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Phenolic compounds, antioxidant capacity and physicochemical properties of Brazilian *Apis mellifera* honeys

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ABSTRACT

Forty-nine honey samples from five monofloral (eucalyptus, mastic, wildflower, Japanese grape and quitoco) and six polyfloral honeys were analyzed for their physicochemical and antioxidant compounds. Levels of total phenolics and flavonoids as well as individual composition were analyzed by HPLC. The oxygen radical absorption capacity (ORAC), the ferric reducing antioxidant power (FRAP), and the 2,2-diphenyl-1-picrylhydrazyl free radical assays (DPPH) were performed to determine antioxidant capacity. The highest antioxidant capacity was observed in eucalyptus and mastic honeys; gallic acid levels were greatest in both of them while *p*-coumaric acid was highest in Japanese grape honey. Physicochemical and antioxidant properties of honey were found to be dependent on its botanical origin. Linear discriminant analysis was able to differentiate 90% of honeys in terms of the seasons when they were collected and the main discriminant responses were *p*-coumaric acid, titratable acidity, diastase activity, and total flavonoids content. LDA correctly classified 96% of honeys according to their botanical origin.

1. Introduction

Honey is a supersaturated solution of sugars, of which fructose and glucose are the major components along with water. Other sugars, proteins, enzymes, carotenoids, phenolic compounds, free amino acids, organic acids, vitamins and minerals are also found in honey (Das et al., 2015; Habib, Meqbali, Kamal, Souka, & Ibrahim, 2014; Vilhena & Almeida-Muradian, 1999).

Although best known as a sweetener, honey is a high-quality food rich in beneficial substances essential for ensuring balanced biological processes. For this reason, its functional potential has lately been investigated (Das et al., 2015; Gasic et al., 2014). Studies around the world have demonstrated the beneficial properties of honey of different botanical origins (Gasic et al., 2014; Silva et al., 2013) as well as their association with the levels of bioactive compounds, mainly polyphenols or phenolic compounds, present in it.

Phenolic compounds are biologically active secondary metabolites from plants that act at molecular level and are potent natural antioxidants (Martins, Petropoulos, & Ferreira, 2016; Silva, Costa, Santana, & Koblitz, 2010). They have been reported to be the main constituents in honey responsible for its health beneficial properties and to be capable of inhibiting or reducing the formation of free radicals. Evidence indicates that free radicals can induce oxidative damage to molecules such as proteins, carbohydrates, lipids and DNA, which can lead to the development of various diseases (Alvarez-Suarez et al., 2012). For this reason, bioactive compounds, particularly polyphenols, have been investigated for beneficial uses and proved to have positive effects on chronic diseases such as cardiovascular disease, cancer, obesity and diabetes (Carocho & Ferreira, 2013; Irondi, Oboh, Akindahunsi, Boligon, & Athayde, 2015). In addition, polyphenols have been studied for their antimicrobial, anti-inflammatory, antimutagenic, antitumor, and many other beneficial effects on human health. Thus, identifying and quantifying them in honey can significantly contribute to better understanding its overall bioactivity. Phenolic compounds can also be used to classify honeys according to their botanical origin (Keckes et al., 2013).

As well-established in the literature, honey composition varies greatly as it depends on botanical and geographical origins, bee species

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and climatic conditions of the production site (Alvarez-Suarez et al., 2018; Attanzio, Tesoriere, Allegra, & Livrea, 2016; Boussaid et al., 2014). This fact makes the search for honeys of different places and botanical origins interesting. Southern Brazil differs from other Brazilian regions in climate and flora and accounts for up to half of the country's honey production; Rio Grande do Sul state is the largest producing state. The main objective of this study was to characterize *Apis mellifera* honeys produced in the state of Rio Grande do Sul, Brazil in terms of their phenolic profile and antioxidant and physicochemical properties. It secondarily aimed to authenticate the origin of honey samples by using linear discriminant analysis (LDA).

2. Materials and methods

2.1. Chemical compounds

2,2′-Azobis (2-amidinopropane) dihydrochloride, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sugar standards and phenolic compounds, Folin-Ciocalteu's phenol reagent, 5-hydroxymethylfurfural (HMF), and fluorescein were obtained from Sigma Aldrich Co.® (St. Louis, MO, USA). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was obtained from Fluka Chemie AG® (St Louis, MO, USA). *n*-Butanol p.a. reagents were purchased from Sigma Aldrich-Vetec, *para*-aimonbenzoic acid (PABA) from Synth and sodium nitrite, aluminum chloride and HPLC grade reagents (Formic acid, methanol and acetonitrile) were obtained from Merck® (Darmstadt, Germany).

2.2. Honey samples

Forty-nine samples of *Apis mellifera* honey of different botanical origins were acquired at Casa do Mel of the Beekeepers Association of Rio Grande do Sul (Viamão, RS state, Brazil) in 2014 and 2015. The monofloral honey samples were: eucalyptus (*Eucalyptus*), Japanese grape (*Hovenia dulcis*), mastic (*Schinus terebinthifolius*), quitoco (*Pluchea Sagittalis*), wildflower (*Gaya Macrantha*) and there were also samples of six polyfloral honeys. They were all collected from different regions in Rio Grande do Sul state, Brazil.

2.3. Melissopalynological analysis

The analysis was performed according to the standardized method described in Maurizio and Louveaux (1965). The results were analyzed in terms of pollen type dominance taking into account the over- and under-representation of its botanical species in each honey sample.

2.4. Physicochemical properties

Physicochemical analyses were performed by using AOAC (1990) methods and in compliance with the relevant Brazilian Legislation (Brasil, 2000). Moisture content was determined using a digital refractometer ($DR_{201\text{-}95}$, Kruss) and ash content was gravimetrically determined after combustion of samples in a muffle furnace at 550 °C. Electrical conductivity was measured using a conductivity meter (Crison Instruments, EC-Meter) and free acidity was measured by simple titration with a pH meter (Hanna Instruments). Color measurement was performed using a colorimeter (Hanna Instruments, C_{221}) and the determination of diastase activity was carried out by spectrophotometry.

Sugars were determined as described in Bogdanov (1999) by HPLC using a Zorbax NH_2 normal phase column (Agilent, 250 mm × 4.6 mm, 5 μm) and a refractive-index detector (RID). The column was operated under the following conditions: mobile phase, acetonitrile and water (80:20, v/v); isocratic flow rate, 1.3 mL.min^{-1}; temperature, 37 °C.

HMF content was determined as described in Ajlouni and Sujirapinyokul (2010). The HPLC system used was equipped with a reversed phase C_{18} column (Phenomenex, 250 mm × 4.6 mm, 5 μm) and operated isocratically at 0.75 mL.min^{-1} of the mobile phase water and methanol, 90:10 (v/v). The standard curve was constructed using HMF standard solutions (1–50 mg.L^{-1}). Detection was performed by using an ultraviolet detector at 285 nm.

2.5. Determination of total phenolic content (TPC) and total flavonoids content (TFC)

TPC and TFC were measured on a Synergy H1 Multi-Mode Spectrophotometric Reader (Biotek instruments Inc., VT, USA). Aliquots of aqueous solutions of honey (0.2 g.mL^{-1}) were analyzed on a 96-well polystyrene microplate and absorbance was measured at 760 nm and 415 nm, respectively.

TPC analysis was performed using the Folin-Ciocalteu method, as described in Zhang, Shen, Silva, Dennis, and Barrow (2006). The standard curve was generated with gallic acid (10.5–210 μg.mL^{-1}).

The determination of TFC was performed as described in Xu and Chang (2007) with the following modifications: 180 μL of honey solution and 15 μL of 2.5% $NaNO_2$ were loaded directly into each well. At t = 6 and 11 min, 15 μL of 10% $AlCl_3$ and 50 μL of 1 mol.L^{-1} NaOH were added, respectively. The minimum time necessary for the formation of the flavonoid-chloride aluminum complex at room temperature was 10 min. The standard curve was plotted using quercetin (0.1–50 μg.mL^{-1}).

2.6. Determination of total antioxidant capacity

ORAC, DPPH and FRAP assays were spectrophotometrically performed on a Synergy H1 Multi-Mode 96-well microplate Reader (Biotek instruments Inc., VT, USA).

ORAC was determined as described in Prior et al. (2003) and Prior, Sintara, and Chang (2016) using a hydromethanolic solution of honey (0.05 g.mL^{-1}). A standard curve was plotted using different concentrations of Trolox (6.25 and 100 μmol/L).

The DPPH assay was performed as described in Bobo-Garcia et al. (2015) using an aqueous solution of honey (0.2 g.mL^{-1}). Absorbance reading was performed at 517 nm and results were expressed as a percentage of EC_{50} (minimum concentration required for the antioxidant to reduce the initial concentration of DPPH by 50%).

FRAP was carried out as described in Benzie and Strain (1996) using an aqueous solution of honey (0.2 g.mL^{-1}). The FRAP reagent was prepared by mixing 300 mmol.L^{-1} sodium acetate buffer (pH 3.6), 10 mmol.L^{-1} TPTZ solution and 20 mmol.L^{-1} ferric chloride solution in a ratio of 10:1:1 v/v in a water bath at 37 °C. Absorbances were read at 593 nm. A standard curve of Trolox ranging from 25 to 800 μmol.L^{-1} was prepared.

2.7. Analysis of phenolic compounds by HPLC

A liquid-liquid extraction was performed as described in Spilioti et al. (2014). The identification and quantification of phenolic compounds were carried out by HPLC using a reversed phase C_{18} column (Merck Lichrospher 100 RP 18E, 250 mm × 4.6 mm, 5 μm) maintained at 25 °C. The mobile phase consisted of water with 5% formic acid (A) and methanol (B) and was run at a flow rate of 0.8 mL.min^{-1}. The linear gradient employed was as follows: 2% B at 0.01 min; 10% B at 3 min; 40% B at 30 min; 50% B at 44 min; 100% B at 53min; 100% B for 2 min (53–55 min) for column cleaning. The initial conditions of the mobile phase were then resumed and maintained for 10 min for stabilization of the column.

The phenolic compounds were monitored between 240 and 400 nm. The standards used (purchased from Sigma-Aldrich) were gallic acid, protocatecuic acid, *para*-hydroxybenzoic acid, (+)-catechin, genic acid, vanillic acid, caffeic acid, chlorogenic acid, (-)-epicatechin, *p*-coumaric acid, ferulic acid, benzoic acid, synaptic, *o*-coumaric acid, rutin,