

Original article

## Comparative study of the physicochemical and palynological characteristics of honey from *Melipona subnitida* and *Apis mellifera*

Ligia B. de Almeida-Muradian,<sup>1</sup> Klaus M. Stramm,<sup>1</sup> Andreia Horita,<sup>1</sup> Ortrud M. Barth,<sup>2</sup> Alex da Silva de Freitas<sup>2</sup> & Leticia M. Estevinho<sup>3\*</sup>

<sup>1</sup> Pharmaceutical Science School, University of Sao Paulo, Av. Prof. Lineu Prestes, 580, bloco 14, CEP 05508-900, São Paulo, Brazil

<sup>2</sup> Instituto Oswaldo Cruz, FIOCRUZ, Avenida Brasil 4365, Rio de Janeiro, 21040-900, Brazil

<sup>3</sup> CIMO-Mountain Research Center, Agricultural College of Bragança, Polytechnic Institute of Bragança, Campus Santa Apolónia, Bragança, E 5301-855, Portugal

(Received 14 October 2012; Accepted in revised form 25 February 2013)

**Summary** Twenty-four samples of *Apis mellifera* honey and twenty-four samples of *Melipona subnitida* (Jandaira) honey were collected in the northeast of Brazil. Moisture, hydroxymethylfurfural, free acidity, insoluble solids in water, diastase activity, ashes, electrical conductivity, proteins, lipids, total carbohydrates, energy and sugars were the parameters analysed. The efficiency of the qualitative tests (Fiehe's test, Lugol's reaction, Lund's reaction) was tested. Pollen types and the corresponding plant species were identified in all samples (3 in *Apis* and 1 in *Melipona*). *Apis mellifera* honey samples demonstrated parameters in accordance with the Brazilian Legislation, while the *Melipona subnitida* honey samples displayed moisture (24.80%) and diastase activity (null) in discordance with the established by the regulation for *Apis mellifera* honeys. *Apis* honey samples presented higher values of electric conductivity ( $284.00 \mu\text{S cm}^{-1}$ ) than the obtained from the Jandaira honey samples ( $102.77 \mu\text{S cm}^{-1}$ ) as well as a darker colour (26.67 mmPfund) when compared with Jandaira honey (7.00 mmPfund). The concentration of the glucose, fructose and sucrose was higher in the *Apis* honeys than in the Jandaira honey. The characteristics of the two types of honey were very different, highlighting the need of developing specific legislation for stingless bees' honey.

**Keywords** *Apis mellifera*, Melipona honey, *Melipona subnitida*, nutritional value, palynological analysis, physicochemical analysis.

### Introduction

Honey's elaboration starts with the nectar collected from many plants, which honeybees transform and combine with their own specific substances, store and leave to mature in honeycombs. This natural product is generally composed of a complex mixture of carbohydrates and other less frequent substances, such as organic acids, amino acids, proteins, minerals, vitamins, lipids, aroma compounds, flavonoids, vitamins, pigments, waxes, pollen grains, several enzymes and other phytochemicals (Gomes *et al.*, 2010; Lazar-*evic et al.*, 2010). However, the specific composition depends on many factors, such as the nectar composition of the source plant, bees' species, the climate, environmental and seasonal conditions, agricultural practices and treatment of honey during extraction and storage (Marchini *et al.*, 2006; Iglesias *et al.*, 2012).

According to Basualdo *et al.* (2007), the physical and chemical properties strongly influence the healing capacity of honey. It has been used not only in foods and beverages as a sweetener and flavouring, but also in medicine since the early humans. The role of this product in the treatment of burns, gastrointestinal disorders, respiratory illnesses, infected and chronic wounds, skin ulcers and cancer has been studied recently by many researchers (Castaldo & Capasso, 2002; Orhan *et al.*, 2003; Ramalhosa *et al.*, 2011).

Some authors even state the higher activity of honey over well-known antibiotics (Malika *et al.*, 2005; Estevinho *et al.*, 2011). The antimicrobial effectiveness reported have been evaluated with diverse sets of methodologies, degrees of sensitivity and microbial strains, what leads to difficulties comparing results from work teams (Vargas *et al.*, 2007).

Two types of honey are produced and commercialised in Brazil: the traditional *Apis mellifera* honey

\*Correspondent: E-mail: leticia@ipb.pt

and that produced by the stingless bees (e.g. *Melipona subnitida*). The first, the Africanised honeybee, is the most widespread species in Brazil (Araújo *et al.*, 2006) and is the most intensely studied (Nogueira-Neto *et al.*, 1986). The latter is a bee specie from the northeast of Brazil (Jandaira region), being found in beehives and in rural and urban zones (Carvalho *et al.*, 2001).

Stingless bees' honey is completely different from that produced by the bees of the genus *Apis* (Vit *et al.*, 2004). The demand for this product has increased recently, being the commercial value higher than the one of *Apis mellifera* honey. However, the lack of complete studies regarding the physicochemical characteristics of the stingless bees' honey hampers the definition of quality patterns and standards (Kerr *et al.*, 1996). Indeed, the current legislation, both internationally and in Brazil, concerning the beehive products is only directed to the *Apis mellifera* products. In addition, little information is found in the literature about the botanical sources that stingless bees utilise to collect nectar and pollen (Barth *et al.*, 2012).

This study aimed to evaluate the composition of *Melipona subnitida* and *Apis mellifera* honeys through the use of physicochemical analyses. In parallel, palynological analysis was carried out. It was also intended to find out whether the obtained parameters for the stingless bees' honey were in concordance with the *Apis mellifera* honey's Legislations established both nationally and internationally. Indeed, the quality control of honey has two principle purposes: to verify its genuineness and to reveal possible frauds such as artificial honeys and adulterations. The detailed characterisation performed in this work will contribute to the enhancement of legal specifications regarding the stingless bees' honey as well as to improve the current regulations for *Apis mellifera* honey.

## Materials and methods

### Chemicals and materials

All the chemical reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade. The water was purified using a Milli-Q purification system (Millipore, Bedford, MA, USA). The equipments were as follows: Shimadzu UV-Visible Spectrophotometer – UV-1650 PC; Krüss-Digital Hand-held Refractometer DR 201-95; Hanna C221 honey color analyzer; conductivimeter Crison-EC-Meter Basic 30+, micro Kjehdahl system, Tecnal-TE-0363; Soxhlet extraction using Tecnal-SEBELIN TE-188 and the HPLC Shimadzu- Prominence with Refractive Index detector model RID-10A for sugar analysis.

### Honey samples

Twenty-four samples of *Melipona subnitida* honey were harvested from beehives located in the city of Jandaira (05°21'21" S, 36°07'40" W), and twenty-four samples of *Apis mellifera* honey were collected in the same botanical region. The samples were homogenised and kept in glass flasks of 250 mL. After honeys' harvest, the samples were delivered to the laboratory, where they were stored in a dark place at room temperature ( $\pm 20$  °C) until analysis, which occurred in no more than one month after the extraction from the hives by beekeepers. All the samples showed no sign of fermentation or spoilage.

### Palynological analysis

The samples were subjected to qualitative pollen analysis, as recommended by Louveaux *et al.* (1978), Barth (1989, 2005) and Von der Ohe *et al.*, 2004;. Ten grams of each sample were dissolved in distilled water, and the sediment was concentrated by repeated centrifuging 15' at 1500 rpm. The precipitate was washed in distilled water, centrifuged and glycerine water (1:1) was added for 30'. After this stage, the material was centrifuged, decanted, and the sediment was prepared using not-stained glycerine jelly. Pollen grains slides were observed using light and polarised light microscopy at 400 $\times$  magnification, in order to identify the pollen types. The recognition of the pollen types was based on the reference collection of the Palynology Laboratory of the Federal University of Rio de Janeiro, Brazil and on specific literature (Barth, 1989; Roubik & Moreno, 1991; Moreti *et al.*, 2002). The definition of pollen classes presented by Zander (Louveaux *et al.*, 1978) was used for qualitative and quantitative analyses. The following terms were used for pollen frequency classes: predominant pollen (PP, more than 45% of pollen grains counted), secondary pollen (SP, 16–45%), important minor pollen (IMP, 3–15%) and minor pollen (<3%).

### Physicochemical analyses

Physicochemical parameters were analysed using The *Official Methods of Analysis* of Association of Official Analytical Chemists (AOAC, 1990), The *Harmonised Methods* of the European Honey Commission (Bogdanov *et al.*, 1997) and the Codex Alimentarius Codex Alimentarius (CAS, 2001). These methodologies are also the recommended by Brazilian legislation (Almeida-Muradian *et al.*, 2002). Samples were analysed in triplicate and during the same time period to ensure uniform conditions and comparability.

### Colour analyses

In the determination of the honey's colour, it was used a photometer with direct readout in mmPfund. Honey colour is measured in millimeters on the Pfund scale compared to an analytical standard scale of reference on the graduation of glycerin. The use of this meter removes all the guesswork commonly associated with honey colour measurement, providing accurate and repeatable results.

### Moisture content

Moisture was determined using the indirect refractometric method of Chataway. All measurements were taken using an Abbe refractometer, and the percentage of moisture ( $\text{g } 100 \text{ g}^{-1}$  honey) was obtained from the refractive index of the honey sample by consulting a standard table for the purpose (Chataway table).

### Hydroxymethylfurfural (HMF)

Five grams of honey were dissolved in 25 mL of distilled water. The absorbance was measured at 284 and 336 nm against a filtered solution treated with  $\text{NaHSO}_3$ . HMF was determined following the equation, where  $D$  = dilution factor and  $W$  = sample weight in grams:

$$\text{HMF}(\text{mg/kg of honey}) = (\text{Abs}_{284} - \text{Abs}_{336}) \times 149.7 \times 5 \times \frac{D}{W}$$

### Free acidity

Free acidity was determined by potentiometric titration. Ten grams of honey were then dissolved in 75 mL of distilled water, and alcoholic solution of phenolphthalein added. The solution was titrated with 0.1 N NaOH. The milliequivalents of acid per kg of honey were determined as 10 times the volume of NaOH used in titration.

### Water insoluble solids content

For the determination of the insoluble solids in water, the gravimetric method was used. Twenty grams of honey were diluted with water, filtered and washed carefully, until free from sugars. The presence of sugars was tested by the addition of 1% phloroglucinol in ethanol to some filtrate and few drops of concentrated sulphuric acid, because sugars produce colour at the interface. The crucible was dried at  $135^\circ\text{C}$  for an hour. The following equation was used (where

$m$  = mass of dried insoluble matter and  $m_1$  = mass of honey taken):

$$\text{Insoluble Matter}(\%) = \frac{m}{m_1} \times 100$$

### Diastase activity

The spectrophotometrical method was used to assess the diastase activity. Diastase activity was determined using a buffered solution of soluble starch and honey incubated in a thermostatic bath at  $40^\circ\text{C}$ . The diastase value was calculated using the time taken for the absorbance to reach 0.235, and the results were expressed in Gothe degrees as the amount (mL) of 1% starch hydrolysed by an enzyme in 1 g of honey in 1 h.

### Ash content

The gravimetric methodology was used for the determination of ash content. Ten grams of the sample were transferred to the crucible and two drops of olive oils were added. Afterwards, the sample was heated in a hot plate until carbonised. The sample was kept in the preheated furnace, at  $550^\circ\text{C}$ , for at least 5 h. The following equation was used in the determination of the ash content:

$$\text{Ash content}(\%) = \frac{\text{difference of crucible's weight}}{\text{total weight of the sample}}$$

### Electrical conductivity

The electrical conductivity of a solution of 20 g dry matter of honey in 100 mL of distilled water was measured using an electrical conductivity cell. This analysis is based on the measurement of the electrical resistance, of which the electrical conductivity is the reciprocal.

### Proteins

The nitrogen content was determined using micro Kjeldahl method. The crude protein content was calculated using the conversion factor of 6.25 ( $\text{N} \times 6.25$ ).

### Lipids

The crude fat was determined by gravimetry after extraction with diethyl ether using a Soxhlet equipment (Tecnal model SEBELIN TE-188).

### Total carbohydrates

The total carbohydrate contents were obtained by difference (Estevinho *et al.*, 2012):

$$\text{Total carbohydrates} = 100 - (\text{ashes} + \text{proteins} + \text{lipids})(\%)$$

### Energy

The total energy (in kcal and kJ) was estimated according to the following relation (Barros *et al.*, 2010):

$$\begin{aligned} \text{Energy(kcal)} &= 4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g lipid}) \\ \text{Energy(kJ)} &= 4.184 \times \text{Energy(kcal)} \end{aligned}$$

### Sugars

Analysis of sugars (glucose, fructose and sucrose) was performed by High Pressure Liquid Chromatography (HPLC Prominence System from Shimadzu) with a Refractive Index detector (RID-10A Shimadzu). The column, Shim-Pack CLC-NH<sub>2</sub> (6.0 × 150 mm), 5 µm was eluted by use of isocratic system with acetonitrile (pump A – LC 20AT Shimadzu) and water (pump B – LC 20AT Shimadzu) (80:20, v/v), previously filtered through a 0.45-µm filter. The separation was performed at a flow rate of 1.3 mL min<sup>-1</sup>, with the column and detector temperature set at 30 °C using auto sampler (SIL20A-Shimadzu). Quantification was achieved by external calibration method and the calibration curves ranged from 50 to 500 µg mL<sup>-1</sup> for glucose, fructose and sucrose. Sugar contents were further expressed in g per 100 g of dry weight.

### Fiehe's test

This test was performed according to Instituto Adolfo Lutz (IAL, 2008). Two grams of honey were dissolved in 10 mL of water, extracted with 30 mL of diethyl ether in a separating funnel, and the layer was concentrated to 5 mL. Two millilitre of freshly prepared resorcinol solution (1 g of resublimed resorcinol in 100 g mL of hydrochloric acid) was added. The solution was shaken. A cherry red colour appearing in a minute indicates the presence of commercial inverted sugar. Yellow and other shades have no significance.

### Lugol's reaction

This test was performed according to Instituto Adolfo Lutz (Instituto Adolfo Lutz, 2008). Twenty millilitre of water was added to 10 g of honey. The solution was kept in the water bath for 1 h, cooled to room temperature, and 0.5 mL of Lugol solution was added. In the presence of commercial glucose or sugar syrups,

the solution gets stained (blue). The intensity of the colour reflects the quality and quantity of dextrans or starch present in the adulterated sample.

### Lund's reaction

This test was performed according to Instituto Adolfo Lutz (Instituto Adolfo Lutz, 2008). Two grams of each sample were weighted and transferred to a volumetric flask (50 mL). Twenty millilitre of water and 5 mL of tannic acid (0.5%) were added. Water was added to complete the volume to 40 mL. The mixture was shaken and placed for 24 h. In the presence of pure honey, a precipitate (0.6–3.0 mL) is developed.

### Statistical analysis

All the experiments were performed in triplicate ( $n = 3$ ), and the results were expressed as mean ± standard deviation. The studies were conducted in a fully randomised manner, and all the obtained data were tested regarding normal distribution (Shapiro–Wilk test) and homogeneity of variances (Levene and Brown–Forsythe tests). Upon finding that the conditions were met for the application of parametric statistical tests of mean comparison, the comparisons between the different honey varieties (*Melipona subnitida* and *Apis mellifera*) were performed by the One-dimensional Variance Analysis (One-way ANOVA) followed by Tukey test. In the data sets that did not follow normal distributions and homogeneity of variances, it was used the non-parametric test of Mann–Whitney. All the statistical analysis were carried out using the program STATISTICA 8.0 adopting the significance level of 5% ( $P < 0.05$ ).

## Results and discussion

### Palynological analysis

One of the fundamental aspects that influence the commercial value of honey is its botanical and geographical declaration of origin (Estevinho *et al.*, 2012). The palynological analysis is the most frequently used method of honey identification (Louveaux *et al.*, 1978; Anklam, 1998; Von der Ohe *et al.*, 2004). However, according to Hermosin *et al.* (2003) it is, sometimes, difficult to establish their exact botanical origin. In Table S1, it presented the results of the palynological analysis of the two types of honey (*Melipona subnitida* and *Apis mellifera*). The predominant botanical species of the stingless bees' honey was *Alternanthera* sp., with a mean value of 86.5 and a standard deviation of 7.0. This species and *Mimosa verrucosa* can be considered dominant pollen, since the percentage is higher than 45%.



Concerning the *Apis mellifera* honeys, three important species of the Mimosaceae family were found: *Mimosa verrucosa* ( $49.2 \pm 2.6\%$ ); *Mimosa caesalpiniaefolia* ( $40.4 \pm 3.0\%$ ) and *Piptadenia moniliformis* ( $5.0 \pm 0.8\%$ ) (Fig. S1). As such, it can be inferred that botanical species visited by the two honeybees were completely different. It is hard to compare these results with the obtained by other authors because the palynological analysis of stingless bees' honey is not very common (Barth *et al.*, 2012).

### Physicochemical analyses

The results obtained for the physicochemical parameters are presented in the Table S2.

### Colour analyses

Honey can have different colours from straw yellow to amber, and from dark amber to almost black with a hint of red. This property is related to the mineral content, pollen and phenolic compounds present in the honey and, as such, varies according to the geographical origin and botanical varieties visited by the bees (Ramalhosa *et al.*, 2011). The method of production and agricultural practices also influence the colour. Some changes also occur during storage; browning/darkening of honey is due to Maillard reactions, caramelisation of fructose and polyphenolic reactions, depending on storage temperature and/or duration (Bertoncelj *et al.*, 2007). The results of the colour analyses were  $26.67 \pm 0.58$  mmPfund (*Apis mellifera*) and  $7.00 \pm 0.01$  mmPfund (*Melipona subnitida*) (Table S2). Concerning the honey from *Apis mellifera*, 79.17% of the samples were white ( $16.5\text{--}34.0$  mmPfund), while 20.83% were extra light amber ( $34.0\text{--}50.0$  mmPfund). Regarding the one from *Melipona subnitida* 66.67% of the samples were extra white ( $8.0\text{--}16.5$  mmPfund) and 33.33% were white ( $16.5\text{--}34.0$  mmPfund). Significant differences were found between the two types of honey ( $P < 0.05$ ), being the honey from *Melipona subnitida* lighter than the one from *Apis mellifera*. This is corroborated by the studies of Azeredo *et al.* (2003), Sousa *et al.* (2008) and Anacleto *et al.* (2009). There is no any legislation or regulation concerning honeys' colour, what justifies the need for further studies.

### Moisture content

Honey's moisture content depends on the environmental conditions and the beekeepers' manipulation at the harvest period (Acquarone *et al.*, 2007). This parameter is the only composition criteria, which as a part of the Honey Standard has to be fulfilled in world honey trade. Honey having high water content is more likely

to ferment, making the preservation and storage more difficult (Iglesias *et al.*, 2012). The stingless bees' honey presented a mean moisture content of  $24.80 \pm 1.01\%$  (ranged from 23.86% to 25.88%), while the mean content of the other type of honey was  $18.27 \pm 0.40\%$  (ranged from 17.86% to 18.66%). The first did not comply with the existent legislation, directed to the *Apis mellifera* products, both in Brazil and in Europe, which recommends 20% as the maximum limit. Significant differences were found between the two honeys under study ( $P < 0.05$ ). The values hereby reported have the same order of magnitude as that found by Gomes *et al.* (2010), for *Apis* honey. In addition, our results are in agreement with the studies of Anacleto *et al.* (2009), Alves *et al.* (2008), who have also found higher values of moisture content in stingless bees' honey. On the other hand, Almeida-Muradian *et al.* (2012) found lower mean values ( $15.64 \pm 1.03\%$ ). However, despite the higher moisture, for reasons that are not yet clear, *Melipona* honeys are fairly resistant to spoilage by unwanted fermentation (Vit *et al.*, 2004).

### Hydroxymethylfurfural (HMF)

The hydroxymethylfurfural content is widely recognised as a parameter of honey samples freshness, as it is absent in fresh honeys immediately stored by bees and tends to increase during processing and/or ageing of the product. This compound is produced from simple sugars, especially fructose, by the action of acids; thus, it provides an indication of storage in poor conditions (Gomes *et al.*, 2010). Several factors have been reported to influence the levels of HMF, such as temperature and time of heating, storage conditions, pH and floral source (Fallico *et al.*, 2006). The results obtained for the HMF were significantly different between the two types of honey ( $P < 0.05$ ). For *Apis mellifera*, the mean value was  $10.82 \pm 0.46$  mg Kg<sup>-1</sup>, ranging from 10.32 to 12.27 mg Kg<sup>-1</sup>; and for the *Melipona subnitida*, it was  $7.56 \pm 0.26$  mg Kg<sup>-1</sup>. The minimum value detected for this honey was 7.18 mg Kg<sup>-1</sup> and the maximum was 8.22 mg Kg<sup>-1</sup>. Both values were within the recommended by the Brazilian (maximum of 50 mg Kg<sup>-1</sup>) and European legislation (maximum of 40 mg Kg<sup>-1</sup>). The results here reported are in agreement with the referred by Anacleto *et al.* (2009). The studies conducted by Souza *et al.* (2007) also corroborate the values hereby presented. According to these authors, the HMF content of *Apis mellifera*'s honey is generally superior to that produced by *Melipona subnitida*.

### Free acidity

The free acidity of honey may be explained by taking into account the presence of organic acids in

equilibrium with their corresponding lactones, or internal esters, and some inorganic ions, such as phosphate (Gomes *et al.*, 2010). High acidity can be indicative of fermentation of sugars into organic acids. The values obtained for the *Apis mellifera*'s honey ranged from 25.99 to 27.21 mEq Kg<sup>-1</sup>, with a mean value of 26.47 ± 0.46 mEq Kg<sup>-1</sup>. Regarding the stingless bees' honey, the mean value was 32.49 ± 1.13 mEq Kg<sup>-1</sup>, ranging from 31.79 to 33.19 mEq Kg<sup>-1</sup>. Significant differences, adopting the significance level of 5%, were found between the honeys from *Apis* and *Melipona*. All the values obtained were in agreement with the current legislation for *Apis* honey. The free acidity of stingless bees' honey is generally higher, what justifies the more acidic flavour of this type of honey, responsible for the higher consumers' preference (Vit *et al.*, 2004). Mesquita *et al.* (2007) found much higher values (mean 81.27 mEq Kg<sup>-1</sup>), which are not within the limits allowed, for stingless bees' honey.

#### Water insoluble solids content

Honey insoluble matter includes bee pollen, honeycomb debris, bee and filth particles. As such, this parameter is a criterion of cleanness, as it allows detecting honeys' impurities (Bogdanov *et al.*, 1997). The values obtained for the two types of honey were very low and were within the established by the present legislation (European and Brazilian regulation for *Apis mellifera*), revealing that the product' manipulation was adequate. This is one of the few parameters in which no significant differences were observed. Few studies have analysed the insoluble solids content of stingless bees' honey, what difficult comparisons. Regarding the insoluble solids content of *Apis* honey, our results are corroborated by Almeida-Muradian *et al.* (2002) and Sousa (2008).

#### Diastase activity

Diastase is the common name of the enzyme alpha-amylase, naturally present in honey, which is logically weakened and destroyed by heat (White, 1994). In this context, diastase activity is an indicator of the freshness and is a useful tool to detect heat-induced defects and improper storage of honey (Ramalhosa *et al.*, 2011). In this study, the mean value of the diastase activity obtained for *Apis mellifera* was 42.87 and the standard deviation was 2.85. The minimum value obtained was 41.45 and the maximum was 45.00. Highly significant differences ( $P < 0.001$ ) were found between the two types of honey, as no diastase activity was detected in stingless bees' honey. In this particular case, the absence of diastase activity does not mean adulteration or lack of

quality, but is a specific characteristic of this type of honey, as stated by Cortopassi-Laurino & Gelli (1991). This difference between the diastase content is associated with the fact that this enzyme is added by the bees and does not come from the botanical sources (Vit *et al.*, 2004). The diastase activity of the *Apis* honey was higher than Gothe's scale (8), minimum value recommended by the current legislation, confirming the freshness and good quality of the product.

#### Ash content

The mineral content in honey is generally small and depends on nectar composition of predominant plants in their formation. The soil type in which the original nectar-bearing plant was located also influences the quantity of minerals present in the ash. As such, the variability in ash contents has been associated in a qualitative way with different botanical and geographical origins of honeys (Felsner *et al.*, 2004). The determination of this parameter gives an insight of the honeys' quality, as the blossom honeys have a lower ash content than the honeydew honeys (Andrade *et al.*, 1999). For this parameter, all the honeys under analysis fall within imposed limits. However, the results for *Apis mellifera*'s honey (0.18 ± 0.01%) were much higher (highly significant differences,  $P < 0.001$ ) than that obtained for *Melipona subnitida*'s honey (0.02 ± 0.00%). Similar values (0.01%) were obtained by Alves *et al.* (2008), who analysed stingless bees' honey. Concerning the *Apis* honey, the ash content was slightly inferior to the obtained by Estevinho *et al.* (2012) who analysed 75 samples. Cano *et al.* (2001) who studied Brazilian honey samples, found similar results.

#### Electrical conductivity

According to Acquarone *et al.* (2007), electrical conductivity is directly related to the concentration of mineral salts, organic acids and proteins, being very useful in the determination of the floral origin. None of the analysed honey types showed electrical conductivity values superior to 800 µS cm<sup>-1</sup> (*Apis* honey mean value of 284.00 ± 5.00 µS cm<sup>-1</sup>; *Melipona* honey mean value of 102.77 ± 1.31), suggesting that all samples are from nectar honey, which is corroborated by the content of total ashes inferior to 0.6% (BRASIL, 2000, EU, 2001). Significant differences were found between the two types of honey under study ( $P < 0.05$ ).

#### Brute protein

Relatively little is known about the nitrogen content of honey. According to Anklam (1998), the proteins in honey might originate from the plant nectar, from the honeybee, or from pollen. They have an important role

in the formation of the honey. Thus, their reduction or absence in adulterated, overheated or excessively stored honeys serves as an indicator of freshness.

Iurlina & Fritz (2005) refer that the protein content of honey is usually around 0.3%. A small portion of this fraction consists of enzymes, notably invertase, diastase, amylase, glucose oxidase, catalase,  $\alpha$ -glucosidase and  $\beta$ -glucosidase (Won *et al.*, 2008). The brute protein content showed statistical differences between the two types of honey. For *Apis* honey, the value obtained was  $0.49 \pm 0.01\%$ , while for *Melipona* honey, it was  $0.28 \pm 0.01\%$ . There is no any regulation or legislation imposing limits for the proteins in honey, but is needed for the labelling of this product.

### Lipids

Lipids are partly responsible for the physicochemical properties of food and those that are of major nutritional interest are the fatty acids esters (Estevinho *et al.*, 2012). For *Apis mellifera*'s honey, the total fat oscillated between 0.37% and 0.39%. The values detected for lipids in *Melipona subnitida*'s honey were very small ( $<0.000$ ). Significant differences were found, adopting the significance level of 5% ( $P < 0.05$ ). It is important to notice that the determination of the lipids, which may have origin in bee pollen, is not very common, what difficults the results' comparison.

### Total carbohydrates and energy

Both the carbohydrate contents and the energetic values were statistically similar across the studied types of honey. The total carbohydrates were  $98.95 \pm 0.19\%$  (for *Apis mellifera*) and  $99.70 \pm 0.24$  (for *Melipona subnitida*'s honey). The energy determined for *Apis* honey was  $401.18 \pm 3.03 \text{ kcal } 100 \text{ g}^{-1}$  ( $1678.54 \pm 12.68 \text{ kJ}$ ) and for *Melipona* was  $399.92 \pm 2.58$  ( $1673.27 \pm 10.79 \text{ kJ}$ ).

### Sugars

Carbohydrates are the major constituents of honey, corresponding to 95–99% of the dry matter (Olaitan *et al.*, 2007). These sugars are composed mainly of fructose, glucose and sucrose. The other carbohydrates in honey constitute about 12% by mass (Iurlina & Fritz, 2005). They include disaccharides, such as maltose, isomaltose, trisaccharides and tetrasaccharides (Anklam, 1998). The sugars of honey were determined by HPLC and the chromatograms are presented in Fig. S2. Concerning the amount of glucose, the two types of honey showed some homogeneity ( $P > 0.05$ ). The value obtained for *Apis mellifera*'s honey was  $23.50 \pm 0.73\%$  and for *Melipona subnitida*'s honey was  $21.76 \pm 1.22\%$ . On the other hand, significant dif-

ferences were found in the percentages of fructose and sucrose between the types of honey ( $P < 0.05$ ).

For honey produced by the genus *Apis*, the percentage of fructose was  $38.78 \pm 0.69$  and the percentage of sucrose was  $5.72 \pm 0.23$ . Regarding the stingless bees' honey, it was obtained the percentages of  $29.21 \pm 1.81$  and  $4.86 \pm 0.15$  for fructose and sucrose, respectively. Similar results were obtained by Marchini *et al.* (2006) and Almeida-Muradian & Matsuda (2007). On the other hand, Sousa (2008) found higher values for glucose (between 29.49% and 37.45%), fructose (between 41.52% and 47.53%) and lower values of sucrose (between 0 and 2.68%).

### Fiehe's test

Fiehe's test is qualitative and is based on the detection of Hydroxymethylfurfural that results from the dehydration of fructose, obtained by the acidic hydrolysis of sucrose. This furfural's derivative reacts with resorcinol, forming a colour. The test is considered positive when the colour is red (Almeida-Muradian & Matsuda, 2007). In the present study, the test was negative for all the samples of the two types of honey (Table S3), confirming the results obtained in the quantitative test of HMF, which reveals the freshness of this natural product.

### Lugol's reaction

This determination is based on the reaction between iodine and potassium iodide in the presence of glucose, resulting in a stained solution (red-purple to blue). The intensity of the colour depends on the amount of dextrines of glucose. The reaction is considered positive when the stained solution is blue (Almeida-Muradian & Matsuda, 2007). The Lugol's reaction was negative for all the samples under study (Table S3), confirming the absence of adulteration. In this context, this rapid test corroborates the results obtained in the quantitative tests.

### Lund's reaction

This reaction is based on the precipitation of natural occurring honey's proteins by the tannic acid. The reaction is considered positive, indicating the presence of pure honey, when the precipitate varies from 0.6 to 3.0 mL (Almeida-Muradian & Matsuda, 2007). Concerning the *Apis mellifera*'s honey, the values found for the deposits of proteins after Lund test application were within the range established by the Instituto Adolfo Lutz (Instituto Adolfo Lutz, 2008). However, stingless bees' honeys precipitate was  $0.50 \pm 0.01 \text{ mL}$  (Table S3), what could suggest that the honey is not pure. Considering the results obtained in the quantities



tests for *Melipona subnitida*'s honey, that guarantee its pureness, it can be claimed that this rapid test is not very reliable for the analysis of stingless bees' honey.

## Conclusions

The palynological profile of the two types of honey under study was completely different, suggesting that the two bees' species does not visit the same plants. The results obtained for the physicochemical analysis of *Apis mellifera*'s honey were within the established by the European and Brazilian legislation. As consequence, it can be concluded that the product was of good quality. Concerning the *Melipona subnitida*'s honey, all the parameters apart from moisture and diastase activity were in accordance with the legislation, totally directed to *Apis* honey. Significant differences were found between the two honeys under study for almost all the physicochemical parameters, highlighting the need for the development of standards and regulations exclusively directed to stingless bees' honey. The increasing demand for stingless bees' product also justifies further studies and more complete approaches.

Having into account the results obtained in this study, the authors consider that, for stingless bees products, the maximum moisture content should be 30%, higher than it is for *Apis*, the maximum allowed for the electrical conductivity should be around  $500 \mu\text{S cm}^{-1}$ , smaller than for *Apis mellifera* honey. Special attention should be paid to the minimum value of diastase activity, because stingless bees' honey has much lower enzymatic activity. Despite the importance of diastase activity as a freshness indicator parameter for *Apis* honey, it does not have the same role in the stingless bees' product.

In this study, it was also verified that, despite its efficiency in the rapid analysis of *Apis* products, the Lund's reaction should not be used to test the quality of stingless bees' product, to avoid false results. Much of the information hereby conveyed is completely new and with applications not only in the academic world but also in the practical life.

## Acknowledgments

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support and scholarship to the fourth author. The authors are also grateful to Elias Araújo, who helped in the chromatographic analysis. The authors declare that there are no any conflicts of interest.

## References

Acquarone, C., Buera, P. & Elizalde, B. (2007). Pattern of pH and electrical conductivity upon honey dilution as a complementary

tool for discriminating geographical origin of honeys. *Food Chemistry*, **101**, 695–703.

Almeida-Muradian, L.B. & Matsuda, A.H. (2007). Physicochemical parameters of Amazon *Melipona* honey. *Química Nova*, **30**, 707–708.

Almeida-Muradian, L.B., Pamplona, L., Bera, A. & Vilhena, F. (2002). Composição nutricional de méis comercializados no Estado de São Paulo. In: *Congresso Brasileiro de Apicultura, Campo Grande*. Anais (edited by Apacame) Pp. 27. Campo Grande: Confederação Brasileira de Apicultura.

Almeida-Muradian, L.B., Luginbühl, W., Badertscher, R. & Gallmann, P. (2012). Generalizability of PLS calibrations with FT-IR ATR spectrometry for the prediction of some physicochemical measurands of honey. *ALP Science*, **541**, 1–15.

Alves, D.F.S., Cabral, F.C. Jr, Cabral, P.P.A.C., Oliveira, R.M. Jr, Rego, A.C.M. & Medeiros, A.C. (2008). Efeitos da aplicação tópica do mel de *Melipona subnitida* em feridas infectadas de ratos. *Revista do Colégio Brasileiro de Cirurgiões*, **35**, 188–193. URL: <http://www.scielo.br/rcbc>.

Anacleto, D.A., Souza, B.A., Marchini, L.C. & Moreti, A.C.C.C. (2009). Composição de amostras de mel de abelha Jataí (*Tetragonisca angustula latreille*, 1811). *Ciência e Tecnologia de Alimentos*, **29**, 535–541.

Andrade, P.B., Amaral, M.T., Isabel, P., Carvalho, J.C.M.F., Seabra, R.M. & Cunha, A.P. (1999). Physicochemical attributes and pollen spectrum of Portuguese heather honeys. *Food Chemistry*, **66**, 503–510.

Anklam, E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry*, **63**, 549–562.

AOAC. 1990. *Official Methods of Analysis*. 15th edn. Pp. 770–771. Virginia, USA: Association of Official Analytical Chemists, Inc..

Araújo, M.B., Thuiller, W. & Pearson, R.G. (2006). Climate warming and the decline of amphibians and reptiles in Europe. *Journal of Biogeography*, **33**, 1712–1728.

Azeredo, L.C., Azeredo, M.A.A., Souza, S.R. & Dutra, V.M.L. (2003). Protein contents and physicochemical properties in honey samples of *Apis mellifera* of different origins. *Food Chemistry*, **80**, 249–254.

Barros, L., Heleno, S.A., Carvalho, A.M. & Ferreira, I.C.F.R. (2010). Lamiaceae often used in Portuguese folk medicine as a source of powerful antioxidants: vitamins and phenolics. *LWT – Food Science and Technology*, **43**, 544–550.

Barth, O.M. (1989). *O pólen no mel brasileiro*. Rio de Janeiro: Gráfica Luxor.

Barth, O.M. (2005). Análise polínica de mel: avaliação de dados e seu significado. São Paulo. *Mensagem Doce*, **81**, 2–6.

Barth, O.M., Freitas, A.S. & Almeida-Muradian, L. (2012). Palynological analysis of Brazilian stingless bee pot-honey. In: *Stingless Bees Process Honey and Pollen in Cerumen Pots* (edited by P. Vit & D.W. Roubik) Pp. 1–8. Mérida, Venezuela: SABER-ULA, Universidad de Los Andes. <http://www.saber.ula.ve/handle/123456789/35292>.

Basualdo, C., Sgroi, V., Finola, M.S. & Marioli, J.M. (2007). Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. *Veterinary Microbiology*, **124**, 375–381.

Bertoncelj, J., Doberšek, U., Jamnik, M. & Golob, T. (2007). Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. *Food Chemistry*, **105**, 822–828.

Bogdanov, S., Martin, P. & Lüllman, C. (1997). Harmonised methods of the European Honey Commission. *Apidologie Extra Issue*, 1–59.

Cano, C.B., Felsner, M.L., Matos, J.R., Bruns, R.E., Whatanabe, H.M. & Almeida-Muradian, L.B. (2001). Comparison of methods for determining moisture content of citrus and eucalyptus Brazilian honeys by refractometry. *Journal of Food Composition and Analysis*, **14**, 101–109.

Carvalho, C.A.L., Moreti, A.C.C.C., Marchini, L.C., Alves, R.M.O. & Oliveira, P.C.F. (2001). Pollen spectrum of honey of “uruçu”



- bee (*Melipona scutellaris* Latreille, 1811). *Revista Brasileira de Biologia*, **61**, 63–67.
- Castaldo, S. & Capasso, F. (2002). Propolis, an old remedy used in modern medicine. *Fitoterapia*, **73**, S1–S6.
- Codex Alimentarius (2001). Revised Codex Standard for Honey, Codex STAN 12–1981, Rev. 1 (1987), Rev. 2.
- Cortopassi-Laurino, M. & Gelli, D.S. (1991). Analyse pollinique, propriétés physico-chimiques et action des miels d'abeilles africainisées *Apis mellifera* et de *Meliponinés* du Brésil. *Apidologie*, **22**, 61–73.
- Estevinho, L.M., Afonso, S.E. & Feás, X. (2011). Antifungal effect of lavender honey against *Candida albicans*, *Candida krusei* and *Cryptococcus neoformans*. *International Journal of Food Science and Technology*, **48**, 640–643.
- Estevinho, L.M., Feás, X., Seijas, J.A. & Vázquez-Tato, M.P. (2012). Organic honey from Trás-Os-Montes region (Portugal): chemical, palynological, microbiological and bioactive compounds characterization. *Food and Chemical Toxicology*, **50**, 258–264.
- EU (2001). Council Directive 2001/110/CE relating to honey. *Official Journal of European Community*, **L10**, 47–52.
- Fallico, B., Arena, E., Verzera, A. & Zappalà, M. (2006). The European Food Legislation and its impact on honey sector. *Accreditation and Quality Assurance*, **11**, 49–54.
- Felsner, M.L., Cano, C.B., Bruns, R.E., Watanabe, H.M., Almeida-Muradian, L.B. & Matos, J.R. (2004). Characterization of monofloral honeys by ash contents through a hierarchical design. *Journal of Food Composition and Analysis*, **17**, 737–747.
- Gomes, S., Dias, L., Moreira, L., Rodrigues, P. & Estevinho, L.M. (2010). Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. *Food and Chemical Toxicology*, **48**, 544–548.
- Hermosin, I., Chicon, R.M. & Cabezudo, M.D. (2003). Free amino acid composition and botanical origin of honey. *Food Chemistry*, **83**, 263–268.
- Iglesias, A., Feás, X., Rodrigues, S. et al. (2012). Comprehensive study of honey with protected denomination of origin and contribution to the enhancement of legal specifications. *Molecules*, **17**, 856–8577.
- Instituto Adolfo Lutz (2008). Apostila Instituto Adolfo Lutz. Métodos Físico-Químicos para Análise de Alimentos – 4ª Edição<sup>1a</sup> Edição Digital. Instituto Adolfo Lutz © 2008.
- Iurlina, M.O. & Fritz, R. (2005). Characterization of microorganisms in Argentinean honeys from different sources. *International Journal of Food Microbiology*, **105**, 297–304.
- Kerr, W.E., Carvalho, G.A. & Nascimento, V.A. (1996). *Abelha urucu – biologia, manejo e conservação*. Pp. 144. Belo Horizonte: Fundação Acangau.
- Lazarevic, J.S., Palic, R.M., Radulovic, N.S., Ristic, N.R. & Stojanovic, G.S. (2010). Chemical composition and screening of the antimicrobial and antioxidative activity of extracts of *Stachys* species. *Journal of the Serbian Chemical Society*, **75**, 1347–1359.
- Louveaux, J., Maurizio, A. & Vorwohl, G. (1978). Methods of melissopalynology. *Bee World*, **59**, 139–157.
- Malika, N., Mohammed, F. & Chakib, E. (2005). Microbiological and Physico-chemical properties of Moroccan honey. *International Journal of Agriculture and Biology*, **7**, 773–776.
- Marchini, L.C., Reis, V.D.A. & Moreti, A.C.C.C. (2006). Composição físico-química de amostras de pólen coletado por abelhas africanizadas *Apis mellifera* (Hymenoptera: Apidae) em Piracicaba, Estado de São Paulo. *Ciência Rural*, **36**, 949–953.
- Mesquita, L.X., Samakamoto, S.M., Maracajá, P.B., Pereira, D.S. & Medeiros, P.V.Q. (2007). Análises físico-químicas de amostras de mel de Jandaira puro (*Melipona subnitida*) e com misturas. *Mossoró. Revista Verde*, **2**, 65–68.
- Moreti, A.C.C.C., Marchini, L.C., Souza, V.C. & Rodrigues, R.R. (2002). *Atlas do Pólen de Plantas Apícolas*. Rio de Janeiro: Papel Virtual.
- Nogueira-Neto, P., Imperatriz-Fonseca, V.L. & Kleinert-Giovannini, A. (1986). *Biologia e manejo das abelhas sem ferrão*. Pp. 54. São Paulo: Tecnapi.
- Olaitan, P.B., Adeleke, O.E. & Ola, I.O. (2007). Honey: a reservoir for microorganisms and an inhibitory agent for microbes. *African Health Sciences*, **7**, 159–165.
- Orhan, F., Sekerel, B.E., Kocabas, C.N., Sackesen, C., Adalioğlu, G. & Tuncer, A. (2003). Complementary and alternative medicine in children with asthma. *Annals of Allergy, Asthma, and Immunology*, **90**, 611–615.
- Ramalhosa, E.E., Gomes, T.T., Pereira, A.P., Dias, T.T. & Estevinho, L.M. (2011). Mead production tradition versus modernity. *Advanced Food Nutritional Research*, **63**, 101–118.
- Roubik, D.W. & Moreno, J.E. (1991). *Pollen and spores of Barro Colorado Island*. St. Louis: Missouri Botanical Garden.
- Sousa, G.L. (2008). Estudo comparativo sobre a composição e qualidade de méis de *Apis mellifera* e méis de abelha jataí (*Tetragonisca angustula*). São Paulo, Brazil: Tese de mestrado em Ciências dos Alimentos, Universidade de São Paulo.
- Sousa, G.L., Barion, F., Fand, I.B., Barth, O.M., Freitas, A.S. & Almeida-Muradian, L.B. (2008). Caracterização físico-química e botânica de méis de *Tetragonisca angustula* (Jataí) do estado de São Paulo. In: Congresso Brasileiro de Apicultura, 17, Meliponicultura.
- Souza, B., Roubik, D., Barth, O. et al. (2007). Composition of stingless bee honey: setting quality standards. *Interciencia*, **31**, 867–875.
- Vargas, C., Sousa, S.M., Smânia, E.F.A. & Smânia, A.J. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, **38**, 369–380.
- Vit, P., Medina, M. & Enriquez, M.E. (2004). Quality standards for medicinal uses of Meliponinae honey in Guatemala, Mexico and Venezuela. *Bee World*, **85**, 2–5.
- Von der Ohe, W., Persano Oddo, L., Pianca, L., Morlot, M. & Martin, P. (2004). Harmonized methods of melissopalynology. *Apidologie*, **35**, 518–525.
- White, J. (1994). The role of HMF and diastase assays in honey quality evaluation. *Bee World*, **75**, 104–117.
- Won, S.R., Lee, D.C., Ko, S.H., Kim, J.W. & Rhee, H.I. (2008). Honey major protein characterization and its application to adulteration detection. *Food Research International*, **41**, 952–956.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Main pollinic types found in the honey samples. 1 – *Alternanthera* sp.; 2 – *Mimosa verrucosa*; 3 – *Mimosa caesalpiniaefolia*; 4 – *Piptadenia gonoacantha*; Black bar = 10 µm.

**Figure S2.** Chromatograms of carbohydrates for the two types of honey under study: 1 – Sugar standards; 2 – *Apis mellifera* honey5 sugars; 3 – *Melipona subnitida* honey sugars.

**Table S1.** Palynological spectrum of the two types of honey (*Melipona subnitida* and *Apis mellifera*).

**Table S2.** Color, physicochemical composition and energetic value of the two types of honey (mean ± SD, n = 24 of each type of honey).

**Table S3.** Results of the qualitative analyses (Fiehe, Lund and Lugol) of the two types of honey