

## ORIGINAL ARTICLE

# Antioxidant activity and physicochemical characteristics of honeys from the eastern Amazon region, Brazil

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## ABSTRACT

Honey is a natural product that has several beneficial properties for health, such as anti-inflammatory, antioxidant and antimicrobial activities, which depend on its composition. In this context, physicochemical properties (colour, pH, ash, moisture, sugars, and total phenolic contents) and antioxidant activity (DPPH radical scavenging ability) of *Apis mellifera* honeys from Santarém, the main honey production area in the eastern Amazon region, were evaluated. Most samples were dark in colour and acidic. The ash and moisture contents ranged from 0.112 to 0.318 and from 14.751 to 17.514, respectively. The reducing sugars content was 62.873–91.563%. The total amount of phenolic compounds was higher than normally reported, ranging between 15.22 and 16.51 mg g<sup>-1</sup> and 17.70 and 18.94 mg g<sup>-1</sup>, for honey after and before exposure to light, respectively. Quercetin was found only in honey that had been protected from light, with values ranging from 0.24 to 0.43 mg g<sup>-1</sup>. The absence of quercetin in the samples of honey exposed to light suggests that UV radiation has degraded this compound. All samples presented maximum radical scavenging capacity close to 50%. Our results showed inverse correlations between the colour and pH, ash, reducing sugars, and moisture content, and positive correlations between the colour and the concentration of phenolic compounds, and antioxidant activity.

**KEYWORDS:** *Apis mellifera*, quercetin, DPPH radical, flavonoids

## Atividade antioxidante e características físico-químicas de méis da Amazônia Oriental, Brasil

### RESUMO

O mel é um produto natural que apresenta várias propriedades benéficas para a saúde, tais como atividade antiinflamatória, antioxidante e antimicrobiana, as quais dependem de sua composição. Neste contexto, as propriedades físico-químicas (cor, pH, conteúdo de cinzas, umidade, açúcares e compostos fenólicos totais) e a atividade antioxidante (capacidade de remoção do radical DPPH) de méis de abelhas *Apis mellifera* de Santarém, na principal região de produção de mel na Amazônia Oriental, Brasil, foram avaliadas. A maioria das amostras teve cor escura e apresentaram-se ácidas. Os teores de cinzas e a umidade variaram de 0,112 a 0,318 e de 14,751 a 17,514, respectivamente. O teor de açúcares redutores variou entre 62,873 e 91,563%. O teor total de compostos fenólicos foi mais elevado que os já reportados na literatura, variando entre 15,22 e 16,51 mg g<sup>-1</sup> e 17,70 e 18,94 mg g<sup>-1</sup> para amostras de mel protegidas e expostas à radiação UV, respectivamente. A quercetina foi encontrada apenas no mel que foi protegido da luz, com teores variando entre 0,24 e 0,43 mg g<sup>-1</sup>. A ausência de quercetina nas amostras de mel expostas à luz sugere que a radiação UV pode ter degradado esse composto. Todas as amostras apresentaram máxima capacidade de remover o radical DPPH próxima a 50%. Houve correlação inversa entre a cor e o pH, cinzas, açúcares redutores e teor de umidade, e correlação positiva entre a cor e o teor de composto fenólicos e a atividade antioxidante.

**PALAVRAS-CHAVE:** *Apis mellifera*, quercetina, radical DPPH, flavonóides

## INTRODUCTION

According to the *Codex Alimentarius* (2001), honey is a sweet and viscous substance having a characteristic but variable odour, which is produced by bees from the nectar of flowers (floral honey) or induced as plant secretions by the action of sucking insects (melato honey). Regulatory bodies in the USA and Brazil define honey as a product from a specific species of bee, *Apis mellifera* L. (FAO 2015). Honey is a rich source of carbohydrates for humans, and also provides a great variety of minor nutrients such as proteins, minerals, and vitamins. Honey has been part of human diet for centuries, and has found increasing medicinal use due its antibiotic, anti-caries, anti-inflammatory and other properties (Abadio Finco *et al.* 2010), as well as a supplementary natural food free of synthetic substances. Therefore, it is important to determine the overall composition of honey in order to prove its efficiency as a complete natural food.

The composition of the honey can be evaluated by physicochemical and sensory analyses. Flavour and quality parameters have been used to discriminate between monofloral and polyfloral honeys, as have the identification and counting of pollen grains (Escuredo *et al.* 2012), but these methods can generate ambiguous results. Therefore, the most-commonly reported parameters to characterize the physicochemical properties of honey are the pH, sugar content, electrical conductivity, enzymatic activity, and water content (Abadio Finco *et al.* 2010). Other parameters include the colour, speed of crystallization (related to glucose content), and hygroscopicity (related to fructose content) (Venir *et al.* 2010). Finally, instrumental analytical techniques can be used for compositional analysis; these include high performance liquid chromatography (HPLC), thermal analysis, and molecular and atomic spectroscopic methods (Pulcini *et al.* 2006).

HPLC has been an important analytical tool in studies involving the determination of the phenolic, flavonoid, and other antioxidant compounds in honeys. This compositional determination is relevant because honey can contain a number of compounds that are also detected in many fresh fruits and vegetables (Gheldof *et al.* 2002). The presence of this great variety of components characterizes honey as a functional food. For example, the polyphenolic and flavonoid components may contribute to the prevention of cardiovascular diseases (Abadio Finco *et al.* 2010). The use of honey as an alternative health treatment, known as “apitherapy”, can be associated with beneficial flavonoid-mediated activities (Vit *et al.* 2008). These and other salutary honey properties are related mainly to the presence of phenolic acids and flavonoids, but also depend on minor constituents such as polyphenols and other phytochemicals, ascorbic acid, amino acids, proteins, vitamins, and essential metals (Silva *et al.* 2016).

The amount and distribution of phenolic compounds reflect directly on the colour, sensory features, and antioxidant

activity of the honey (Silva *et al.* 2016). The phenolic content depends on the botanical and geographical origin of the flowers from which the bees collected the nectar (Escuredo *et al.* 2014). Several studies have attempted to discern the origin of botanical honey, or pure from adulterated honey, using multivariate analysis with spectroscopic data, mainly on FT-IR and Raman techniques (Gok *et al.* 2015; Corvucci *et al.* 2015). Natural and artificial honeys are discriminated through the analysis of parameters such as antioxidant activity, pH, colour, and bioactive compounds determined by chemometrics tools (Sant’Ana *et al.* 2012). Several authors have determined the bioactive compounds in honey through HPLC with diode array detection (HPLC-DAD) (Escuredo *et al.* 2012; Hayes *et al.* 2014; Karabagias *et al.* 2014), but did not make an integrated analysis of the data using multivariate analysis. Thus, the combination of HPLC and multivariate analysis represents an improved way of interpreting the analytical parameters of honey.

Although a number of studies have attempted to classify honeys from some countries such as Italy, Spain and Greece based on their physicochemical parameters and chemical compositions (Pulcini *et al.* 2006; Escuredo *et al.* 2012; Karabagias *et al.* 2014), little work has been carried out with Amazonian honeys.

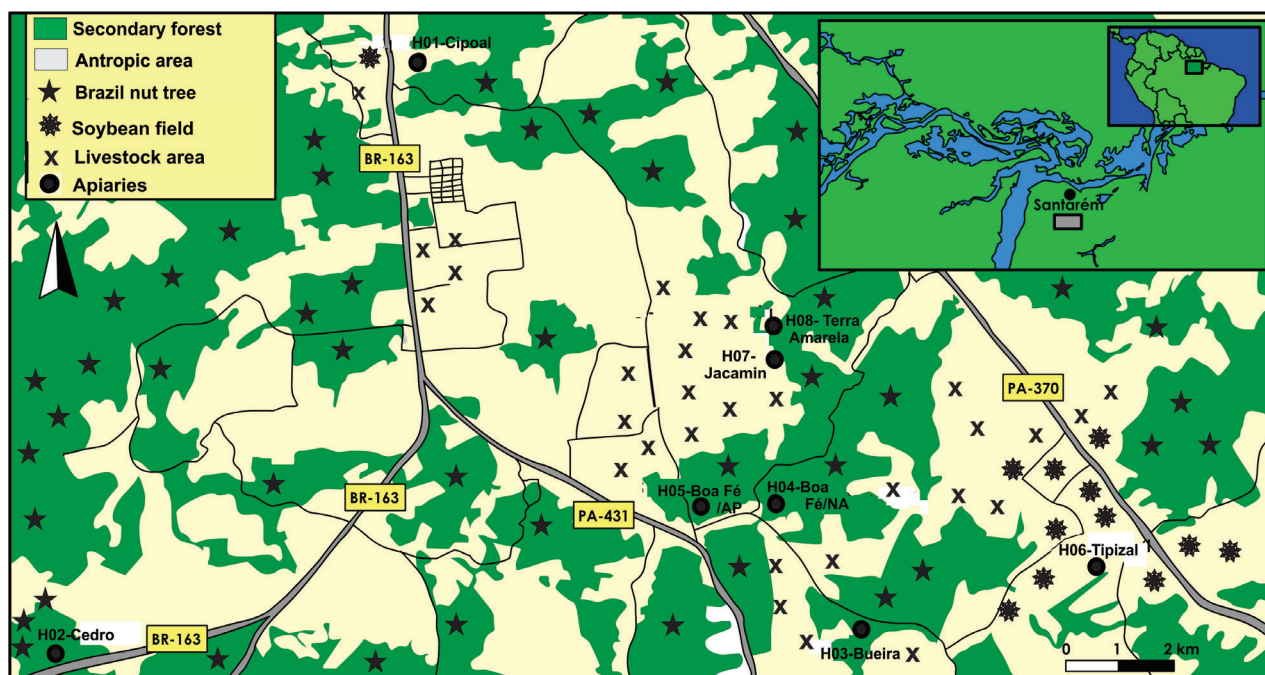
Beekeeping is very promising in the northern region of Brazil, although it has been maintained as a secondary subsistence activity by most producers (Costa 2009). Santarém is the largest city in the western state of Pará, in the eastern Brazilian Amazon, and is an economic pole for the surrounding municipalities. In the municipality of Santarém honey is the fourth most commercialized natural product, one of the reasons being its use as a popular therapeutic alternative (Gonçalves *et al.* 2012).

In this context, the aim of this study was to characterize *A. mellifera* honeys of Santarém. We analyzed the physicochemical properties and antioxidant activity of honeys from different apiaries. The major phenolic compounds of the honey samples were determined by HPLC-DAD and those compounds were compared in honey stored exposed and protected from UV radiation.

## MATERIALS AND METHODS

### Honey samples

Samples of *A. mellifera* honey from diverse floral sources were obtained in November 2014 from eight apiaries located in the municipality of Santarém, state of Pará, in the eastern Amazon region of Brazil (02°35’20.78”S and 54°41’0.80”W) (Figure 1). One sample (in triplicate) was collected at each apiary (H01-H08), directly from beehives through manual extraction without any prior treatment. The vegetational characteristics of the surroundings of each apiary within a 1-km radius (corresponding to the maximum average distance traveled by bees) are described in Table 1.



**Figure 1.** Map depicting the locations of sampled apiaries in the municipality of Santarém, state of Pará, northern Brazil, in the eastern Amazon region. This figure is in colour in the electronic version.

**Table 1.** Descriptive characteristics of the surrounding area of each apiary sampled for *Apis mellifera* honey in Santarém (Pará, Brazil). Apiaries are identified by their location codes in Figure 1.

Apiary	Main vegetation matrix	Additional vegetation characteristics
H01	Secondary forest	Soybean field at 300 m from apiary, presence of few trees with low height
H02	Secondary forest	Pasture area at 500 m many Brazil nut trees and babassu ( <i>Attalea ssp.</i> ) palm trees
H03	Secondary forest	Soybean cropland and pasture area at 500 m, Brazil nut trees and babassu ( <i>Attalea ssp.</i> ) palm trees
H04	Secondary forest	Passion fruit plantation at 300 m, Brazil nut trees and babassu ( <i>Attalea ssp.</i> ) palm trees
H05	Secondary forest	Brazil nut trees and babassu ( <i>Attalea ssp.</i> ) palm trees
H06	Secondary forest	Soybean cropland at 500 m, few trees with low height, babassu ( <i>Attalea ssp.</i> ) palm trees
H07	Secondary forest	Pasture at 500 m, Brazil nut trees and babassu ( <i>Attalea ssp.</i> ) palm trees
H08	Secondary forest	Vegetable garden at 1000 m, pasture at 1500 m, Brazil nut trees and babassu ( <i>Attalea ssp.</i> ) palm trees

Soon after the collection, each sample was divided into two sub-samples packed into polyethylene Falcon tubes, one of them exposed to ambient light and the other wrapped in aluminum foil and kept protected from UV radiation. The samples were stored during three months under room temperature (20 to 30 °C) until analysis.

### Physicochemical characteristics

The colour of each honey sample was measured according to a modified Pfund classifier (Belay *et al.* 2015). The honey samples were diluted to 50% (v/v) with deionized water and the resulting solutions were filtered. The absorbance was measured at 635 nm using a Nova 3300 spectrophotometer (Nova Instruments, Piracicaba, Brazil). Measurements were

performed in triplicate for each sample and the quantitative mm Pfund scale was transformed into a qualitative colour value using the approved colour standards of the United States Department of Agriculture (USDA 1985).

The pH, ash and moisture contents of the honeys were measured according to the AOAC method (Horwitz and Latimer 2006) with some modifications. The pH was determined for a sample (10 g) diluted in deionized water (90 mL) using a pH meter (model mPA-210, MS Tecnopon, Piracicaba, Brazil). For ash content a sample (~2 g) was weighed into an ash crucible and dried in an oven at 100 °C for 4 h. After cooling, the ensemble was weighed, incinerated at 600 °C in a muffle furnace for 3 h,

and then reweighed. The ash content was then calculated by subtracting the difference between the initial and post-combustion weights. For the moisture content a sample (-2.5 g) was weighed and heated in an oven at 60 °C until a constant weight was obtained. The moisture content was calculated by subtracting the difference between the initial and post-heating weights (Horwitz and Latimer 2006).

The reducing sugar content analysis was performed using a glucose liquiform solution (2.0 mL, Merck, Darmstadt, Germany) and honey solution (200 µL, 1:2500, v/v). The solutions were mixed, let stand for 30 min at room temperature, and then the absorbance was measured at 555 nm using a Nova 3300 spectrophotometer (Nova Instruments, Piracicaba, Brazil). A five-point calibration curve was obtained using standard glucose solutions with the following concentrations: 0.00, 0.25, 0.50, 0.75, and 1.00 µg mL<sup>-1</sup>.

### HPLC analysis

All chemicals were analytical grade. Methanol, phosphoric acid, gallic acid, caffeic acid, chlorogenic acid, rosmarinic acid, and *p*-coumaric acid were purchased from Merck (Darmstadt, Germany). Quercetin, kaempferol, apigenin, and chrysin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-DAD was performed with a Shimadzu Prominence Autosampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector, and LC solution 1.22 SP1 software. The samples were immediately separated into two fractions upon collection that were either protected from or exposed to ambient light.

HPLC was performed using a Phenomenex C<sup>18</sup> analytical column (4.6 mm × 250 mm, 5 µm particle size). Gradients were generated using 0.05% phosphoric acid in water (A) and methanol (B), starting at 95% A; decreasing to 85% A in 10 min, to 70% A in 5 min, to 60% A in 5 min, to 55% A in 10 min, to 40% A in 20 min, and to 20% A in 2 min; and holding for 8 min (Gheldof *et al.* 2002). The flow rate was 0.6 mL min<sup>-1</sup> and the injection volume was 40 µL. The honey samples were prepared as dilute solutions in methanol/water (1:5, v/v) and were filtered prior to use. Stock solutions of standards were prepared in the HPLC mobile phase mixture at concentrations of 0.050–0.500 mg mL<sup>-1</sup>. Quantification was based on integration of the peaks using the external standard method, at 254 nm for gallic acid; 327 nm for chlorogenic, caffeic, *p*-coumaric, and rosmarinic acids; and 366 nm for quercetin, chrysin, apigenin, and kaempferol. The identities of the analytes were confirmed by comparing their retention times with those of the reference standards and their DAD spectra (200 to 500 nm). The calibration curve for each standard is described as follows: gallic acid:  $Y = 11967x + 1305.9$  ( $r$

$= 0.9998$ ); chlorogenic acid:  $Y = 12549x + 1184.2$  ( $r = 0.9995$ ); caffeic acid:  $Y = 12527x + 1419.3$  ( $r = 0.9999$ ); *p*-coumaric acid:  $Y = 13546x + 1093.6$  ( $r = 0.9997$ ); rosmarinic acid:  $Y = 13067x + 1273.5$  ( $r = 0.9998$ ); chrysin:  $Y = 11863x + 1127.1$  ( $r = 0.9999$ ); quercetin:  $Y = 14053x + 1182.3$  ( $r = 0.9994$ ); kaempferol:  $Y = 13713x + 1258.0$  ( $r = 0.9998$ ); and apigenin:  $Y = 12357x + 1087.3$  ( $r = 0.9996$ ). All chromatography operations were carried out in triplicate at ambient temperature. The limit of detection (LOD) and limit of quantitation (LOQ) values were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon *et al.* (2015). The LOD and LOQ were calculated as 3.3 and 10  $\sigma/S$ , respectively, where  $\sigma$  is the standard deviation of the response and  $S$  is the slope of the calibration curve.

### DPPH free radical scavenging activity

The antioxidant activity of the honeys was tested for DPPH radicals. The assay is a very useful parameter for correlating the phenolic compound content with the antioxidant properties of biological samples. The free radical scavenging activity of the honeys was determined with 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Fluka, Steinheim, Germany) using the method of Escriche *et al.* (2014) with some modification.

Honey samples (50 µL; 0.12, 0.36, and 0.60 mg) were dissolved in ethanol (200 µL) and mixed with a solution of DPPH in methanol (25 µL, 1.2 mM). For the blank samples, solutions of honey (50 µL; 0.12, 0.36 and 0.60 mg) in ethanol (225 µL) were prepared.

The mixtures were left for 30 min at room temperature; then, the absorbance was measured at 555 nm in an ELISA plate reader. The ability of the honey samples to scavenge DPPH radicals, given by the  $SA_{DPPH}$ -value, was calculated using the following equation:

$$SA_{DPPH}(\%) = 100 \times \frac{(A_0 - A_x)}{A_0}$$

where  $A_0$  and  $A_x$  are the absorbance of the blank and the sample, respectively.

The antioxidant activity was determined against a standard curve for  $\alpha$ -tocopherol (vitamin E, 0–2.50 mg L<sup>-1</sup>,  $R^2 = 0.992$ ), and the results were expressed in  $\alpha$ -tocopherol equivalents antioxidant (mg TEA.100 g<sup>-1</sup>).

### Statistical analysis

The association among total phenolic compounds and physicochemical parameters was analyzed using Pearson's correlation. Principal component analysis (PCA) was used to evaluate the phenolic acid and flavonoid composition in honeys exposed to and protected from light. All analyses were performed using the software Minitab 14 (Minitab, State College, PA, USA). Significance level was 5% ( $p < 0.05$ ) in all tests.

## RESULTS

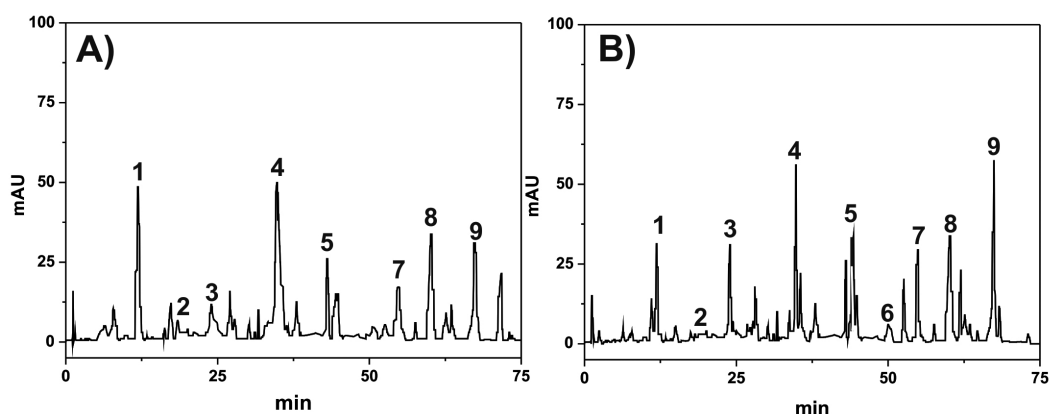
Antioxidant activity was expressed as DPPH inhibition (%) against a blank control. All honey samples had a maximum DPPH radical quenching capacity close to 50%. The maximum inhibitory activity for undiluted honey ranged from 49.394 to 61.061% (Table 2).

Nine phenolic compounds were identified: five phenolic acids (gallic acid, average retention time ( $t_R$ ) = 11.83 min; chlorogenic acid,  $t_R$  = 18.37 min; caffeic acid,  $t_R$  = 23.96 min; *p*-coumaric acid,  $t_R$  = 34.78 min, and rosmarinic acid,  $t_R$  = 44.15 min), and four flavonoids (quercetin,  $t_R$  = 50.11 min; kaempferol,  $t_R$  = 54.83 min, apigenin,  $t_R$  = 60.14 min, and chrysin,  $t_R$  = 67.32 min) (Figure 2). The total amount of phenolic compounds ranged between 15.22 and 16.51 mg g<sup>-1</sup>, and 17.70 and 18.94 mg g<sup>-1</sup> for honeys that were exposed to and protected from light, respectively (Table 3).

Phenolic acids were the most abundant phenolic derivatives in all samples. In the honey exposed to light the contribution, in descending order, was of *p*-coumaric acid, gallic acid, apigenin, and chrysin (Table 3). In the honey protected from light, the relative abundance of phenolic acid compounds varied, in decreasing order, from chrysin, *p*-coumaric acid, apigenin, kaempferol, caffeic acid, to gallic acid (Table 3). It is important to note that the presence of quercetin, in amounts above the detection level of the method, was observed only when the honey samples were not exposed to light (Table 3).

### Statistical analysis

The honey's colour was negatively correlated with pH, ash, reducing sugars, and moisture contents, and positively correlated with the total phenolic compounds and antioxidant activity (Table 4). The low correlations between the colour and antioxidant activity and, between ashes and moisture



**Figure 2.** Representative chromatograms of one honey sample (H01) from Santarém (Pará, Brazil) exposed to light (A) and protected from light (B). The peaks are indicated as follows: **1** - gallic acid; **2** - chlorogenic acid; **3** - caffeic acid; **4** - *p*-coumaric acid; **5** - rosmarinic acid; **6** - quercetin; **7** - kaempferol; **8** - apigenin; and **9** - chrysin.

**Table 2.** Physicochemical parameters and maximum antioxidant activity (results are expressed as the mean  $\pm$  standard deviation) of honeys samples from Santarém (Pará, Brazil) exposed to UV radiation. Apiaries are identified by their location codes in Figure 1.

Parameters	H01	H02	H03	H04	H05	H06	H07	H08
Colour	Dark amber	Dark amber	Amber	Dark amber	Light amber	Amber	Light amber	Amber
pH	3.653 $\pm$ 0.064 <sup>b</sup>	3.686 $\pm$ 0.114 <sup>ab</sup>	3.869 $\pm$ 0.154 <sup>ab</sup>	3.800 $\pm$ 0.071 <sup>ab</sup>	4.264 $\pm$ 0.321 <sup>ab</sup>	4.016 $\pm$ 0.149 <sup>ab</sup>	4.073 $\pm$ 0.187 <sup>ab</sup>	4.316 $\pm$ 0.443 <sup>a</sup>
Ash (%)	0.147 $\pm$ 0.036 <sup>a</sup>	0.112 $\pm$ 0.013 <sup>a</sup>	0.168 $\pm$ 0.061 <sup>a</sup>	0.190 $\pm$ 0.056 <sup>a</sup>	0.318 $\pm$ 0.043 <sup>a</sup>	0.181 $\pm$ 0.076 <sup>a</sup>	0.224 $\pm$ 0.111 <sup>a</sup>	0.260 $\pm$ 0.096 <sup>a</sup>
Moisture (%)	15.401 $\pm$ 0.020 <sup>bc</sup>	14.751 $\pm$ 0.316 <sup>c</sup>	16.057 $\pm$ 0.124 <sup>abc</sup>	16.470 $\pm$ 0.402 <sup>abc</sup>	15.526 $\pm$ 0.767 <sup>bc</sup>	16.498 $\pm$ 0.200 <sup>abc</sup>	16.986 $\pm$ 0.615 <sup>ab</sup>	17.514 $\pm$ 0.761 <sup>a</sup>
Reducing sugars (%)	80.936 $\pm$ 2.233 <sup>abcd</sup>	64.274 $\pm$ 1.380 <sup>bcd</sup>	62.002 $\pm$ 1.070 <sup>cd</sup>	88.570 $\pm$ 2.993 <sup>a</sup>	60.866 $\pm$ 2.007 <sup>d</sup>	84.582 $\pm$ 1.225 <sup>abc</sup>	85.800 $\pm$ 1.416 <sup>ab</sup>	79.435 $\pm$ 2.176 <sup>abcd</sup>
Antioxidant activity (%)	61.061 $\pm$ 1.481 <sup>ab</sup>	57.055 $\pm$ 2.992 <sup>abc</sup>	50.518 $\pm$ 2.992 <sup>c</sup>	58.672 $\pm$ 1.647 <sup>abc</sup>	61.624 $\pm$ 0.760 <sup>a</sup>	51.212 $\pm$ 1.097 <sup>c</sup>	49.183 $\pm$ 2.642 <sup>c</sup>	54.795 $\pm$ 0.609 <sup>bc</sup>
Antioxidant activity (mgTEA.100 g <sup>-1</sup> ) <sup>1</sup>	863.78 $\pm$ 22.05 <sup>ab</sup>	804.12 $\pm$ 44.55 <sup>abc</sup>	706.79 $\pm$ 44.55 <sup>c</sup>	828.20 $\pm$ 24.52 <sup>ab</sup>	872.15 $\pm$ 11.32 <sup>a</sup>	717.12 $\pm$ 16.33 <sup>c</sup>	686.90 $\pm$ 39.34 <sup>c</sup>	770.47 $\pm$ 10.37 <sup>bc</sup>

**Table 3.** Phenolic acid and flavonoid contents (results are expressed as the mean ± standard deviation) of honeys samples from Santarém (Pará, Brazil) exposed and protected from light. Apiaries are identified by their location codes in Figure 1. LOD = limit of detection, LOQ = limit of quantification. Unshaded lines present values for samples exposed to light; shaded lines present values for samples exposed to light.

Compound mg g <sup>-1</sup>	H01	H02	H03	H04	H05	H06	H07	H08	LOD mg g <sup>-1</sup>	LOQ mg g <sup>-1</sup>
Gallic acid	3.15 ± 0.02 <sup>a</sup>	3.20 ± 0.03 <sup>a</sup>	3.17 ± 0.01 <sup>a</sup>	3.28 ± 0.01 <sup>a</sup>	3.40 ± 0.01 <sup>a</sup>	3.14 ± 0.01 <sup>a</sup>	3.11 ± 0.04 <sup>a</sup>	3.25 ± 0.01 <sup>a</sup>	0.011	0.037
	1.78 ± 0.03 <sup>a</sup>	1.80 ± 0.04 <sup>a</sup>	1.54 ± 0.01 <sup>a</sup>	1.65 ± 0.01 <sup>a</sup>	1.91 ± 0.04 <sup>a</sup>	1.72 ± 0.01 <sup>a</sup>	1.65 ± 0.01 <sup>a</sup>	1.98 ± 0.03 <sup>a</sup>	0.011	0.037
Chlorogenic acid	0.27 ± 0.01 <sup>b</sup>	0.31 ± 0.02 <sup>b</sup>	0.25 ± 0.03 <sup>b</sup>	0.26 ± 0.01 <sup>b</sup>	0.23 ± 0.01 <sup>b</sup>	0.30 ± 0.03 <sup>b</sup>	0.28 ± 0.01 <sup>b</sup>	0.29 ± 0.03 <sup>b</sup>	0.023	0.076
	0.23 ± 0.04 <sup>b</sup>	0.27 ± 0.01 <sup>b</sup>	0.32 ± 0.02 <sup>b</sup>	0.27 ± 0.04 <sup>b</sup>	0.30 ± 0.02 <sup>b</sup>	0.22 ± 0.03 <sup>b</sup>	0.31 ± 0.01 <sup>b</sup>	0.26 ± 0.01 <sup>b</sup>	0.023	0.076
Caffeic acid	0.30 ± 0.01 <sup>b</sup>	0.29 ± 0.01 <sup>b</sup>	0.32 ± 0.01 <sup>b</sup>	0.30 ± 0.04 <sup>b</sup>	0.29 ± 0.03 <sup>b</sup>	0.27 ± 0.01 <sup>b</sup>	0.25 ± 0.01 <sup>b</sup>	0.30 ± 0.02 <sup>b</sup>	0.018	0.059
	1.81 ± 0.01 <sup>a</sup>	1.85 ± 0.01 <sup>a</sup>	2.07 ± 0.01 <sup>c</sup>	1.85 ± 0.03 <sup>c</sup>	1.87 ± 0.02 <sup>a</sup>	1.90 ± 0.01 <sup>c</sup>	1.97 ± 0.03 <sup>c</sup>	1.85 ± 0.01 <sup>a</sup>	0.018	0.059
p-Coumaric acid	3.68 ± 0.04 <sup>c</sup>	3.72 ± 0.01 <sup>c</sup>	2.98 ± 0.01 <sup>c</sup>	2.71 ± 0.01 <sup>c</sup>	3.74 ± 0.04 <sup>c</sup>	3.68 ± 0.01 <sup>c</sup>	3.41 ± 0.02 <sup>c</sup>	3.94 ± 0.01 <sup>c</sup>	0.009	0.030
	4.03 ± 0.01 <sup>c</sup>	3.96 ± 0.02 <sup>c</sup>	4.16 ± 0.03 <sup>d</sup>	3.99 ± 0.01 <sup>d</sup>	3.95 ± 0.01 <sup>c</sup>	4.06 ± 0.02 <sup>d</sup>	4.11 ± 0.04 <sup>d</sup>	4.01 ± 0.02 <sup>c</sup>	0.009	0.030
Rosmarinic acid	1.79 ± 0.03 <sup>d</sup>	1.63 ± 0.01 <sup>d</sup>	1.72 ± 0.04 <sup>d</sup>	1.68 ± 0.02 <sup>d</sup>	1.85 ± 0.01 <sup>d</sup>	1.45 ± 0.01 <sup>d</sup>	1.47 ± 0.03 <sup>d</sup>	1.73 ± 0.01 <sup>d</sup>	0.025	0.084
	1.95 ± 0.01 <sup>a</sup>	2.04 ± 0.03 <sup>d</sup>	1.97 ± 0.03 <sup>c</sup>	2.15 ± 0.01 <sup>e</sup>	1.48 ± 0.01 <sup>d</sup>	1.73 ± 0.04 <sup>a</sup>	1.54 ± 0.01 <sup>a</sup>	2.62 ± 0.03 <sup>d</sup>	0.025	0.084
Quercetin	-	-	-	-	-	-	-	-	0.016	0.053
	0.26 ± 0.02 <sup>b</sup>	0.31 ± 0.01 <sup>b</sup>	0.39 ± 0.01 <sup>b</sup>	0.43 ± 0.04 <sup>f</sup>	0.26 ± 0.03 <sup>b</sup>	0.25 ± 0.01 <sup>b</sup>	0.24 ± 0.01 <sup>b</sup>	0.27 ± 0.01 <sup>b</sup>	0.016	0.053
Kaempferol	1.42 ± 0.02 <sup>e</sup>	1.50 ± 0.04 <sup>d</sup>	1.49 ± 0.03 <sup>e</sup>	1.45 ± 0.01 <sup>e</sup>	1.46 ± 0.04 <sup>e</sup>	1.49 ± 0.02 <sup>d</sup>	1.51 ± 0.01 <sup>d</sup>	1.39 ± 0.03 <sup>e</sup>	0.010	0.034
	1.79 ± 0.01 <sup>a</sup>	1.98 ± 0.01 <sup>d</sup>	1.56 ± 0.02 <sup>e</sup>	1.89 ± 0.02 <sup>c</sup>	1.93 ± 0.04 <sup>a</sup>	1.81 ± 0.01 <sup>a</sup>	1.83 ± 0.02 <sup>c</sup>	1.93 ± 0.01 <sup>a</sup>	0.010	0.034
Apigenin	2.81 ± 0.01 <sup>f</sup>	2.83 ± 0.01 <sup>e</sup>	2.80 ± 0.01 <sup>f</sup>	2.79 ± 0.04 <sup>f</sup>	2.96 ± 0.03 <sup>f</sup>	2.86 ± 0.01 <sup>e</sup>	2.94 ± 0.02 <sup>e</sup>	2.84 ± 0.01 <sup>f</sup>	0.027	0.089
	2.15 ± 0.03 <sup>d</sup>	2.01 ± 0.01 <sup>d</sup>	2.04 ± 0.04 <sup>c</sup>	2.07 ± 0.01 <sup>e</sup>	2.01 ± 0.01 <sup>a</sup>	1.97 ± 0.02 <sup>a</sup>	2.76 ± 0.04 <sup>e</sup>	2.08 ± 0.01 <sup>a</sup>	0.027	0.089
Chrysin	2.79 ± 0.04 <sup>f</sup>	2.69 ± 0.03 <sup>f</sup>	2.75 ± 0.01 <sup>f</sup>	2.75 ± 0.03 <sup>f</sup>	2.58 ± 0.01 <sup>a</sup>	2.78 ± 0.03 <sup>e</sup>	2.36 ± 0.01 <sup>f</sup>	1.82 ± 0.01 <sup>d</sup>	0.015	0.048
	4.11 ± 0.01 <sup>c</sup>	4.08 ± 0.03 <sup>c</sup>	4.03 ± 0.01 <sup>f</sup>	3.96 ± 0.01 <sup>d</sup>	4.05 ± 0.01 <sup>c</sup>	3.94 ± 0.03 <sup>d</sup>	3.85 ± 0.05 <sup>d</sup>	3.94 ± 0.04 <sup>c</sup>	0.015	0.048

**Table 4.** Pearson's correlation coefficients for total phenolic compounds and physicochemical parameters for mean values of honey samples from eight apiaries in Santarém (Pará, Brazil).

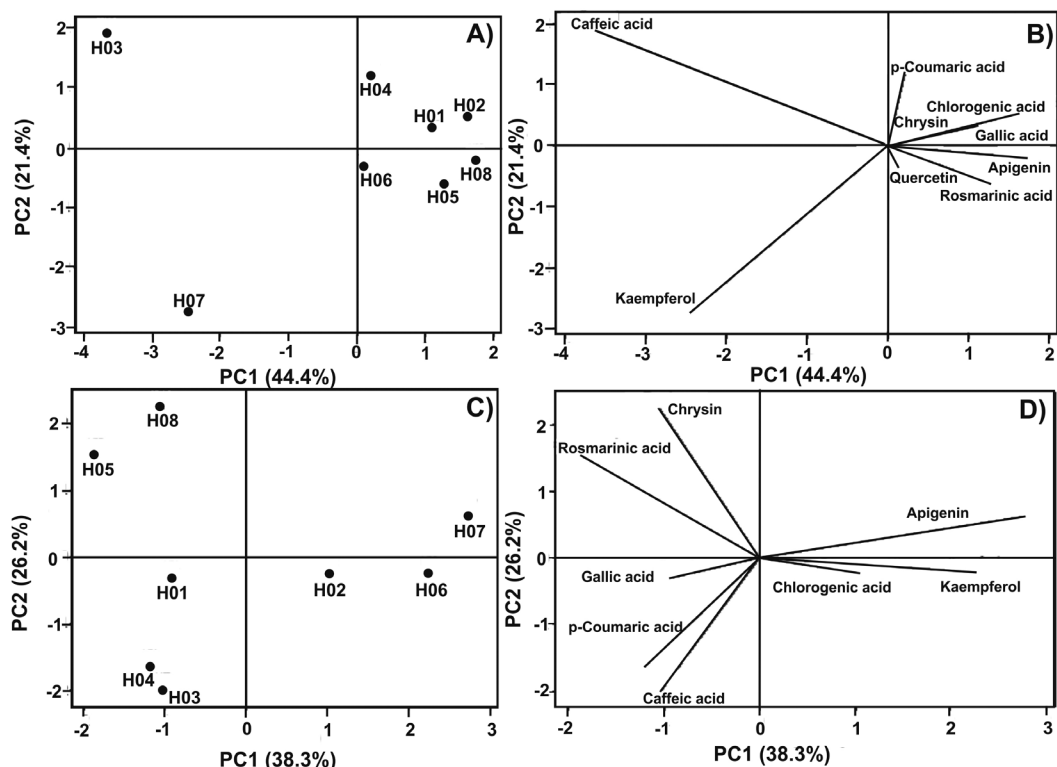
	Ash	Reducing sugars	Colour	pH	Moisture	Antioxidant activity
Total phenolic compounds	0.077 <sup>e</sup>	-0.523 <sup>c</sup>	0.122 <sup>e</sup>	-0.022 <sup>e</sup>	-0.700 <sup>c</sup>	0.569 <sup>c</sup>
Ash	-	-0.079 <sup>e</sup>	-0.796 <sup>b</sup>	0.903 <sup>a</sup>	0.445 <sup>d</sup>	0.041 <sup>e</sup>
Reducing sugars	-	-	-0.090 <sup>e</sup>	-0.041 <sup>e</sup>	0.597 <sup>c</sup>	-0.244 <sup>e</sup>
Colour	-	-	-	-0.828 <sup>b</sup>	-0.599 <sup>c</sup>	0.409 <sup>d</sup>
pH	-	-	-	-	0.620 <sup>c</sup>	-0.326 <sup>d</sup>
Moisture	-	-	-	-	-	-0.665 <sup>c</sup>

a. Very strong correlation. b. Strong correlation. c. Moderate correlation. d. Low correlation. e. Very low correlation.

were supported by Pearson coefficients of 0.409 and 0.445, respectively. Colour also had a low positive correlation with total phenolic compounds (0.122).

The pH exhibited high and moderate positive correlations with the ash (0.903) and moisture (0.620) levels, respectively. The reducing sugars had a moderate positive correlation only with the moisture content (0.597).

In both PCA analyses the first two axes (PC1 and PC2) explained around 65% of the variance in the data (Figure 3). Honey samples protected from light had a more homogeneous composition, except H03 and H07, which had divergent values of caffeic acid and kaempferol, respectively (Figure 3a,b), while samples exposed to light tended to disperse in the multivariate space, with main loads associated to different bioactive compounds (Figure 3c,d).



**Figure 3.** Scores and loadings of the PCA for data on phenolic acid and flavonoids contained in honey samples from Santarém (Pará, Brazil) not exposed to light (A,B) and exposed to light (C,D).

## DISCUSSION

### Physicochemical characteristics

Colour is the first attractive attribute for a commercial honey, and its intensity depends on its ash content and storage conditions (e.g. temperature, moisture) (Silva *et al.* 2016). Interestingly, dark honeys have stronger flavours and are more appreciated in countries such as Germany, Austria, and Switzerland (Bogdanov *et al.* 2004). The variation in colour found in the honey analysed in this study was also observed by other authors and attributed to several factors. Moreti *et al.* (2006) evaluated 346 honey samples from six Brazilian states and found a predominant light amber colour in samples from Bahia, Tocantins, and Santa Catarina, and an extra light amber colour in samples from Piauí. The darker colours of our honeys from Santarém may be associated with the presence of high concentrations of minerals and/or carotenoids and flavonoids, which are associated with the botanical origin of the honey (Silva *et al.* 2016) and its antioxidant activity (Moniruzzaman *et al.* 2014).

All samples were acidic and within the official standard limits, which indicates that honey samples may have a longer shelf life. Lower acidity may be related to highest antimicrobial activity in the honey, because the optimum pH for growth of most microorganisms lies between 7.2 and 7.4 (Karabagias *et al.* 2014).

On the other hand, a low pH and low storage temperature favour the growth of fungi and reduce the shelf-life of a honey (Escriche *et al.* 2014). An increased pH may also indicate adulteration of pure honey through the addition of fructose corn syrup (Ribeiro *et al.* 2014). Honeys from Ceará, in northeaster Brazil, had pH between 3.40 and 5.30 (Moreti *et al.* 2006). The variation in pH of honeys among regions can be associated with differences in the pH of the nectar of the plants visited by bees in different regions, as well as to variations in the pH of soil, temperature and rainfall, all of which can affect the concentration of different acids and ash content of the honey (Gheldof *et al.* 2002).

The ash content is an indirect measure of the mineral content of the honey and is related to environmental pollution and the botanical and geographical origin of the honey (Karabagias *et al.* 2014; Silva *et al.* 2016). The ash content in our samples was similar to that found in other regions of Brazil (Marchini *et al.* 2004; Silva *et al.* 2004; Evangelista-Rodrigues *et al.* 2005; Araújo *et al.* 2006; Moreti *et al.* 2009) and could be related to the generally low nutrient content in Amazonian soils (Sombroek 1966).

The moisture content in the honey samples from Santarém did not exceed 20%, which could prolong the honey's shelf-life (Codex Alimentarius 2001). Moisture levels of the honey can vary with ambient humidity along seasonal patterns (specially

in regions with rainy and dry seasons), as well as with processing and/or storage conditions (Karabagias *et al.* 2014).

The most abundant sugars in honey are the monosaccharides fructose and glucose, which comprise, respectively, about 39 and 31% of honey weight, with a small contribution from complex sugars. Generally the fructose/glucose ratio is around 1.2, but is influenced by many factors (Escuredo *et al.* 2014). The Brazilian Ministry of Agriculture, Livestock, and Supply established minimum reducing sugar contents of 65% and 60% (w/w), respectively, for floral and melato honeys (Brazil 2001). Only our samples H03 and H05 presented levels below this value. Differences in the reducing sugars content may have been associated with the different botanical origin and premature harvesting of the honey, so that sucrose was not totally dissociated in glucose and fructose by the action of the enzyme invertase (Vargas 2006; Ajlouni and Sujirapinyokul 2009).

### HPLC and antioxidant analysis

In addition to sugars, honey contains enzymes and polyphenols such as caffeic acid, ferulic acid, benzoic acid, gallic acid, quercetin, luteolin, chrysin, myricetin, pinocembrin, and kaempferol (Silva *et al.* 2013). The total amount and distribution of phenolic compounds depend on the botanical origin and geographic origin of the honey (Karabagias *et al.* 2014). Phenolic compounds are non-specific markers that can be found in honeys produced from plants of different botanical families (Silva *et al.* 2016). Other studies have also found higher amounts of polyphenols than flavonoids in honey samples (Khalil *et al.* 2012; Oliveira *et al.* 2012; Moniruzzaman *et al.* 2014).

High phenolic content (in the 17–66 mg g<sup>-1</sup> range) was also found in honey produced by native *Melipona (Michmelia) seminigra merrillae* (Silva *et al.* 2013). Honey produced from chestnut (*Castanea* sp.) has a content of total phenolic compounds about 5.60 mg g<sup>-1</sup> (Pulcini *et al.* 2006), which is consistent with the concentration of phenolic compounds we found in the honey from Santarém. Our sampled apiaries were located within bee flying distance of a number of Brazil nut (*Bertholletia excelsa*) trees (see Figure 1 and Table 1), and the main pollinators of Brazil nut are medium to large bee species (Tonini 2011). The honey from our samples was produced during the period of flowering of Brazil nut trees (mainly from December to May, but also extending over other months; Rathcke and Lacey 1985). It is thus possible that Brazil nut has the same effect on phenolic compound content of honeys as chestnut.

The decrease in the flavonoid content in the light-exposed honey samples may be associated with their oxidation and degradation, as these compounds are susceptible to oxidation due to their large number of conjugated double bonds (Meléndez-Martínez *et al.* 2004). Some samples of chestnut honey analysed by Bertonecelj *et al.* (2011) did not contain quercetin, although others did; however, no information about protection from light was relayed in their report. It is possible that quercetin would be degraded in honeys exposed to light, since UV radiation

can interact with quercetin (Meléndez-Martínez *et al.* 2004). Quercetin is the most abundant flavonoid consumed in the human diet, and is thought to function as a potent antioxidant (Behling *et al.* 2004).

The maximum inhibitory activity for undiluted honey found in this study was in agreement with Estevinho *et al.* (2008), who found higher values for DPPH inhibition for dark honeys (above 70%) and lower values for light honeys (below 40%).

### Statistical analysis

The positive correlation of colour with phenolic compounds reflects the low contribution of these compounds to the colour of honeys, which is directly indicative of the presence of phenolic acids and flavonoids (Belay *et al.* 2015). Samples with different levels of phenolic compounds and flavonoids likely reflect differences in their botanical composition. For example, H05 and H07 were both classified as “light amber” honeys, but the PCA analysis indicated that they differed in their kaempferol content (Table 3, Figure 3).

Honey protected from light H06, H02 and H01 (Figure 3a,b) have contents of kaempferol, chlorogenic acid and gallic acid responsible to separate them from the others samples. The contents of kaempferol, chlorogenic acid are close each other, which may indicate similar floral origin in those. The gallic acid content is different, and the three parameters are close to zero in PC2, showing the poor segregation capacity of this PC, therefore, the variability of those parameters is 38.3%, explained mostly by PC1. Samples H07 and H01 presented opposition in eigenvectors showed in loadings plot, this represents antagonistic relationship between these parameters to separate those samples, which may indicate different floral origin.

Likewise, samples H03, H08 and H06 were classified as light amber, but differed with respect to its caffeic acid content. On the other hand, samples H01, H02 and H04, which were classified as dark amber, were grouped in the PCA analysis for samples exposed to light in association with the loadings of *p*-coumaric, chlorogenic and gallic acid contents. These samples come from apiaries located close together (see Figure 1) and their floristic composition is therefore likely very similar.

Contrary to our results, other authors have reported a negative relationship between the pH and moisture content of honey, and used these variables to discern the botanical origin of the honeys (Silva *et al.* 2016). The ash content, which represents the fixed mineral residue of the honey, is positively and highly correlated with the pH, most likely because the mineral uptake by plants depends on acidity. The use of neural networks to discriminate the botanical origin of honeys found an 81% variation in ash content among samples (Anjos *et al.* 2015).

The negative correlation between reducing sugars and other parameters, except moisture content may be explained by the fact that reducing sugars are good indicators of maturation grade,



which has a direct relationship with the moisture content in food matrices (Pires *et al.* 2009).

In addition to the presence-absence of quercetin, the comparison of the PCA results for light-protected and light-exposed honey showed that the samples exposed to light tended to be less homogen in their phenolic and flavonoid compound composition than the samples kept in the dark. This is probably related to a stochastic effect of UV-radiation on the chemical compounds in each sample, and suggests that protecting honey from light may be a simple and cost-effective method to preserve the original chemical composition of the honey for a longer time, and should be further studied.

## CONCLUSIONS

The current study showed that all the analysed honey samples from Santarém (Pará, Brazil) presented physicochemical parameters in agreement with the *Codex Alimentarius* (2001). In addition, all the samples contained higher amounts of total phenolic compounds than most of the studies described in the literature, as well as high antioxidant activity (above 50% for undiluted honey). The phenolic compounds present in the honey samples influenced their colour and antioxidant activity. Honey samples exposed to light did not present noticeable amounts of the flavonoid quercetin in comparison with samples protected from light-exposure. Also, light-protected samples had a more homogen chemical phenolic and flavonoid compound composition. Since quercetin is an important flavonoid in the human diet, and it can be degraded by the action of UV radiation, it is recommended that the honey produced in Santarém be stored in dark containers, which will also probably preserve the overall original chemical composition of the honey for a longer time.

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