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REVIEW ARTICLE

Standard methods for *Apis mellifera* propolis research

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Propolis is one of the most fascinating honey bee (*Apis mellifera* L.) products. It is a plant derived product that bees produce from resins that they collect from different plant organs and with which they mix beeswax. Propolis is a building material and a protective agent in the bee hive. It also plays an important role in honey bee social immunity, and is widely used by humans as an ingredient of nutraceuticals, over-the-counter preparations and cosmetics. Its chemical composition varies by geographic location, climatic zone and local flora. The understanding of the chemical diversity of propolis is very important in propolis research. In this manuscript, we give an overview of the available methods for studying propolis in different aspects: propolis in the bee colony; chemical composition and plant sources of propolis; biological activity of propolis with respect to bees and humans; and approaches for standardization and quality control for the purposes of industrial application.

Métodos estándar para investigar el própolis de *Apis mellifera*

El própolis es uno de los productos más fascinante de la abeja de la miel (*Apis mellifera* L.). Es un producto derivado de plantas que las abejas producen a partir de resinas que recogen en diferentes órganos de la planta y que mezclan con la cera de abejas. El própolis es un material de construcción y un agente protector en la colmena de abejas. También juega un papel importante en la inmunidad social de la abeja de la miel, y es ampliamente utilizado por los seres humanos como un ingrediente de nutracéuticos, preparados de venta no regulada y cosméticos. Su composición química varía según la ubicación geográfica, la zona climática y la flora local. La comprensión de la diversidad química del própolis es muy importante en su investigación. En este manuscrito, damos una visión general de los métodos disponibles para el estudio del própolis en diferentes aspectos: própolis en la colonia de abejas; composición química y fuentes vegetales del própolis; actividad biológica del própolis con respecto a las abejas y los seres humanos; y enfoques para la normalización y control de calidad para los fines de aplicación industrial.

蜜蜂蜂胶实验标准方法

摘要

蜂胶是一种重要而特别的蜂产品。蜂胶是通过蜜蜂采集树脂后，混合蜂蜡酿造而成。蜂胶是蜂巢的重要建造材料。蜂胶能够抑制病菌生长，对蜜蜂的社会免疫起到重要作用。不仅如此，蜂胶还广泛用于功能食品和化妆品。不同蜂胶的化学成分也不相同，这主要是和当地的气候与植物群落有关。了解蜂胶的化学成分对于蜂胶研究非常重要。本文将从不同角度概述蜂胶的研究方法：蜜蜂蜂群内的蜂胶分析；蜂胶成分和植物源分析；蜂胶对蜜蜂和人的功能性分析；蜂胶产业化应用和质量标准分析。

Keywords: COLOSS; *BEEBOOK*; honey bee; *Apis mellifera*; propolis; chemical composition; plant sources; biological activity; standardization; quality control

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1. Introduction

Western honey bees (*Apis mellifera* L.) produce propolis (also called bee glue) from resins that they collect from different plant organs and with which they mix beeswax. The term "propolis" is of Greek origin: "pro" meaning "in front of/for" and "polis" meaning "city", that is, in front (or for defense) of the city. Propolis is used by bees as a building material in their hives, for blocking holes and cracks, repairing combs, and strengthening the thin borders of the comb (Ghisalberti, 1979). Feral bees inhabiting tree cavities cover the inside of the cavity with a layer of propolis called the "propolis envelope" (Seeley & Morse, 1976). Propolis plays the role of chemical defense against microorganisms and as an embalmer of larger, dead intruders (insect, small animals) that have died in the hive and are too large to be removed by the bees (Ghisalberti, 1979).

The valuable therapeutic properties of propolis were recognized by human beings millennia ago; historical records suggest the use of propolis dates back to the ancient Egyptians, Romans, and Greeks (Crane, 1999). It is still used as a popular homemade remedy in many countries all over the world, but also as a constituent of food additives, cosmetics and over-the-counter preparations (de Groot, 2013; Sforcin & Bankova, 2011; Suárez, Zayas, & Guisado, 2005).

The biological activity of propolis is due to its chemical composition which, in turn, depends on the source plant(s) from which bees collect the resin. A number of chemical types of propolis have been registered according to their plant source. The understanding of propolis chemical diversity plays a core role in propolis studies.

In this manuscript, an overview is presented of the available methods for studying propolis in different aspects: propolis in the bee colony, chemical composition and plant sources of propolis, biological activity of propolis with respect to bees and humans, and approaches for standardization and quality control for the purposes of industrial application.

2. Resin and propolis: sampling and harvesting

Propolis collected from the hive may contain a mixture of resins from various plant sources and beeswax. If individual sources of resin are needed for chemical analysis, it may be necessary to collect the resin from plant tissue or from the hindlegs of returning resin foragers. The procedures described below first describe how to collect resins from plants and individual bees, and then how to collect propolis from within a colony.

2.1. Resin sample collection

2.1.1. Sampling resin from plant tissue

Identify resinous plants in your area. The most comprehensive guide to resinous plants available is Langenheim (2003), while the most comprehensive guide to resinous plants used by bees is Crane (1990). Also see Bankova, Popova, and Trusheva (2006).

(1) Collect resin from individual plants. If the target resins are foliar, use clean pruning shears to detach 4–6 resinous buds/leaves and place all in a 15 ml screw-top EPA vial. If resins are internal, collect fresh resin from existing or generated wounds.
(2) The number of individual plants sampled will vary by apiary due to availability. Try to collect resin from at least three different individuals per plant species if possible.

2.1.2. Sampling resin from foragers in the field

(1) Individual resin foragers carrying pure resin can be captured returning to the hive (Figure 1). Block the hive entrance with a mesh screen and observe for 15 min. Capture resin foragers clustering on the hive entrance in wire cages or a suitable screened container and maintain captured bees out of the sun. It is easiest to

Figure 1. Honey bees with resin (on left) and pollen (on right) on hind legs. The resin loads of foragers are semi-translucent and shiny, whilst pollen is opaque and powdery in texture.
Photo: M. Simone-Finstrom.

collect resin foragers from small colonies that are situated on hive stands (see Section 2.2).
(2) Collect samples twice per day (once in the morning and once in the afternoon) as required.
(3) Anesthetize caged bees on ice for 5 min, then remove them from the cage. Remove resin from bee corbiculae using an insect pin. Resin foragers may be marked (see the *BEEBOOK* paper on miscellaneous honey bee research methods by Human et al. (2013)) and released as desired.
(4) Place resin globules from an individual bee inside a small, screw-top glass vial and store on ice while in the field. Place the resin in the freezer (−10 °C) until needed for further use.

2.2. *Harvesting propolis from hives*

2.2.1. *Commercial traps*

The major commercial beekeeping supply companies sell "propolis traps." These usually are thick sheets of plastic with a series of 1.6 mm grooved slits over the entire surface. This is the width that encourages honey bees to deposit more propolis and less wax to close the opening (Crane, 1990).

(1) Place the propolis trap directly over the top frames of the uppermost box (super) of a colony (Crane, 1990) and cover with a standard colony lid.
(2) Trap success can be improved by increasing air flow and light through the trap (Crane, 1990; Krell, 1996). This can be done easily by placing a wooden rim with holes drilled into its sides over the propolis trap and under the outer cover. Using a migratory cover (a flat cover that does not have an overhang covering the holes in the rim) further supports this process. While this extra step is not necessary, it will increase resin collection (Borba, Simone-Finstrom, Spivak, personal observation).
(3) It is important to note that the amount and quality of propolis collected will vary greatly across colonies based on genetics, environment and colony strength (Butler, 1949; Wilson, Brinkman, Spivak, Gardner, & Cohen, 2015). A strong, high resin-collecting colony can fill a trap full of propolis in a couple of weeks. Other colonies will never close all gaps completely or will use mostly wax to seal the gaps (Borba, Simone-Finstrom, Spivak, personal observation), as there is a genetic component to the level of propolis collection exhibited by bees (e.g. Manrique & Soares, 2002; Nicodemo, Malheiros, De Jong, & Couto, 2014).
(4) To harvest the propolis from the traps, it is best to freeze the traps so that the propolis becomes hard and brittle (Krell, 1996). It then can be knocked or scraped out of the traps.

2.2.2. *Non-commercial propolis traps*

Many different materials can be utilized to collect propolis (Krell, 1996). The key is making sure that the bees cannot chew away the material and that the gaps are appropriately sized to encourage resin deposition.

(1) One suitable option includes mesh (burlap) bags, like those used for storing corn, potatoes and other crops. These bags doubled-over and placed on top of the colony in the same way as the commercial traps (Section 2.2.1) work particularly well. Landscape cloth also can be used.
(2) Similar to commercial traps (Section 2.2.1), it is best to freeze the cloth prior to harvesting the propolis. Rolling the cloth on a hard surface will release the propolis from the gaps.

2.2.3. *Hive scrapings*

The most common way for propolis to be harvested in the apicultural setting is simply by scraping propolis from the frame rests, frame edges and from the bottom boards or insides of boxes (Ellis & Hepburn, 2003; Krell, 1996). This is typically done at the end of the season to clean up the boxes for use in the following year and can easily generate a significant amount of propolis. Scrapings may contain propolis from multiple seasons, and it is unknown how age affects propolis quality. More research is needed to determine if the antimicrobial properties of propolis diminish over time.

2.2.4. *African-derived bee colonies in Brazil*

Honey bees of African origin, such as those found in the tropics of Brazil, deposit large amounts of propolis in tree cavities as well as in commercial bee boxes (Manrique & Soares, 2002). Brazilian beekeepers have developed methods to harvest large quantities of propolis by introducing slats of wood with 4cm gaps to the sides of the hive boxes (Figure 2(a)). The large opening stimulates African-derived bees to fill the slats with propolis. When the gap is completely filled with a thick layer of propolis, the wood slats can be removed and the propolis harvested using a knife to cut out the sheet (Figure 2(b)).

3. Propolis chemical analysis

Propolis consists of plant resins and beeswax and the chemical analysis of propolis is directed to the plant derived compounds as they are the components responsible for the bioactivity of propolis. The compounds also indicate the plant(s) that bees have visited for resin collection. The chemical information is important with respect to quality control and standardization purposes. Also, if the propolis type is new and unexplored, it may contain new valuable bioactive compounds.

(a) (b)

Figure 2. Brazilian propolis trap. (a) The sides of a hive box are replaced with removal wooden slats, containing 4 cm gaps. (b) The slats are removed for harvesting once they are filled with propolis. The propolis sheet can be cut from the wood with a knife. The bees leave holes in the sheet of propolis naturally.
Photo: R. Borba.

3.1. *Extraction of propolis*

3.1.1. *General extraction procedure*

The aim of the extraction is to remove the major plant secondary metabolites from any impurities, such as beeswax, for further analysis or for biotests. This is achieved by extraction with 70% ethanol, as noted below.

(1) Keep propolis overnight in a freezer (−20 °C). Powder the frozen propolis using a coffee mill or other similar grinding device to achieve a particle size of about 10–80 μm.
(2) Measure a sample of the powdered propolis, add 70% ethanol (1:30 w:v) and keep it for 24 h at room temperature. Alternatively, sonicate the suspension (propolis in 70% ethanol) for 20 min in an ultrasonic bath at 20 °C.
(3) Filter the resulting suspension at room temperature using a paper filter and repeat the procedure with the part trapped in the filter, extracting the residue again under the same conditions. Experiments have shown that a third extraction under the same conditions is not necessary since the third extract yielded a negligible amount of dry propolis (Popova et al., 2004).
(4) The concentration C of the extract (i.e. the amount of propolis) is determined by evaporating 2 ml of the extract to dryness *in vacuo* to constant weight g and using the formula $C = g/2$ mg/ml (average of three replicates).

The obtained extract can be evaporated to dryness for further use or used as is in further experiments. Alternative extraction procedures might be applied depending on the analysis for which the propolis extract is to be used. For biological tests, a variety of solvents have been used, including methanol, different ethanol-water mixtures (80, 90, and 96%), absolute ethanol, glycerol, water (Park & Ikegaki, 1998; Sforcin & Bankova, 2011), and even DMSO (Netíková, Bogusch, & Heneberg, 2013). It is important to note that water dissolves less than 10% of the weight of propolis.

3.1.2. *Extraction of propolis for mass spectrometry fingerprinting*

(1) Extract ground propolis by maceration for 7 days in an orbital shaker at a temperature of 30 °C, with 10 ml of absolute ethanol (Merck; Darmstadt, Germany) for every 3 g of crude propolis.
(2) Separate the insoluble portion by filtration; keep the ethanolic solutions in a freezer at −16 °C overnight and filter again at this temperature to reduce the wax content of the extracts.

3.2. *Extraction of propolis volatiles*

Propolis volatile constituents are responsible for the specific pleasant aroma of propolis and contribute to its biological activity, although their amount is seldom greater than 1% of the weight of the sample. They also may play an important role as olfactory cues during resin collection by honey bees (Leonhardt, Zeilhofer, Bluthgen, & Schmitt, 2010). Different methods have been used to extract propolis volatiles: steam distillation, hydrodistillation (Clevenger), distillation-extraction (Likens-Nikerson), solvent extraction (including ultrasound-assisted and microwave-assisted extraction), and static and dynamic head-space, solid-phase microextraction. The method of extraction significantly affects the chemical composition of the volatile constituents of propolis (Bankova, Popova, & Trusheva, 2014). Here, we describe one of the most often used approaches for propolis volatile extraction, distillation-extraction (Bankova, Boudourova-Krasteva, Popov, Sforcin, & Funari, 1998). A review of volatile extraction procedures for hive components in general can be found in Torto et al. (2013).

(1) Keep propolis overnight in a freezer (−20 °C). Powder the frozen propolis using a coffee mill to achieve a particle size of 10–80 μm (Section 3.1.1).
(2) Put 3 g powdered propolis in a 100 mL round-bottom flask and add 80 ml distilled water.
(3) Put 50 ml *n*-pentane - diethyl ether 1:1 (v/v) in another 100 ml round-bottom flask and dip it in an ice bath.
(4) Distill for 4 h in a Likens-Nickerson apparatus (Figure 3, Queiroga, Madruga, Galvão, & Da Costa, (2005)).
(5) After the distillation is over, remove the water layer using a separatory funnel. Keep the organic layer in refrigerator until further processing.
(6) Wash the water layer with 5 ml ice cold *n*-pentane - diethyl ether 1:1 (v/v).
(7) Dry the organic layer over anhydrous Na_2SO_4: add 3 g of anhydrous Na_2SO_4, shake the flask for 5 min and filter the liquid using a filter paper. Wash the solid on the filter with 1 ml ice cold *n*-pentane - diethyl ether 1:1 (v/v).
(8) Evaporate the solvent under reduced pressure without heating using a rotatory evaporator.

The obtained volatiles can be analyzed further using GC, GC-MS or subjected to biological tests.

3.3. *Gas chromatography-mass spectrometry analysis of propolis*

Gas chromatography-mass spectrometry (GC-MS) is one of the so-called hyphenated analytical techniques extensively used for the chemical analysis of complex mixtures such as propolis. GC-MS combines the features of gas chromatography for compound separation and mass spectrometry to identify different substances. This method is used for chemical profiling of propolis for the needs of comparative analysis, quality control and standardization.

3.3.1. GC-MS analysis of non-volatile propolis constituents

Prior to the GC-MS analysis, derivatization of the propolis extracts is required because propolis contains metabolites that are not volatile enough for gas chromatography (Greenaway, Scaysbrook, & Whatley, 1987). One of the most widely used derivatization reagents is N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) (Bankova, Dyulgerov, Popov, & Marekov, 1987; Greenaway & Whatley, 1990). Silyl derivatives (trimethylsilyl ethers) obtained from propolis are less polar and more volatile than their parent compounds and are suitable for analysis by GC-EIMS (gas chromatography – electron impact mass spectrometry).

3.3.1.1. Sample preparation. Dry propolis extracts obtained according to Section 3.1.1 are analyzed by GC-MS after derivatization. The derivatization (conversion to trimethylsilyl derivatives) is performed, as follows:

(1) Mix 5 mg of the propolis extract obtained per Section 3.1.1 with 50 μl of dry (water-free) pyridine.
(2) Add 75 μl of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) to the mixture.
(3) Heat the mixture at 80 °C for 20 min.
(4) Subject the silylated extract to GC–MS analysis (see Section 3.3.1.2).

3.3.1.2. GC-MS analysis. The GC–MS analysis should be performed with a proper instrument such as a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system (Trusheva et al., 2011).

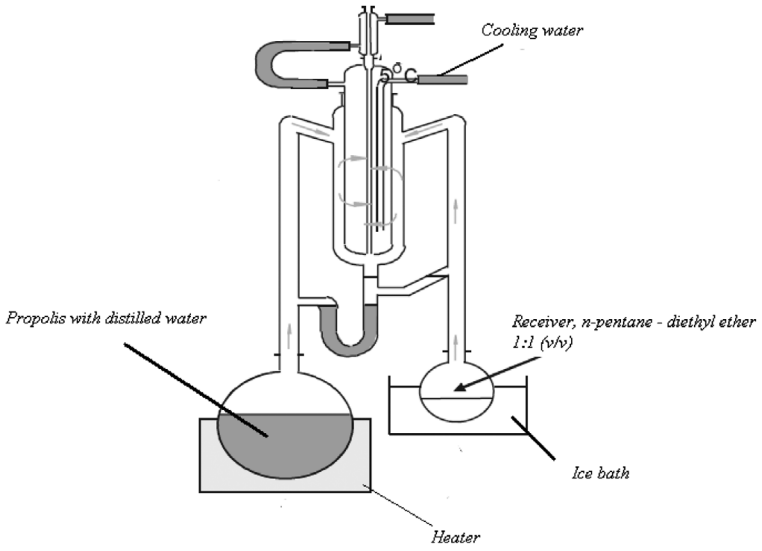

Figure 3. Likens–Nickerson apparatus for distillation-extraction of volatiles.

(1) Use a 30 m long, 0.25 mm ID, and 0.5 μm film thickness HP5-MS capillary column. Other columns with similar characteristics also can be used depending on analytical need.
(2) Program the temperature from 60 to 300 °C at a rate of 5 °C/min, and a 10 min hold at 300 °C.
(3) Helium is used as a carrier gas at a flow rate of 0.8 ml/min.
(4) The split ratio should be 1:10.
(5) The injector temperature should be 280 °C.
(6) The interface temperature should be 300 °C.
(7) The ionization voltage should be 70 eV.
(8) Every extract should be analyzed in duplicate.

The GC conditions can vary depending on the apparatus used and with respect to optimization of chromatographic separation (Isidorov, Szczepaniak, & Bakier, 2014).

3.3.2. GC-MS analysis of propolis volatile constituents

The GC–MS analysis should be performed with a proper instrument such as a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system (Bankova et al., 1998).

(1) Use a 30 m long, 0.25 mm ID, and 0.25 μm film thickness SPB-1 capillary column. Other columns with similar characteristics can be also used depends on analytical needs.
(2) Program the temperature from 40 to 280 °C at a rate of 6 °C/min.
(3) Helium is used as a carrier gas at a flow rate of 0.8 ml/min.
(4) The split ratio should be 1:10.
(5) The injector temperature should be 280 °C.
(6) The interface temperature should be 300 °C.
(7) The ionization voltage should be 70 eV. Every extract should be analyzed in duplicate.

The GC conditions can vary depending on the apparatus used and with respect to optimization of chromatographic separation (Cheng, Qin, Guo, Hu, & Wu, 2013; Kaškonienė, Kaškonas, Maruška, & Kubilienė, 2014; Nunes & Guerreiro, 2012).

3.3.3. Identification and quantification of compounds

The identification of individual compounds (such as trimethylsilyl derivatives) can be performed using computer searches on commercial libraries (such as NIST 14, Wiley 10, etc.), comparison with spectra and retention characteristics of authentic samples, and literature data. If no reference spectra are available, identification can be performed based on the characteristic mass-spectral fragmentation, in such cases the compounds are described as "tentative structures".

The quantification of individual constituents is based on internal normalization. This is a general approach used in cases where it is impossible to use other methods such as the internal standard method. The internal normalization method is based on the assumption that all detector response factors are unity, and the following equation should be applied:

$$\%\text{Analyte} = \frac{A_a}{\sum A_i} \times 100$$

where ΣA_i is the sum of all the peak areas in the chromatogram. Thus, the percentage of the individual compounds refers to percent of the Total Ion Current (TIC), and the result should not be considered as quantitative in absolute terms (IOFI Working Group on Methods of Analysis, 2011).

3.4. LC-MS chemical profiling of propolis

3.4.1. Introduction

The relatively polar nature of propolis constituents (with several hydroxyl groups in their structure), combined with soft ionization techniques compatible with liquid chromatography, make HPLC-DAD and LC-MS the favorite methods for analysis of propolis balsamic content (Sforcin & Bankova, 2011). In the structural identification of new compounds, both mass spectrometry with electrospray ionization (ESI-MS) in the negative (Falcão et al., 2010) or positive ion mode (Piccinelli et al., 2011) studies are satisfactory.

High performance liquid chromatography (HPLC) was and still is the preferred separation technique for the analysis of natural products (Steinmann & Ganzera, 2011). Recent developments of new stationary phases and pumping devices enabling pressures up to 1300 bar are further supporting this trend (Steinmann & Ganzera, 2011). Different detectors can be used, depending on the analytes investigated. The most commonly used detectors for analyzing propolis are DAD and MS detectors.

3.4.2. Separation and analysis of propolis by liquid chromatography-mass spectrometry (LC-MS)

The use of LC–MS for the qualitative and quantitative analysis of constituents in propolis has increased steadily over the last years.

(1) The extraction of propolis is performed as described in Section 3.1.1.
(2) Dissolve the dry ethanolic extract (10 mg) in 1 ml of 80% of ethanol.
(3) Filter the sample through a 0.2 μm Nylon membrane (Whatman)
(4) Injected 10 μl of the solution into the chromatograph.

The following sub-Section describes in detail the parameters for LC and MS that could be applied for the analysis of propolis.

3.4.2.1. LC parameters. HPLC separation is largely dependent on the different affinities between the propolis compounds and the stationary phase. For a particular application, the chemical properties of the packing and physical properties of the column (e.g. particle size and column dimensions) need to be taken into account.

Reversed phase HPLC is doubtlessly the most widely used chromatographic method in propolis analysis (Falcão et al., 2010; Gardana, Scaglianti, Pietta, & Simonetti, 2007; Pellati, Orlandini, Pinetti, & Benvenuti, 2011; Piccinelli et al., 2011; Righi, Negri, & Salatino, 2013; Volpi & Bergonzini, 2006). Most appropriate are octadecylsilane columns (ODS or C18). Nucleosil C18 250 × 4 mm ID, 5μm particle diameter (Falcão et al., 2010); Luna C18 column 150 × 2.0 mm ID, 5 μm (Piccinelli et al., 2011); and CLC-ODS 150 × 6.0 mm ID (Midorikawa et al., 2001) can also give good results. Due to the complex nature of the matrix, a drawback for the use of these columns is the long runs needed, frequently above 50 min per run.

A fast and ultra-fast separation can be achieved with columns packed with sub-2 μm particles operating at ultra-high pressure systems. Ultra-high-performance liquid chromatography (UHPLC) is quite versatile and can be used to increase throughput, particularly suitable for the analysis of complex samples such as plant extracts or their metabolites (Nicoli et al., 2005). Recent work has been performed with propolis in equivalent columns of Waters BEH C18 (50 mm × 2.1 mm ID × 1.7 μm particle size) reducing the time run to 12 min (Novak et al., 2014).

The chromatographic conditions of the HPLC methods include, almost exclusively, the use of UV–Vis diode array detector (DAD) with spectral data for all peaks acquired in the range of 200–600 nm, although 280 nm is the most generic wavelength for phenolic compounds due to the high molar absorptivity of the different phenolic classes at that wavelength.

The eluent is composed of a binary solvent system containing acidified water (solvent A) combined with a polar organic solvent (solvent B). Gradient elution has usually been mandatory in recognition of the complexity of the propolis chemical profile. 0.1% formic or acetic acid can be added to water (as solvent A) and acetonitrile or methanol (as solvent B) are commonly used in propolis analysis. 0.1% formic acid is the most suitable when using a MS detector. The flow rate is dependent on the type of column used, but for the above parameters it is recommended to be 1 ml min^{-1}. Temperature control of the column should also be considered to achieve a better peak separation, between 25 and 40 °C, with 30 °C being the most suitable for propolis compound separation. For a flow rate of 1 ml min^{-1}, a post-column split of 0.2 ml min^{-1} to MS should be applied (Falcão et al., 2013a).

Table 1 presents the guidelines needed to achieve a good separation and analysis of the phenolic compounds present in propolis.

3.4.2.2. MS parameters. Given the unique characteristics of different mass spectrometers, it is critical to choose the suitable MS parameters. Table 1 summarizes the best conditions for the MS analysis of propolis phenolic compounds.

The ion source used should be electron-spray ionization (ESI). ESI is a soft ionization technique for a wide range of compounds (slight fragmentation but adducts are often observed), where ionization is achieved by applying a high electric charge to the sample needle, with voltage between 3 and 5 kV and the capillary temperature between 300 and 350 °C. ESI can be operated in the negative or positive full scan ion mode, although, and concerning the phenolic compounds, a higher sensitivity and better fragmentations can be achieved with the negative ion, thus resulting in more structural information (Cuyckens & Claeys, 2004). A more recent development is atmospheric pressure photoionization (APPI). If the compounds are poorly ionized by ESI and APCI, APPI should be considered as an alternative (Ignat, Volf, & Popa, 2011).

Concerning the mass analyzers, the ion trap is the one most recommended for the profiling of propolis composition since it is specially designed for multiple fragmentation steps (MS^n). Regarding target analysis, a tandem-MS detection over a single-stage MS operation is recommended because of the much better selectivity and the wider-ranging information that can be obtained (de Rijke et al., 2006). In linear ion traps, ions are isolated and accumulated due to a special arrangement of hyperbolic and ring shaped electrodes as well as oscillating electric fields. Then the ions can be fragmented by collision-induced decomposition (CID) (Ignat et al., 2011). The MS^n data is simultaneously acquired for the selected precursor ion. The collision induced decomposition (CID)–MS–MS and MS^n experiments should be performed using helium as the collision gas, with collision energy (CE) of 20–40 eV. The CE is dependent on the molecule stability under study. In the negative ion mode, collision energies of 20 eV for phenolic acids and 20–40 eV for flavonoids are suitable (Pellati et al., 2011).

3.4.3. *Identification of phenolic compounds*

Propolis chemical composition is a rich pool of phenolic compounds. Those, often referred to as polyphenols, embody a class of widely distributed and chemically diverse secondary metabolites synthesized in plants at different developmental stages (Steinmann & Ganzera, 2011). Polyphenols possess at least one aromatic ring with one or more hydroxyl functional groups. Flavonoids,

Table 1. Experimental guidelines for the propolis LC-MS analysis.

LC parameters	MS parameters
Column Reversed-phase HPLC octadecylsilane (ODS or C18) with standard measures 250 mm × 4 mm ID, 5 μm particle diameter UHPLC C18 alternative: 50mm × 2.1mm, 1.7 μm particle diameter	*Ionization technique* Electron-spray ionization (ESI) in the negative ion mode Capillary voltage: 3–5 kV Capillary temperature: 300–350 °C
Column temperature 30 °C	*Mass analyzer* Ion-trap The collision induced decomposition (CID) –MS–MS and MS^n experiments should be performed using helium as the collision gas, with a collision energy of 20 eV for phenolic acids and between 20–40 eV for flavonoids
Eluents Mobile phases comprising solvent (A) 0.1% formic acid in water and solvent (B) acetonitrile with 0.1% of formic acid, previously degassed and filtered *Solvent gradient* Start with 80% A and 20% B, reaching 30% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min, followed by the return to the initial conditions	
Flow 1 ml/min	
Detection UV–vis DAD detection in the range 200–600 nm, with 280 being the most common wavelength used in the study of phenolic compounds	

Table 2. Propolis compounds characterized by LC-DAD-MS.

Compounds	λ_{max} (nm)	*m/z* (ESI polarity)	MS^2 (% base peak)	Reference
Phenolic acids				
Quinic acid		179 (+)	143	(a)
Chlorogenic acid	325	353 (−)	179, 135, 191	(b)
Caffeic acid	292, 322	179 (−)	135	(c)
Dicaffeoylquinic acid	325	515 (−)	179, 135, 191	(b)
Ellagic acid	253, 367	301 (−)	301 (100), 257 (77), 229 (96)	(d)
p-Coumaric acid	310	163 (−)	119	(c)
Ferulic acid	295sh, 322	193 (−)	177 (16), 149 (47), 133 (100)	(c)
Dicaffeoylquinic acid	325	515 (−)	179, 135, 191	(b)
Isoferulic acid	298, 319	193 (−)	177 (16), 149 (47), 133 (100)	(c)
Tricaffeoylquinic acid	325	677 (−)	179, 135, 191	(b)
Benzoic acid	229	121 (−)		(d)
3,4-Dimethyl-caffeic acid	295sh, 322	207 (−)	163 (60), 102 (100)	(c)
Cinnamic acid	277	147 (−)	103	(c)
p-Coumaric acid methyl ester	307	177 (−)	163 (100), 119 (15)	(d)
Cinnamylidenacetic acid	310	173 (−)	129	(d)
Drupanin (3-prenyl-*p*-coumaric acid)	311	232 (−)	187, 133	(b)
Caffeic acid isoprenyl ester	298, 325	247 (−)	179 (100), 135 (15)	(d)
Caffeic acid isoprenyl ester (isomer)	298, 325	247 (−)	179 (100), 135 (15)	(d)
Caffeic acid benzyl ester	298, 325	269 (−)	178 (100), 134 (32), 161 (12)	(d)
Caffeic acid phenylethyl ester	295, 325	283 (−)	179 (100), 135 (28)	(d)
p-Coumaric acid isoprenyl ester	294, 310	231 (−)	163 (100), 119(12)	(d)
p-Coumaric acid benzyl ester	298, 312	253 (−)	162, 145, 118	(e)
p-Coumaric acid isoprenyl ester (isomer)	294, 310	231 (−)	163 (100), 119 (12)	(d)
Caffeic acid cinnamyl ester	295, 324	295 (−)	178 (100), 134 (24)	(d)
Caffeic acid cinnamyl ester (isomer)	295, 324	295 (−)	178 (100), 134 (24)	(d)
p-Coumaric acid cinnamyl ester	296, 310	279 (−)	162, 118	(e)
Artepillin C	311	299 (−)	255, 163, 151, 107	(b)
3-Prenyl-4-(2-methylpropionyl-oxy)-cinnamic acid	279.5	315 (−)	271	(b)
3-(2,2-Dimethyl-3,4-dehydro-8-prenyl-1-benzopyran-6-yl-propenoic acid	310	297 (−)	253, 149	(b)
3-Prenyl-4-(dihydrocinnamoyloxi)-cinnamic acid	279.5	363 (−)	319, 187, 149, 131	(b)
p-Methoxi cinnamic acid cinnamyl ester	279	293 (−)	177, 133	(b)
p-Coumaric acid-4-hydroxyphenylethyl ester dimer	289, 345	565 (−)	455 (10), 417 (36), 283 (100), 269 (43)	(b)
Di-hidroflavonols				
Pinobanksin-5-methyl-ether	286	285 (−)	267 (100), 252 (13), 239 (27)	(d)
Pinobanksin-5-methyl-ether-3-*O*-acetate	289	327 (−)	285 (100), 267 (18), 239 (31)	(d)
Pinobanksin	292	271 (−)	253 (100), 225 (26), 151 (10)	(d)
Pinobanksin-5,7-dimethyl-ether	292	299 (−)	285, 253, 139	(b)
Pinobanksin-3-*O*-acetate	292	292 (−)	271 (18), 253 (100)	(d)
Pinobanksin-3-*O*-acetate-5-*O*-*p*-hydroxyphenylpropionate	292	292 (−)	443 (68), 401 (75), 351 (100), 291 (55), 253 (2)	(d)
Pinobanksin-3-*O*-propionate	289	289 (−)	271 (9), 253 (100)	(d)
Pinobanksin-5-methyl-ether-3-*O*-pentanoate	289	289 (−)	285 (53), 267 (65), 239 (100)	(d)
Pinobanksin-7-methyl-ether-5-*O*-*p*-hydroxyphenylpropionate	292	292 (−)	433 (9), 415 (100), 400 (8), 253 (<1)	(d)
Pinobanksin-3-*O*-butyrate or isobutyrate	292	292 (−)	271 (5), 253 (100)	(d)
Pinobanksin-3-*O*-pentenoate	292	292 (−)	271 (7), 253 (100)	(d)
Pinobanksin-3-*O*-pentanoate or 2-methylbutyrate	292	292 (−)	271 (5), 253 (100)	(d)
Pinobanksin-*O*-hexenoate	292	292 (−)	271 (100), 253 (45)	(d)
Pinobanksin-3-*O*-phenylpropionate	292	292 (−)	271 (16), 253 (100)	(d)
Pinobanksin-3-*O*-hexanoate	292	292 (−)	271 (14), 253 (100)	(d)
Flavonols				
Quercetin	256, 370	301 (−)	179 (100), 151 (60)	(d)
Quercetin-3-methyl-ether	256, 355	315 (−)	300	(d)
Kaempferol	265, 364	285 (−)	285 (100), 257 (13), 151 (20)	(d)
Isorhamnetin	253, 370	315 (−)	300	(d)
Kaempferol-methyl-ether	265, 352	299 (−)	284	(d)
Kaempferol-methoxy-methyl-ether	265, 340	329 (−)	314	(d)

(*Continued*)

Table 2. (*Continued*).

Compounds	λ_{max} (nm)	*m/z* (ESI polarity)	MS^2 (% base peak)	Reference
Quercetin-dimethyl-ether	253, 355	329 (−)	314	(d)
Quercetin-tetramethyl-ether	256, 349	359 (−)	344	(d)
Galangin-5-methyl-ether	265, 300sh, 352	283 (−)	268 (100), 239 (60), 211 (10)	(d)
Rhamnetin	256, 367	315 (−)	300 (34), 193 (76), 165 (100)	(d)
Quercetin-dimethyl-ether	256, 355	329 (−)	314	(d)
Galangin	265, 300sh, 358	269 (−)	269 (100), 241 (61), 227 (20), 197 (22), 151 (20)	(d)
Kaempferide	265, 364	299 (−)	284, 151 (<1)	(d)
Kaempferol-dimethyl-ether	265, 346	313 (−)	299 (10), 298 (100)	(d)
Myricetin-3,7,4′,5′-tetramethyl-ether		375 (+)	360, 345, 315	(f)
Flavonol glycosides				
Quercetin-3-*O*-rutinoside	256, 352	609 (−)	301(100), 300 (87)	(d)
Quercetin-3-*O*-glucuronide	256, 355	477 (−)	301	(d)
Quercetin-3-*O*-glucoside	256, 355	463 (−)	301(100), 300 (64)	(d)
Kaempferol-3-*O*-rutinoside	265, 349	593 (−)	285	(d)
Isorhamnetin-3-*O*-rutinoside	253, 355	623 (−)	315 (100), 300 (22)	(d)
Isorhamnetin-*O*-pentoside	253, 346	447 (−)	315 (100), 300 (8)	(d)
Quercetin-3-*O*-rhamnoside	256, 349	447 (−)	301(100), 300 (47)	(d)
Isorhamnetin-*O*-glucuronide	253, 346	491 (−)	315	(d)
Kaempferol-methyl-ether-*O*-glucoside	265, 343	461 (−)	446 (91), 299 (100), 284 (11)	(d)
Isorhamnetin-*O*-acetylrutinoside	253, 352	665 (−)	623 (18), 315 (100), 300 (14)	(d)
Rhamnetin-*O*-glucuronide	256, 349	491 (−)	315	(d)
Quercetin-dimethyl-ether-*O*-rutinoside	253, 349	637 (−)	329 (100), 314 (18)	(d)
Quercetin-dimethyl-ether-*O*-glucuronide	253, 349	505 (−)	329 (100), 314 (18)	(d)
Kaempferol-*O*-*p*-coumaroylrhamnoside	265, 322	577 (−)	431 (6), 285 (100)	(d)
Flavones				
Luteolin	253, 268sh,349	285 (−)	285 (100), 267 (54), 241 (63), 175 (52)	(d)
Apigenin	268, 337	269 (−)	225 (100), 151 (29)	(d)
Luteolin-5-methyl-ether	266, 350	299 (−)	284, 256, 151	(d)
Chrysin-5-methyl-ether	268, 313	267 (−)	253 (100), 224 (25)	(d)
Chrysin	268, 313	268, 313 (−)	225 (17), 209 (100), 151 (5)	(d)
Acacetin	268, 331	268, 331 (−)	269	(d)
6-Methoxychrysin	265, 300sh, 350sh	283 (−)	269	(d)
Chrysoeriol-methyl-ether	250, 268sh, 343	313 (−)	298	(d)
Chrysin-5,7-dimethyl-ether	265, 311sh	281 (−)	267, 165	(b)
Flavanones				
Pinocembrin-5-methyl-ether	286	269 (−)	255 (48), 227 (100), 165 (30)	(d)
Liquiritigenin	280, 310	257 (+)	137 (62), 147 (72), 211 (19), 239 (100), 242 (36)	(g)
Pinocembrin	289	255 (−)	213 (100), 211 (32), 151 (48)	(d)
Naringenin	289	271 (+)	153 (100), 149 (100)	(g)
Pinocembrin-5-*O*-3-hydroxy-4-methoxyphenylpropionate	295	295 (−)	415 (3), 401 (31), 323 (15), 309 (100)	(d)
3-Hydroxy-5-methoxyflavanone	289	289 (−)	254 (100), 251 (54), 165 (22)	(d)
Chalcone				
Isoliquiritigenin	309, 372sh	257 (+)	242 (34), 239 (100), 171 (2), 147 (78), 137 (69)	(g)
Dimethylkuraridin		425 (+)	285	(a)
Isoflavonoids				
Formononetin	248, 302	269 (+)	254 (100), 237 (39), 213 (35)	(g)
Biochanin A	362, 326sh	285 (+)	270 (51), 257 (11), 253 (22), 229 (19)	(g)
Vestitol	280	273 (+)	137 (100), 123 (74)	(g)
Neovestitol	280	273 (+)	137 (100), 123 (70)	(g)

(*Continued*)

Table 2. (*Continued*).

Compounds	λ_{max} (nm)	*m/z* (ESI polarity)	MS^2 (% base peak)	Reference
7-*O*-methylvestitol		287 (+)	163 (10), 137 (100)	(g)
Mucronulatol	280, 340	303 (+)	167 (100), 149 (19), 123 (23)	(g)
7,3´-Dihydroxy-5´-methoxi-isoflavone	295	285 (+)	270 (100), 253 (55), 225 (18)	(g)
Retusapurpurin B	285, 470	523 (+)	399 (61), 387 (100), 385 (53)	(g)
Retusapurpurin A	285, 480	523 (+)	399 (61), 387 (100), 385 (59)	(g)
Pterocarpans				
Medicarpin	290	271 (+)	161(44), 137 (100)	(g)
Homopterocarpin		285 (+)	137 (100), 161 (51), 137 (100)	(g)
Vesticarpan		287 (+)	153 (100), 177 (19)	(g)
3,8-dihydroxy-9-methoxy-pterocarpan		287 (+)	269 (36), 255 (40), 177 (100), 153 (59)	(g)
3,4-dihydroxy-9-methoxy-pterocarpan		287 (+)	161 (23), 139 (100), 137 (55)	(g)
3-dihydroxy-8,9-dimethoxy-pterocarpan		301 (+)	191 (100), 167 (87), 153 (13)	(g)
Polyisoprenylated benzophenones				
Nemorosone		501 (−)	432	(a)
Guttiferone E/xanthochymol	250, 355	603 (+)	467 (85), 411 (25), 343 (21)	(g)
Oblongifolin A	250, 355	603 (+)	467 (41), 411 (8), 399 (32), 343 (24)	(g)
Prenylated benzophenone		407 (−)	338	(a)
Diterpenes				
Cupressic acid		$(M\text{-}H_2O+H)^+$: 303 (+)	285, 257, 247	(f)
Isocupressic acid		$(M\text{-}H_2O+H)^+$: 303 (+)	257, 247, 193	(f)
Imbricatoloic acid		323 (+)	305, 287, 277, 259, 181	(f)
Torulosal		$(M\text{-}H_2O+H)^+$: 287 (+)	269, 259, 177, 163	(f)
Isogathotal		$(M\text{-}H_2O+H)^+$: 287 (+)	269, 259, 163, 149	(f)
Torulosol		$(M\text{-}H_2O+H)^+$: 289 (+)	271, 243, 233, 215, 193, 179	(f)
Agathodiol		$(M\text{-}H_2O+H)^+$: 289 (+)	271, 243, 231, 215, 193, 179	(f)
Cistadiol		$(M\text{-}H_2O+H)^+$: 291 (+)	273, 235, 221, 209, 181, 163	(f)
18-Hydroxy-cis-clerodan-3-ene-15-oic acid		$(M\text{-}H_2O+H)^+$: 305 (+)	287, 269, 235, 223, 195, 177	(f)

Notes: (a) Zhang et al. (2014); (b) Gardana et al.(2007); (c) Falcão et al. (2010); (d) Falcão et al. (2013a); (e) Pellati et al. (2011); (f) Piccinelli et al. (2013); (g) Piccinelli et al. (2011).

whose structures are based on a C6-C3-C6 skeleton, are the most abundant group of phenolic compounds, and are sub-divided into several classes differing in the oxidation state of the central heterocyclic ring (Veitch & Grayer, 2008). These comprise chalcones, flavones, flavonols, flavanones, isoflavonoids, anthocyanidins and flavanols (catechins and tannins). Non-flavonoids comprise simple phenols, phenolic acids, coumarins, xanthones, stilbenes, lignins and lignans. Phenolic acids are further divided into benzoic acid derivatives, based on a C6-C1 skeleton, and cinnamic acid derivatives, which are based on a C6-C3 skeleton (Veitch & Grayer, 2008). The variability of propolis chemical composition contains large numbers of phenolics from different classes including, unexpectedly, glycoside phenolic compounds, clearly highlighting the challenges associated with their analysis.

The structural elucidation of different classes of propolis compounds is achieved by comparing their chromatographic behavior, UV spectra and MS information, to those of reference compounds. When standards are not available, the identity of the compounds can be achieved through comparison of the product ion spectra and retention times with pure compounds isolated from propolis or, alternatively, combining UV data with MS fragmentation patterns previously reported in the literature (Falcão et al., 2013a). Table 2 shows the UV data and MS fragmentation of many compounds described in the literature as propolis constituents. Only compounds with all the information regarding MS fragmentation are present.

Fragmentation patterns are specific for a given compound or class of compounds. For example, for the negative ion mode, phenolic acids demonstrated a common fragmentation pattern, with a loss of the carboxyl

group (CO_2, −44 Da) (Falcão et al., 2010). In the case of flavonoids, the distinct flavonoids classes differ in their pattern of substitution, which strongly influences the fragment pathway, the interpretation of MS/MS data provides specific structural information about the type of molecules. The MS^2 spectrum of many of these flavonoids (Table 2) revealed the fragments at *m/z* 151 or at *m/z* 165, which are resultant from the retro Diels-Alder mechanism (Cuyckens & Claeys, 2004). Also, neutral losses commonly described to occur in these compounds, such as the small molecules CO (−28 Da), CO_2 (−44 Da), C_2H_2O (−42 Da), as well as the successive losses of these molecules, were also observed (Cuyckens & Claeys, 2004). In accordance with Cuyckens and Claeys (2004), methylated flavonoids presented a significant $[M\text{-}H\text{-}CH_3]^{-\bullet}$ product ion.

Attention has to be taken to experimental conditions used, such as the type of ion source and mass analyzer, when comparing literature data, since different fragments can be found when different experimental set-up and/or operating conditions are applied. The mass spectra of flavonoids obtained with quadrupole and ion-trap instruments typically are closely similar, even though relative abundances of fragment ions and adducts do show differences. Therefore, direct comparison of spectra obtained with these two instruments is allowed. The main advantage of an ion-trap instrument is the possibility to perform MS^n experiments (Steinmann & Ganzera, 2011).

3.4.4. Concluding remarks

LC-MS is a powerful tool that can be used to overcome the difficult task of propolis chemical profiling, due to the high diversity of the resin floral sources collected by honey bees. To enhance the amount of structural information given by the technique, the most important features to be considered in LC-MS propolis chemical profiling are to:

(1) Chose the right LC parameters for the analysis such as a reversed-phase C18 HPLC column, which is the most selective in propolis analysis (Section 3.4.2.1).
(2) Use mobile phases comprising (A) 0.1% formic acid or acetic acid in water and (B) acetonitrile or methanol, (Section 3.4.2.1).
(3) Acquire spectral data with the UV–Vis DAD set at 280 nm, which is the most generic wavelength for phenolic compounds identification (Section 3.4.3).
(4) Use a ESI source and a ion trap mass analyzer, with helium as the collision gas, with CE of 20–40 eV (Section 3.4.2.2).
(5) Compare the UV spectra and MS information to those of reference compounds. If standards are not available, the identity of the compounds can be achieved through comparison of the product ion spectra and retention times with pure compounds isolated from propolis or combining UV data with MS fragmentation patterns previously reported in the literature (Section 3.4.3).

The fast technical evolution of the LC-MS systems, particularly in respect to the mass analyzers, will continue to allow new findings within the chemical composition of propolis.

3.5. *Mass spectrometry fingerprinting of propolis*

MS fingerprinting is a qualitative analytical tool used to discern between different types of propolis and to compare the composition of propolis samples to those of plant resins. MS fingerprints are proposed as characteristic of the composition of samples and can be used as a guide for their therapeutic uses. The method used in one study (Sawaya et al., 2004) was only slightly modified in the subsequent applications and can be considered as the standard method for propolis extraction for MS fingerprinting.

3.5.1. *Electrospray ionization mass spectrometry (ESI-MS) fingerprinting of propolis samples*

(1) Extract propolis as described in Section 3.1.1.
(2) Evaporate the solvent (ethanol) on a water bath at a temperature of 50 °C to obtain dry extracts of propolis.
(3) Dissolve these dry extracts in a 70% (v/v) methanol/water solution, containing 50 ng of dry propolis extract per ml of methanolic solution and 5 µl of ammonium hydroxide.
(4) Infuse these solutions directly into the ESI-source of a hybrid high resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer, via a syringe pump (Harvard Apparatus) at a flow rate of 15 µl/min. The MS conditions should be capillary −3.0 kV, cone 30 V.

Due to the prevalence of acid compounds, the negative ion mode fingerprints result in the clearest discrimination between the groups of propolis samples. This pattern was confirmed by subsequent studies of propolis fingerprinting conducted by Sawaya, da Silva, Cunha, and Marcucci (2011).

A simple chemometric evaluation is applied with Principal Component Analysis (PCA) performed using the 2.60 version of Pirouette software (Infometrix, Woodinville, WA, USA) (see the *BEEBOOK* manuscript on statistical guidelines for more information on using PCA, Pirk et al., 2013). Only the two most characteristic negative ion markers of each sample are selected and expressed as the intensities of these individual ions (variables). The data are preprocessed using auto scale and analyzed using PCA.

Samples are grouped according to their geographic origin (Sawaya et al., 2004). Furthermore, tandem mass

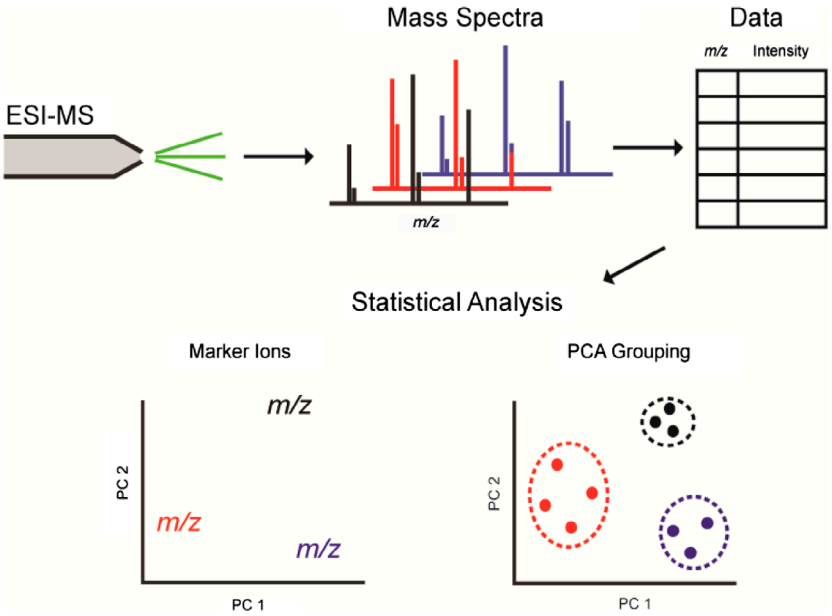

Figure 4. Genaral process used in ESI-MS fingerprinting studies: ionization and anlaysis by ESI-MS, extraction of the *m/z* and intensity of selected ions, statistical analysis of the data via PCA to group samples and indicate the marker ions for each group.

spectrometry with collision induced dissociation (CID) allowed on-line structural identification of certain marker ions such as dicaffeoylquinic acid, 3,5-Diprenyl-4-hydroxycinnamic acid, Pinocembrin, Chrysin, 3-Prenyl-4-hydroxycinnamic acid, 2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopyran and *p*-Coumaric acid (Sawaya et al., 2004). The general flow of these ESI-MS fingerprinting studies is shown in Figure 4.

Using the same extraction and analysis procedures, propolis samples can be compared to the plant sources of their resins. This could allow one to link the resin producing source plant to the propolis from these regions (Marcucci, Sawaya, Custodio, Paulino, & Eberlin, 2008).

3.5.2. *Concluding remarks*

MS fingerprinting may be applied to propolis samples to characterize their composition, identify the plant sources, and indicate their potential therapeutic application. Besides ESI, a new ionization source, named easy ambient sonic ionization (EASI), has been used for this purpose as well (Sawaya et al., 2010). The use of chemometric methods such as PCA to analyze the results is frequently necessary due to the large number of ions observed in each spectrum. The results of the analyses are capable of grouping similar samples, indicating their marker ions and, in some cases, correlating with the biological activity of samples.

3.6. *NMR analysis of propolis*

3.6.1. *Introduction*

Since its discovery, the phenomenon of Nuclear Magnetic Resonance (NMR) has been widely exploited as a research tool in analytical laboratories throughout the world. NMR spectroscopy is used to study the structure of molecules (Kwan & Huang, 2008). It also is well known that NMR can be used to analyze complex mixtures such as herbal extracts, foods, biological fluids, etc. (Forseth & Schroeder, 2011). In particular, NMR is used increasingly in the evaluation of food and in the quality assurance of natural products, although all its potential has not been fully exploited. The amount of information available in an NMR spectrum and the ease of sample preparation make this spectroscopic technique very attractive for the assessment of product quality.

One of the main advantages of this technique over that of other methods is its ability to furnish structural and quantitative information on a wide range of chemical species in a single NMR experiment. The mixture analysis by NMR is complex, but potentially very informative (Lin & Shapiro, 1997).

In recent years, the use of much higher magnetic fields and the greater sensitivity and spectral resolution that they bring, have stimulated interest in 1D and 2D NMR spectroscopy as a routine method for the analysis of complex mixtures (Charlton, Farrington, & Brereton, 2002; Fan, 1996).

There are two main strategies for analyzing mixtures via NMR: (a) separate components of the mixture prior to NMR analysis; and (b) analyze the mixture as it is. The first strategy is used when the goal of the work is the characterization of an isolated compound and it is not the subject of this discussion. The second strategy allows one to obtain an overall image of the mixture in question, without any further type of pre-treatment of the sample, except the eventual solubilization in a

suitable deuterated solvent. The obtained spectra will be considered as chemical fingerprints of the product under investigation. In this case, the analysis of the spectra, that usually appear very complex, requires tools for the pre-treatment of the signal and for the analysis of the results, normally based on multivariate statistical techniques (Papotti, Bertelli, Plessi, & Rossi, 2010).

3.6.2. Sample preparation

Since propolis is a solid material, it requires an initial extraction procedure using 70% ethanol (see Section 3.1.1). Obviously, if the extract is analyzed as is, very intense signals related to the solvent will be present in the obtained spectra. To avoid this problem, it is preferable to eliminate the solvents under a light nitrogen stream operating at low temperature. This procedure can be conducted directly in NMR tubes, and by dissolving the solid residue in an appropriate volume of the selected deuterated solvents. The most important thing to remember when choosing the most suitable solvent is that if D_2O is chosen, all the signals relating to alcoholic, phenolic or carboxylic hydroxyls, that are very abundant in propolis, will be lost in the spectrum. If one is interested to observe the signals related to these functional groups, a solvent that does not exchange deuterium with hydroxyls should be used. The most suitable in the case of propolis is the DMSO-d_6 (deuterated dimetyl sulfoxyde) (Papotti et al., 2010). There is no ideal ratio of propolis extract and the amount of solvent used; each one must find what works best in each case.

(1) Transfer 1ml of propolis extract (see 3.1.) to an NMR tube and evaporate to dryness at room temperature using a flow of nitrogen gas.
(2) Dissolve the dry residue in 0.5 ml of methyl sulphoxide-d6 (DMSOd6).
(3) Add 20 µl of tetramethylsilane (TMS) as a reference compound.
(4) Use the sample immediately for NMR experiments.

3.6.3. NMR analysis of propolis

A typical ^{1}H NMR spectrum of a propolis hydroalcoholic extract in DMSO-d_6 is reported in Figure 5. There are a high number of signals present in many spectral regions, particularly in the area between 1 and 8 ppm.

For this reason, a simple interpretation of this kind of spectra is rarely possible. Nevertheless, a preliminary assignment of the principal signals is often necessary to permit a correct interpretation of the results. The assignments can be performed using data obtained from one-dimensional NMR experiments and comparing them with literature data or with data obtained from pure standard compounds. The final correct assignment can be obtained using the most informative two-dimensional experiments such as COSY, HSQC and HMBC. In Table 3, the assignments of some well-known propolis components are reported. An example of this application is reported in Bertelli, Papotti, Bortolotti, Marcazzan, and Plessi (2012).

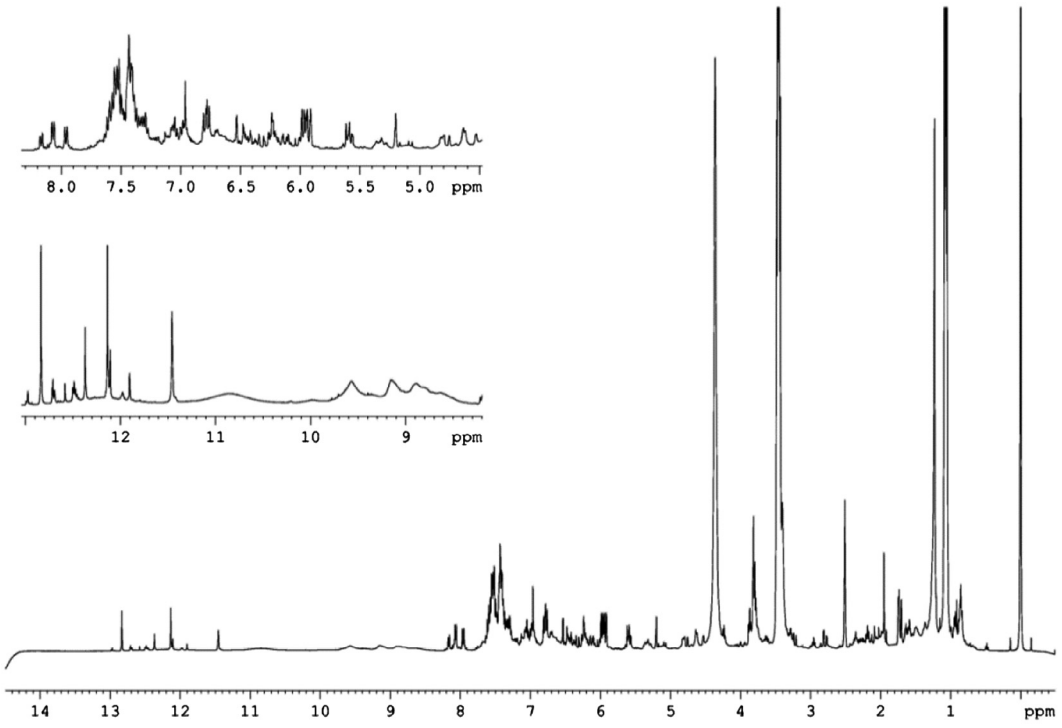

Figure 5. Typical ^{1}H NMR spectrum of propolis extracts in DMSO-d6 (Papotti et al., 2010).

Table 3. ^{1}H and ^{13}C NMR[a] chemical shifts of some flavonoids and phenolic acids, found in European poplar type propolis.

Flavones and flavonols

	Apigenin		Chrysin		Galangin		Kaempferol		Quercetin	
Position	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C
2	–	164.59	–	163.60	–	146.11	–	147.28	–	147.27
3	6.75 (s)	103.31	6.94 (s)	105.63	9.59 (s) *OH*	137.52	9.35 (s) *OH*	136.11	9.18 (s) *OH*	136.18
4	–	182.19	–	182.30	–	176.68	–	176.36	–	176.30
5	12.96 (s) *OH*	161.62	12.82 (s) *OH*	161.94	12.31 (s) *OH*	161.19	12.48 (s) *OH*	161.18	12.48 (s) *OH*	161.18
6	6.19 (d) (1.5)	99.30	6.22 (d) (1.8)	99.49	6.16 (d) (2.0)	98.74	6.20 (d) (2.2)	98.67	6.19 (d) (1.9)	98.64
7	10.75 (s) *OH*	164.19	10.90 (s) *OH*	164.91	10.59 (s) *OH*	164.65	10.78 (s) *OH*	164.35	10.75 (s) *OH*	164.33
8	6.46 (d) (1.5)	94.42	6.51 (d) (1.8)	94.58	6.40 (d) (2.0)	93.99	6.44 (d) (2.2)	93.94	6.41 (d) (1.9)	93.81
9	–	157.77	–	157.91	–	156.83	–	156.65	–	156.60
10	–	104.18	–	104.44	–	103.65	–	103.52	–	103.47
1′	–	121.66	–	131.19	–	131.38	–	122.15	–	122.42
2′; 6′	7.90 (d) (8.8)	128.90	8.04 (d) (8.8)	126.84	8.08 (d) (8.8)	127.94	8.05 (d) (8.8)	129.97	2′; 7.73 (d) (2.2) 6′; 7.54 (dd) (8.4; 2.2)	115.53 120.44
3′; 5′	6.92 (d) (8.8)	116.41	3′; 4′; 5′ 7.58 (m)	129.56	3′; 4′; 5′ 7.44 (m)	128.88	6.93 (d) (8.8)	115.90	3′; 9.45 (s) *OH* 5′; 6.89 (d) (8.8)	145.51 116.06
4′	10.40 (s) *OH*	161.93		132.42		130.28	10.10 (s) *OH*	159.65	9.59 (s) *OH*	148.16

Flavanones

Position	Naringenin		Pinocembrin		Pinostrobin	
	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C
2	5.46 (dd) (12.8; 2.2)	78.88	5.58 (dd) (12.5; 2.8)	78.21	5.62 (dd) (12.7; 3.0)	79.03
3	3α; 3.27 (dd) (17.1; 12.8) 3β; 2.67 (dd) (17.1; 2.2)	42.45	3α; 3.23 (dd) (17.1; 12.8) 3β; 2.79 (dd) (17.1; 3.2)	42.77	3α; 3.29 (dd) (17.1; 12.8) 3β; 2.83 (dd) (17.1; 3.0)	42.63
4	–	196.82	–	196.45	–	196.92
5	12.16 (s) *OH*	163.95	12.13 (s) *OH*	164.10	12.12 (s) *OH*	163.71
6	5.89 (d) (2.2)	96.24	5.91 (d) (2.2)	96.50	6.11 (d) (2.2)	95.24
7	10.79 (s) *OH*	167.10	10.79 (s) *OH*	167.23	3.80 (s) OCH_3	167.94
8	5.89 (d) (2.2)	95.42	5.95 (d) (2.2)	95.62	6.15 (d) (2.2)	94.34
9	–	163.40	–	163.65	–	163.09
10	–	102.23	–	102.30	–	103.10
1′	–	129.31	–	139.64	–	138.99
2′; 6′	7.31 (d) (8.8)	128.77	7.52 (d) (7.8)	127.12	7.54 (d) (8.8)	127.06
3′; 5′	6.79 (d) (8.8)	115.62	3′; 4′; 5′7.41 (m)	129.13	3′; 4′; 5′7.42 (m)	129.06
4′	9.59 (s) *OH*	158.19				
OCH_3	–	–	–	–	–	56.33

Phenolic acids

Position	Caffeic acid		Cinnamic acid		p-Coumaric acid		Ferulic acid	
	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C	δ_H[b] (*J* in Hz)	δ_C
1	12.10 (s) *COOH*	168.34						
2	6.17 (d) (16.0)	115.58	12.39 (s) *COOH*	168.00	12.09 (s) *COOH*	168.38	12.07 (s) *COOH*	168.42
3	7.42 (d) (16.0)	145.04	6.53 (d) (16.0)	119.70	6.29 (d) (16.0)	115.81	6.36 (d) (16.0)	116.09
1′	–	126.16	7.59 (d) (16.0)	144.38	7.51 (m)	144.60	7.49 (d) (16.0)	144.95
2′	7.03 (d) (1.8)	115.09	–	134.70	–	130.53	–	126.24
3′	9.13 (s) *OH*	146.02	7.68 (m)	128.65	7.51 (m)	125.74	7.28 (d) (2.0)	111.66
4′	9.52 (s) *OH*	148.59	3′; 4′; 5′ 7.42 (m)	129.36	6.81 (d) (8.8)	116.20	3.82 (s) OCH_3	148.38
5′	6.76 (d) (8.0)	116.21		130.67	9.97 (s) *OH*	160.04	9.54 (s) *OH*	149.55
6′	6.96 (dd) (8.0; 2.0)	121.60		129.36	6.81 (d) (8.8)	116.20	6.79 (d) (8.0)	115.99
OCH_3	–	–	7.68 (m)	128.65	7.51 (m)	125.74	7.08 (dd) (8.0; 2.0)	123.27
			–	–	–	–	–	56.17

[a]Assignments were from HSQC and HMBC experiments.
[b]Multiplicity in parentheses. (Bertelli et al., 2012).

The application of the NMR technique to propolis samples generates very complicated spectra that need to be processed before spectral calculations and subsequently analyzed by chemometric methods. The NMR signals can be used as intensity or can be integrated.

If the choice is to use spectra as intensity, an ideal preprocessing should include the steps that follow.

(1) Calibrate phased spectra by placing the signal of the standard compound TMS to 0 ppm.
(2) Each spectrum generates a file containing several thousand data points corresponding to the time domain that is the number of points acquired and digitalized by the instrument along the spectral width. Export these files and assemble them in a data-set.
(3) Solve misalignment problems a posteriori using suitable software. A good example of this kind of software is the open source Icoshift program running in Matlab environment (Savorani, Tomasi, & Englesen, 2009). Although the chemical shift of a nucleus is generally assumed to be rather stable, it is necessary to consider that some experimental factors (pH, ionic strength, solvent, field inhomogeneity, temperature) can affect the absolute and the relative position of an NMR signal, producing slight or significant variations in chemical shifts along the spectral width (Bertelli et al., 2012). Unresolved peaks in one spectrum can be resolved or more overlapped in another spectrum. This is particularly important in the analysis of complex mixtures, such as propolis extracts, in which a high number of similar compounds are present.
(4) To reduce the number of data points, do not consider all the spectral regions devoid of signals and the solvent signals, and subsequently remove them. If the number of spectral variables remains very high, reduce it further by lowering the spectral resolution.

3.6.4. Statistical analysis of NMR spectra

Multivariate chemometric methods can be applied on the data-set containing spectra. There are a number of multivariate techniques that can be used in the analysis of NMR spectra (Brereton, 2013):

(1) Principal Component Analysis (PCA): PCA is an unsupervised technique and allows one to express a large portion of the data's total variance with a smaller number of variables which can be used to represent graphically the population of samples and to identify the most significant original factor(s).
(2) Discriminant Analysis (DA): DA is a supervised technique used to determine whether a given classification of cases into a number of groups is appropriate. DA can be used, for instance, to test whether a particular clustering of cases obtained from a unsupervised method like PCA or Cluster analysis is likely. Also, this analysis can be used to classify unknown samples.
(3) Partial Least Square Discriminant Analysis (PLS-DA): PLS-DA can be described as the regression extension of PCA, giving the maximum covariance between measured data (NMR spectral intensities distribution) and the response variable (represented in this case by the possible classification of samples).

Normally, some statistical pre-treatment should be done before performing one of the above mentioned methods on NMR-generated data. The most useful to improve the results are normalization, mean centering and autoscaling. It is essential that one has a large number of samples in order to cross-validate the obtained models and also to have a test set for external validation. To date, there have not been many published reports where NMR was used to study propolis extracts as mixtures. Meneghelli et al. (2013) used NMR to identify some components of Brazilian propolis using the extracts directly without any kind of isolation and purification steps. They used one- and two dimensional NMR to study the chemical profile of the samples. (Meneghelli et al., 2013).

Two different studies report the use of NMR to compare different types of propolis. Cuesta-Rubio et al. (2007) studied three different varieties of Cuban propolis using ^{1}H and ^{13}C one-dimensional NMR as chemical fingerprint technique, HPLC-PDA and HPLC-MS. A similar work was published in 2010 by the same authors (Hernandez et al., 2010).

Watson et al. (2006) used NMR and PCA to build a model for the classification of propolis of different geographical origins. In this case, the authors used the bucketing technique. This technique consists of dividing the spectra in different small regions, following which the signals present in each region are integrated and the area results are used as spectral variables. The obtained model was able to classify samples from different areas of the world.

Papotti et al. (2010) published an article regarding the use of NMR to classify propolis samples according to their production procedure. In this work, the authors used not only ^{1}H NMR but also ^{1}H-^{13}C HMBC spectra. In the first case, the spectra were used as intensity and after integration of principal signals. In the latter, the volume of two-dimensional spectra signals were calculated adding together the intensity of the points located in previously manually defined areas surrounding the correlations and all spectra were processed using the same map of regions of interest. On the different obtained data-set, general discriminant analysis (GDA) was used to classify propolis according to their NMR fingerprint (Papotti et al., 2010).

In conclusion, NMR represents a very powerful tool for the study of propolis and the use of NMR coupled with an appropriate data processing procedure and multivariate statistical methods enables the development of sufficiently effective and appropriate models for classifying propolis. It is interesting to note that the best results are normally obtained using the ^{1}H NMR which is the simplest and fastest technique.

3.7. Propolis type dereplication

3.7.1. Introduction

Propolis from different locations always demonstrates considerable biological activity even though the chemical composition may vary (Kujumgiev et al., 1999; Seidel, Peyfoon, Watson, & Fearnly, 2008). For this reason, the chemical diversity of different propolis samples also has the potential to provide valuable leads to active components. Thus, the future discovery of new types of propolis from unexplored regions is important with respect to uncovering new biologically active compounds with important pharmacological effects. Investigating propolis from currently unstudied regions is important as it would allow one to determine if the new propolis belongs to an already known propolis type. The rapid identification (dereplication) of known propolis types avoids re-isolation and identification of known propolis constituents and is crucial for fast discovery of new natural/propolis compounds. Dereplication is rapid identification of known bioactive metabolites from chemical profiling of plants and other natural sources.

3.7.2. GC-MS as a strategy for propolis type dereplication

GC-EI MS is a powerful analytical platform for dereplication, combining the unprecedented resolving power of capillary GC with the structural information provided by EI mass spectra and supported by rich spectral libraries. In propolis research, GC-MS is one of the most common methods used and thus it is an excellent tool for propolis chemical type dereplication. Propolis ethanol extracts are subjected to GC-MS analysis after silylation (Section 3.3.1).

The first outcome of the GC-MS analysis is the TIC chromatogram. In the case of propolis, this is usually a complicated chromatogram containing several dozen peaks (Figure 6). Although sometimes the practiced eye is able to recognize a characteristic pattern, the analysis of the mass spectra is inevitable. After obtaining the TIC chromatogram, attention is directed towards the most prominent peaks and their mass spectra are analyzed. Let us assume that this analysis has resulted in identification of the major peaks. As soon as the major peaks in the TIC chromatogram are identified, it is necessary to check the characteristic constituents of the known propolis types and determine if these major constituents match one of them. In this case, the dereplication process has been completed.

In this Section 3.7.2, the most important markers for positive identification of the most widespread and well known propolis types are presented. Data about propolis types in Australia, the Middle East, Africa and to some extent North America are scarce and demonstrate diverse chemistry. Thus it is hard to formulate propolis types for these regions.

3.7.2.1. Poplar type propolis. Poplar type propolis, originating from *Populus* spp, is characterized by flavonoids, phenolic acids and their esters as bioactive constituents (Ahn et al., 2007; Greenaway, Scaysbrook, & Whatley, 1990; Marcucci, 1995). The most intensive peaks in the TIC chromatogram of a poplar propolis sample typically belong to pinocembrin, chrysin, galangin, pinobanksin 3-acetate and pinobanksin. These compounds are characteristic of propolis originating from the bud exudates of the black poplar *Populus nigra* (Bankova, de Castro, &

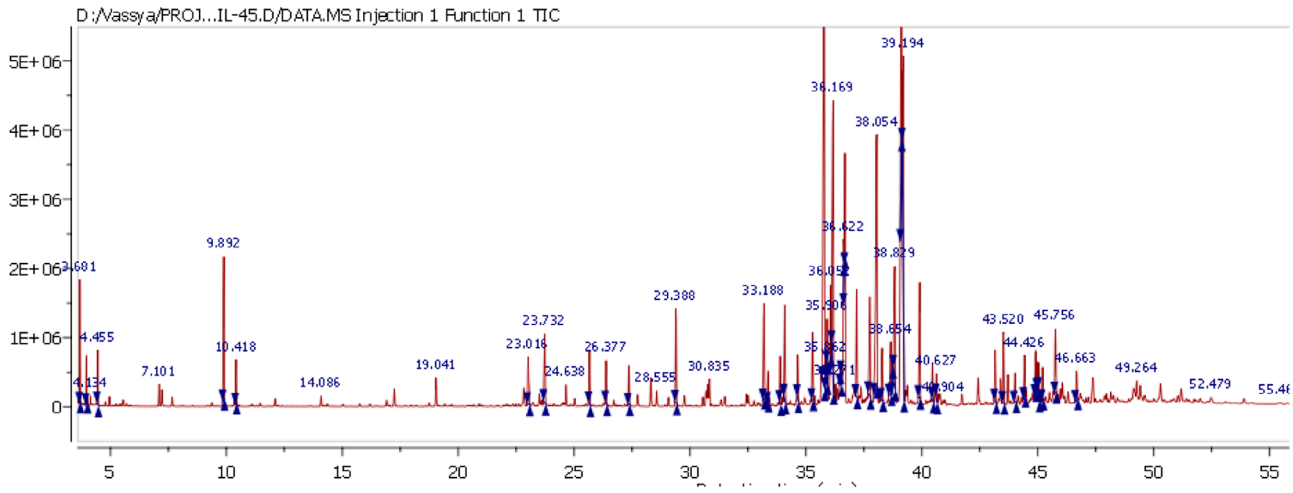

Figure 6. TIC chromatogram of a typical poplar propolis sample (Popova et al., Unpublished data: internal database).

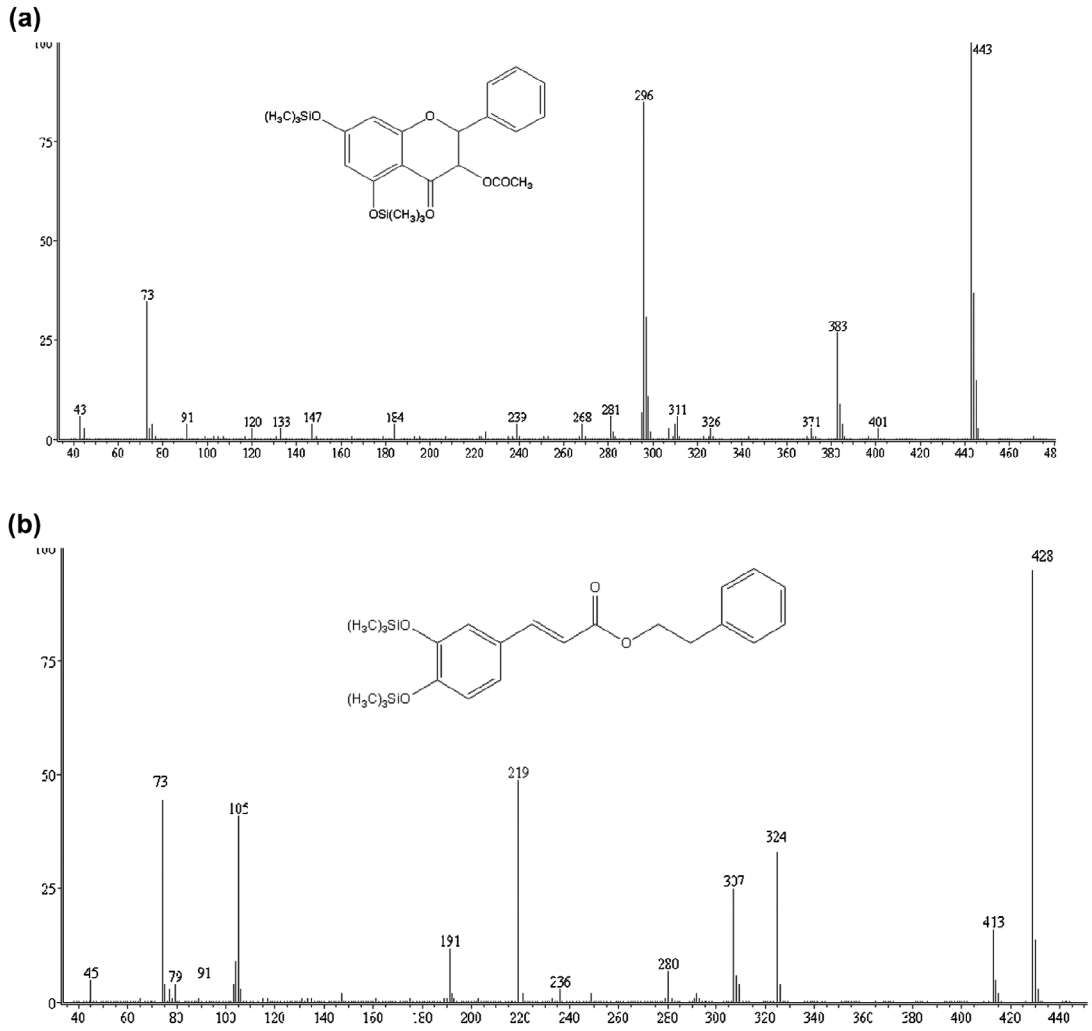

Figure 7. EIMS spectra of the TMS derivatives (a) pinobanksin 3-acetate, $(M)^+$ at *m/z* 458 and (b) phenylethyl caffeate (CAPE), $(M)^+$ at *m/z* 428. (Popova et al., Unpublished data: internal database).

Marcucci, 2000). For positive confirmation of poplar propolis, it is necessary to confirm the presence of the taxonomic markers of the black poplar – esters of substituted cinnamic acids, and especially penteny caffeates and phenylethyl caffeate, as well as pinobanksin 3-acetate. Their mass spectra are presented in Figure 7.

3.7.2.2. Aspen type propolis. In northern regions of Europe, the trembling aspen (European aspen) *Populus tremula* is used by bees as a propolis plant source (Bankova, Popova, Bogdanov, & Sabatini, 2002; Isidorov et al., 2014; Popravko, Sokolov, & Torgov, 1982). In the case of aspen propolis, major peaks in the TIC chromatogram belong to *p*-coumaric, ferulic, and benzoic acids, benzyl *p*-coumarate and benzyl ferulate. The minor but discriminant markers of aspen bud exudates are the glycerol esters of substituted cinnamic acids (phenolic glycerides) as 2-acetyl-1,3-di-p-coumaroylglycerol and 1-acetyl-3-feruloyl glycerol (Figure 8).

3.7.2.3. Brazilian green propolis. Brazilian green propolis is another well studied propolis type. Its main bioactive constituents include phenolic acids, prenylated phenolic acids and flavonoids which are characteristic for *Baccharis dracunculifolia*, the most important botanical source of Southeastern Brazilian propolis (Bankova et al., 1999; Kumazawa et al., 2003). For this propolis type, the major peaks in TIC chromatogram belong to artepillin C (Figure 9), drupanin, p-coumaric acid and dihydrocinnamic acid. Minor, but important markers are 2,2-dimethyl-6-carboxyethyl prenylbenzopyrane and aromadendrine 4′-methyl ether (Figure 9).

3.7.2.4. South American red propolis. The biologically active constituents of red propolis from Cuba and Brazil are isoflavans, isoflavons and pterocarpans (López, Schmidt, Eberlin, & Sawaya, 2014; Lotti et al., 2010; Piccinelli et al., 2011; Trusheva et al., 2006). This type has as major constituents vestitol (Figure 10), medicarpin (Figure 10), neovestitol, 7-O-methylvestitol (isosativan),

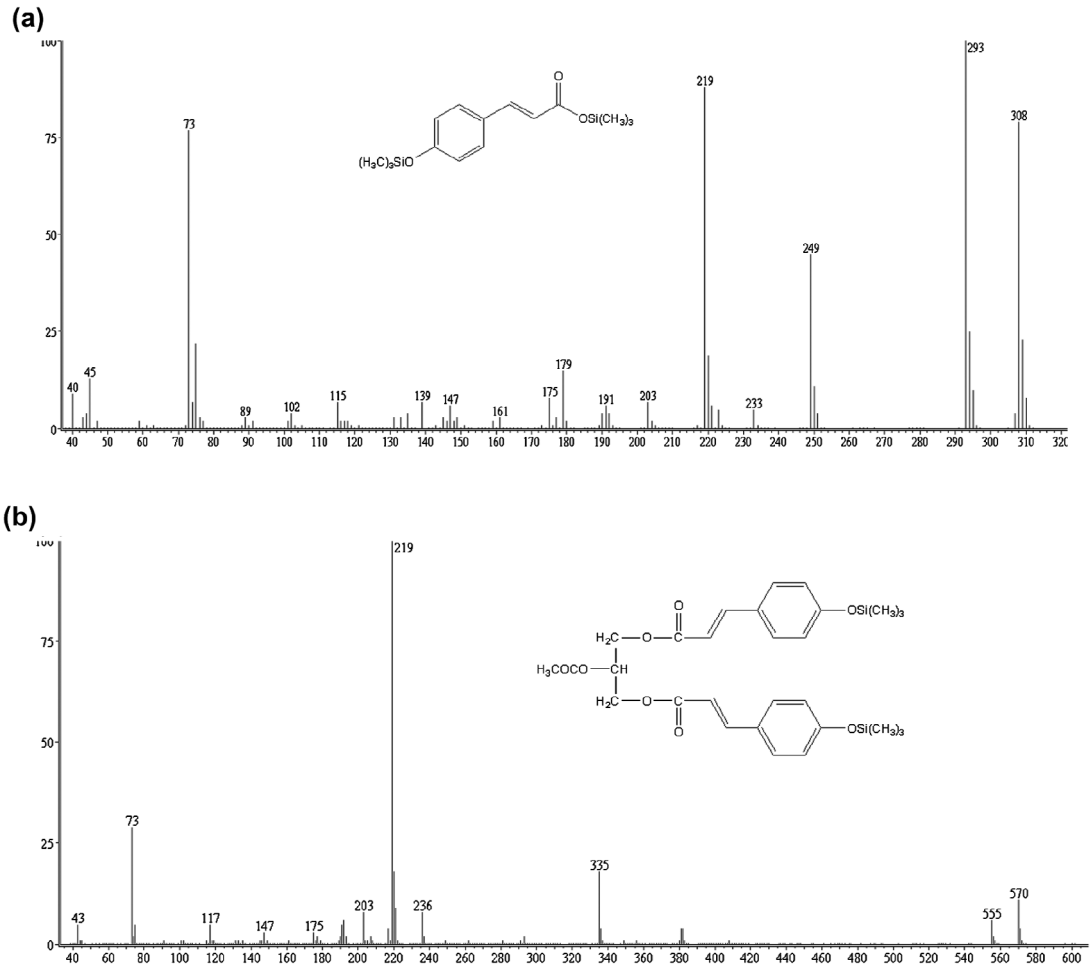

Figure 8. EIMS spectra of the TMS derivatives (a) *p*-coumaric acid, $(M)^+$ at *m/z* 308 and (b) 2-acetyl-1,3-di-p-coumaroylglycerol, $(M)^+$ at *m/z* 570. (Popova et al., Unpublished data: internal database).

and formononetin, all of them taxonomic markers of *Dalbergia ecastophyllum*.

3.7.2.5. Mediterranean type propolis. This type is characteristic for propolis samples originating from the Mediterranean region and its major constituents are diterpenes typical for the resin of the cypress tree *Cupressus sempervirens* (Popova, Graikou, Chinou, & Bankova, 2010; Popova et al., 2012). Isocupressic acid (Figure 11), pimaric acid, agathadiol, isoagatholal and totarol (Figure 11) give the major peaks in the TIC chromatogram. The only phenolic compounds in typical cypress propolis are the phenolic diterpenes totarol and totarolone. Cypress propolis usually does not contain flavonoids and phenolic acids.

3.7.2.6. Pacific type propolis. This propolis type is characteristic for samples from Pacific islands (Taiwan, Okinawa, Indonesia) (Huang et al., 2007; Kumazawa et al., 2008; Trusheva et al., 2011). Its dereplication includes identification of the prenylated flavanones (propolins) propolin C, propolin D (Figure 12) and propolin F as major peaks in TIC chromatogram. The plant source of these compounds is *Macaranga tanarius*.

3.7.2.7. Mangifera indica type propolis. The main bioactive metabolites of this propolis type are a series of phenolic lipids: cardanols, cardols and anacardic acid derivatives – all resin biomarkers of the tree *Mangifera indica* (mango) (Knödler et al., 2008; Trusheva et al., 2011). Among them, heptadecenyl-recorcinol (Figure 13), nonadecenyl-recorcinol, nonadecyl-anacardic acid and heptadecenyl-anacardic acid correspond to the most prominent peaks in TIC chromatogram. Minor, but characteristic constituents are triterpenes from cycloartane type as cycloartenol, mangiferolic acid (Figure 13) and 24-hydroxyisomangiferolic acid.

3.7.2.8. Mixed propolis types. In many cases, bees collect resins from two or even three plant sources. In such cases, the characteristic markers of the particular

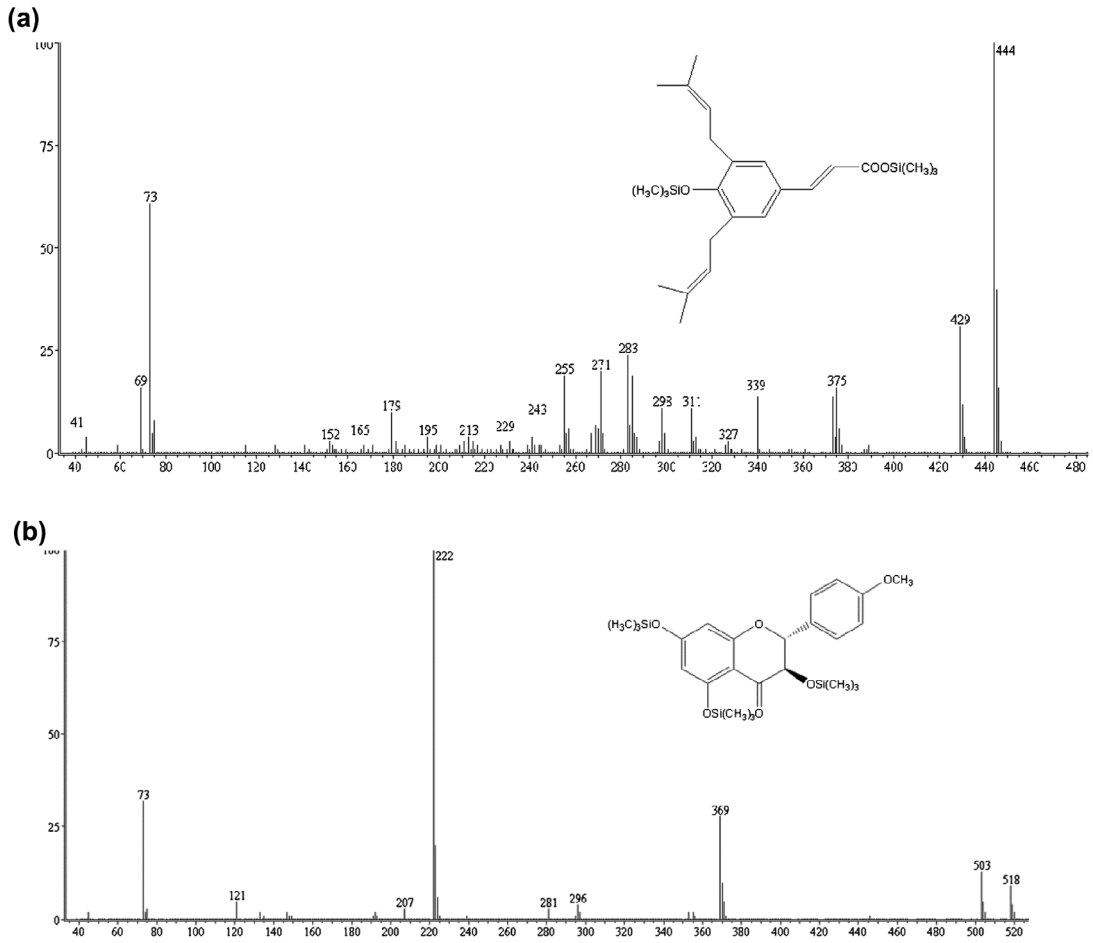

Figure 9. EIMS spectra of the TMS derivatives (a) artepillin C, $(M)^+$ at *m/z* 444 and (b) aromadendrine 4′-methyl ether, $(M)^+$ at *m/z* 518. (Popova et al., Unpublished data: internal database).

source plants can be detected by GC-MS. For this reason, a more detailed analysis of the total ion chromatogram is necessary, in order to consider more than just a limited number of prominent peaks.

Several mixed propolis types have been detected, for example aspen-poplar, *Cupressus*-poplar (Bankova et al., 2002), and Pacific (*Macaranga*)-*Mangifera indicia* propolis (Trusheva et al., 2011).

3.7.3. *Other possibilities for dereplication*

Other analytical methods also offer the possibility to perform dereplication of the propolis type: LC-MS (Section 3.4), ESI-MS fingerprinting (Section 3.5.1), NMR analysis (Section 3.6), and HPTLC (Morlock, Ristivojevic, & Chernetsova, 2014; Ristivojevic et al., 2014). The important point is to identify the corresponding markers that allow unambiguous positive identification of the source plant(s). If the results of such analyses do not allow the dereplication of propolis type, the metabolomic approach described in Section 3.7.4 should be applied in order to determine the botanical sources of propolis and, respectively, its chemical type based on the chemistry of the source plant. Alternatively, a very recent publication (Jain, Marchioro, Mendonca, Batista, & Araujo, 2014) reports on the application of DNA analysis for determining the botanical origin of red Brazilian propolis.

3.7.4. *LC-MS-based metabolomic analysis to determine the botanical sources of propolis*

Direct observation of resin forager behavior in the field can be extremely difficult or impossible, as foraging can occur over a large area and in the canopy of trees. This makes analytical analyses an attractive alternative, but one must consider several challenges. First, bees typically have many resinous plants from which to choose in a given environment and these available species may be closely related. For example, six species of *Populus* (a known resin source for honey bees) and numerous hybrids occur in the state of Minnesota, USA, and their resins have some degree of similarity. Second, resins from most species remain uncharacterized and characterization itself is a very labor intensive process. Lastly, further complications occur in the hive where resins from several plant species may be mixed. Therefore, any universal method developed to determine the

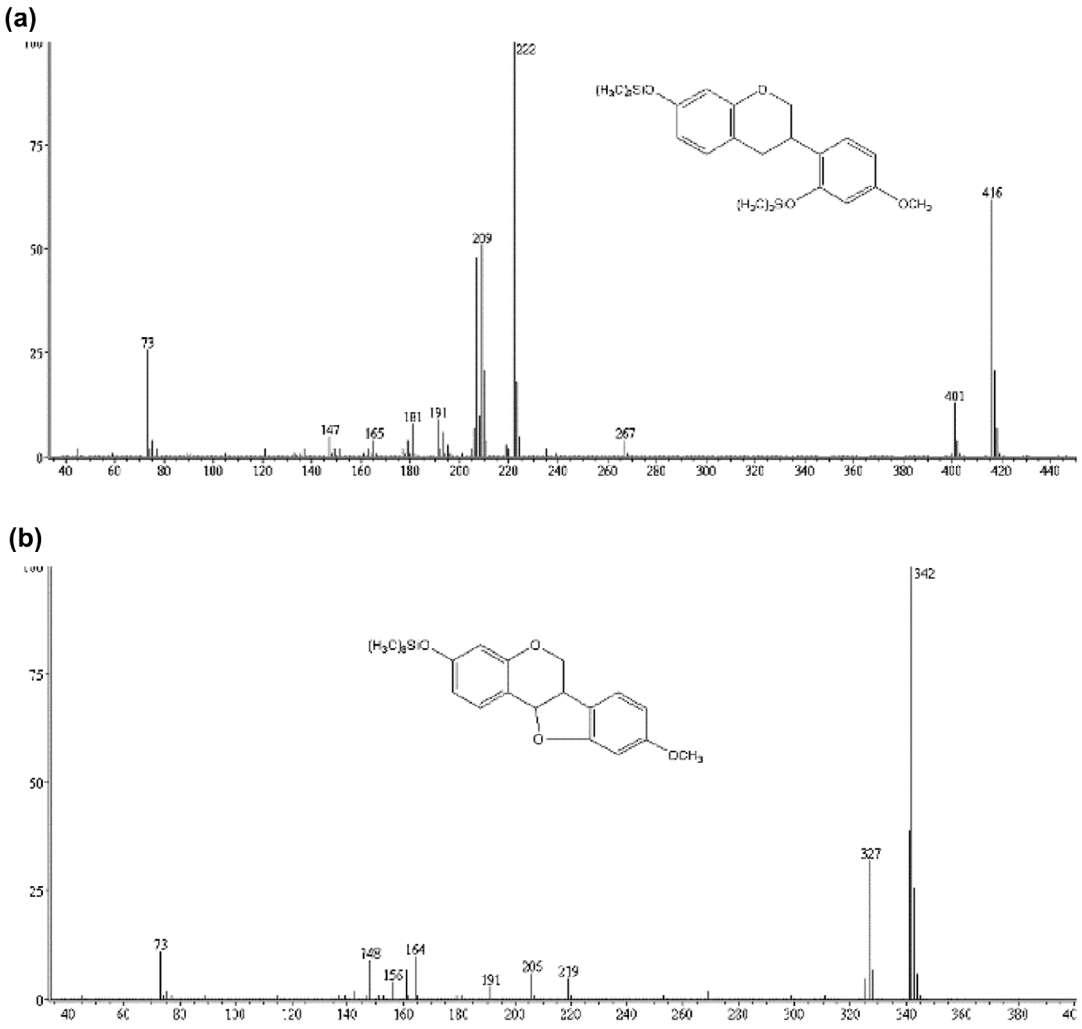

Figure 10. EIMS spectra of the TMS derivatives (a) vestitol, $[M]^+$ at *m/z* 416; (b) medicarpin, $[M]^+$ at *m/z* 342. (Popova et al., Unpublished data: internal database).

botanical sources of propolis must: (1) be powerful enough to discriminate between resins from closely related species; (2) work effectively with uncharacterized resins; and (3) be sensitive enough to sample at the level of individual bees carrying pure resin.

Traditional analytical methods will generally fail to meet our second criteria because comparisons are made regarding specific characterized compounds. Metabolomics is an approach that compares the global pattern of metabolite signals among samples using powerful statistical analyses without regard for the identities of specific compounds. LC-MS based metabolomics analysis fulfils all of our criteria in that: (1) LC-MS can easily generate hundreds of chemical signals that can be used to discriminate between closely related species; (2) metabolomics makes powerful comparisons between sample "fingerprints" without requiring any chemical characterization; and (3) sampling of individual resin foragers can be performed. LC-MS instruments equipped with an auto-sampler have the added capacity to run tens to hundreds of samples easily. Herein, we describe the metabolomics methods used in Wilson, Spivak, Hegeman, Rendahl, and Cohen (2013) to track the resin foraging behavior of individual honey bees.

3.7.4.1. Sample preparation for LC-MS

(1) Metabolomics works best with many samples; however, increasing the sample number increases analytical time and difficultly. It is generally reasonable to collect up to 100 samples of resin in total, directly from bees and from plants (Section 2.1).
(2) Weigh resin globules from bees, place in LC-MS vials, and dissolve in HPLC-grade acetonitrile. The final concentration of your samples is highly dependent on your instrumentation; however, we have found that a sample concentration of 1 mg/ml works well for a variety of high and low resolution instruments (Wilson et al., 2013; Wilson, Brinkman, Spivak, Gardner, & Cohen, 2015).

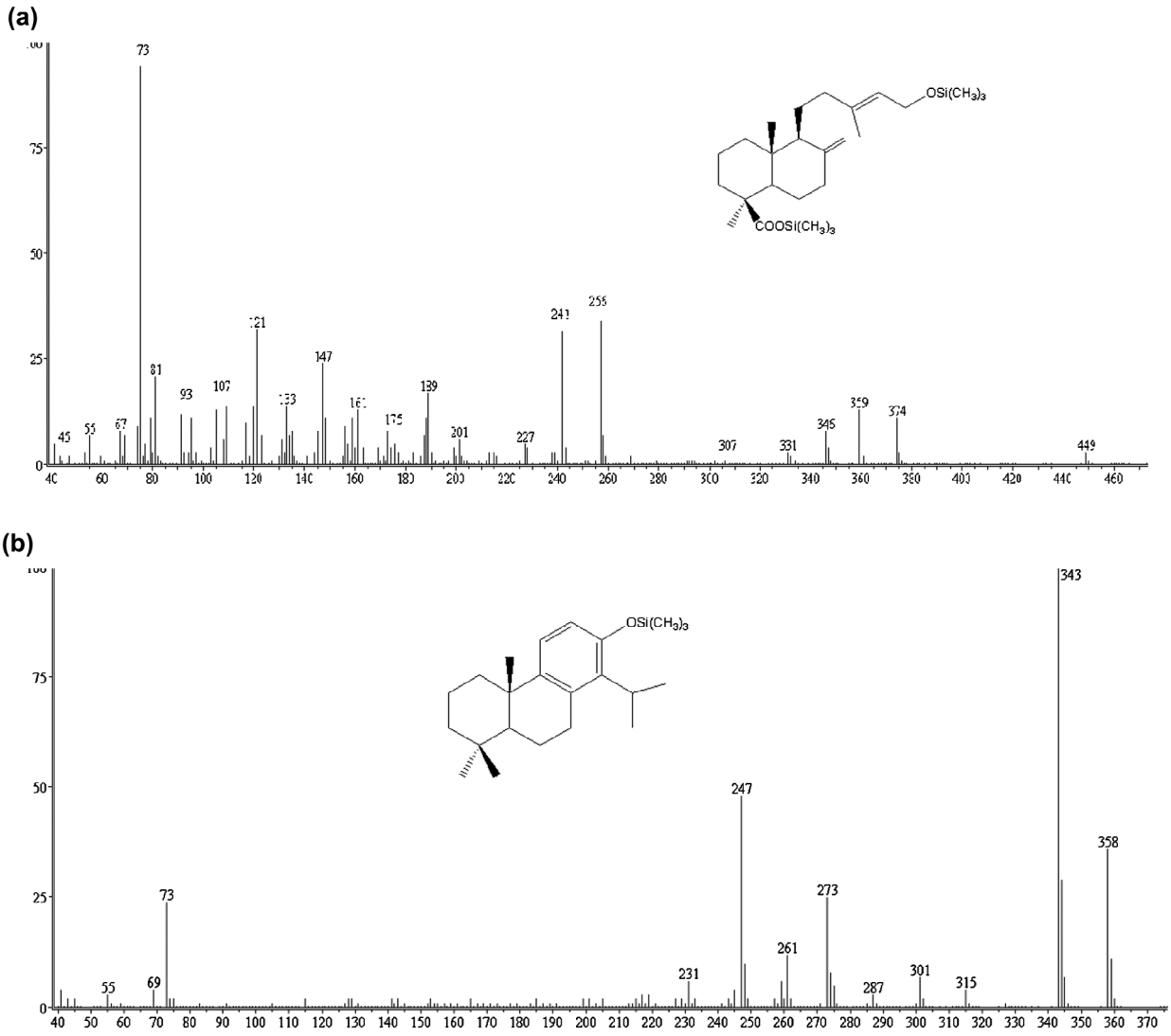

Figure 11. EIMS spectra of the TMS derivatives (a) isocupressic acid, $(M)^+$ at *m*/*z* 464 and (b) totarol, $(M)^+$ at *m*/*z* 358. (Popova et al., Unpublished data: internal database).

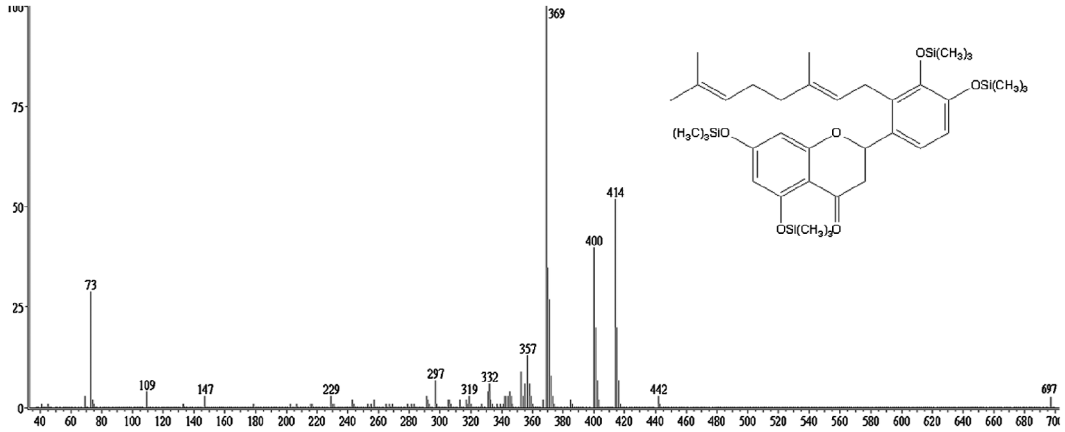

Figure 12. EIMS spectra of the TMS derivative of propolin D, $(M)^+$ at *m*/*z* 712. (Popova et al., Unpublished data: internal database).

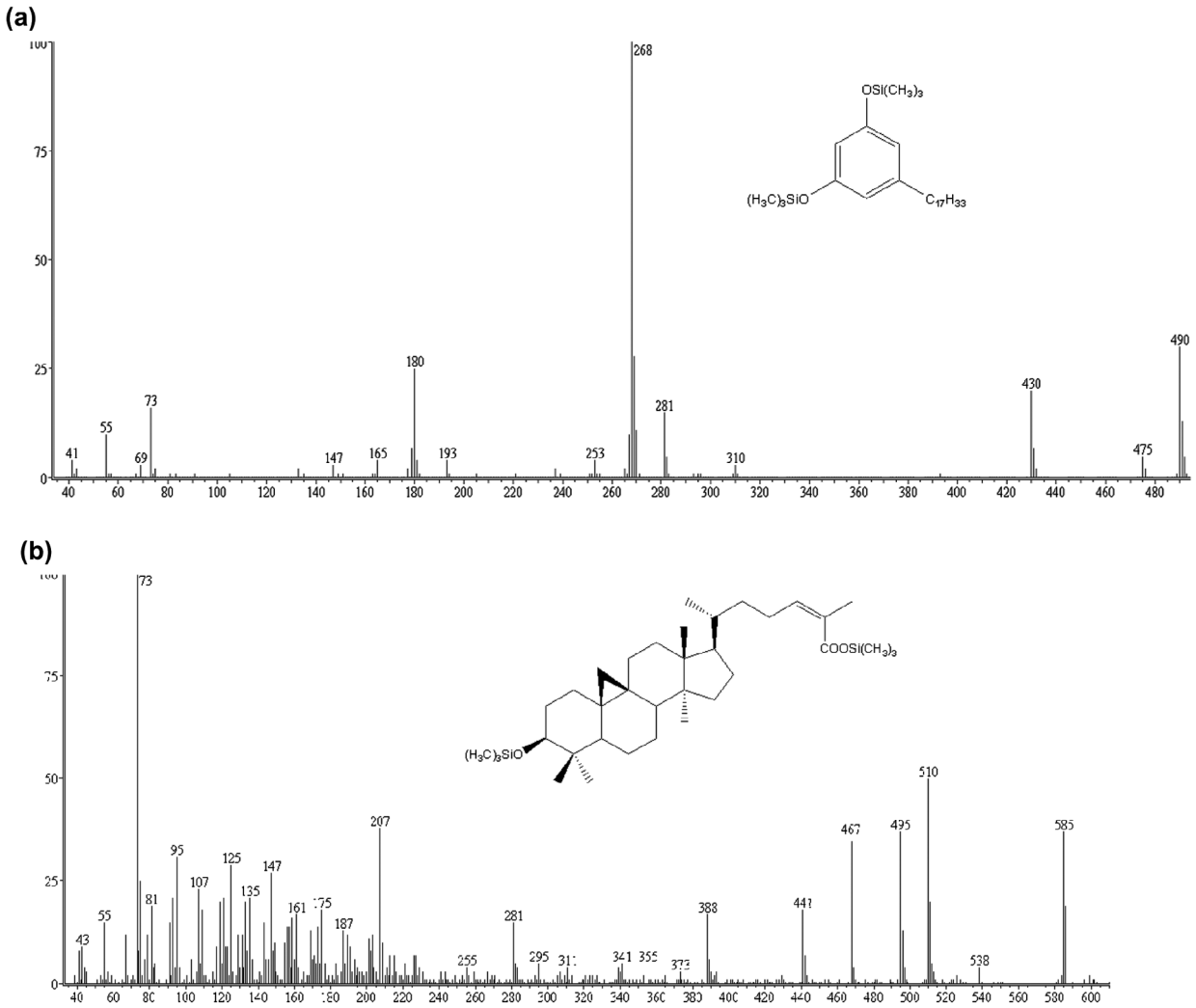

Figure 13. EIMS spectra of the TMS derivatives (a) 5-heptadecenyl-recorcinol, $(M)^+$ at *m/z* 490 and (b) mangiferolic acid, $(M)^+$ at *m/z* 600. (Popova et al., Unpublished data: internal database).

Table 4. General LC method for metabolomics analysis.

Column: Agilent Zorbax C_{18}, 2.1 × 100 mm, 1.8 μm particle size
Flow rate: 0.45 ml/min

Time (min)	% A (water w/0.1% formic acid)	% B (acetonitrile w/0.1% formic acid)
0	90	10
1.5	90	10
17.5	5	95
19.5	5	95
20.5	90	10

(3) Add 5 ml of HPLC-grade acetonitrile to plant tissues or collected resin. Rock gently for 15 min to wash resins off of tissues, then remove tissues using clean forceps. Be careful not to cross-contaminate samples.
(4) Determine the concentration of resin samples from plants using vacuum centrifugation.
(5) Dilute resin samples from plants to 1 mg/ml for analysis.
(6) Create a composite sample for quality control by adding equal volume amounts of each biological sample into a new vial (e.g. If you have 100 total samples from plants and bees, take 10 μl from each and add to a new vial). Since a composite sample

made in this way contains essentially all of the signals that could be produced in all of the biological samples, technical replicates of the composite sample can be used to filter out non-reproducible LC-MS signals during data analysis.

3.7.4.2. LC-MS data collection. It is important to recognize that the chemistry of unknown resins cannot be accounted for in the analytical method preemptively. Therefore, we present a general reversed-phase C_{18} approach developed for a Waters Acuity UPLC system connected to either a Waters SQD mass spectrometer (low resolution) or a Waters G2 Synapt mass spectrometer (high resolution) as used in Wilson et al. (2013, 2015) (Table 4). Data can be collected in either negative ion mode, positive ion mode, or both simultaneously, but the composite sample should be run every 5–10 samples, and at least three times during the course of the entire LC-MS run. Remember to utilize best practices for LC-MS analysis. (Viswanathan et al., 2007).

3.7.4.3. LC-MS data analysis

(1) Convert data files to CDF format. Waters instruments come with a program called Databridge that will perform this function. This will not be necessary if you plan on using proprietary metabolomics data analysis software.
(2) Smith, Want, O'Maille, Abagyan, and Siuzdak (2006) developed a freely available R script to analyze metabolomics data in CDF format which utilizes XCMS to produce a table of mass/retention time pairs and their intensities by sample for the entire data-set (data matrix). Please refer to Wilson et al. (2013) for a full description. Other metabolomics data analysis software can be used to perform this task, but few can utilize quality control samples in the manner described here, which may result in low quality signals being carried into subsequent analyses.
(3) Perform principle component analysis (PCA) on the data matrix (Pirk et al., 2013). Points representing samples will scatter on the PCA graph based on their LC-MS peak patterns, with samples showing similar peak patterns clustering together (see Wilson et al., 2013). If samples of bee collected resin cluster with samples of plant collected resin, this is a strong indication that bees foraged from this plant.

3.8. Spectrophotometric analysis of propolis

Spectrophotometric methods are very useful for fast and easy quantitative determination of phenolic compounds in propolis and for routine control of propolis preparations. There are efficient, precise and reliable spectrophotometric methods that are aimed at the determination of total flavonoids or total phenolics content. Phenolics and flavonoids are major constituents and most important bioactive ingredients of several propolis types and spectrophotometric methods are useful in their rapid characterization.

3.8.1. Spectrophotometric analysis of poplar type propolis

The analysis of poplar type propolis consists of the spectrophotometric quantitative determination of the following groups of phenolic compounds: (Popova et al., 2004): flavones and flavonols; flavanones and dihydroflavonols; and total phenolics.

3.8.1.1. Extraction and sample preparation

(1) Perform propolis extraction as described in Section 3.1.1. Extract 1 g propolis and make up the volume to 100 ml (volumetric flask). The resulting extract is designated as solution A.
(2) Transfer 1 ml from each of three parallel extracts into a volumetric flask and dilute to 50 ml using methanol. The resulting solution is designated as solution B.
(3) Prepare three parallel extracts for every analyzed sample.

3.8.1.2. Total flavone and flavonol content. Total flavone and flavonol content is measured using a spectrophotometric assay based on aluminum chloride complex formation (Bonvehi & Coll, 1994). Methanolic solutions of galangin are used as references to obtain a calibration graph. The analytical procedure for measuring total flavones and flavonols is performed the following way:

(1) To prepare a calibration graph with galangin as the standard, prepare a stock standard solution of galangin 32 μg/ml by dissolving 3.2 mg in methanol in a 100 ml volumetric flask.
(2) Prepare a series of working reference solutions by appropriate dilution of the stock standard solution with methanol (in volumetric flasks) to give a concentration range of 4–32 μg/ml (16.0; 8.0; 6.4; 4.0 μg/ml).
(3) Mix 1 ml of each one of the reference solutions, 10 ml methanol and 0.5 ml 5% $AlCl_3$ in methanol (w/v) in a volumetric flask and make up the volume to 25 ml with methanol.
(4) Let the mixture sit for 30 min and measure the absorbance at 425 nm.
(5) For a blank, use 1ml methanol instead of galangin solution in analogues procedure.
(6) Each reference solution should be analyzed in triplicate.
(7) To obtain the regression, absorbance should be plotted against concentration (International Conference on Harmonization, [ICH], 1996).

(8) For analysis of the propolis sample solution, use B (Section 3.8.1.1), or, if necessary, solution B with additional dilution, and apply the same procedure as described for the reference (steps 3–5).
(9) Perform calculation using the calibration equation for galangin (step 7):

$$c = aA + b$$

where c – concentration, mg/ml; A – absorbance; a – slope of the calibration graph; b – intercept of the calibration graph.

(10) From this value, the percentage of flavones and flavonols in the propolis sample is calculated after the equation:

$$P = \frac{c \times 100 \times 50}{3\bar{M}} \times 100\%$$

where P – percentage in raw propolis; c – concentration, mg/mL (from step 9); $\bar{M}$– mean value of the weight of the three parallel propolis samples, extracted for analysis, mg (Section 3.8.1.1).

(11) In instances when an additional dilution of solution B is provided, it should be reflected in the equation.

3.8.1.3. Total flavanone and dihydroflavonol content. For flavanones and dihydroflavonols determination, the colorimetric method from DAB9 was modified for propolis (Nagy & Grancai, 1996; Popova et al., 2004). Methanolic solutions of pinocembrin are used as references to obtain a calibration graph.

(1) To prepare a calibration graph with pinocebmrin as the standard, prepare a stock standard solution of pinocembrin 1.8 mg/ml by dissolving 18.0 mg in methanol in 10 ml volumetric flask.
(2) Prepare a series of working reference solutions by appropriate dilution of the stock standard solution with methanol (in volumetric flasks) to give concentration range of 0.18–1.8 mg/ml (0.9; 0.45; 0.22; 0.18 mg/ml).
(3) Dissolve 1 g of dinytrophenylhydrazine (DNP) in 2 ml 96% sulfuric acid and dilute to 100 ml with methanol (volumetric flask).
(4) Mix 0.5 ml of each one of the reference pinocembrin solutions and 1 ml of the DNP solution.
(5) Heat the mixture at 50 °C for 50 min (water bath).
(6) Cool the mixture to room temperature and dilute it to 5 ml with 10% KOH in methanol (w/v).
(7) Add 0.5 ml of the resulting solution to 10 ml methanol, dilute to 25 ml with methanol (volumetric flasks), and measure absorbance at 486 nm.
(8) As a blank, use 0.5 ml methanol instead of pinocembrin solution in analogues procedure (steps 4–7).
(9) Each reference solution should be analyzed in triplicate.
(10) To obtain the regression, absorbance should be plotted against concentration (International Conference on Harmonization, 1996).
(11) For analysis of the propolis sample, use 0.5 ml of each of the test solutions of each of the three parallel extractions, prepared as described in Section 3.1.1, and apply the same procedure as described for the reference (steps 4–8).
(12) Perform calculation using the regression obtained for pinocembrin. (step 10).

$$c = aA + b$$

where c – concentration, mg/ml; A – absorbance; a – slope of the calibration graph; b – intercept of the calibration graph.

(13) From this value, calculate the percentage of flavanones and dihydroflavonols in the propolis sample using the equation:

$$P = \frac{c \times 100}{\bar{M}} \times 100\%$$

where P – percentage in raw propolis; c – concentration, mg/ml; $\bar{M}$– mean value of the weight of the three parallel samples, extracted for analysis, mg (Section 3.8.1.1).

3.8.1.4. Total phenolic content. The Folin–Ciocalteu's method is used for the quantification of total phenolics (Waterman & Mole, 1994) and it is modified for poplar type propolis (Popova et al., 2004). As a reference, methanolic solutions of a mixture of pinocembrin-galangin at a 2:1 ratio (w/w) in the range 25–300 μg/ml are used to obtain a calibration graph.

(1) To prepare a calibration graph with pinocebmrin:galangin 2:1 (w/w) as the standard, prepare a stock standard solution by dissolving of 2.2 mg pinocembrin and 1.1 mg galangin in methanol in a 10 ml volumetric flask. The concentration of the stock solution is 0.33 mg/ml of the mixture pinocebmrin:galangin 2:1.
(2) Prepare a series of working reference solutions by appropriate dilution of the stock standard solution with methanol (in volumetric flasks) to give a concentration range of 33–330 μg/ml (165; 82.5; 41.2; 33 μg/ml) for the mixture pinocebmrin:galangin 2:1.
(3) Transfer 0.5 ml of the reference solution into a 25ml volumetric flask, containing 7.5 ml distilled water.

(4) Add 2 ml of the Folin–Ciocalteu's reagent and 3ml of a 20% sodium carbonate solution in distilled water.
(5) Make up the volume to 25 ml with distilled water and wait for 2 h (±3 min) at room temperature.
(6) Measure the absorbance at 760 nm using a UV–vis spectrophotometer.
(7) As a blank 0.5 ml methanol instead of reference mixture is used following the same procedure (steps 3–6).
(8) Each reference solution should be analyzed in triplicate.
(9) To obtain the regression, the absorbance should be plotted against concentration (International Conference on Harmonization, 1996).
(10) For analysis of the propolis samples, use 0.5 ml of the solution B (Section 3.8.1.1) in analogues procedure (steps 3–6). Every assay is carried out performed in triplicate.
(11) Perform the calculation using the regression obtained for the reference mixture pinocembrin-galangin (2:1, step 9).
(12) Perform calculations using the regression obtained for pinocembrin-galangin (2:1).

$$c = aA + b$$

where c – concentration, mg/ml; A – absorbance; a – slope of the calibration graph; b – intercept of the calibration graph.

(13) From this value, calculate the percentage of total phenolics in the propolis sample using the equation:

$$P = \frac{c \times 100 \times 50}{3M} \times 100\%$$

where P – percentage in raw propolis; c – concentration, mg/ml; $\bar{M}$– mean value of the weight of the three parallel samples, extracted for analysis, mg (Section 3.8.1.1).

3.8.2. *Spectrophotometric analysis of Brazilian green propolis*

The analysis of Brazilian green propolis consists in the spectrophotometric quantitative determination of the following groups of phenolic compounds: flavonoids; and total phenolics.

3.8.2.1. Extraction of propolis. The procedure described in Section 3.8.1.1 is used for the extraction of green propolis.

3.8.2.2. Total flavonoid content. The procedures described in Section 3.8.1.2 are followed to determine total flavonoid content. Methanolic solutions of quercetin are used for calibration (Woisky & Salatino, 1998).

3.8.2.3. Total phenolic content. The procedures described in Section 3.8.1.4 are followed to determine total phenolic content. Methanolic solutions of gallic acid are used for calibration (Woisky & Salatino, 1998).

3.8.3. *Spectrophotometric analysis of Pacific type propolis*

Since the main components and biologically active compounds in the Pacific type propolis are prenylated flavanones, the analysis of this type propolis is made on the basis of their quantification.

3.8.3.1. Extraction of propolis. The procedure described in Section 3.8.1.1 is used for the extraction of Pacific type propolis.

3.8.3.2. Total flavanones content. The procedures described in Section 3.8.1.3 are followed to determine total flavanone content. However, methanolic solutions of a mixture of propolin C-propolin D 4:1 (wt/wt) are used for calibration (Popova, Chen, Chen, Huang, & Bankova, 2010).

4. Quality criteria and standards

Propolis is a bee product of plant origin, so the standardization of propolis is similar to that of medicinal plants: it has to be based on the concentration of biologically active constituents. Different propolis types are characterized by their distinct chemical profiles and obviously there cannot be any uniform chemical criteria for standardization and quality control in this respect. Specific criteria based on the concentration of bioactive secondary metabolites should be formulated for particular propolis chemical types. The International Honey Commission suggests the values for the concentration of biologically active constituents for the two most wide-spread propolis types, European poplar type propolis (Poplar type) and Brazilian green propolis (*Baccharis* type), determined as described in Sections 3.8.1 and 3.8.2. For Brazilian green propolis, the values are determined by Brazilian legislation (Sawaya et al., 2011).

4.1. *Specific criteria and standard values for particular propolis chemical types*

The specific criteria and standard values for the most popular and most commercialized propolis types: poplar and green Brazilian propolis, are summarized in Table 5.

Important: Prior to the analysis, the chemical type of propolis should be determined by one of the analytical methods/dereplication strategies listed in Sections 3.3, 3.4, 3.5, 3.6, and 3.7. It is possible to apply by default the specific methodology and criteria for propolis from well-known geographic origins where it has been proved over the years to be of constant plant origin.

Table 5. Specific criteria and standard values for the content of bioactive constituents in propolis.

Propolis type		Minimum % by weight in raw propolis	Reference
Poplar propolis	Total phenolics	21	(Popova et al., 2004)
	Total flavones and flavonols	4	(Popova et al., 2004)
	Total flavanones and dihydroflavonols	4	(Popova et al., 2004)
Brazilian green propolis	Total phenolics	5	(Sawaya et al., 2011)
	Total flavonoids	0.5	(Sawaya et al., 2011)

In the recent years, the problem of poplar propolis adulteration with poplar extracts emerged, connected mainly to Chinese propolis. An HPLC method was developed, based on detection of catechol as a marker for propolis adulteration (Huang et al., 2014).

4.2. *Criteria and standards common for all propolis types*

There are other quality parameters that can be applied to any propolis sample, no matter its plant origin and content of secondary plant metabolites. These include content of matter soluble in 70% ethanol (balsam content), water content, wax content, mechanical impurities, and ash content. The limits of their acceptable values, as suggested by the IHC follow:

Balsam – minimum 45% (Popova et al., 2007; http://www.ihc-platform.net/bankova2008.pdf).

Wax content – Different national standards suggest different values.

Mechanical impurities – maximum 6% (Popova et al., 2007; http://www.ihc-platform.net/bankova2008.pdf).

Water content – maximum 8% (Popova et al., 2007; http://www.ihc-platform.net/bankova2008.pdf).

Ash content – maximum 5% (Falcão, Freire, & Vilas-Boas, 2013b).

For Brazilian green propolis, Brazilian legislation determines a minimum of 35% ethanol extractable substances and a maximum of 25% wax (Sawaya et al., 2011).

4.2.1. *Amount of matter soluble in 70% ethanol (balsam)*

(1) Perform extraction as described in Section 3.1.1.
(2) From each of the three parallel extracts, evaporate 2 mL *in vacuo* to dryness to constant weight *g*.
(3) Calculate the percentage of balsam *P* in the propolis sample using the following formula.

$$P = \frac{g \times 100}{2M} \times 100\%$$

where *g* – the weight of the residue after evaporation of 2 ml of propolis 70% ethanol extract; *M* – the weight of the raw propolis sample, g.

4.2.2. *Water content*

Water content is determined according to Woisky and Salatino (1998).

(1) Heat 10 g of powdered raw propolis (see Section 3.1.1, step 1) in an oven at 105 °C for 5 h.
(2) Cool to room temperature and place in a desiccator until constant weight is achieved.
(3) Calculate the percentage of water content *P* in the propolis sample using the following formula.

$$P = \frac{M_0 - M_1}{M_0} \times 100\%$$

where M_0 – the weight of the raw propolis sample before heating, g; M_1 – the weight of the propolis residue after heating, g.

Figure 14. Determining the wax content of propolis by Soxhlet extraction.
Photo: B. Trusheva.

A mean of the three measurements should be calculated.

4.2.3. *Wax content*

4.2.3.1. Wax content measurement by extraction. The wax content is determined according to the procedures described by Woisky and Salatino (1998).

(1) Treat 3 g of the powdered propolis sample (powdered per Section 3.1.1, step 1) with chloroform in a Soxhlet for 6 h (Figure 14), using a weighed cartridge.
(2) Concentrate the extract to dryness under reduced pressure and add 120 ml of hot methanol to the residue.
(3) Boil the mixture until there is a clear solution on top and a small oily residue on the bottom of the flask. The residue should solidify upon cooling.
(4) Filter the methanolic phase through filter paper, taking care to avoid transferring the oily residue. Transfer the methanolic phase, while hot, to a previously weighed 150 ml flask.
(5) Cool the flask containing the methanolic phase to 0 °C and filter the content through a filter paper that has been weighed and the weight recorded.
(6) Wash the flask and the residue with 25 ml cold methanol.
(7) After drying in the air, transfer the flask and the residue to a desiccator until constant weight.
(8) Calculate the percentage of wax content P_w in the propolis sample using the following formula.

$$P_w = \frac{M_w}{M} \times 100\%$$

where M_w – the weight of the wax obtained, g; M – the weight of the propolis sample, g.

(9) The analysis should be performed in duplicate.

4.2.3.2. Wax content measurement based on differences in specific density. An alternative procedure for measuring the wax content of propolis has been described by Hogendoorn, Sommeijer, and Vredenbregt (2013).

(1) Add 25 ml de-ionized water to 20 g powdered propolis (powdered per Section 3.1.1, step 1) in a tube with screw-cap. When adding the water to the powdered sample, it is necessary to stir the mixture constantly and carefully to avoid propolis powder floating on the water surface.
(2) Tighten the screw cap loosely to prevent pressure building up while heating and place the tubes vertically in a household microwave apparatus set at medium.
(3) Adjust the time of heating so that the temperature rises to about 100 °C but without the boiling of the water phase (usually about 1 min).
(4) Cool down the sample to room temperature. A three layer system is formed in the tube: the beeswax (upper layer), then water (middle layer), and de-waxed propolis at the bottom.
(5) With a small stainless steel spatula, transfer the beeswax in the upper layer to a weighed paper tissue for the removal of the remaining water.
(6) Weigh the amount of extracted beeswax and calculate the wax content as a percentage of the weight of the original sample.
(7) The analysis should be performed in duplicate.

4.2.4. *Mechanical impurities*

Follow the procedure below in order to determine the amount of mechanical impurities in a propolis sample.

(1) Extract the rest of the propolis sample (i.e. that which remained in the cartridge after the procedure described in Section 4.2.3.1) in the same Soxhlet with ethanol for 4 h (until the extract becomes colorless).
(2) Transfer the weighed cartridge together with the residue (the mechanical impurities), after drying it in the air, to a desiccator until constant weight.
(3) Calculate the percentage of mechanical impurities P_{mi} in the propolis sample using the formula that follows.

$$P_{mi} = \frac{M_{mi}}{M} \times 100\%$$

where M_{mi} – the weight of the residue after extraction, g; M – the weight of the propolis sample, g.

(4) The analysis is performed in duplicate.

4.2.5. *Ash content*

The ash content is determined according to the AOAC method (Association of Official Analytical Chemists, 2000).

(1) Place the crucible and lid in the furnace at 550 °C overnight to ensure that impurities on the surface of the crucible are burnt off.
(2) Cool the crucible in a desiccator for 30 min.
(3) Weigh the crucible and lid to 3 decimal places.
(4) Weigh about 5 g of the powdered propolis sample (Section 3.1.1 step 1) into the crucible. Heat over a low Bunsen flame with the lid half covering the crucible. When fumes are no longer produced, place crucible and lid into the furnace.

(5) Heat at 550 °C overnight. During heating, do not fully cover the crucible with the lid. After heating is complete, fully place the lid over the crucible to prevent the loss of fluffy ash. Cool the crucible down in a desiccator.
(6) Weigh the ash with crucible and lid when the sample turns gray. If the sample does not turn gray, return the crucible and lid to the furnace for the further ashing.
(7) Calculate the ash content using the formula that follows.

$$\text{Ash}(\%) = \frac{\text{Weight\_of\_ash}}{\text{Weight\_of\_sample}} \times 100$$

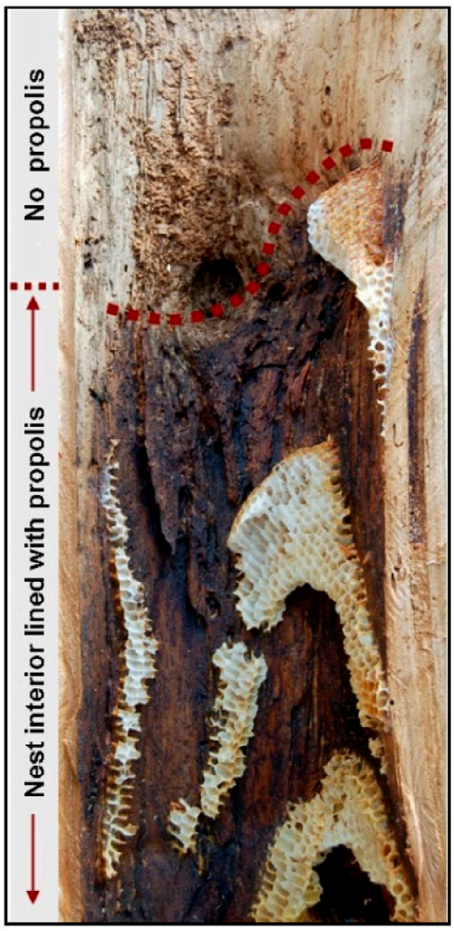

Figure 15. A cross-section of a feral honey bee hive within a tree cavity found September 2009 in the residential area of Bloomington, Minnesota, USA. The nest interior, where comb is present, is coated with a thin layer of propolis creating a "propolis envelope" around the colony. The upper portion of the cavity had not been lined with propolis, as the colony had not begun to use that space. Mold can be seen growing above the propolis envelope From: Simone-Finstrom and Spivak (2012).

5. Health benefits of a propolis envelope to bees

In a natural tree cavity, honey bees line the inside of the cavity with propolis in a contiguous sheet called a propolis "envelope" (Seeley & Morse, 1976). In a tree, the propolis envelope is particularly thick around the entrance and extends from where the combs attach at the top of the nest as far down as the combs are constructed (Simone-Finstrom & Spivak, 2012). Above and below the envelope, molds and fungi can be observed in the tree (Figure 15), which suggests that one purpose of the propolis envelope is to prevent the growth of molds inside the nest. The propolis envelope is an anti-microbial layer surrounding the colony and has quantifiable benefits to the bees' immune systems, and pathogen defense (Simone, Evans, & Spivak, 2009; Simone-Finstrom & Spivak, 2012).

The smooth and solid inner surfaces of standard beekeeping wooden boxes do not elicit resin collection behavior and further construction of a propolis envelope by bees. Instead, the bees deposit propolis in cracks and crevices, such as between boxes and under the frame rests, making it difficult to pry apart boxes and remove frames for beekeeping inspections without use of a hive-tool (Haydak, 1953; Huber, 1814; Ghisalberti, 1979). For this reason, many beekeepers do not like the difficulty that sticky propolis presents in the colony, and over many years, it is likely that queen producers have selected for colonies that do not deposit large quantities of propolis in the nest (Fearnley, 2001). At the same time, some beekeepers have harvested propolis from bee colonies for uses in human medicine (Burdock, 1998; Castaldo & Capasso, 2002; Krell, 1996).

The effects of a propolis envelope on honey bee immunity and on pathogen defense within the colony can be studied in two ways: (1) guide the bees to naturally deposit propolis throughout the nest interior; or (2) apply a propolis extract to the hive walls.

5.1. Forming a propolis envelope within standard beekeeping equipment

5.1.1. A naturally-deposited propolis envelope

A colony of bees can be encouraged to build a natural propolis envelope within standard beekeeping equipment by modifying the inner walls of bee boxes. If the inside of the bee box is built using unfinished, rough lumber the bees will apply a layer of propolis over the rough surfaces. The inner walls of bee boxes can be scraped with a wire brush; the rougher the surface, the more propolis the bees will deposit on the walls (Simone-Finstrom & Spivak, personal observation). Alternatively, commercial propolis traps, used to harvest propolis, (see Section 2.2.1) can be cut to fit the four inside walls of the hive boxes and stapled with the smooth side of the trap facing the wood and the rough side facing the colony (Borba & Spivak, personal observation; Figure 16). It is recommended to manage

Figure 16. Propolis traps stapled to inside walls of hive to create a propolis envelope.
Photo: R. Borba.

colonies using nine frames instead of ten when using this method in standard 10-frame Langstroth equipment.

5.1.2. Experimental or artificial propolis envelope

For experimental purposes when it is necessary to quantify the quantity or concentration of the propolis envelope, a propolis envelope can be painted on the inside surface of the box using an extract of propolis (Simone et al., 2009; Figure 17).

(1) Propolis is harvested using any combination of the methods described below (Section 2.2).
(2) Extraction of propolis – (13% propolis in 70% ethanol, e.g. Simone et al. (2009); see section 3.1 for further details and discussion).
(3) The extracts then can be painted on as a "varnish" for the interior hive walls. Based on the determined concentrations of the extracts ~50 g (for a nucleus colony, 5-frame Langstroth) or ~100 g (for a single deep, 10-frame Langstroth) of propolis should be applied evenly to the 4 side hive walls and the bottom board and cover (Simone et al., 2009; Simone-Finstrom & Spivak, 2012).
(4) In order to apply enough grams of propolis to the hive interior, multiple coats of the propolis extracts may need to be applied to the surfaces if the extract is not sufficiently strong or of high enough concentration for a single coat.
(5) The same volume of solvent used for the propolis extract should be applied to control colonies to account for any effects from the solvent alone.

Figure 17. Example of painting the hive interior with propolis extract to create a propolis envelope. The top box was painted with 70% ethanol, the middle with an extract of Brazilian green propolis and the bottom with MN propolis extract.
Photo: M. Simone-Finstrom.

5.2. Effect of propolis envelope on the immune system of bees

The honey bee immune response varies with age, so when comparing immune-related gene expression among treatments, it is important to sample bees of the same age. Young bees have greater fat body mass, therefore higher capacity to synthesize antimicrobial peptides, compared to older bees (Wilson-Rich, Spivak, Fefferman, & Starks, 2009). As honey bees age and switch from in-hive tasks to foraging, immune function can be altered both by age and task performance (Schmid, Brockmann, Pirk, Stanley, & Tautz, 2008; Wilson-Rich et al., 2009).

Previous studies on the role of propolis as a social immune trait have focused on younger, in-hive bees (e.g. Simone et al., 2009). However, investigators focusing on environmental effects on immunocompetence should consider collecting samples from other life stages and among behavioral tasks when possible (Human et al., 2013).

Once individuals are collected based on the colony treatments, RNA can be extracted for analysis of gene expression via real-time PCR (Evans et al., 2013; Simone et al., 2009). From current and previous work, gene expression for the antimicrobial peptide hymenoptaecin seems to be affected consistently by exposure to a propolis-enriched environment (e.g. Simone et al., 2009). However, continued work finds other genes involved in cellular immunity and representatives of each of the immune pathways, providing a more robust analysis of immune gene expression.

5.3. *Effect of propolis envelope on pathogens and pests in the hive*

In addition to indirect effects of propolis envelope on bee health through the immune system, research is underway to explore if the propolis envelope has direct effects on bee pathogens (e.g. Simone-Finstrom & Spivak, 2012) and pests. Colonies provided with a propolis envelope (either an extract or natural), can be challenged with *Ascosphaera apis*, *Paenibaciullus larvae*, other pathogens, small hive beetles (*Aethina tumida*), varroa (*Varroa destructor*), and other pests as described in *BEEBOOK* Vol II (e.g. De Graaf et al., 2013; Dietemann et al., 2013; Jensen et al., 2013; Neumann et al., 2013). Comparing challenged colonies with unchallenged controls allows quantification of the potential effects of propolis on the pest/pathogen in question.

5.4. *Self-medication: monitoring colony-level changes in resin-collection*

Colonies challenged with *A. apis* have been shown to collect significantly more resin after challenge (Simone-Finstrom & Spivak, 2012). Since a resin-enriched environment also reduces overall colony-level infection of this pathogen, resin foragers are self-medicating at the colony level against at least particular pathogens.

High variation across colonies in the number of resin foragers can be an issue when conducting this experiment. The appropriate sample size needs to be calculated carefully. Half of the colonies would be treated or challenged with a pathogen and the other half would remain unchallenged. An experiment to address the question of resin use as self-medication in honey bees combines the methods described above in Sections 2.1 and 5.3.

Statistical analysis of the change in resin foraging after exposure to pathogens can be done following various methods. One method previously used (Simone-Finstrom & Spivak, 2012), determined the change in resin foraging for each colony (total number of resin foragers pre-challenge subtracted from the total number counted post-challenge per colony). The change in resin foraging was then compared across pathogen-challenged and unchallenged colonies. A matched pairs analysis could also be used with treatment (challenged vs. unchallenged) as a factor in the statistical analysis.

The most accurate and direct indicator of increased resin use is by observing foraging rates (Simone-Finstrom & Spivak, 2012). However alternative methods of the assessment of propolis deposition in hives pre- and post-challenge could possibly be used to determine if resin collection rate increases in response to pathogen exposure. Deposition on commercial propolis traps (see Section 2.2.1) could be examined by weight or amount of coverage, although the amount of wax that is incorporated into resins varies highly across colonies and would greatly influence this measure. Similarly, the deposition of propolis on frame edges and in the hive itself, as described in the introduction to Section 5, could be analyzed but this has similar issues in terms of difficultly for accurate quantification (Borba, Simone-Finstrom & Spivak, personal observations).

6. Testing the biological activity of propolis *in vitro*

The most studied biological activities of propolis are the antimicrobial and antioxidative ones. Here, tests against both human and bee pathogens will be described.

6.1. *Testing the antibacterial activity*

6.1.1. *Activity against human pathogens*

6.1.1.1. Bacterial strains. Antibacterial tests have been used to analyze bacterial sensitiveness to propolis. One may compare, for example, its effect on Gram positive and Gram negative bacteria, e.g. *Staphylococcus aureus* and *Escherichia coli* strains. American Type Culture Collection (ATCC) strains should be used in the assays.

6.1.1.2. Susceptibility tests (macrodilution). Susceptibility tests are performed by dilution in agar as recommended by the Clinical and Laboratory Standards Institute and minimal inhibitory concentration (MIC) values are determined (Alves et al., 2008; Clinical & Laboratory Standards Institute - CLSI/National Committee for Clinical Laboratory Standards – NCCLS, 2005).

(1) Inoculate bacterial strains in Brain Heart Infusion (BHI – Difco, USA) at 35 °C for 24 h and standardize at 0.5 on the McFarland scale in sterile saline (Sutton, 2011). Perform dilutions of each sample to obtain bacterial suspensions with 1×10^6 colony-forming units (CFU)/ml.

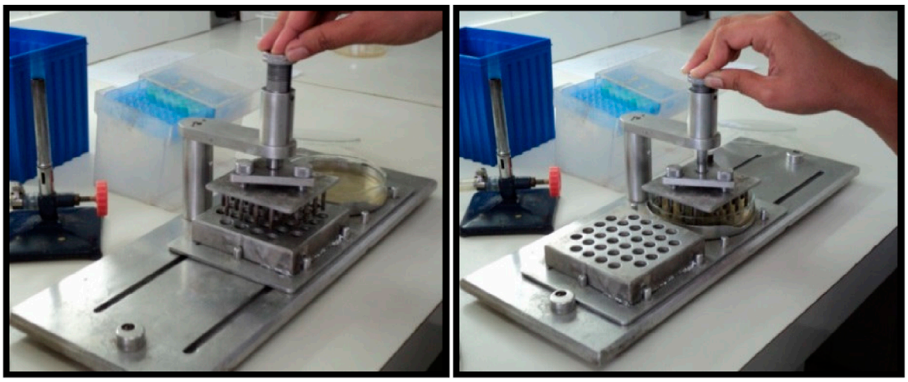

Figure 18. Steer's multiple inoculator used for bacterial inoculation in the plates.

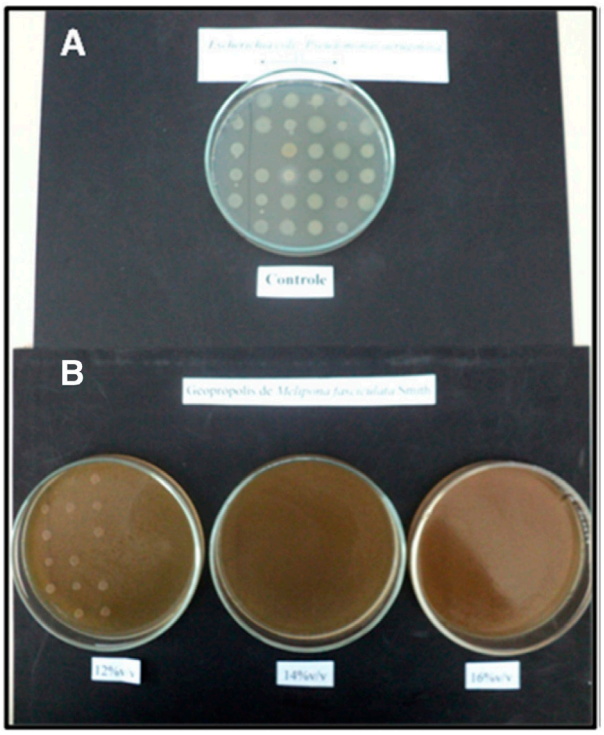

Figure 19. (A) Control plate showing bacterial growth. (B) Plates incubated with propolis showing the partial bacterial growth at left and inhibition of bacterial growth in the plates containing MIC (center and right).

(2) Add propolis to Petri dishes containing Mueller Hinton Agar (MHA) (Difco, USA) at different concentrations, such as: 3, 6, 9, 12, 14, 16, 18 and 20% v/v. Control plates contain only 70% ethanol at the same concentrations found in propolis.
(3) Inoculate bacterial strains in Petri dishes containing different concentrations propolis and 70% ethanol, using a Steer's multiple inoculator (Figure 18), and incubated at 35 °C for 24 h.
(4) MIC_{90} is considered as the lowest concentration of propolis able to inhibit 90% of microorganisms, showing no visible growth or haze on the surface of the culture medium (Figure 19).

6.1.1.3. Susceptibility tests (microdilution)

(1) Incubate bacterial strains in BHI at 35 °C for 24 h and standardize at 0.5 on the McFarland scale (Sutton, 2011) in sterile saline. Perform dilutions of each sample to obtain bacterial suspensions with 1×10^6 CFU/ml.
(2) Add 100 µl of BHI medium containing different concentrations of propolis or ethanol 70% to 96 well plates and then 100 µl of the bacterial suspension. Incubate plates at 35 °C for 24 h (Figure 20).
(3) Read the plates by observing the turbidity of the solution in each well by adding the dye resazurin (50 µl). Record the MIC values of propolis for each strain (Figure 21). Resazurin (7-hydroxy-3H-phenoxazine-3-one-10-oxide) is a redox indicator used to check for the presence of viable cells in microdilution method. It naturally is blue or purple in color. In the presence of viable cells, it oxidizes to resofurin, which is red and promotes the observation of microbial growth (Alves et al., 2008).

6.1.1.4. Time kill curve. The time kill curve of bacteria is carried out to observe the bactericidal or bacteriostatic action of propolis over time, using the MIC_{90} values.

(1) Inoculate bacterial suspensions (1×10^6 CFU/ml) in tubes or Erlenmeyer flasks (20 ml) containing BHI plus Tween 80% (0.5% v/v) and the MIC_{90} of propolis or 70% ethanol. Bacterial suspensions in BHI plus Tween 80% (0.5% v/v) alone are considered as control.
(2) After 3, 6, 9 and 24 h of incubation at 35 °C, take aliquots (50 µl) of each culture and plate on Plate Count Agar (PCA – Difco; USA) by the pour plate method which is used to count the bacteria. Put 50 µl of each solution in a dish and

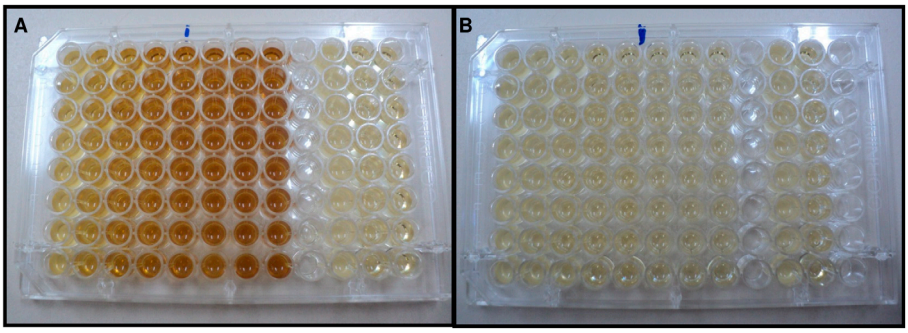

Figure 20. Plates for the microdilution test. In the 8 columns: BHI + propolis in different concentrations (A) or ethanol 70% (B). Column 10 (A and B): positive control (bacteria + BHI) and column 11 (A and B): negative control (BHI alone).

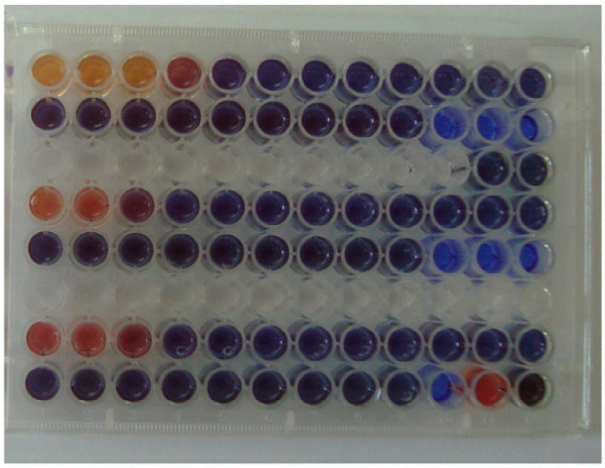

Figure 21. MIC of propolis. Blue color indicates absence of viable cells, while red color indicates the presence of viable ones.

mix with 15 ml of plate count agar (PCA). CFU are counted after incubation at 35 °C for 24 h.

(3) Calculate the survival percentage (Sforcin, Fernandes, Lopes, Bankova, & Funari, 2000) according to the formula:

$$\text{Survival percentage} = \text{CFU sample} \times 100/\text{CFU control}$$

6.1.2. *Testing against bee pathogens: American foulbrood (*Paenibacillus larvae*)*

Described here is a high-throughput susceptibility assay published in Wilson et al. (2015) for testing antimicrobial activity against active *Paenibacillus larvae* cultures in 96-well plate format. Liquid *P. larvae* culturing techniques were adapted from Bastos, Simone, Jorge, Soares, & Spivak, (2008) and De Graaf et al. (2013). This protocol views antimicrobial activity as treated bacterial growth relative to untreated bacterial growth, and includes the equations for making good statistical comparisons of antimicrobial activity between propolis samples.

6.1.2.1. *Culturing* P. larvae

(1) Obtain target strains of *P. larvae*. Many reference strains can be obtained from the USDA Agricultural Research Service culture collection (http://nrrl.ncaur.usda.gov/) and are discussed in De Graaf et al. (2013). Field strains can be isolated from infected larvae according to De Graaf et al. (2013).

(2) Grow stock *P. larvae* cultures in liquid brain/-heart infusion media (BHI) supplemented with 1 mg/l thiamine by shaking and incubating at 37 °C. A 30 ml stock culture started from lyophilized cells or isolated spores needs to be grown for 48 h.

(3) Split the stock culture into three 10 ml aliquots and add 10 ml glycerol to each aliquot and store at −20 °C. These 50% glycerol cultures should last for several months.

(4) Inoculate 29.5 ml of liquid BHI with 0.5 ml of glycerol culture. Shake and incubate at 37 °C for 48 h.

6.1.2.2. *Preparing 96-well plates*

(1) Add propolis extracts (per Section 3.1.1) to flat-bottom 96-well plates in desired dilutions, and then dry extracts to residue under nitrogen. Experiments should include a range of propolis concentrations, with at least 3 replicates per treatment. Negative and positive growth controls should be included in the experiment.

(2) Add 100 µl of liquid BHI media to each propolis-treated well. Cover, shake, and incubate microplates at 37 °C for 15 min to solubilize propolis residue; however, propolis residue is unlikely to be completely soluble if concentrations are too high.

(3) Dilute the 48 h *P. larvae* culture started from glycerol stock 1:50 and add 100 µl of this dilute culture to each well. Measure the initial optical density (OD) at 600 nm with a spectrophotometer, which should be ~0.13 AU in untreated

controls. Cover, shake, and incubate at 37 °C.

(4) Measure final OD_{600nm} at 6 h, which should be ~0.6 AU in untreated controls.

6.1.2.3. *Data analysis*

(1) Subtract the initial OD_{600nm} of each well from the final OD_{600nm} of each well to normalize the growth data.

(2) Bacterial growth can be interpreted relative to untreated controls as a percent:

$$\%\text{Relative}\quad \text{growth} = \frac{\text{treated average OD}_{600\text{nm}}}{\text{untreated}\quad \text{average}\quad \text{OD}_{600\text{nm}}}$$

Error needs to be propagated between the two means used to calculated relative growth:

$$\%\text{Standard}\quad \text{error} = \sqrt{\left(\frac{SE_a}{a}\right)^2 + \left(\frac{SE_b}{b}\right)^b}$$

where '*a*' is the treated average OD_{600nm}; '*b*' is the untreated average OD_{600nm}; SE_a is the standard error of '*a*'; SE_b is the standard error of '*b*'.

(3) If bacterial growth inhibition is dose-responsive, you should observe a sigmoidal growth curve with less growth at high propolis concentrations and more growth at low propolis concentrations. It is best if experiments are developed so that several of the highest propolis concentrations completely inhibit growth and several of the lowest propolis concentrations allow growth similar to untreated controls.

(4) Sigmoidal growth curves can be fit with a four-parameter logistic equation to calculate IC_{50} values and their standard errors for individual propolis samples,

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{IC_{50}}\right)^{-\text{Hillslope}}}$$

This operation can be done by many statistical analysis programs, such as SigmaPlot.

(5) IC_{50} values can be compared pair-wise using confidence intervals:

$$''\text{CI} = z \pm \left[1.96\left(''\sqrt{(x^2 + y^2)}\right)\right]$$

where x is the standard error of $IC_{50(1)}$; y is the standard error of $IC_{50(2)}$; z is the difference between $IC_{50(1)}$ and $IC_{50(2)}$.

If the confidence interval of the difference between $IC_{50(1)}$ and $IC_{50(2)}$ does not include 0, then the difference between the two IC_{50} values can be taken as significant. α = 1.96 in the equation above, which is the value used to test at 95% confidence.

6.2. *Antifungal activity*

6.2.1. *Testing against human pathogens*

6.2.1.1. Yeasts. Antifungal tests have been carried out to compare the sensitiveness of yeasts to propolis. As an example, pathogens isolated from human infections such as *Candida albicans*, *Candida guilliermondii* and *Candida tropicalis* may be used (Fernandes, Sugizaki, Fogo, Funari, & Lopes, 1995; Sforcin, Fernandes, Lopes, Bankova, & Funari, 2001). Microorganisms should be identified by current standard microbiological methods and ATCC strains should be used in the assays.

6.2.1.2. Susceptibility tests (macrodilution). Susceptibility tests may be performed by dilution in agar as recommended by the Clinical and Laboratory Standards Institute and MIC values are determined (Clinical & Laboratory Standards Institute - CLSI/National Committee for Clinical Laboratory Standards – NCCLS, 2005).

(1) Grow yeast strains in Sabouraud Dextrose Agar (Difco) at 35 °C/24 h. After incubation, suspend five colonies of each strain in 5 mL of sterile phosphate buffer solution (PBS) and dilute 1/100 in PBS to get a final inoculum of approximately 5×10^4 cells/ml.

(2) Make serial concentrations (% v/v) of propolis from each sample on plates containing Sabouraud Dextrose Agar to achieve 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 9.0, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5, 13.0, and 14.0%.

(3) Prepare a duplicate set of plates containing culture medium plus ethanol in order to obtain 5.0, 10.0, and 15.0% concentrations of solvent as control.

(4) Perform the inoculation procedures using a multiloop replicator, incubate the plates at 35 °C for 24 h and read MIC endpoints as the lowest propolis concentration that results in no visible growth or haze on the surface of the culture medium. Perform population analyses of data by calculating the MIC for 50 and 90% of the strains of each group of microorganisms.

6.2.2. *Testing against bee pathogens: chalkbrood fungus (*Ascophaera apis*)*

Described here is a high-throughput susceptibility assay published in Wilson et al. (2015) for testing antimicrobial activity against *Ascophaera apis* spores in 96-well plate format. Liquid culture and propagation techniques are based on those described in Jensen et al. (2013).

This protocol views antimicrobial activity as treated fungal growth relative to untreated fungal growth, and includes the equations for making good statistical comparisons of antimicrobial activity between propolis samples.

6.2.2.1. *Culturing* A. apis

(1) Obtain target strains of *A. apis*. Reference strains can be obtained from the USDA Agricultural Research Service Entopathogenic Fungal Culture Collection (http://www.ars.usda.gov/is/np/systematics/fungibact.htm). USDA #7405 (+ mating type) and USDA #7406 (- mating type) were used in Wilson et al. (2015). Field strains can be isolated from chalkbrood mummies according to Jensen et al. (2013).
(2) Grow and mate strains on solid MY-20 media and then harvest spores into sterile water all according to Jensen et al. (2013). Store spore solution at 4 °C.
(3) Count spores under a microscope with a hemocytometer. There will be a high risk of contamination if spores were isolated from mummies, so proper steps must be taken to ensure that *A. apis* is the organism that grows in assay cultures. For PCR methods to identify *A. apis*, please refer to Jensen et al. (2013).

6.2.2.2. *Preparing 96-well plates*

(1) Add propolis extracts to flat-bottom 96-well plates in desired dilutions, and then dry extracts to residue under nitrogen. Experiments should include a range of propolis concentrations, with at least 5 replicates per treatment. Negative and positive growth controls should be included in the experiment.
(2) Add 180 µl of liquid MY-20 media to each propolis-treated well. Cover, shake, and incubate microplates at 31 °C for 15 min to solubilize propolis residue; however, propolis residue is unlikely to be completely soluble if concentrations are too high.
(3) Add approximately 2.0×10^6 *A. apis* spores in 20 µl sterile water to each well. Measure initial OD at 600 nm with a spectrophotometer, which should be ~0.13 AU in untreated controls. Cover, shake, and incubate at 31 °C.
(4) Measure final OD_{600nm} at 65 h, which should be ~0.8 AU in untreated controls. It takes ~50 h for spores to germinate, but near maximum growth should be achieved by 72 h.

6.2.2.3. *Data analysis*

(1) Subtract the initial OD_{600nm} of each well from the final OD_{600nm} of each well to normalize the inhibition data.
(2) Bacterial growth can be interpreted relative to untreated controls as a percent:

$$\%\text{Relative growth} = \frac{\text{Treated average } OD_{600nm}}{\text{Untreated average } OD_{600nm}}$$

(3) Error needs to be propagated between the two means used to calculated relative growth:

$$\%\text{Standard error} = \sqrt{\left(\frac{SE_a}{a}\right)^2 + \left(\frac{SE_b}{b}\right)^b}$$

where '*a*' is the treated average OD_{600nm}; '*b*' is the untreated average OD_{600nm}; SE_a is the standard error of '*a*'; SE_b is the standard error of '*b*'.

(4) If fungal growth inhibition is dose-responsive, you should observe a sigmoidal growth curve with less growth at high propolis concentrations and more growth at low propolis concentrations. It is best if experiments are developed so that several of the highest propolis concentrations completely inhibit growth and several of the lowest propolis concentrations allow growth similar to untreated controls.
(5) Sigmoidal growth curves can be fit with a four-parameter logistic equation to calculate IC_{50} values and their standard errors for individual propolis samples.

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{IC_{50}}\right)^{-\text{Hillslope}}}$$

This operation can be done by many statistical analysis programs, such as SigmaPlot.

$$''\text{CI} = z \pm \left[1.96\left(''\sqrt{(x^2 + y^2)}\right)\right]$$

(6) IC_{50} values can be compared pair-wise using confidence intervals.

$$''\text{CI} = z \pm \left[1.96\left(''\sqrt{(x^2 + y^2)}\right)\right]$$

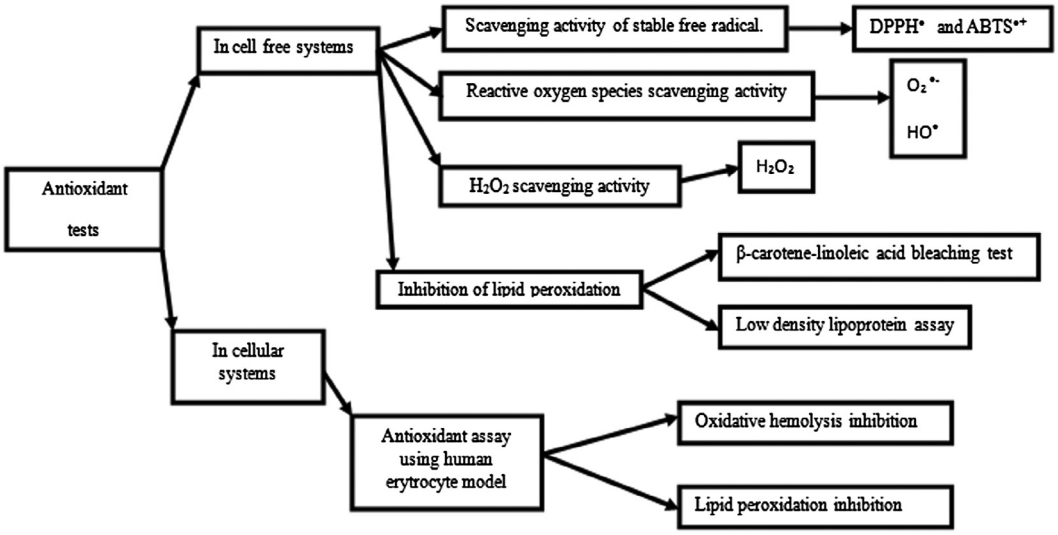

Figure 22. Methods for determination of antioxidant activity of propolis samples.

where x is the standard error of $IC_{50(1)}$; y is the standard error of $IC_{50(2)}$; z is the difference between $IC_{50(1)}$ and $IC_{50(2)}$.

If the confidence interval of the difference between $IC_{50(1)}$ and $IC_{50(2)}$ does not include 0, then the difference between the two IC_{50} values can be taken as significant. $\alpha = 1.96$ in the equation above, which is the value used to test at 95% confidence.

6.3. Testing the antioxidant activity of propolis

6.3.1. Introduction

Oxidative stress, originated from an increase in free radical production or from a decrease in the antioxidant network, is characterized by the inability of endogenous antioxidants to counteract the oxidative damage on biological targets. In this context, it has been suggested that the intake of antioxidant is inversely associated with the risk to develop some pathologies like cancer, inflammatory process, cardiovascular diseases, and others (Lobo, Patil, Phatak, & Chandra, 2010; Pisoschi & Pop, 2015; Siti, Kamisah, & Kamsiah, 2015). Thus, attention has been paid to the antioxidant capacity of natural products such as bee products (honey, propolis), medicinal plant extract, and functional food (fruits and vegetable). Different *in vitro* assays have been developed to determine the antioxidant capacity of natural products (Figure 22). However, considering the complexity of *in vivo* antioxidant action mechanisms, several *in vitro* assays have also been used to study the potential antioxidant of natural products.

6.3.2. Evaluation of the antioxidant activity in cell free system

6.3.2.1. Scavenging activity toward stable free radicals (DPPH•, ABTS•+) by quantitative methods

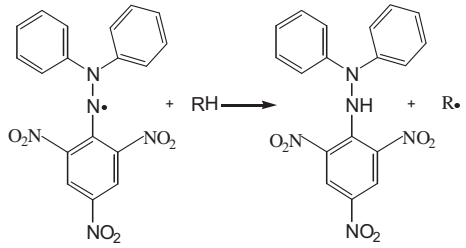

Figure 23. DPPH• radical scavenging process, leading to decoloration which is registered spectrophotometrically in the DPPH assay.

6.3.2.1.1. DPPH free radical scavenging activity. The 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay is one of the most extensively used antioxidant assays for propolis samples. DPPH• is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH• through the addition of a radical species or an antioxidant that decolorizes the DPPH• solution (Figure 23). The antioxidant activity is then measured by the decrease in absorption at 515 nm according to Nieva Moreno, Isla, Sampietro, and Vattuone (2000) and Yamaguchi, Takamura, Matoba, and Terao (1998).

6.3.2.1.1.1. DPPH quantitative analysis using macro-method

(1) Prepare a solution of DPPH• in 96% ethanol to obtain a 300 μM DPPH• solution.
(2) Add 1.5 ml of this solution to 0.5 ml of different concentrations of dry propolis extract (see Section 3.1.1) dissolved in 96% ethanol.
(3) Maintain during twenty minutes at 25 °C and then measure the absorbance at 517 nm in a

spectrophotometer. A decrease in the absorbance (>20%) of the reaction mixture indicates free radical scavenging activity of the propolis samples.

(4) Calculate the percentage of radical scavenging activity (RSA%) using the following equation:

$$\text{RSA}\% = \left[\frac{A_0 - A_s}{A_0}\right] \times 100$$

where A_0 is the absorbance of the control; A_s is the absorbance of the samples at 515 nm.

SC_{50} values denote the μg GAE/ml or μg dry weight of propolis extract/ml required to scavenge 50% DPPH free radicals. Quercetin, an antioxidant natural product or BHT, a synthetic antioxidant, are used as positive controls.

6.3.2.1.1.2. DPPH quantitative analysis using micromethod. Reaction mixtures containing different concentrations of propolis extract (0 to 50 μg dry weight of propolis extract (see Section 3.1.1) dissolved in 5 μl DMSO) and 95 μl of $DPPH^{\bullet}$ solution (0.125 mg/ml) in a 96-well microtiter plate are incubated at 25 °C for 30 min. Absorbance is measured at 550 nm in a microplate spectrophotometer. Scavenging activity (SC_{50} values) of different propolis samples is determined by comparison with a DMSO control (Solórzano et al., 2012).

6.3.2.1.2. ABTS free radical scavenging activity. Along with the DPPH method (Section 6.3.2.1.1), the ABTS radical cation ($ABTS^{\bullet+}$) scavenging method is one of the most extensively used antioxidant assays for propolis samples. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, and its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 734 nm (Re et al., 1999).

$ABTS^{\cdot+}$ is generated by reaction of 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature (23 °C) in the dark for 16 h. The $ABTS^{\cdot+}$ solution is obtained by diluting the stock solution to an absorbance of 0.70 at 734 nm in ethanol, or PBS pH 7.4 according the solvent used to extract preparation.

6.3.2.1.2.1. ABTS quantitative analysis using macromethod

(1) Add $ABTS^{\cdot+}$ solution (1 ml) to 0.5 ml propolis extract (see Section 6.3.2.1.1.1 step 2) and mix thoroughly.
(2) The absorbance should be recorded at 734 nm after 6 min.
(3) Calculate the percentage of inhibition using the following formula:

$$\%\text{Inhibition} = \left[\frac{A_0 - A_s}{A_0}\right] \times 100$$

where A_0 is the absorbance of the control (blank, without propolis sample); A_s is the absorbance in presence of propolis extract.

SC_{50} values denote the μg GAE/ml required to scavenge 50% ABTS free radicals. This assay measures the total antioxidant capacity in both lipophilic and hydrophilic substances. Trolox, a water-soluble analog of Vitamin E, or quercetin, an antioxidant natural product is used as a positive control.

6.3.2.1.2.2. ABTS quantitative analysis using micromethod. Different concentrations of propolis dry extract (see Section 3.1.1) dissolved in ethanol or buffer (20 μl) and 180 μl of $ABTS^{\bullet+}$ are incubated at 25 °C for 6 min. Absorbance is measured at 734 nm in a microplate spectrophotometer. Scavenging activity (SC_{50} values) of different propolis samples is determined by comparison to an ethanol or buffer control.

6.3.2.1.3. Scavenging activity toward stable free radicals ($DPPH^{\bullet}$, $ABTS^{\bullet+}$) by qualitative methods: autographic assay with $DPPH^{\bullet}$ and $ABTS^{\bullet+}$

(1) Separate the chemical components of the propolis extract (see Section 3.1.1) by thin layer chromatography (TLC, 4 × 4 cm silica gel plate) using as mobile phase a solvent system such as toluene: chloroform:acetone 4.5:2.5:3.5 v/v/v.
(2) Air-dry the TLC plate.
(3) Distribute 3 ml of medium containing agar 0.9% and 1 ml $ABTS^{\bullet+}$ solution (Figure 23) or $DPPH^{\bullet}$ solution on TLC plates (Vera et al., 2011; Zampini, Ordoñez, & Isla, 2010).

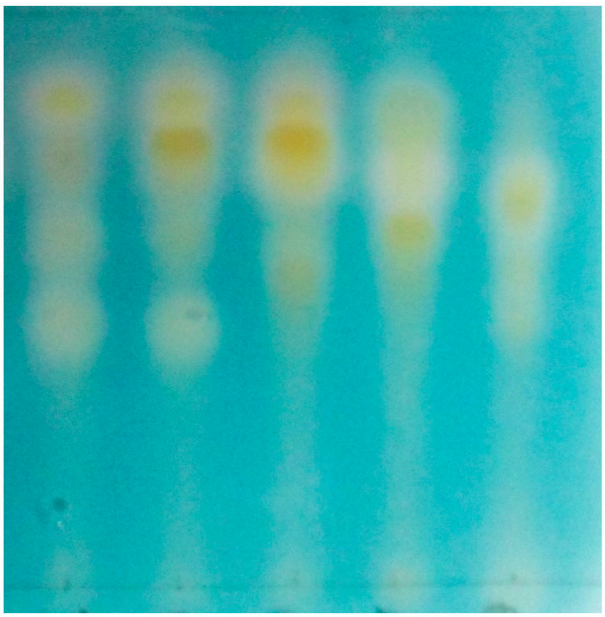

Figure 24. Autographic assay of $ABTS^{\bullet+}$ scavenging activity in propolis samples. The yellow spots on the thin layer chromarography correspond to compounds which scavenge $ABTS^{\bullet+}$ radicals.

(4) Incubate the plate at room temperature for 1 min in the dark.
(5) The antioxidant compounds are visualized as bright areas on a purplish (DPPH) or green blue ($ABTS^{\bullet+}$) background (Figure 24).

6.3.2.2. *Scavenging activity of reactive oxygen species*

6.3.2.2.1. Superoxide radical scavenging activity-non-enzymatic assay. Superoxide radicals are generated by the NADH/PMS (phenazine methosulfate) system following a method as described Valentão et al. (2002) and modified by Danert et al. (2014).

(1) Mix a total of 50 µl of the tested propolis extract (see Section 3.1.1) with 40 µl of NADH (2 mM), 20 µl of NBT (nitroblue tetrazolium) (1 mM) and 40 µl of PMS (60 µM).
(2) Dissolve all the reagents in a phosphate buffer (19 mM, pH 7.4).
(3) Dissolve the extracts in DMSO (final concentration of 0.1%).
(4) Incubate the reaction mixture for 30 min at 37 °C and measure the absorbance at 550 nm in a microplate reader. SC_{50} values denote the µg GAE/ml required to scavenge 50% of superoxide free radicals and are obtained from doses-response curves.

6.3.2.2.2. Hydroxyl radical scavenging activity. Hydroxyl radical scavenging is carried out by measuring the competition between deoxyribose and each extract for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The attack of the hydroxyl radical on deoxyribose leads to thiobarbituric acid reactive species formation. The reaction is performed according to Chobot (2010) with modifications according to Danert et al. (2014).

(1) Add various concentrations of propolis extract (see Section 3.1.1) to the reaction mixture containing 50 µl of a 10.4 mM 2-deoxy-D-ribose solution, 100 µl of $FeCl_3$ (50 µM) and 100 µl of 52 µM EDTA.
(2) Add 50 µl of 10 mM H_2O_2, 50 µl of 1.0 mM ascorbic acid and 50 mM phosphate buffer (pH 7.4) making up a final volume of 0.5 ml, to start the Fenton reaction.
(3) Incubate the reaction mixture at 37 °C for 1 h.
(4) Dissolve 500 µl of 1% 2-thiobarbituric acid in 3% trichloroacetic acid (w/v) and add to each test tube and maintained at 100 °C for 20 min.
(5) To remove the reaction product, add 700 µl of *n*-butanol and vigorously vortex the mixture.
(6) Separate the *n*-butanol layers, each 600 µl, and measure the absorbance at 532 nm.
(7) Assays are performed in triplicate.

Reaction mixtures without the test compound serve as positive controls (100% malodialdehyde). The negative control should contain the full reaction mixture except 2-deoxy-D-ribose. Controls without either EDTA or ascorbic should be performed. IC_{50} values are obtained from dose-response curves.

6.3.2.2.3. *Hydrogen peroxide scavenging activity*

(1) Prepare a solution (4 mM) of hydrogen peroxide in phosphate buffer (PBS, pH 7.4).
(2) Determine hydrogen peroxide concentration spectrophotometrically from absorption at 230 nm using the molar absorptivity 81 M^{-1} cm^{-1}.
(3) Add the propolis sample to the hydrogen peroxide solution (0.6 ml).
(4) Measure the absorbance of hydrogen peroxide with and without propolis extract at 230 nm (Aruoma, Grootveld, & Halliwell, 1987; Zampini et al., 2008).

6.3.2.3. *Inhibition of lipid oxidation*

6.3.2.3.1. β-Carotene-linoleic acid bleaching assay. The β-carotene linoleic acid bleaching assay is one of the antioxidant assays suitable for propolis samples. In this assay, the antioxidant capacity is determined by the formation of conjugated diene hydroperoxides arising from linoleic acid oxidation, which results in the discolouration of β-carotene. The reaction is carried out according to Velioglu, Mazza, Gao, and Oomah (1998) with slight modifications according to Danert et al. (2014).

(1) Add β-carotene (10 mg) in 50 µl of chloroform to 40 µl of linoleic acid and 400 µl of Tween 40 emulsifier mixture.
(2) After evaporation of the chloroform under vacuum, add 10 ml of distilled water with vigorous shaking.
(3) Add 60 ml of 14 mM H_2O_2, transfer 1ml of this mixture into test tubes containing different concentrations of dry propolis extract (see Section 3.1.1) or positive controls (100 µl).
(4) As soon as the emulsion is added to each tube, the zero time point absorbance is measured at 470 nm using a spectrophotometer.
(5) Incubate the emulsion for 2 h at 50 °C. A blank, devoid of β-carotene is prepared and a control of β-carotene and propolis. Quercetin, BHT and α-tocopherol are used as standards.

6.3.2.3.2. Inhibition of oxidation of low density lipoprotein. At present, it is well known that reactive oxygen species (ROS) can play a pivotal role in the initiation, propagation

and termination reactions of the low density lipoprotein (LDL) peroxidation processes (Lobo et al., 2010; Pisoschi & Pop, 2015; Siti et al., 2015). *In vitro* assays usually employ cupric sulfate or cupric chloride as initiators of LDL oxidation and the lipid peroxidation processes should be followed with the formation of diene conjugates by UV spectroscopy at 234 nm. The kinetic is characterized by the presence of a lag time associated with the presence of endogenous antioxidants (mainly vitamin E and coenzyme Q) in the LDL particle. After that period, the peroxidation of lipids is evidenced as an increase in the absorbance at 234 nm. In the presence of antioxidants, this lag time is increased. The main advantage of this *in vitro* assay is the use of a biologically relevant target.

(1) Obtain blood by vein puncture of a forearm vein from 12 h fasted individuals.
(2) Receive the blood into tubes without anticoagulant and centrifuge at 1000× *g* for 20 min at 4 °C.
(3) Recover the serum and use it immediately for the assays.
(4) Incubate human serum samples containing 1.23 mg of protein/ml; 0.035 mg of LDL-cholesterol/ml; 0.04 mg of protein of LDL/ml in 10 ml of PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl) at 37 °C with or without $CuCl_2$ (final concentration 11.7 mM) and with or without propolis extract (see Section 3.1.1) (final concentration 1–50 μg/ml) for 2 h.
(5) Terminate the oxidation by the addition of 100 μmol of EDTA or 10 μM butylated hydroxytoluene (BHT) and refrigeration at 4 °C (Aviram, 1996; adapted by Isla, Nieva Moreno, Sampietro, & Vattuone, 2001).
(6) The formation of conjugated dienes is followed by the absorbance at 234 nm. Determine the concentration of dienes using the difference in absorbance at zero time and at the end of experiment, using the molar absorption coefficient $\varepsilon_{234} = 29{,}500\ M^{-1}\ cm^{-1}$ for conjugated dienes (Abuja, Murkovic, & Pfannhauser, 1998). Lag times (min) should be determined from the intercept of lines drawn through the linear portions of the lag phase and propagation phase.

6.3.3. *Evaluation of the antioxidant activity of propolis in cellular systems*

Oxidative stress can be induced in whole cell suspension by hydrophilic compounds such as H_2O_2 or 2,2′-Azobis-(2-amidinopropane) dihydrochloride (AAPS). H_2O_2 that is normally generated *in vivo* mainly by the autoxidation of hemoglobin and dismutation of superoxide gives rise to radicals like hydroxyl ions. AAPH generates peroxyl radicals outside the membrane.

6.3.3.1. *Inhibitory efficiency of propolis extracts on H_2O_2-induced lipid peroxidation*

(1) Obtain blood (5–10 ml) from healthy non-smoker adult individuals after informed consent. Isolate human erythrocytes from citrated blood immediately by centrifugation at 1500 rpm for 10 min at 4 °C.
(2) After removal of plasma and buffy coat, wash the erythrocytes three times with phosphate-buffered saline (PBS; pH 7.4) at 4 °C, and, finally, resuspend in PBS to obtain erythrocyte suspensions at 5%.
(3) Dissolve initially the dry propolis extract (collected per Section 3.1.1) in DMSO to obtain stock solutions and further dilute in PBS to obtain different final concentrations of propolis. From these serial dilutions, the DMSO final concentration is never higher than 0.08%.
(4) To study the protective effects of propolis extracts against H_2O_2-induced lipid peroxidation, pre-incubate 0.5 ml of an erythrocyte suspension at 5% in PBS suspension, with 10 μl of propolis extract in presence or absence of 4 mM sodium azide, a catalase inhibitor, for 20 min at 37 °C before inducing oxidative stress.
(5) After incubation, centrifuge the mixture, wash with PBS, re-suspend with 0.5 ml of PBS and treat with 0.5 ml of 0.5, 2 and 8 mM of H_2O_2 for 4 h at room temperature (Senturk et al., 2001). A negative control (erythrocytes in PBS), a positive control (erythrocytes in PBS with ascorbic acid), and extract controls (erythrocytes in PBS with each extract) are necessary.
(6) Estimate the extension of lipid peroxidation using a modified thiobarbituric acid (TBA) assay. Briefly, take 500 μl of erythrocyte suspensions and incubate at 95 °C for 45 min with 1 ml of TBA–TCA–HCl (0.375% (w/v) TBA, 15% (w/v) TCA, 0.25 M HCl).
(7) Cool at room temperature and centrifuge at 1000× g for 10 min.
(8) Measure the absorbance of the supernatant at 532 nm. Use a standard curve to quantify the amount of MDA.

6.3.3.2. *Protective effect of propolis extracts on H_2O_2-induced oxidative hemolysis*

(1) Pre-incubate 0.5 ml of an erythrocyte suspension at 5% in PBS, with 10 μl of propolis extract (collected per Section 3.1.1) in presence or absence of 4 mM sodium azide, a catalase inhibitor, for 20 min at 37° C before inducing oxidative stress.
(2) After incubation, centrifuge the mixture, wash it with PBS, re-suspend with 0.5 ml of PBS and

treat with 0.5 ml of 0.5, 2 and 8 mM of H_2O_2 for 4 h at room temperature (Senturk et al., 2001). A negative control (erythrocytes in PBS) and extract controls (erythrocytes in PBS with each extract) are necessary.

(3) Take out aliquots of the reaction mixture at each hour during 4 h of incubation, dilute with saline, and centrifuge at 1000× g for 10 min to separate the erythrocytes.

(4) Determine the percentage of hemolysis by measuring the absorbance of the supernatant (A) at 545 nm and compare with that of complete hemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water.

(5) Calculate the hemolysis percentage using the formula: $A/B \times 100$. IC_{50} values at time 3 h are determined from a concentration–response curve obtained by plotting the percentage of hemolysis inhibition vs. the extract concentration. Use ascorbic acid as the reference antioxidant compound.

6.4. *Antiparasitic activity: action against varroa*

The methods are described in Dietemann et al. (2013).

6.5. *Other tests, including clinical tests*

Propolis extracts have been tested for many different types of biological and pharmacological activities (Burdock, 1998; Farooqui & Farooqui, 2010; Sforcin & Bankova, 2011), including in clinical trials (Henshaw et al., 2014; Hoheisel, 2001; Paulino, Coutinho, Coutinho, & Scremin, 2014; Soroy, Bagus, Yongkie, & Djoko, 2014; Vaz Coelho et al., 2007). Most significant is the number of clinical trials in dentistry (Anauate-Netto et al., 2014; Pereira et al., 2011; Prabhakar, Karuna, Yavagal, & Deepak, 2015; Purra, Mushtaq, Acharya, & Saraswati, 2014; Torwane et al., 2013). It is impossible to describe standard methods for these numerous and diverse tests here.

However, it is essential to emphasize the importance of using chemically characterized and standardized propolis in any biological and/or clinical test performed with propolis extracts and preparations containing propolis. The fact that propolis chemical composition varies dramatically with the geographic and plant origin makes any pharmacological research done with propolis without chemical characterization irreproducible and completely irrelevant.

7. Conclusion

Propolis has been attracting the attention of researchers for over five decades, due to its wide range of valuable pharmacological activity and potential for prevention and treatment of numerous diseases. Only recently have scientists begun to recognize the importance of propolis for honey bees and its significance as a component of their social immunity. Appropriate methods should be developed further for in-depth studies of this aspect of propolis function.

Future studies on propolis should also be directed to the development of procedures for the standardization of propolis types other than poplar type and green Brazilian propolis, and to conduct research on propolis from different geographic regions in order to characterize them chemically and discover their plant source(s). Studies of biological and pharmacological activities of propolis have to be performed only with chemically characterized and standardized propolis in order to get meaningful, reliable and reproducible results. Metabolomics approaches should be applied in combination with biological tests in order to get a holistic picture of the composition-activity relationship.

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