 50 microplate well and seal the wells.
2.	Incubate at 65 °C until the negative control changes color or when the absorbances for the negative control are between 0.2 and 0.4 (absorbance = difference of measurement at 590 nm (filter 1) and 650 nm (reference filter)).
3.	Interpret the end color: <ul style="list-style-type: none"> • visual reading: yellow is negative; purple is positive; intermediate color is doubtful. • optical reading (measurement of absorbance = difference of measurement at 590 nm (filter 1) and 650 nm (reference filter)): absorbance > absorbance for negative control + 0.2: positive.

Table 13. Extraction and test protocol for the Premitest for honey (Gaudin et al., 2013).

Extraction protocol	
1.	Weigh 2 g of honey in a 15 mL centrifuge tube.
2.	Add 5 mL of acetonitrile/acetone (70/30).
3.	Mix for 30–40 s (vortex).
4.	Sonicate for 5 min.
5.	Vortex for 30–40 s.
6.	Centrifuge at 4500 rpm for 10 min at 4 °C.
7.	Remove the supernatant.
8.	Evaporate under a nitrogen stream at 40–45 °C.
9.	Resuspend the residue in Lab Lemco broth 8 g per L (Oxoid Cat No. CM0015) and mix well.
Test protocol	
1.	Transfer 100 µL of this mixture into the PremiTest ampoule.
2.	Incubate at 64 °C until the negative control changes color.
3.	Interpret the end color: <ul style="list-style-type: none"> • visual reading: yellow is negative; purple is positive; intermediate color is doubtful. • instrumental reading with flatbed scanner and Premiscan software: z-value <0: negative; z-value \geq0: positive.

Table 14. Extraction and test protocol for the Chloramphenicol Residue Rapid Test Device.

Extraction procedure

1. Weigh 4 g of honey into a 15 mL centrifuge tube.
2. Add 1 mL PBS A and 1 mL PBS B orderly, shake to dissolve fully.
3. Add 8 mL ethyl acetate, cap and mix by inversion for approx. 8 min.
4. Keep still for clear separation, transfer 5 mL of supernatant into a 5 mL centrifuge tube.
5. Dry it at 65 °C with a stream of air evaporator or nitrogen evaporator.
6. Dissolve the dried residue in 160 µL of CAP PBST buffer.

Test protocol

1. Use a dropper to collect at least 3 drops (100 µL) of prepared sample, hold the dropper vertically and transfer 3 full drops (around 100 µL) of solution to the specimen well (S) of the test kit.
2. Start the timer.
3. Wait for red bands to appear.
4. Interpret the result; result should be read in approximately 3–5 min:
 - visual reading – the test line (T) is the same as or darker than the control line (C): negative; the test line (T) is lighter than the control line (C) or there is no test line, it is positive; reference line fails to appear: invalid.

ATCC6633 (Khismatoullin et al., 2003), and *Micrococcus luteus* ATCC 9341 (Khismatoullin et al., 2004), respectively. However, the preparation of the test medium and the testing procedures themselves are not fully and clearly described.

6.3. Immunological and receptor assays

6.3.1. Lateral flow devices

For use at the apiary, on-site honey tests in the format of lateral flow devices based on the technology of Colloidal Gold Immune Chromatographic Strip Assay (GICA) are commercially available for the detection of residues of several veterinary drugs in honey from Nankai Biotech Co., Ltd. (Binjiang District, Hangzhou, Zhejiang, China). These test kits are individually packed and contain all the reagents needed. Different test devices are available for Chloramphenicol, Streptomycin, Tetracyclines, Sulfadiazine, Sulfaguanidine, Sulfamethazine, Sulfathiazole, Sulfonamides, Furazolidone Metabolite (AOZ), Furaldone Metabolite (AMAZ), Furacilin Metabolite (AHD), Nitrofurans Metabolite, Fluoroquinolones, Quinolones, Gentamicin, Kanamycin, and Tylosin Residue Rapid Test Device. The following SmarKIT kits are available from the same company: Gentamicin, Kanamycin, Penicillin, Streptomycin, Sulfadiazine, Sulfamethazine, Sulfathiazole, Sulfonamides, Furaldone Metabolite (AMAZ), Nitrofurazone (SEM), Furantoin (AHD), Furazolidone (AOZ), Nitrofurans 4-in-1, and Fluoroquinolones Rapid Test Kit. Another Chinese company (Shenzhen Bioeasy Biotechnology Co., Ltd., Shenzhen, Guangdong, China) is producing the Chloramphenicol and Tetracycline Rapid Test Kit for Honey.

The tests are utilizing gold conjugated antibodies as signal reagents and a drug protein conjugate as a solid phase capture reagent. As the sample flows through the absorbent sample pad, the liquid reconstitutes the dried monoclonal gold conjugate. The drug in the sample will bind to the conjugate antibody and will migrate further

up the membrane to the test line. If there is no drug in the sample, the free antibody conjugate will bind to the test line giving a negative result. In case the sample contains drug residues, the antibody conjugate will not bind to the test line giving a positive result. There is a short sample pretreatment for honey but without the need of instrumentation for most of the tests. With all these test devices, a result is obtained within 3–5 min and visual interpretation of the result is possible. As an example, the test protocol of the Chloramphenicol Residue Rapid Test Device (Nankai Biotech Co., Ltd.) is given in Table 14.

The TetraSensor Honey KIT008 (25 tests)/KIT009 (100 tests) (Unisensor s.a., Liège, Belgium) sensitively (<10 µg/kg) detects the four most important tetracyclines in honey in 30 min, without any special equipment, making analysis at the production site possible (Alfredsson et al., 2005; Reybroeck et al., 2007; Gaudin et al., 2013). The test procedure of the TetraSensor Honey is given in Table 15. During this first incubation period, tetracyclines possibly present in the honey bind with the specific receptor forming a stable complex. Afterward, during the second incubation, the liquid is absorbed by the dipstick; and while flowing over the dipstick, the liquid passes through the green capture lines. In case the honey is free from tetracycline residues, the first line captures the remaining active receptor and a strong red line will appear. The second line, serving as a control line, takes a certain amount of the excess of reagent that passed through the first line. A red control line should always become visible; otherwise, the test is invalid. Results can be read visually or by means of a ReadSensor, comparing the color intensity of both capture lines.

During this first incubation period, tetracyclines possibly present in the honey bind with the specific receptor. Afterward, the dipstick is dipped into the vial and a second incubation at room temperature occurs for 15 min. When the liquid passes through the green

Table 15. Extraction and test protocol for the TetraSensor Honey KIT008/009.

Sample pretreatment

1. Fill the lid of the plastic 'Honey Dilution Tube' with honey (around 600 mg).
2. Reclose the plastic 'Honey Dilution Tube'.
3. Shake well until all honey is dissolved in the buffer.

Test protocol

1. Add 200 μ L of this mixture to the lyophilised receptor in the glass vial.
2. Mix well.
3. Incubate at room temperature ($20 \pm 5^\circ\text{C}$) for 15 min.
4. Bring the dipstick into the glass vial.
5. Continue incubating at room temperature ($20 \pm 5^\circ\text{C}$) for 15 min.
6. Interpret the result:
 - visual reading: intensity of the test line is stronger compared to the reference line: negative result; intensity of the test line is equal or weaker compared to the reference line: positive result.
 - instrumental reading (ReadSensor): the instrument is calculating the ratio test line/reference line. Ratio ≤ 1.10 (cut-off): negative; $0.90 \geq \text{ratio} < 1.10$: low positive; ratio < 0.90 : positive; reference line fails to appear: invalid.

Table 16. Extraction and test protocol for the Sulfasensor Honey KIT033.

Extraction protocol

1. Fill the lid of the empty plastic 'Honey Tube' with honey (around 650 mg).
2. Add 600 μ L of ACID Buffer in the empty tube.
3. Reclose the tube.
4. Shake well until complete honey dissolution.
5. Incubate the tubes 5 min in boiling water ($90\text{--}100^\circ\text{C}$).
6. Add 600 μ L of NEUTRALIZING Buffer, close the tube and vortex.
7. Add 1800 μ L of HONEY-II Buffer.

Test protocol

1. Place the reagent microwell in the Heatsensor at 40°C .
2. Add 200 μ L of sample and mix 5–10 times until complete reagent dissolution.
3. Incubate for 5 min at 40°C .
4. Bring the dipstick into the microwell.
5. Continue incubating for 15 min at 40°C .
6. Remove the dipstick, remove the filter pad from the dipstick and interpret the result:
 - visual reading: intensity of the test line is stronger compared to the reference line: negative result; intensity of the test line is equal or weaker compared to the reference line: positive result.
 - instrumental reading (ReadSensor): the instrument is calculating the ratio test line/reference line. Ratio ≤ 1.20 (cut-off): negative; $1.00 \geq \text{ratio} < 1.20$: low positive; ratio < 1.00 : positive; reference line fails to appear: invalid.

capture lines, a red color appears. The first line captures the remaining active receptor and the second line takes a certain amount of the excess reagent that passed through the first line. The second line serves as a control line and always has to become visible; otherwise the test is invalid. Results can be read visually or by means of a ReadSensor, comparing the color intensity of both capture lines.

Only limited laboratory equipment is required to run the Sulfasensor Honey KIT033 (Unisensor s.a.), a generic monoclonal antibody test, for the detection of sulfonamides in honey in 20 min. A sample pretreatment (acid hydrolysis) by heating a mixture of honey sample and buffer at 95°C for 5 min is needed to release the sulfonamides that are chemically bound to the sugars (Chabottaux et al., 2010; Gaudin et al., 2012; Reybroeck & Ooghe, 2010). The kit manufacturer claims a detection of several sulfa drugs at $25\ \mu\text{g}/\text{kg}$ in honey. The extraction procedure and test protocol are shown in Table 16.

With the competitive multiplex dipstick Bee4Sensor KIT059 (Unisensor s.a.), the screening for tylosin, (fluoro)quinolones, sulfonamides, and chloramphenicol in honey is possible (Heinrich et al., 2013). The test could be used in two different ways: as a field test or as a lab test with better detection capabilities. The sample extraction and test protocol of the field test is described in Table 17. To run the lab test, one aliquot (A) is dissolved using acid hydrolysis, whereas the other aliquot (B) is dissolved in water. After liquid/liquid partitioning of both aliquots with ethyl-acetate, the organic layers are evaporated until dry under nitrogen. After reconstitution in a buffer, aliquots A and B are combined and applied to the well of a Bee4Sensor test kit for 5 min at 40°C , as one sample extract. Afterward, a dipstick is then incubated in this prepared well for 15 min at 40°C . The dipstick can be assessed visually or instrumentally via the ReadSensor.

Table 17. Extraction and test protocol (field test) for the Bee4Sensor KIT059.

Extraction protocol

- 1a. Fill the lid of an empty plastic 'Honey Tube' (A) with honey (around 650 mg).
 - 2a. Add 300 μ L of ACID Buffer (Solution 1) in the empty tube A.
 - 3a. Reclose the tube.
 - 4a. Shake well until complete honey dissolution.
 - 5a. Incubate the tube 5 min in boiling water (90–100 °C).
 - 6a. Add 300 μ L of NEUTRALIZING Buffer (Solution 3)(preheated), close the tube and mix by inversion.
 - 7a. Let the tube cool down.
 - 8a. Add Dipstick Buffer (Solution 2) to a final extract volume of 3.5 mL and mix well by inversion.
- 1b. Fill the lid of an empty plastic 'Honey Tube' (B) with honey (around 650 mg).
 - 2b. Add 2 mL of Dipstick Buffer (Solution 2) in the empty tube B.
 - 3b. Reclose the tube.
 - 4b. Shake well until complete honey dissolution.
 - 5b. Incubate the tube 5 min in boiling water (90–100 °C).
 - 6b. Let the tube cool down.
 - 7b. Add Dipstick Buffer (Solution 2) to a final extract volume of 3.5 mL and mix well by inversion.

Test protocol

1. Mix A& B in 1:1 ratio.
2. Take a reagent microwell and open the microwell.
3. Add 200 μ L of mixture of sample extract (A&B) and mix 5–10 times until complete reagent dissolution.
4. Incubate for 5 min at room temperature.
5. Bring the dipstick into the microwell.
6. Continue incubating for 20 min at 40 °C.
7. Remove the dipstick, remove the filter pad from the dipstick and interpret the result visually.

6.3.2. Enzyme-linked immunosorbent assays (ELISA)

There are many commercial and noncommercial ELISA's developed for the detection of antibiotic residues in different matrices, including honey. Table 18 summarizes existing ELISA kits (the list is nonexhaustive). The sample preparation for honey ranges from very simple, just a dilution followed by a filtration, to more complex, including acidic hydrolysis, derivatization, solvent extraction, or purification on a SPE column. The technical brochures of the different kit manufacturers should be followed. Validation studies of ELISA kits have been published regarding the detection of chloramphenicol (Scortichini et al., 2005), tylosin and tilmicosin (Peng et al., 2012), nitrofurans metabolites (Elizabetha et al., 2012), and tylosin en streptomycin (Gaudin et al., 2013) (Figure 8).

Competitive enzyme immunoassays are used in most cases for the detection of small molecular weight components such as antimicrobial residues. Such assays use either an enzyme-linked antibody or an enzyme-linked analyte to detect a particular antigen (drug). As an example, the sample preparation and test procedure for the detection of chloramphenicol in honey by means of the Chloramphenicol ELISA of EuroProxima (Arnhem, The Netherlands) is shown in Table 19. In this kit, the walls of the wells of the microtiterplate are precoated with sheep antibodies to rabbit IgG. A specific antibody (rabbit anti-CAP), enzyme labeled CAP (enzyme conjugate) and CAP standard or sample are added to the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilized antibodies. At the same time, free CAP (present in the standard solution or sample) and

enzyme conjugated CAP compete for the CAP antibody binding sites. After an incubation time of 1 h, the nonbound (enzyme labeled) reagents are removed in a washing step. The amount of CAP enzyme conjugate is visualized by the addition of a chromogen substrate. The bound enzyme conjugate elicits a chromogenic signal. The substrate reaction is stopped to prevent eventual saturation of the signal. The color intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the CAP concentration in the sample.

In some laboratories, noncommercial ELISA methods are used to monitor honey for the presence of antimicrobial residues (Heering et al., 1998; Jeon & Rhee Paeng, 2008). A microplate and magneto iELISA was developed for the detection of sulfonamides in honey using magnetic beads to reduce nonspecific matrix interferences (Muriano et al., 2015).

6.3.3. Enzyme-linked aptamer assays (ELAA)

Assays were developed using DNA or RNA aptamers to get specific binding to streptomycin (Zhou et al., 2013) or tetracyclines (Wang et al., 2014) in honey.

6.3.4. Radio-labeled receptor/antibody techniques (Charm II tests)

The Charm II (Charm Sciences Inc., Lawrence, MA) is a scintillation-based detection system for chemical families of drug residues utilizing class-specific receptors or an antibody in immune-binding assay format. The sample preparation is mostly just a dilution of the honey with an extraction buffer supplied as part of

Table 18. Commercial and non-commercial ELISA kits for the detection of antibiotic residues in honey (the list is non-exhaustive).

Drug (family)	ELISA Kit	Kit manufacturer or reference
AHD (1-aminohydantoin)	Nitrofurantoin (AHD) ELISA Test Kit	Unibiotest Co.,Ltd (Wuhan, China)
	MaxSignal Nitrofurantoin (AHD) ELISA Kit	Bioo Scientific (Austin, TX)
AMOX (3-amino-5-morpholinomethyl-2-oxazolidone)	Furaltadone (AMOX) ELISA	Abraxis (Warminster, PA)
	I'screen AMOX v2	Tecna s.r.l. (Trieste, IT)
	MaxSignal Furaltadone (AMOX) ELISA Test Kit	Bioo Scientific (Austin, TX)
	Nitrofurantoin (AMOX) ELISA Kit	Unibiotest Co.,Ltd (Wuhan, China)
AOZ (3-amino-2-oxazolidone)	Furazolidone (AOZ) ELISA	Abraxis (Warminster, PA)
	Nitrofurantoin (AOZ) ELISA Kit	Unibiotest Co.,Ltd (Wuhan, China)
Chloramphenicol	Chloramphenicol (CAP) ELISA Kit	Unibiotest Co.,Ltd (Wuhan, China)
	Chloramphenicol ELISA	Abraxis (Warminster, PA)
	Chloramphenicol ELISA	EuroProxima bv (Arnhem, NL)
	Chloramphenicol ELISA	Randox (Crumlin, UK)
	I'screen CAP	Tecna s.r.l. (Trieste, IT)
	I'screen CAP v2	Tecna s.r.l. (Trieste, IT)
	MaxSignal Chloramphenicol (CAP) ELISA Test Kit	Bioo Scientific (Austin, TX)
	RIDASCREEN Chloramphenicol	r-Biopharm (Darmstadt, DE)
Doxycycline	MaxSignal Doxycycline ELISA Kit	Bioo Scientific (Austin, TX)
Enrofloxacin	Enrofloxacin ELISA	EuroProxima bv (Arnhem, NL)
	MaxSignal Enrofloxacin ELISA Test Kit	Bioo Scientific (Austin, TX)
Erythromycin	Erythromycin ELISA	EuroProxima bv (Arnhem, NL)
	Erythromycin ELISA Test Kit	Unibiotest Co.,Ltd (Wuhan, China)
Flumequine	Flumequine ELISA	Randox (Crumlin, UK)
Fluoroquinolones	Fluoroquinolones (Generic) ELISA	EuroProxima bv (Arnhem, NL)
	Fluoroquinolones II ELISA	EuroProxima bv (Arnhem, NL)
	MaxSignal Fluoroquinolone ELISA Kit	Bioo Scientific (Austin, TX)
	QUINOLONONES ELISA KIT	Tecna s.r.l. (Trieste, IT)
	Quinolones (QNS) ELISA Kit	Unibiotest Co.,Ltd (Wuhan, China)
Lincomycin	MaxSignal Lincomycin ELISA Test Kit	Bioo Scientific (Austin, TX)
Norfloxacin	MaxSignal Norfloxacin ELISA Test Kit	Bioo Scientific (Austin, TX)
Oxytetracycline	MaxSignal Oxytetracycline ELISA Kit	Bioo Scientific (Austin, TX)
	Oxytetracycline ELISA	Randox (Crumlin, UK)
SEM (semicarbazide)	Nitrofurantoin (SEM) ELISA Test Kit	Unibiotest Co.,Ltd (Wuhan, China)
Streptomycin	I'screen STREPTO	Tecna s.r.l. (Trieste, IT)
	MaxSignal Streptomycin ELISA Test Kit	Bioo Scientific (Austin, TX)
	MaxSignal Streptomycin ELISA Test Kit For Honey Samples	Bioo Scientific (Austin, TX)
	RIDASCREEN Streptomycin	r-Biopharm (Darmstadt, DE)
	Streptomycin ELISA	EuroProxima bv (Arnhem, NL)
	Streptomycin ELISA	Randox (Crumlin, UK)
Sulfadiazine	MaxSignal Sulfadiazine ELISA Test Kit	Bioo Scientific (Austin, TX)
Sulfamethazine	MaxSignal Sulfamethazine ELISA Test Kit	Bioo Scientific (Austin, TX)
Sulfamethoxazole	MaxSignal Sulfamethoxazole ELISA Test Kit	Bioo Scientific (Austin, TX)
Sulfaquinoxaline	MaxSignal Sulfaquinoxaline (SQX) ELISA Test Kit	Bioo Scientific (Austin, TX)
Sulfonamides	B ZERO SULFA	Tecna s.r.l. (Trieste, IT)
	I'screen SULFA	Tecna s.r.l. (Trieste, IT)
	I'screen SULFA QL	Tecna s.r.l. (Trieste, IT)
	MaxSignal Sulfonamide ELISA Test Kit	Bioo Scientific (Austin, TX)
	Total Sulfonamides ELISA kit	Unibiotest Co.,Ltd (Wuhan, China)
Tetracyclines	B ZERO TETRA HS	Tecna s.r.l. (Trieste, IT)
	MaxSignal Tetracycline (TET) ELISA Test Kit	Bioo Scientific (Austin, TX)
	RIDASCREEN Tetracycline	r-Biopharm (Darmstadt, DE)
	SuperScreen TETRA	Tecna s.r.l. (Trieste, IT)
	SuperScreen Tetra HS	Tecna s.r.l. (Trieste, IT)
	Tetracycline ELISA	EuroProxima bv (Arnhem, NL)
	Tetracyclines ELISA	Abraxis (Warminster, PA)
	Tetracyclines ELISA	Randox (Crumlin, UK)
	Tetracyclines(TCs) ELISA Test Kit	Unibiotest Co.,Ltd (Wuhan, China)
Tilmicosin	MaxSignal Tilmicosin ELISA Test Kit	Bioo Scientific (Austin, TX)
Tylosin	I'screen TYLOSIN	Tecna s.r.l. (Trieste, IT)
	MaxSignal Tylosin ELISA Test Kit	Bioo Scientific (Austin, TX)
	Tylosin Plate Kit	Abraxis (Warminster, PA)

Note: AOZ: 3-amino-2-oxazolidone; AHD: 1-aminohydantoin; SEM: semicarbazide; AMOX: 3-amino-5-morpholinomethyl-2-oxazolidone.

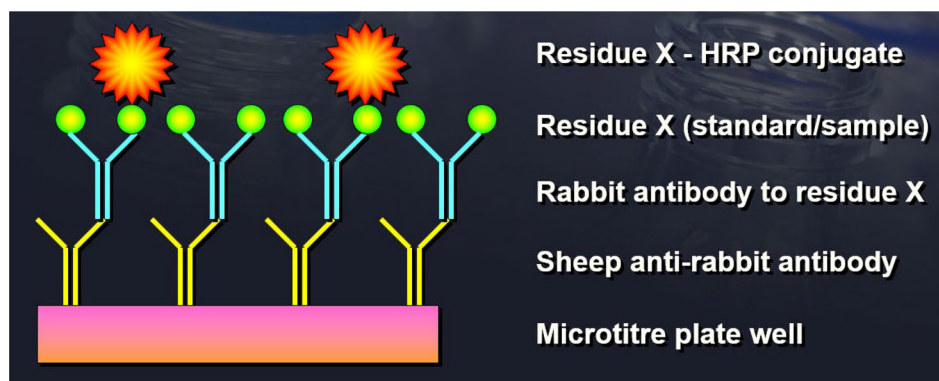


Figure 8. Format of a competitive ELISA. Residue X = target molecule; HRP = horseradish peroxidase enzyme.

the kit. The assay itself takes < 30 min to complete. Charm II kits are available for the detection of sulfonamides, tetracyclines, macrolides (and lincosamides), aminoglycosides ((dihydro)streptomycin), amphenicols (chloramphenicol), nitrofurans AOZ metabolite, and beta-lactams. Some Charm II assays were improved or adapted by the integration of solid phase extraction as extract clean-up to limit false positive results due to matrix quenching effects (McMullen et al., 2004). As an example, the procedure of the Charm II Macrolide Test for Honey is given in Table 20. This screening test detects macrolides and lincosamides in honey.

6.3.5. Biochip-based methods

Some biochip-based methods such as Biacore (GE Healthcare Europe GmbH, Freiburg, DE) and Anti Microbial Arrays (Randox Laboratories Limited, Crumlin, UK) allow the detection of multiple drug residues in honey (McAleer et al., 2010).

The Biacore biosensor system is based on surface plasmon resonance. Analytical Qflex Kits are offered for use with the Biacore Q-instrument for screening for the antibiotics chloramphenicol, streptomycin, sulfonamides, and tylosin (Caldow et al., 2005). The sample preparation includes dissolving the honey in an aqueous buffer and filtering. Analysis for chloramphenicol requires an extra LLE. For sulfonamides, a hydrolysis step is needed to release the sulfonamides bound to sugars. With this system, a high throughput and a rapid (around 5 min) multi-analyte screening in honey is possible (Weigel et al., 2005). However, the instrument costs are high.

Randox Laboratories Limited offers a multi-analyte quantitative testing platform, the Evidence Investigator using Biochip Array Technology for the monitoring of honey for antimicrobials (Daniela et al., 2012; Gaudin et al., 2014; O'Mahony et al., 2010; Popa et al., 2012). An overview of available reagents for the monitoring of honey on antimicrobials is given in Table 21. The sample preparation typically takes about 20 min for dilution. This does not include the Antimicrobial Array III which

requires 4 h due to the derivation step or 50 min if CAP only due to extraction step. The incubation and assay times take 2 h for all arrays. Arrays for the detection of nitroimidazoles in honey are in development.

6.3.6. Methods using MIPs and other immunotechniques

Molecularly imprinted polymers (MIP) with specific recognition sites are sometimes used as a preconcentration step as for the determination of chloramphenicol in honey (Thongchai et al., 2010). MIP sensors based on electropolymerized oligothioaniline-functionalized gold nanoparticles are prepared and applied to the detection of tetracycline in honey (Bougrini et al., 2016). Furthermore, there are publications on the development of an immunosensor for the detection of residues of sulfonamides in honey (Muriano et al., 2013; Valera et al., 2013).

6.4. Physico-chemical methods (chromatographic techniques)

Methods for the detection of residues of veterinary drugs in honey based on chromatographic techniques have been described in several publications (Benetti et al., 2004, 2006; Carrasco-Pancorbo et al., 2008; Dubreil-Chéneau et al., 2014; Edder et al., 1998; Kaufmann et al., 2002; Kivrak et al., 2016; Maudens et al., 2004; Nozal et al., 2006; Sporns et al., 1986; Van Bruijnsvoort et al., 2004; Verzegnassi et al., 2002) and in a review article (Bargańska et al., 2011). In the 1990s, the use of HPLC was popular; but today, confirmation of antibiotic residues in honey is performed by LC-MS, mainly LC-MS² (tandem mass spectrometry) (Blasco et al., 2007). When mass fragments are measured using techniques other than full scan, the system of identification points (IP) is applied. Confirmatory methods must fulfil the criteria listed in Commission Decision 2002/657/EC (European Union Commission, 2002) and must be based on molecular spectrometry providing direct information concerning the molecular structure of the

Table 19. Extraction and test protocol (laboratory test) for the Chloramphenicol ELISA (EuroProxima B.V.).

Extraction protocol

1. Bring 10 ± 0.1 g of honey into a centrifugation tube of 50 mL; fill also a centrifugation tube with the same amount of blank honey, honey doped with $0.3 \mu\text{g}/\text{kg}$ of chloramphenicol, and honey doped with $0.1 \mu\text{g}/\text{kg}$ of chloramphenicol, respectively.
2. Add to each sample 5 mL of distilled water.
3. Vortex the samples until all honey is dissolved.
4. Add 5 mL of ethyl acetate.
5. Mix (head over head) for 15 min.
6. After centrifugation (5 min at 2,600 g), pipette 2 mL of the upper layer (ethyl acetate) into a glass test tube.
7. Evaporate at $45 \pm 5^\circ\text{C}$ under a mild stream of nitrogen.
8. Dissolve the residue in 2 mL of *n*-hexane for defatting purposes and vortex.
9. Add 1 mL of reconstitution buffer and vortex for 20 s.
10. After centrifugation (10 min at 2600 g), pipette the layer underneath (reconstitution buffer, $\pm 800 \mu\text{L}$) into a short glass test tube.
11. Add again 1 mL of *n*-hexane and vortex for 20 s.
12. Add 1 mL of reconstitution buffer and vortex for 20 s.
13. After centrifugation (10 min at 2,600 g), pipette the layer underneath (reconstitution buffer, $\pm 800 \mu\text{L}$) into a short glass test tube.
14. Use the extract in the ELISA (extract could be stored for one day at $4 \pm 2^\circ\text{C}$).

Test protocol

1. Before starting the test, the reagents should be brought up to ambient temperature by taking them out of the refrigerator ~ 20 min before use. Keep the substrate away from light. After analysis, store the remaining reagents as soon as possible in the refrigerator.
2. Identify the wells of the microtiterstrip upon the plate configuration sheet.
3. Pipette 100 μL of reconstitution/zero standard buffer into well A1 (blank).
4. Pipette 50 μL of reconstitution/zero standard buffer into well A2 (zero standard).
5. Pipette 50 μL of chloramphenicol-free extract buffer into wells B1 and B2 (blank control sample).
6. Pipette 50 μL of extract of samples doped at $0.1 \mu\text{g}/\text{kg}$ into wells C1 and C2 (positive control sample 0.1 ppb).
7. Pipette 50 μL of extract of samples doped at $0.3 \mu\text{g}/\text{kg}$ into wells C1 and C2 (positive control sample 0.3 ppb).
8. Pipette 50 μL of each sample extract in duplicate into the remaining wells of the microtiter plate.
9. Add 25 of conjugate (CAP-HRPO) into all wells except in well A1.
10. Add 25 of antibody solution into all wells except in well A1.
11. Cover the plate with aluminum foil and shake the plate for 1 min.
12. Incubate for 1 h in the dark (refrigerator) at $2\text{--}8^\circ\text{C}$.
13. Discard the solution from the microtiter plate and wash three times with rinsing buffer. Fill all the wells each time with rinsing buffer to the rim. Place the inverted plate on absorbent paper and tap the plate firmly to remove residual washing solution. Take care that none of the wells dry out before the next reagent is dispensed.
14. Shake the substrate solution before use. Pipette 100 μL of substrate solution into each well. Cover the plate and shake the plate slowly.
15. Incubate for 30 min in the dark at room temperature ($20\text{--}25^\circ\text{C}$).
16. Add 10 μL of stop solution into each well.
17. Read the absorbance values (OD) immediately at 450 nm in a spectrophotometer.

Interpretation of results

1. Subtract the optical density (OD) value of the blank well (A1) from the individual OD of the other wells.
2. Calculate the Cut-off by adding $3 \times \text{SD}$ of repeatability for the positive control sample spiked at 0.3 ppb (value calculated out of the validation data) to the mean of both corrected OD values for the two positive control samples spiked at 0.3 ppb.
3. Interpret the control samples: the negative control sample should test negative and the positive control sample spiked at 0.1 ppb should give an OD below the OD of the negative control and higher than the OD of the positive control sample at 0.3 ppb. The run is invalid if the control samples are not giving correct results.
4. Compare the corrected value for each sample to the cut-off value. If the corrected OD of the sample is equal to or below the cut-off: the sample is considered as suspect for the presence of chloramphenicol at $0.3 \mu\text{g}/\text{kg}$; if the corrected OD of the sample is higher than the cut-off: the sample is free from residues of chloramphenicol at $0.3 \mu\text{g}/\text{kg}$.

analyte under examination, such as GC-MS and LC-MS (De Brabander et al., 2009).

Some compounds such as nitroimidazoles (Polzer et al., 2010), fumagillin (Daeseleire & Reybroeck, 2012; Kanda et al., 2011; Tarbin et al., 2010), and nitrofurans (Khong et al., 2004; Lopez et al., 2007; Tribalat et al., 2006) are mostly directly screened in honey using

LC-MS/MS detection, since no or only few immunochemical methods for the detection in honey have been developed. By the development of multiresidue methods, LC-MS is used more and more for multiclass screening for antimicrobial residues in honey (Azzouz & Ballesteros, 2015; Galarini et al., 2015; Hammel et al., 2008; Lopez et al., 2008).

Table 20. Extraction and test protocol for Charm II Macrolide Honey Test (Charm Sciences Inc.).

Honey sample preparation

1. Label a 50 mL conical centrifuge tube for each sample.
2. Add 20 grams of honey to an appropriately labeled centrifuge tube. Also, prepare two negative control samples by using blank honey and one positive control sample of blank honey spiked with 20 µg/kg of erythromycin A.
3. Add 30 mL MSU Extraction Buffer to each tube. Mix well until honey is completely dissolved by putting the samples for 30 min on a shaker or by vortexing the tubes.
4. Add 9–10 drops of M2-buffer. Check pH with pH indicator strips; pH of extract should be equivalent to 7.5 (–8.0) on pH strip.
5. If the pH is still too low, add M2 Buffer dropwise, mix, and retest pH until the desired pH is reached.
Note: If pH is high, add 0.3 mL (300 µL) 0.1 M HCl, mix, and retest. If pH is still high, add 0.1 M HCl drop-wise, mix, and retest.
6. The extract solutions can be kept for 1 day at 4 ± 2 °C.

Sample filtration

1. Using the syringe assembly, filter the entire sample through a glass fiber filter (e.g., Millipore-Millex AP prefilter of 25 mm).
2. Collect the entire sample into a clean container (50 mL conical tube or beaker).
3. After the sample has been pushed through, detach the filter holder. Rinse syringe and bivalve with 10 mL deionized water.

Clean-up over C18 cartridge

1. Prepare C18 cartridge by attaching the C18 cartridge to adapter, attaching the adapter to the bivalve in the syringe assembly.
2. Activate the C18 cartridge by pushing through 5.0 mL of methanol. The cartridge should be used within 10 min of activation.
3. Wash cartridge with 5.0 mL of deionized or distilled water.
4. Repeat the washing step with 5.0 mL of water.
5. Perform the extraction by adding the filtered honey solution to the syringe. Push the solution slowly through the preactivated C18 cartridge one drop at a time. The sample may be thick and difficult to push through the cartridge. Hold cartridge with two hands to prevent cartridge from popping off due to backpressure. Discard liquid that flows through the cartridge.
6. Wash the cartridge with 5.0 mL distilled water and discard the flow through.
7. Remove the C18 cartridge from the assembly and add 3.0 mL methanol directly into the cartridge. Attach cartridge to the assembly and bring labeled test tube in position.
8. Slowly push methanol one drop at a time through the cartridge and collect the eluate in a labeled test tube.
9. Dry eluate for each sample. Dry under nitrogen or air in a 40–45 °C heat block or water bath and remove from the heat block or water bath when methanol is completely evaporated.
10. Once the methanol is completely evaporated, reconstitute the dried eluate with 5 mL of Zero Control Standard (ZCS) and vortex extensively.
11. Cool the diluted samples on ice for 10 min prior to running the Charm II Macrolide Test Procedure.

Charm II Macrolide test procedure

1. Label test tubes and scintillation vials. Let Charm II reagents reach room temperature.
2. Add the white tablet to the empty test tube.
3. Add 300 ± 100 µL water. Mix 10 s to break up the tablet. Take additional time if required to be sure the tablet is broken up.
4. Add 5 ± 0.25 mL diluted sample or control. Use a new tip for each sample.
Immediately mix by swirling sample up and down 10 times for 10 s.
5. Incubate at 65 ± 2 °C for 2 min.
6. Add green tablet (< 0.19 kBq C¹⁴ labeled erythromycin). Immediately mix by swirling the sample up and down 10 times for 15 s. The tablet addition and mixing of all samples should be completed within 40 s.
7. Incubate at 65 ± 2 °C for 2 min.
8. Centrifuge for 5 min at 1750 G.
9. Immediately pour off liquid completely. While draining, remove fat ring and wipe dry with swabs. Do not disturb the pellet.
10. Add 300 ± 100 µL water. Mix thoroughly to break up the pellet. The pellet must be suspended in water before adding scintillation fluid.
11. Add 3.0 ± 0.5 mL of scintillation fluid. Cap and invert (or shake) until mixture has a uniform cloudy appearance.
12. Count in liquid scintillation counter for 60 s. Read cpm (counts per minute) on [14C] channel. Count within 10 min of adding of scintillation fluid.
13. Calculate the Control Point by taking the highest negative control CPM –35%.
14. Recount if greater than and within 50 cpm of the Control Point.

Interpretation of results

1. The cpm for the positive control sample should be below the Control Point, otherwise the run is not valid.
2. If the sample cpm is greater than the Control Point, the sample is negative. Report as “Not Found”. If the sample cpm is less than or equal to the Control Point, the sample is positive. The presence of macrolides/lincosamides could be confirmed by a confirmatory method (LC-MS/MS).

Table 21. Overview of antimicrobial arrays (Randox Laboratories Limited) for the monitoring for antimicrobials in honey.

Antimicrobial Array I Plus		Antimicrobial Array II	
Assay	Compound(s)	Assay	Compound(s)
Sulfadimethoxine	Sulfadimethoxine	Quinolones	Norfloxacin Pefloxacin Enrofloxacin Ciprofloxacin Ofloxacin Pipemidic Acid Fleroxacin Levofloxacin Nadifloxacin Orbifloxacin Danofloxacin Marbofloxacin Oxolinic Acid Difloxacin Pazufloxacin Sarafloxacin Enoxacin Ceftiofur
Sulfadiazine	Sulfadiazine	Ceftiofur	Desfuoyceftiofur
Sulfadoxine	Sulfadoxine	Thiamphenicol	Florphenicol Thiamphenicol
Sulfamethizole	Sulfamethizole	Streptomycin	Streptomycin
Sulfachlorpyridazine	Sulfachlorpyridazine	Tylosin	Dihydrostreptomycin Tylosin
Sulfamethoxyypyridazine	Sulfamethoxyypyridazine Sulfaethoxyypyridazine	Tetracyclines	Tilmicosin Tetracycline 4-epitetracycline Rolitetracycline 4-epioxytetracycline Oxytetracycline Chlortetracycline Demeclocycline Doxycycline 4-epichlortetracycline Methacycline
Sulfamerazine	Sulfamerazine		Antimicrobial Array III
Sulphisoxazole	Sulphisoxazole	Assay	Compound
Sulfathiazole	Sulfathiazole	AOZ	4-NP-AOZ Furazolidone
Sulfamethazine	Sulfamethazine	AMOZ	4-NP-AMOZ Furaltadone
Sulfaquinoxaline	Sulfaquinoxaline	AHD	4-NP-AHD Nitrofurantoin
Sulfapyridine	Sulfapyridine Sulfasalazine	SEM	4-NP-SEM 5-Nitro-2-furaldehyde
Sulfamethoxazole	Sulfamethoxazole Sulfamethizole Sulfachlorpyridazine	Chloramphenicol	Semicarbazone Chloramphenicol Chloramphenicol glucuronide
Sulfamonomethoxine	Sulfamonomethoxine		Antimicrobial Array III (CAP only)
Trimethoprim	Trimethoprim	Assay	Compound
		Chloramphenicol	Chloramphenicol Chloramphenicol Glucuronide
Antimicrobial Array IV (continued)		Antimicrobial Array IV (continued)	
Assay	Compound	Assay	Compound
Spiramycin/Josamycin	Spiramycin Kitasamycin Spiramycin I Acetylspiramycin Josamycin	Spectinomycin	Spectinomycin

(Continued)

Table 21. (Continued).

Antimicrobial Array IV (continued)		Antimicrobial Array IV (continued)	
Apramycin	Apramycin	Amikacin/Kanamycin	Amikacin Kanamycin A Kanamycin B
Bacitracin	Bacitracin	Lincosamides	Lincomycin Clindamycin Pirlimycin
Neomycin/Paromomycin	Neomycin Paromomycin	Erythromycin	Erythromycin Clarithromycin Roxithromycin N-Demethyl Erythromycin A Streptomycin
Tobramycin	Tobramycin Kanamycin B	Streptomycin/ Dihydrostreptomycin	Dihydrostreptomycin Virginiamycin
Tylosin B/Tilmicosin	Tylosin B Tilmicosin Tylvalosin Tylosin A	Virginiamycin	

Note: AOZ: 3-amino-2-oxazolidone; AHD: 1-aminohydantoin; SEM: semicarbazide; AMOZ: 3-amino-5-morpholinomethyl-2-oxazolidone.

7. Standard method for honey sensory analysis

Sensory analysis of honey describes the organoleptic profile and may be used to assess the quality, as well as the botanical and geographical origin of a honey sample. Furthermore, it may be used to assess the pleasantness to the end-consumers and to monitor the quality of honey during harvesting, packaging, and storage.

In order to evaluate uniflorality, “honey may be designated according to floral or plant source if it comes wholly or mainly from that particular source and has the organoleptic, physicochemical and microscopic properties corresponding with that origin” (Codex 2001). It should be noted that some pollen is either under or over-represented in a honey sample and the percentage of one specific pollen does not necessarily correspond to the amount of nectar from that specific species. Moreover, the pollen entering the nectar during bee collection (primary pollen spectrum, strictly related to botanical origin) can be significantly modified from secondary pollen contamination, that is, pollen grains entering in honey in the hive, during transformation from nectar to honey by bees, or as a consequence of beekeeper operations. Using only pollen analysis for determining uniflorality may be misleading and a unique generalized threshold may be too high for under-represented and too low for over-represented pollens (see, e.g., the descriptive sheets Person Oddo & Piro, 2004). Thus, sensory evaluation, in addition to physico-chemical and melissopalinalogical analyses, is essential for determining uniflorality, also because organoleptic characteristics are the only ones that consumers can identify and evaluate.

Traditional sensory analysis was first applied to honey by Michel Gonnet who trained specialists to evaluate honey on the basis of their sensory experience (Gonnet et al., 1985; Gonnet & Vache, 1979, 1992). Modern techniques relying on a panel of assessors and

controlled protocols are applied more regularly now (Piana et al., 2004). One selected technique for the sensory analysis of honey is presented here, while a wider guide is published in Marcazzan et al. (2018).

The following proposed standard method will be developed by referring to specific articles (Marcazzan et al., 2014; Mucignat-Caretta et al., 2012; Piana et al., 2004; Sabatini et al., 2007) or to ISO standards (ISO 5492, 2008; ISO 6658, 2017). For detailed information regarding testing rooms and the selection and training of assessors or technicians, one can refer to the specific ISO standards (ISO 8586, 2012; ISO 8589, 2007). For a complete list of ISO standards on sensory analysis, see the ISO catalog (<http://www.iso.org>).

7.1. Methods

Many methods for the sensory analysis of honey are available. The general requirements and tasting procedures are common to all methods. The descriptive semiquantitative method will be described in detail, as it requires less training and has a wider use; the other methods are described in Marcazzan et al. (2018), and references herein.

7.2. General requirements

7.2.1. Test room

Evaluations should be conducted under known and controlled conditions with minimal distractions. Individual testing booths should be used (Figure 9) and located in rooms with controlled lighting and temperature, without interfering noises and odors. The general guidance for the design of test rooms is reported in ISO 8589 (2007).



Figure 9. Assessors in testing booths.

7.2.2. Selection and training of assessors and choice of panel

The guidelines for the selection, training, and monitoring of assessors are reported in ISO 8586 (2012). Panel sessions involve at least five specialized expert assessors working as part of a panel managed by a panel leader (ISO 13300, 2006a, 2006b) who oversees the selection, training, monitoring, and performance of the assessors. The panel leader is responsible for organizing and coordinating the tests as well as collecting and processing the results.

7.2.3. Product storage

Recommended temperature for honey sample storage should be $<20^{\circ}\text{C}$ to maintain the stability of honey. Avoid high temperatures ($>35^{\circ}\text{C}$) and exposure to sunlight.

7.2.4. Preparation and presentation of the samples

The sensory analysis is performed in containers as detailed below. It is useful to separate the visual from the olfactory-gustatory assessment: general aspect and certain defects should be assessed in the original container if possible. In any case, the container should not have any sort of identification except a code. Otherwise, honey samples must be presented in the containers mentioned below.

7.2.4.1. Sampling glasses.

The same type of glasses or containers should be used within each tasting session. The glasses/containers should meet the following requirements:

- Containers should be odorless.
- Present samples in a homogeneous and anonymous way (identical containers with no distinguishing marks apart from the identification code).
- Cover samples to prevent the contamination and dispersion of the honey odors.



Figure 10. Balloon stemmed glasses proposed for sensory honey evaluation.

Table 22. Guidelines for assessors.

-
1. In the day of assessment, the assessors should:
 - avoid perfumed toiletries, and
 - avoid anything else that may introduce odors into the tasting room.
 2. For 1 h before the evaluation, the assessors should:
 - avoid smoking, and
 - avoid eating.
 3. During the session, the assessors should:
 - work alone,
 - follow the instructions of the panel leader, and
 - abstain from expressing any comment.
-
- Use a 150/200 mL capacity balloon stemmed wine glass for samples (Figure 10) or other types of glasses if they satisfy the requirements mentioned above, maintaining the sample/volume ratio near 1/5.
 - Use colored or opaque containers if it is necessary to mask the color of the honey. For example, oil sampling beakers defined in the COI/T.20/Doc. No. 5 norm (International Olive Oil Council, 2007) and ISO standard (ISO 16657, 2006) meet these requirements. The color of honey may be masked by using a red light.

7.2.4.2. Preparation of the samples.

- (1) Assign each sample a random three-digit code.
- (2) Pour at least 30 g of the sample (suggested for a 150 mL glass, one for each assessor) into the container.
- (3) Cover samples with a suitable lid (e.g., petri dish, aluminum foil, cling film).
- (4) Use minimal manipulation during transferring of samples to ensure that the subsamples look the same.
- (5) To guarantee anonymity, the technician preparing the samples should not perform the testing.
- (6) Maintain the sample at room temperature, preferably between 20 and 25 °C.
- (7) The evaluation should ideally occur within 24 h after sample preparation.

7.3. Tasting procedures

7.3.1. Guidelines

Each assessor will be given instructions and materials, including evaluation forms and computers, necessary to conduct the sensory analysis. The assessors must have at their disposal neutral spoons (without any odor and/or taste, metallic material should not be used) to stir the honey during the olfactory assessment and to take the product to be tasted. Water and a juicy, slightly acidic apple are suggested to cleanse the mouth between tastings. Also rose-hip tea or low-salt bread may be used. The assessors must follow simple but important rules (ISO 6658, 2017; Piana et al., 2004), see Table 22.

7.3.1.1. Session protocol.

- (1) Limit the number of samples according to the type of honey to prevent oversaturation and fatigue of the senses.
- (2) The evaluation should occur when sensory sensitivity is at its optimum, at least 2 h after a meal.
- (3) Rest at least 30 min between sessions.
- (4) Randomly present the order of the samples to each assessor.

7.3.2. Evaluation of the visual characteristics

- (1) The evaluation is carried out under day light in a well illuminated room or using an artificial illumination as lights with a correlated color temperature of 6 500 °K.
- (2) Evaluate the visual cleanliness and quality of the crystallization of a honey in its original container.
- (3) Evaluate all other characteristics by assessing the sample in a separate glass.

7.3.3. Evaluation of the olfactory characteristics

Evaluate olfactory characteristics before tasting. Avoid receptor desensitization by taking short sniffs of the sample.

- (1) Spread the honey around the inside of the glass using the spoon. This increases the surface area and releases the volatiles.
- (2) Take first sniff to evaluate odor immediately.
- (3) Wait 15–20 s before taking a second sniff, so that the full intensity of the odor is perceived.
- (4) Leave part of crystallized honey untouched for tactile evaluation.

7.3.4. Evaluation of the olfactory-gustatory and tactile characteristics

- (1) Bring to the mouth 1/2 g of honey with a spoon.
- (2) Allow the honey sample to dissolve slowly in the mouth before swallowing.
- (3) Evaluate olfactory-gustatory characteristics immediately.
- (4) Evaluate the taste (sweet, salty, sour, and bitter).

- (5) Evaluate the retro-nasal odor: aroma intensity and quality, persistence, after-taste.
- (6) Evaluate other mouth sensations (viscosity, texture, type of crystals).
- (7) Concentrate on the chemical aspects of the mouth sensations apart from the tactile characteristics.
- (8) Wait 1 or 2 min before taking a second taste, longer in the case of persistent honeys. Eat a slice of apple or bread or cleanse the mouth with water or rose-hip tea if necessary.
- (9) Evaluate tactile characteristics simultaneously. We encourage doing so in a second taste. Press the sample between tongue and palate in order to perceive and assess the viscosity, texture, and crystals characteristics.
- (10) Wait a few minutes between samples, eat a slice of apple or bread or cleanse the mouth with water or rose-hip tea.

7.4. Descriptive semiquantitative analysis

All numbered lists correspond to a scale, while bulleted lists correspond to a nonexhaustive list of descriptors. An example of descriptive card is presented in Figure 11.

7.4.1. Appearance and visual descriptors

The principal visual descriptors for honey are:

- (1) Physical state: liquid or crystallized.
- (2) Color intensity: 1. very light; 2. light; 3. medium; 4. dark; 5. very dark.
- (3) Color tone: normal honey color; bright yellow; grayish; reddish; with orange tone; fluorescent green; dull; bright.
- (4) Presentation: depending on the aim of the evaluation (e.g., competition or checking botanical declaration etc.), the importance of the assessment of some or all of these elements may vary. The following characteristics can be assessed: clarity (for liquid honey); impurities; air bubbles or foam.
- (5) Defects can be assessed as marbling or white spots, incomplete crystallization, phase separation.

Viscosity, cohesion and type of crystals are observed in both visual and tactile evaluation but assessed only in the latter (see Section “Texture and other tactile descriptors” for description).

7.4.2. Olfactory descriptors

- (1) The intensity of the odor may be defined as: 0—absent; 1—weak; 2—medium; 3—strong.
- (2) The description of the odors may be classified in families and subfamilies. These are shown in Table 23. The examples used in the table to describe the families should be considered useful but not exhaustive.

HONEY DESCRIPTION CARD

SAMPLE CODE:

VISUAL ASSESSMENT

Physical aspect Colour intensity 1 2 3 4 5
 Colour tone

OLFACTORY ASSESSMENT:

Intensity of odour:
 absent weak medium strong

Family	Sub-family
floral	subtle <input type="checkbox"/>
	heady <input type="checkbox"/>
fruity	fresh fruit <input type="checkbox"/>
	tropical fruit <input type="checkbox"/>
	syrup <input type="checkbox"/>
	transformed fruit <input type="checkbox"/>
	vine <input type="checkbox"/>
warm	subtle <input type="checkbox"/>
	lactic <input type="checkbox"/>
	caramelized <input type="checkbox"/>
	roasted <input type="checkbox"/>
	malted <input type="checkbox"/>
aromatic	burned <input type="checkbox"/>
	spicy <input type="checkbox"/>
	resinous <input type="checkbox"/>
	woody <input type="checkbox"/>
	camphor <input type="checkbox"/>
	citrus peel <input type="checkbox"/>
chemical	bitter almond <input type="checkbox"/>
	phenolic <input type="checkbox"/>
	medicine <input type="checkbox"/>
	household soap <input type="checkbox"/>
	petrochemical <input type="checkbox"/>
	smoky <input type="checkbox"/>
	vinegar <input type="checkbox"/>
ammonia <input type="checkbox"/>	
vegetal	green <input type="checkbox"/>
	damp <input type="checkbox"/>
	dry <input type="checkbox"/>
animal	sulphur <input type="checkbox"/>
	protein <input type="checkbox"/>
	valerianic <input type="checkbox"/>
	cat's urine <input type="checkbox"/>

AROMA ASSESSMENT:

Intensity of aroma:
 absent weak medium strong

Family	Sub-family
floral	subtle <input type="checkbox"/>
	heady <input type="checkbox"/>
fruity	fresh fruit <input type="checkbox"/>
	tropical fruit <input type="checkbox"/>
	syrup <input type="checkbox"/>
	transformed fruit <input type="checkbox"/>
	vine <input type="checkbox"/>
warm	subtle <input type="checkbox"/>
	lactic <input type="checkbox"/>
	caramelized <input type="checkbox"/>
	roasted <input type="checkbox"/>
	malted <input type="checkbox"/>
aromatic	burned <input type="checkbox"/>
	spicy <input type="checkbox"/>
	resinous <input type="checkbox"/>
	woody <input type="checkbox"/>
	camphor <input type="checkbox"/>
	citrus peel <input type="checkbox"/>
chemical	bitter almond <input type="checkbox"/>
	phenolic <input type="checkbox"/>
	medicine <input type="checkbox"/>
	household soap <input type="checkbox"/>
	petrochemical <input type="checkbox"/>
	smoky <input type="checkbox"/>
	vinegar <input type="checkbox"/>
ammonia <input type="checkbox"/>	
vegetal	green <input type="checkbox"/>
	damp <input type="checkbox"/>
	dry <input type="checkbox"/>
animal	sulphur <input type="checkbox"/>
	protein <input type="checkbox"/>
	valerianic <input type="checkbox"/>
	cat's urine <input type="checkbox"/>

OTHER:

OTHER:

FLAVOUR ASSESSMENT:

TASTE:
Sweetness: absent weak medium strong
Sourness: absent weak medium strong
Saltiness: absent weak medium strong
Bitterness: absent weak medium strong
Aroma/aftertaste persistence: absent short medium long
Aftertaste: absent present
Aftertaste description :

Astringency: absent present
Piquant: absent present
Refreshing: absent present

TEXTURE ASSESSMENT

Consistency/viscosity:
 Crystals:

NOTES (and possible defects)

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Figure 11. Example of descriptive card.

Table 23. Attributes for honey olfactory description.

Attribute	Definition	Reference	
1	Floral	Odor reminiscent of flowers	orange flower (distilled) water, β -ionone (subfamily "fine") rose, ylang ylang, phenylacetaldehyde (subfamily "heady")
2	Fruity	Odor reminiscent of fruit	fruit aroma (strawberry, pear, apple, raspberry) (subfamily "fresh fruit") isoamyl acetate, γ -decalactone, δ -decalactone (subfamily "tropical fruit") grape sugar (concentrated rectified must) (subfamily "syrup") apricot fruit juice, peeled tomatoes (subfamily "processed fruit") white wine (subfamily "fermented fruit")
3	Warm	Odor reminiscent of food characterized by sweet taste	vanillin, benzoin, beeswax (subfamily "fine") concentrated sugared milk, diacetyl (subfamily "lactic") cane sugar, furanone (subfamily "candied") toasted hazel nuts, toasted almonds, toasted peanuts (subfamily "toasted") barley malt syrup, rice syrup (subfamily "malted") toasted barley, toasted bread (charred part) (subfamily "burnt")
4	Aromatic	Odor reminiscent of something fresh, balsamic, cosmetic, not necessarily related to food products	cloves, nutmeg (subfamily "spicy") mugo pine, incense (subfamily "resinous") oak musk (subfamily "woody") peppermint, anise, eucalyptus (subfamily "balsamic") lemon, sweet orange (subfamily "citrus fruit") coumarin (subfamily "bitter almond")
5	Chemical	Odor generally not related to food products, characterized by a certain degree of aggressiveness	phenol, vegetal tar (subfamily "phenolic") castile soap (not perfumed) (subfamily "soap") jute smoke, cigarette butt extract (subfamily "smoky") white wine vinegar (subfamily "vinegar") ammonia (subfamily "ammonia")
6	Vegetal	Odor reminiscent of non-aromatic plants	fresh raw beans, cut grass, fresh ivy leaves (subfamily "green") raw champignon mushrooms, lightly boiled spinach (subfamily "damp") green tea, hay (subfamily "dry")
7	Animal	Odor reminiscent of animal world or decomposition	hardboiled egg, dimethyl sulphide, methionine (subfamily "sulfurized") dried ceps mushrooms, stock cube (subfamily "proteic") isovalerianic acid, butyric acid, hexanoic acid (subfamily "valerianic") cassis (subfamily "cassis")

Table 24. Attributes for honey taste description.

Attribute	Definition	Reference	
1	Sweetness	Basic taste produced by dilute aqueous solutions of natural or artificial substances such as sucrose or aspartame	Sucrose in water 12 g/L
2	Sourness	Basic taste produced by dilute aqueous solutions of mostly acid substances (e.g., citric acid and tartaric acid)	Citric acid in water 0.6 g/L
3	Saltiness	Basic taste produced by dilute aqueous solutions of various substances such as sodium chloride	Sodium chloride in water 2 g/L
4	Bitterness	Basic taste produced by dilute aqueous solutions of various substances such as quinine or caffeine	Caffeine in water 0.27 g/L

7.4.3. Flavor descriptors

Flavor is the "complex combination of the olfactory, gustatory and trigeminal sensations perceived during tasting. It may be influenced by tactile, thermal, painful and/or kinaesthetic effects" (ISO 5492, 2008).

- (1) The intensity of the taste sensation sweetness, sourness, salty, and bitterness (Table 24) is quantified according to the scale: 0. absent; 1. weak; 2. medium; 3. strong. The taste of umami is not considered; if perceived, it is reported as present.
- (2) The odor perceived via retro-nasal pathways (aroma) is described as reported in olfactory description.
- (3) Other sensations we can perceive in the mouth include: (i) piquant: an aggressive and burning sensation perceived by the mucous membranes and/or in

the throat; (ii) metallic: a feeling similar to a slight tingling sensation due to the presence of traces of iron (or perhaps other metals) in ionic form in some honeys. In honey, it indicates a defect.

- (4) Persistence can be evaluated. Persistence refers to the duration of the aroma sensations after swallowing. Less than 30 s is considered "short" persistence, and longer than 5 min, "long" persistence. Persistence is evaluated according to the scale: 0. absent; 1. short; 2. medium; 3. long.
- (5) Aftertaste indicates the presence of aroma sensations in the mouth that remain after swallowing the product and are different from those initially perceived. The quality of the sensations can be described with the same terminology used to explain the olfactory sensations (Table 23).

7.4.4. Texture and other tactile descriptors

Tactile characteristics refer to those perceived in the mouth, although the visual inspection and handling of the sample contribute to its assessment.

- (1) Viscosity is evaluated in liquid honey according to the scale: 1. very fluid; 2. fluid; 3. normal viscosity; 4. viscous; 5. very viscous.
- (2) Texture of the mass in crystallized honeys is evaluated and can be described as: 1. deliquescent; 2. soft; 3. pasty; 4. firm; 5. compact.
- (3) Crystals can be differentiated according to their size: 0. not perceptible; 1. fine; 2. medium; 3. large.
- (4) Crystals can be differentiated depending on the shape and can differ according to solubility, that is, to say the tendency to melt in the mouth more or less easily. The crystals can be described as: sharp, gritty, or roundish.

A further tactile descriptor for honey is astringency, a complex sensation due to substances such as the tannins of persimmon or unripe fruit, which precipitate the proteins in the saliva responsible for lubricating the mucous membranes in the mouth. The astringency is perceived as a lack of lubrication and dryness of mucous membranes; it is often associated with a bitter taste.

In finely crystallized honey, it is common to experience a refreshing sensation. This is not due to the temperature of the product, but it is linked to the solubility of some type of crystals (glucose).

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ORCID

Ligia Bicudo de Almeida-Muradian  <http://orcid.org/0000-0001-5322-1358>

Ortrud Monika Barth  <http://orcid.org/0000-0002-2418-8605>

Vincent Dietemann  <http://orcid.org/0000-0002-0791-9939>

Michael Eyer  <http://orcid.org/0000-0003-1047-1714>

Alex da Silva de Freitas  <http://orcid.org/0000-0002-8665-7649>

Anne-Claire Martel  <http://orcid.org/0000-0002-1179-4286>

Gian Luigi Marcazzan  <http://orcid.org/0000-0002-1695-9713>

Carla Marina Marchese  <http://orcid.org/0000-0002-7495-0688>

Carla Mucignat-Caretta  <http://orcid.org/0000-0001-5307-0414>

Wim Reybroeck  <http://orcid.org/0000-0002-2269-6534>

M. Teresa Sancho  <http://orcid.org/0000-0002-9128-9422>

José Augusto Gasparotto Sattler  <http://orcid.org/0000-0002-7760-0501>

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