# In vitro antibacterial activity and volatile characterisation of organic Apis mellifera ligustica (Spinola, 1906) beeswax ethanol extracts

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A B S T R A C T

Apis mellifera beeswax is synthesized from honey sugars and secreted by specialized glands situated in the ventral side of the abdomen of worker bees from 12 to 18 days old. It is also characterized by several therapeutic properties of great interest for human health with applications for healing bruises, inflammation, burns and with antimicrobial activity. The aims of this investigation were to assess the antibacterial activity of three organic beeswax samples from di□erent locations and to evaluate their volatile composition with HS-SPME/GC-MS analysis. The beeswax's volatile organic compounds (VOC) included 82 molecules belonging to di□erent che- mical classes, such as aldehydes, hydrocarbons, alcohols, esters, ketones, organic acids, and terpenes. The total VOC contents was 35 ± 5 and 40 ± 2 ppm for samples A and B, respectively. Aldehydes were the main class of VOC in the beeswax ethanol extracts, particularly linear aldehydes such as octanal, nonanal and decanal. Minimal inhibitory concentration (MIC) showed that Pseudomonas aeruginosa and Listeria monocytogenes were inhibited by all beeswax samples. Salmonella Typhimurium was the most resistant bacterial strain. Against the 13 Staphylococcus aureus wild strains tested, beeswax samples A was the most e□ective, followed by sample B and C.

## 1. Introduction

Apis mellifera beeswax is a complex substance used to build hon- eycombs and is secreted by worker bees from 12 to 18 days old (Hepburn et al., 1991). Beeswax is synthesized from honey sugars (such as fructose, glucose and sucrose) and secreted in a liquid form by 4 pairs of specialized glands situated on the ventral side of the abdomen. Liquid beeswax in contact with the air immediately solidifies into fine white scales (Bogdanov, 2004a; 2004b, 2009).

Wax scales are miXed by the jaws with other bee products such as honey, pollen and propolis to give a final yellow product. Beeswax of the honeycomb turns dark over time because of oXidation of cocoons and larval faeces, pupal skins and propolis deposition (Bogdanov, 2004a; 2004b).

Hydrocarbons, free fatty acids, alcohols and exogenous substances (residues of propolis, pollen and small pieces of floral components) are also present in beeswax (Bogdanov, 2009; Puleo, 1991).

The environmental and genetic factors and breeding and diet are the

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collect specific beeswax for other uses such as candles, art (lost- wax casts, modelling, glass and metal engraving), varnishes and pol- ishes and emollients and emulsifiers in cosmetics (Bogdanov, 2004b; Fratini et al., 2016a).

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Beeswax has been historically used in medicines for its e□ectiveness against dermatitis, gastrointestinal disease and inflammation; nowa- days it is still present in several traditional and alternative medicines (Lewis et al., 2012; Molina, Mas, & Carbajal, 2015; Stacey, 2011; Zamora et al., 2014).

Honeybee products such as honey, royal jelly, venom, bee pollen and propolis have been studied for their antimicrobial properties with increasing interest as natural remedies (Castaldo & Capasso, 2002; Domenici et al., 2015; Fratini et al., 2016b; Fratini et al., 2017a; Sagona et al., 2017). Only a few studies reported on beeswax's antimicrobial activities (Abdulrhman, Samir Elbarbary, Ahmed Amin, & Saeid Ebrahim, 2012; Al-Waili, 2005; EL Sakka, Abdulrhman, & Shehata, 2013; Ghanem, 2011; Kacániová et al., 2012). Ghanem (2011) reported that both Gram-positive (Staphylococcus aureus, Streptococcus epi- dermidis and Streptococcus pyogenes) and Gram-negative (Pseudomonas aeruginosa, Bacillus subtilis and Escherichia coli) bacteria are a□ected by exposure to beeswax. Kacániová et al. (2012) showed a high inhibitory activity of methanol and ethanol beeswax extracts with several bac- terial strains (S. aureus, Strep. epidermidis, B. subtilis, Salmonella enterica,

E. coli and L. monocytogenes) as well as some yeasts (Candida albicans, C. tropicalis and C. parapsilosis) and molds (Aspergillus niger, A. flavus and

A. fumigatus). Other authors tested antimicrobial activity of beeswax in miXtures with other natural substances such as honey, propolis and olive oil (Abdulrhman et al., 2012; Al-Waili, 2005; EL Sakka et al., 2013).

Beeswax is used in the European Union as a glazing agent, food additive E901 (Regulation EC 1333/2008) and widely used as a carrier for flavors and coatings (EFSA, 2007).

Moreover, e□ectiveness of beeswax against several pathogenic mi- croorganisms suggests a potential future use in food processing as a preservative agent.

The aim of this investigation was to evaluate the volatile composi- tion and antimicrobial activity of ethanol extracts of three di□erent types of Italian organic beeswax.

## 2. Material and methods

### 2.1. Beeswax samples

Three organic Apis mellifera ligustica beeswax samples, obtained from melting of operculum wax (using a solar wax extractor, the tradi- tional method used by the beekeepers), were collected from three Italian organic beekeepers: sample A was produced by a beekeeper in Ragusa (RG), southeast Sicily, Sample B was

collected from the pro- duction of the social cooperative “Il Pungiglione” in Massa (MS), Tuscany and sample C was produced by a beekeeper in Breganze (VI), northeast Italy, Veneto.

All samples were stored in sterile plastic flasks at room temperature (25 °C) in the dark until used for analysis (a maximum of one month).

## 2.2. Beeswax extraction

Beeswax samples were aseptically flaked with a disposable scalpel. and dissolved in ethanol (96%) (ACS grade, Sigma-Aldrich, Milano, Italy) at a concentration 1:2 (w/v) at 70 °C for 24 h. Ethanol was re- moved with a Rotavapor (Waterbooth B-480, BÜCHI, Flawil, Switzerland) at 60 °C.

The supernatants of beeswax extracts, after centrifugation at 5150g (6000 rpm; REMI R-10M, REMI, Mumbai, India) for 15 min at room temperature (25 °C), were stored at 4 °C in the dark until micro- biological evaluations and HS-SPME/GC-MS analysis (within one wk).

## 2.3. Volatile characterisation

The volatile composition of the beeswax ethanol extracts was in- vestigated using a head space solid phase micro-extraction (HS-SPME) technique coupled with GC-MS analyses, according to the protocol of Cirlini et al. (2012) with slight modifications.

For each SPME extraction, 50 mg of beeswax was placed in a 30 ml glass vial. All the extractions were done at 40 °C for 30 min, after 15 min of equilibration at the same temperature. A triphasic fiber coated with 50/30 µm of divinylbenzene-carboXen-polymethylsiloXane (DVB/ CarboXen/PDMS, Supelco, Bellefonte, PA, USA) was used. The fiber was inserted in the sample head space for 30 min; then, the desorption of volatiles was done directly into the GC injector, at 230 °C for 2 min. A Thermo Scientific Trace 1300 gas-chromatograph coupled to a ThermoScientific ISQ mass spectrometer equipped with an electronic impact (EI) source (Thermo Fisher Scientific Inc., San Jose, CA, USA) was used. Analytes were separated on a SUPELCOWAX 10 capillary column (30 m × 0.25 mm, f.t. 0.25 µm; Supelco), applying a temperature gra-dient from 50 °C for 3 min, increasing 5 °C/min to 200 °C and main- taining the final temperature for 12 min with a total run time of 45 min. Helium was used as the carrier gas, at a constant flow of 1 ml/min. The injections were done in splitless mode (the valve was closed for 2 min). The detector temperature was set at 230 °C and the MS acquisition mode was full scan in a range of 40–500 m/z.

Volatile identification was done by comparison of the mass spectra

with those in the instrument's libraries (NIST 14). Moreover, linear retention indices (LRI) were calculated for each GC-MS signal on the basis of a C8eC20 alkane solution analyses applying the following for- mula:

LRI = 100 * z + [100 * ((Ta - Tn-1)/(Tn+1 – Tn-1))]. Where z is the number of carbon atoms of the alkane eluting immediately before the

analyte, Ta is the analyte retention time (min), Tn-1 is the retention time (min) of the alkane eluting immediately before the analyte and Tn+1 is the retention time (min) of the alkane eluting immediately after the analyte.

Calculated LRI were then compared with those found in the litera- ture. The semi-quantification of all detected compounds was done using the internal standard method (toluene; 0.4 ppm) assuming the peak areas represented an equal molar response for all compounds and that there was a linear response with respect to concentration.

### 2.4. Bacterial strains

Twenty bacterial strains were tested. Seven bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA): Staphylococcus aureus ATCC 6538, Listeria monocytogenes ATCC 7644, Enterococcus faecalis V583E, Lactobacillus plantarum ATCC 14917, Escherichia coli ATCC 15325, Pseudomonas aeruginosa ATCC 27853 and Salmonella enterica serovar Typhimurium ATCC 14028.

Thirteen S. aureus wild strains previously isolated from bovine bulk tank milks (named A through M) were obtained from the culture col- lection of the Department of Veterinary Science, University of Pisa, Pisa, Italy.

Strains were stored at −80 °C in a glycerol suspension until their use. Before MIC/MBC determinations, the strains were cultured in brain hearth infusion (BHI, OXoid, Milan, Italy) broth for 24 h at 37 °C using aerobic conditions.

### 2.5. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

One g of beeswax extract from each sample was diluted in 20 ml of a dimethyl sulfoXide (DMSO)-ethanol (50:50 v/v) solution. Beeswax- DMSO-ethanol solution was diluted in BHI at concentrations of 1:10. A twofold dilution method was used for MIC determination in 96-well polypropylene microtiter plates with a final volume of 200 µl according to Fratini, Mancini, et al. (2017b).

Bacterial suspensions were adjusted using the McFarland standard turbidity scale to obtain approXimately $1.5 * 108$ cfu/ml for each strain.

Microplates were incubated at 37 °C for 24 h. The negative control was a solution of DMSO-ethanol tested at the same concentration as the dilutions with beeswax to determine their potential antimicrobial ac-

tivities.

MBC was quantified on bacterial suspension from wells that did not show bacterial growth in the microdilution plate. Suspensions were plated on tryptone soy agar (TSA, OXoid) plates and microbial growth was evaluated after incubation at 37 °C for 24 h.

MIC and MBC evaluations were done in triplicate and results were reported as mode (the value that appears most often).

## 3. Results and discussion

### 3.1. Characterisation of volatile composition

The volatile profile of beeswax resulted to be composed by 82 VOC belonging to di□erent chemical classes as aldehydes, hydrocarbons, alcohols, esters, ketones, organic acids, and terpenes and derivatives (Table 1). The total VOC contents ranged between 35 ± 5 ppm of sample A, 40 ± 2 ppm of sample B and 40 ± 10 ppm of sample C (Fig. 1). The total volatile amount shows no significant di□erences among the three extracts, although di□erences occurred in term of volatile fraction composition. Overall, 82 volatiles were identified across samples that showed di□erent chromatographic profiles. The poorest beeswax extract in terms of volatile compounds resulted the sample A in which found 47 molecules, while the higher number of volatile compounds was found in sample C with 69 di□erent molecules detected. In sample B 63 volatile compounds were observed.

Quantitatively, aldehydes were the main representative class of the volatile profile of beeswax ethanol extracts. Particularly, linear alde- hydes as heptanal, octanal, nonanal, decanal, and dodecanal were found in all the three samples examined, and octanal, nonanal and decanal resulted the most representative. The highest amount of these compounds was observed in sample C in which aldehydes reach about the 60% of the total volatile amount (21 ± 3 ppm), while sample B showed the lowest aldehydes content with about 30% (12 ± 1 ppm). Among this class, furfural, associated with bready note, was detected in samples C and B, while lilac aldehydes (isomers B and D), with their characteristic fresh flowery aroma, were found only in sample B. The presence of these molecules was already reported in honey volatile fraction (Soria, Martínez-Castro, & Sanz, 2003). Hydrocarbons were observed in all three beeswax extracts and the amounts of these com- pounds were comparable among the samples about 15%. Among hy- drocarbons, volatile linear and substituted alkanes and alkenes were identified as decane, dodecane, 1-dodecene, etc, and mainly was the aromatic compound m-di-tert-butyl benzene. This molecule, that can be originated from the thermal degradation of lignin (Quitain, Sato, Daimon, & Fujie, 2003), was observed in all three samples tested:

2.2 ± 1.8 ppm in sample A, 4.5 ± 0.2 ppm in sample B, and

3.8 ± 0.8 in sample C.

Alcohols showed an opposite trend than aldehydes: the percentage of alcohols resulted lower in sample A (about 11% of total volatiles), intermediate in sample C (about 17% of total volatiles) and more concentrated in sample B with a percentage of about 22%. The most abundant alcohols detected in the three di□erent samples were 2,7-di- methyl-1-octanol and 2-isopropyl-5-methyl-1-heptanol. Also, ethanol was identified among volatiles, but even if the concentration was cal- culated on the basis of the internal standard used, its amount was not considered in the alcohols total amount because its presence was probably due to a residue of the solvent used for beeswax extraction procedure.

Esters represented about the 8% of the total volatile amount in the three samples with an average quantity of 3.2 ± 0.7 ppm. Among es- ters, the most abundant were the linear aliphatic ones such as ethyl hexanoate, ethyl octanoate, ethyl nonanoate and ethyl decanoate with their characteristic waxy aromatic note. Ethyl derivatives of hexanoic, octanoic, nonanoic, and decanoic acids have been identified also in thyme honey samples (Karabagias, Badeka, Kontakos, Karabournioti, & Kontominas, 2014).

Acids were observed in samples B and C, but no volatiles pertaining to this chemical class were detected in sample A. The total amount of these compounds in samples B and C is around 1 ppm (1.2 ± 0.1 ppm). Linear acids such as hexanoic, heptanoic, octanoic and nonanoic acid contributed to fatty, cheesy and waxy notes. Volatiles belonging to the class of terpenes were also detected in the beeswax extracts. Slight amount of limonene was observed in sample A, while in samples B and C other terpenes and derivatives were identified, and thymol, asso- ciated with herbal notes, resulted the main representative with con- centrations of 0.4 ± 0.1 and 0.9 ± 0.1 ppm in samples C and B, re-

spectively. Among terpenes, α-terpineol and trans-geranyl acetone resulted distinctive of sample B, while geraniol was detected only in sample C.

### 3.2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

For each beeswax sample, the 20 tested bacterial strains showed the same MIC values for all replicates, especially the 7 international type culture collection strains (Table 2), the DMSO-ethanol (50:50 v/v) solution showed a MIC value < 5 mg/ml, showing an ine□ective anti- bacterial activity. P. aeruginosa and L. monocytogenes were the most susceptible bacterial strains towards antimicrobial compounds in beeswax samples, with 0.63 mg/ml MIC value, followed by S. aureus, with 1.25 mg/ml MIC value. The most resistant strain was Sal. Typhi- murium with MIC value of 5 mg/ml.

Against the 13 S. aureus wild strains tested, the beeswax samples showed di□erent MIC values. Strain G resulted more susceptible to beeswax samples B and C, with MIC value of 0.63 mg/ml, while it showed 1.25 mg/ml MIC value for beeswax sample A. Strain K showed a high variability of MIC values for each sample; MIC resulted 0.63 mg/ ml, for samples A and B, and 2.5 mg/ml for sample C. Mainly, the an- tibacterial activity of beeswax sample A resulted more e□ective against

S. aureus wild strains, followed by sample B; while the beeswax sample C showed the lowest antibacterial activity against S. aureus wild strains. MBC values resulted the same against each strain. The strain J showed the lowest MBC value with beeswax sample A (1.25 mg/ml). The same MBC values for the three beeswax samples resulted for the strains G, with 2.5 mg/ml. The other strains, wild and collection type, showed a value ranged from 2.5 mg/ml to > 5 mg/ml.

For all strains, the MBC values resulted higher than the MIC values. These results suggest that beeswax shows a good bacteriostatic activity but no bactericidal activity against the tested strains.

The antibacterial activity of beeswax against Gram positive and Gram negative bacteria, especially E. coli, P. aeruginosa and S. aureus, described in this investigation, is consistent with Ghanem (2011) and Kacániová et al. (2012).

The antibacterial activity of beeswax is due to unknown factors or compounds. In all three beeswax samples tested the volatile compounds detected in high quantity were aldehydes (octanal, nonanal and decanal) and m-di-tert-butyl benzene, an aromatic compound. Octanal, isolated from other natural products, showed an antibacterial activity against E. coli, S. aureus (Inouye, Takizawa, & Yamaguchi, 2001; Nair et al., 2005) and L. monocytogenes (Nair et al., 2004, 2005). According to these results, it seems possible that the octanal present in beeswax sample A and C contributed to the increased antibacterial e□ectiveness against S. aureus wild strains L and M. Like octanal, nonanal may be involved in beeswax antibacterial activity. Indeed, nonanal has been reported to be e□ective against L. monocytogenes growth, even at small concentrations (Bisignano et al., 2001). Furthermore, nonanal, even at a high concentration, is ine□ective against Sal. spp. (Bisignano et al., 2001), but it has a little antibacterial activity against E. coli and S. aureus (Bisignano et al., 2001; Inouye et al., 2001; Xin, Liu, Zhang, & Gao, 2016). The high amount of nonanal can be involved in a di□erent range of MIC values showed for S. aureus wild strains.

E. coli, P. aeruginosa and S. aureus were more susceptible to decanal

antimicrobial activity, isolated from other natural compound (Bankova, Popova, & Trusheva, 2014; Esin Hames-Kocabas et al., 2013; Shareef, Muhammed, Hussein, & Hameed, 2016). The high amount of this aldehyde may be involved in the MIC value of tested beeswax against P. aeruginosa.

The antibacterial activity of m-di-tert-butyl benzene was studied from the extract of two di□erent marine sponge, Dysidea pallescens (Nazemi et al., 2017) and Axinella donani (Rani Juneius & Selvin, 2012), with high e□cacy against S. aureus and E. coli.

Other volatile compounds may be involved in beeswax antibacterial activity. Limonene and phenylmethyl alcohol, isolated from propolis (Bankova et al., 2014; Esin Hames-Kocabas, Betul, Atac, & Fatih, 2013; Simionatto, Facco, Morel, Giacomelli, & Linares, 2012), another bee- hive product closely related with beeswax, showed antibacterial ac- tivity against a large bacteria spectrum. Limonene was present in small traces in beeswax sample A and may be involved in antibacterial ac- tivity against S. aureus strain A and J.

Dodecanal is an aldehyde defined as an anti-Salmonella volatile compound (Fujita, Chavasiri, & Kubo, 2015), present in lower percen- tage, in all three beeswax samples. The results suggested that the Sal. Typhimurium resistance against beeswax antibacterial activity can be related to the occurrence of dodecanal. Octanoic acid resulted most active against Salmonella spp. (Hulankova, Borilova, & Steinhauserova, 2013; Kinderlerer & Lund, 1992), its occurrence in low amount, like dodecanal, could be associated to ine□ective activity of beeswax sam- ples. Also octanoic acid is involved in antibacterial activity against L. monocytogenes (Kinderlerer & Lund, 1992). Similarly, hexanoic acid showed antibacterial activity against L. monocytogenes (Kinderlerer & Lund, 1992) as well as geraniol against E. coli and S. aureus (Inouye et al., 2001).

Antibacterial activity of thymol obtained from vegetable extracts and essential oils from Labiatae was reported against E. coli (Marchese et al., 2016; Miladinović, Ilić, Kocić, Ćirić, & Nikolić, 2015; Olasupo, Fitzgerald, Gasson, & Narbad, 2003), Sal. Typhimurium (Džamić et al., 2015; Marchese et al., 2016; Mith et al., 2014), L. monocytogenes (Al- Mariri, Swied, Oda, & Al Hallab, 2013; Marchese et al., 2016; Pan, Chen, Davidson, & Zhong, 2014), P. aeruginosa (Džamić et al., 2015; Kavoosi, Dadfar, & Purfard, 2013) and S. aureus (Bogavac et al., 2015; Cirino, Menezes-Silva, Silva, de Souza, & Siqueira-Junior, 2015; Wattanasatcha, Rengpipat, & Wanichwecharungruang, 2012).

As reported for honey, the beeswax antimicrobial activity is not dependent only on its phytochemical nature (Heering, Usleber, Dietrich, & Märtlbauer, 1998; Mulu, Tessema, & Derbie, 2005) but can be attributed to synergic e□ects exerted by its phenolic acid, flavonoids, benzyl-alcohol, 2-hydroXy benzoic acid and other compounds present in small traces.

## 4. Conclusions

To overcome the growing antibiotic resistance phenomenon in re- cent years many researchers have directed their studies towards the antibacterial activity of di□erent natural substances.

Beehive products have long been used in folkloristic and in tradi- tional medicine. The raw materials, crude extracts and also purified active compounds have been found to show interesting properties, such as antimicrobial, anti-inflammatory and antioXidant activities.

Among beehive products, beeswax is undoubtedly one of the least studied as concerns its antimicrobial activity.

In this investigation, the inhibitory activity of organic beeswax samples against some microorganisms was observed.

It is important to analyse the composition of beeswax because, as with other beehive products, chemical diversity of beeswax volatile compounds from di□erent geographic regions and/or di□erent botanic origin, could greatly a□ect its biological properties such as antibacterial activity.

Therefore, it seems desirable to do further studies on organic beeswax samples characterized by other origins and other composi- tions, since they may be used both in the pharmaceutical field and in food industry as bio-preservations.

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Minimum inhibitory concentration (MIC) (mg/ml) and minimum bactericidal concentration (MBC) (mg/ml) values of beeswax samples (A from South Italy, B from Centre Italy, C from North Italy) for all bacterial isolates, international type culture collection and *Staphylococcus aureus* wild strains (A-M).

| Bacteria Strain | MIC (mg/ml) | | | MBC (mg/ml) | | |
|---|---|---|---|---|---|---|
| | Sample A | Sample B | Sample C | Sample A | Sample B | Sample C |
| *E. coli* 15025 | 2.5 | 2.5 | 2.5 | > 5 | > 5 | > 5 |
| *P. aeruginosa* 27853 | 0.63 | 0.63 | 0.63 | > 5 | > 5 | > 5 |
| *S. Typhimurium* 14028 | 5 | 5 | 5 | > 5 | > 5 | > 5 |
| *L. monocytogenes* 7644 | 0.63 | 0.63 | 0.63 | 2.5 | 5 | 2.5 |
| *E. faecalis* V583E | 2.5 | 2.5 | 2.5 | > 5 | > 5 | > 5 |
| *L. plantarum* 14917 | 2.5 | 2.5 | 2.5 | > 5 | > 5 | > 5 |
| *S. aureus* 6538 | 1.25 | 1.25 | 1.25 | 2.5 | 5 | > 5 |
| *S. aureus* A | 1.25 | 2.5 | 2.5 | > 5 | 5 | > 5 |
| *S. aureus* B | 1.25 | 1.25 | 2.5 | > 5 | 5 | > 5 |
| *S. aureus* C | 1.25 | 1.25 | 2.5 | > 5 | > 5 | > 5 |
| *S. aureus* D | 1.25 | 1.25 | 2.5 | > 5 | > 5 | > 5 |
| *S. aureus* E | 1.25 | 1.25 | 2.5 | > 5 | > 5 | > 5 |
| *S. aureus* F | 1.25 | 1.25 | 2.5 | > 5 | > 5 | > 5 |
| *S. aureus* G | 1.25 | 0.63 | 0.63 | 2.5 | 2.5 | 2.5 |
| *S. aureus* H | 1.25 | 1.25 | 2.5 | > 5 | > 5 | > 5 |
| *S. aureus* I | 1.25 | 0.63 | 1.25 | 2.5 | 5 | 2.5 |
| *S. aureus* J | 0.63 | 1.25 | 1.25 | 1.25 | 2.5 | 2.5 |
| *S. aureus* K | 0.63 | 0.63 | 2.5 | > 5 | > 5 | > 5 |
| *S. aureus* L | 1.25 | 2.5 | 1.25 | 2.5 | 5 | 2.5 |
| *S. aureus* M | 1.25 | 2.5 | 1.25 | 2.5 | 5 | 2.5 |