Journal of Apicultural Research

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

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JOURNAL OF APICULTURAL RESEARCH
https://doi.org/10.1080/00218839.2022.2030277

ORIGINAL RESEARCH ARTICLE

Botanical origin, physicochemical characterization, and antioxidant activity of bee pollen samples from the northeast of Portugal

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ABSTRACT

Bee pollen is a beehive product that has increased the attention of both researchers and consumers. The main goal of the present study was to evaluate 69 heterofloral dried bee pollen samples harvested in two regions of the northeast of Portugal (Mogadouro and Vimioso). For such, the botanical origin of the samples and several physicochemical parameters (moisture, pH, water activity, reducing sugar, proteins, lipids, ash, fibers, carbohydrates, energy, total phenolic, and flavonoids) were evaluated and the antioxidant potential was studied. The average percentage of pollen grains from *Erica* spp. and *Cytisus* spp. was higher ($p < 0.05$) in samples from Mogadouro, while *Cistus* spp., *Castanea* spp., and *Echium* spp. were more abundant in Vimioso samples. The pollen harvested in Vimioso presented higher values on proximate parameters as compared to other samples:moisture (4.61 ± 0.84%), lipids (4.94 ± 0.94%), ash (3.28 ± 0.93%), fiber (3.55 ± 1.11%), and carbohydrates (66.60 ± 3.52%).On the other hand, higher values were obtained on Mogadouro samples regarding water activity (0.41 ± 0.11), protein (26.09 ± 2.86%), total phenols (26.71 ± 6.19, expressed as mg of Gallic acid equivalents GAE/g), and antioxidant activity (assessed by β-carotene bleaching [3.35 ± 1.17 mg/mL] or the free radical scavenging assay [2.98 ± 0.63 mg/mL]). All bee pollen samples had a great botanical similarity, yet the dominant pollen types were different between the two regions allowing the classification of the samples according to the geographical origin.

ARTICLE HISTORY
Received 19 December 2018
Accepted 14 October 2021

KEYWORDS
Apis mellifera; antioxidant activity; beehive products; discriminant analysis; physicochemical properties

Introduction

Bee pollen (BP) results from the agglutination of flower pollen grains with nectar and salivary substances carried out by worker honey bees. This beehive product is used as food for all the developmental stages in the hive. The collection of BP is a relatively recent development, dependent primarily on the basic concept of scraping pollen off of the bees' legs as they enter the hive (Feás et al., 2012).

This natural product has been considered to have beneficial properties for human health related to the nutrients and bioactive compounds and has been considered as one of the few foods that contain all the essential amino acids (Gonçalves et al., 2018; Pascoal et al., 2014) and essential minerals (Sattler et al., 2016). BP also contains carbohydrates, lipids, fibers, vitamins, enzymes, coenzymes, and phenolic compounds, such as flavonoids, carotenoids, phytosterols, and terpenes (Campos et al., 2016; de-Melo et al., 2016). The physicochemical composition and, consequently the biological activity of this product, may be strongly influenced by several factors among which geographical origin (Araújo et al., 2017; Estevinho et al., 2012), climatic conditions, soil type, plant source, growth conditions, harvesting month, bee species as well as beekeeping techniques and storage methodologies (Ares et al., 2018; Bárbara et al., 2015; Feás et al., 2012; Karabagias et al., 2017; Morais et al., 2011). Recently, various researchers worldwide have shown gradual interest in the characterization of BP through their physicochemical properties (Ares et al., 2018; Bárbara et al., 2015; de Arruda et al., 2013b; Dias et al., 2016; Gonçalves et al., 2018; Karabagias et al., 2017; Kostić et al., 2015; Pascoal et al., 2014; Vasconcelos et al., 2017). However, there is still little information regarding the parameters that vary the most, and that therefore may aid in discriminating different BP types, especially heterofloral and from different geographical origins.

In this context, this study aimed to determine the physicochemical composition, including the antioxidant activity and phenolic compounds of 69 heterofloral BP from two Portuguese regions (Mogadouro and Vimioso). Also, it was intended to test whether physicochemical variables can be used to classify bee pollen, allowing to distinguish samples from the two geographical provenience.

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Materials and methods

Geographical origin of the samples

Sixty-nine bee pollen (BP) samples, 19 from Mogadouro and 50 from Vimioso – two villages from the northeast of Portugal with different floral sources – were provided by local beekeepers. All samples were harvested in March-April 2017. Upon receipt into the laboratory, none of the samples had signs of any visible contamination namely fermentation, spoilage, or field residues. Samples were kept in the dark at room temperature (±20 °C) until further analysis occurred no more than one month after. In order to ascertain the botanical origin of bee pollen samples, 1000 pollen grains were counted and assessed following the Erdtman method (Erdtman, 1960). The grains were identified by optical microscopy and identified using the reference collection of CIMO-Mountain Research Center and pollen morphology atlas, according to the procedures described by Nogueira et al. (2012).

Physicochemical parameters

No universal legislation exists regarding the physicochemical parameters and the hygienic and sanitary control of BP (Campos et al., 2016). Therefore, the samples were evaluated following the local legislation available in Brazil (Brazil, 2001), France, Switzerland (Bogdanov, 2004), and Argentina (Krell, 1996).

Moisture

The moisture (%) was assessed in agreement with the previously described by Bárbara et al. (2015). For such, approximately two grams (2 g) of each BP sample were dried at a temperature of 105 °C, from 18 to 24 h, until constant weight (weight did not vary more than 0.01%). The moisture was calculated as follows: Moisture (%) $= [(w_1 - w_2)/w_1) \times 100$, where w_1 is the weight (g) of the sample before drying while w_2 represents the weight (g) of the sample after drying.

pH values

This parameter was measured in the aqueous phase obtained after mixing 10 g of BP in 75 mL of distilled water using a combined electrode connected to a pH meter (211 microprocessor-based pH Meter, Hanna Instruments, Póvoa de Varzim, Portugal) (Bárbara et al., 2015).

Water activity

To determine the water activity (a_w), each BP sample was placed directly into a water activity meter (Rotronic HygroPalm, Grindelstrasse, Switzerland), as previously described by Nogueira et al. (2012) and Bárbara et al. (2015).

Reducing sugars

Reducing sugars were determined using the dinitrosalicylic acid method (DNS), following the procedures described by Miller (1959) and Bárbara et al. (2015). For sample preparation, 0.5 mL of concentrated HCl was added and the solution was incubated in a thermostat water bath at 60 °C for 10 min; the mixture was then neutralized with 6 N NaOH and finally rapidly cooled in the ice bath until reaching the room temperature. Then, 0.3 mL of the substrate stock solution (10 mg/mL of 0.1 M Na-acetate buffer) was mixed with 0.3 mL of the enzyme solution (both solutions had been preheated at 50 °C for 5 min). Afterward, the following incubation at 50 °C for 10 min, 0.9 mL of the DNS reagent (containing dinitrosalicylic acid, Rochelle salt, phenol, sodium bisulfite, and sodium hydroxide) was added to the test tube and the mixture was incubated in a boiling water bath for 5 min. After cooling to room temperature, the supernatant's absorbance at 540 nm was measured and reducing sugars (%) quantified using a standard glucose curve whose concentration ranged from 100 to 540 μg (Silva et al., 2003).

Protein content

The nitrogen content (%) was determined by conventional acid hydrolysis and Kjeldahl digestion, using a copper catalyst in 2 g of pulverized bee pollen. The ammonia was distilled and collected in a solution of boric acid, which was then titrated against standard acid. Digestion and distillation were carried out using a 230-Hjeltec Analyser (Foss Tecator, Höganäs, Sweden). The relative nitrogen content (N) of the sample was calculated using the following equation:

$$N\ (\%) = \frac{14.01 \times (y - x) \times N_{HCl}}{w},$$

where w (mg) denotes the total sample amount; x (mL) and y (mL) denote the volume of acid used to titrate the blanks and the sample, respectively; and N_{HCl} represents the normality of the acid (in mol/L), determined by titration of tris-(hydroxymethyl)-amino-methane (Anglov et al., 2003).The crude protein content was then calculated using the conversion factor of 6.25 ($N \times 6.25$) (Bárbara et al., 2015; Nogueira et al., 2012).

Lipids

The lipid content is determined by extracting the fat from the sample using a solvent and then determining the weight of the fat recovered. The percentage of lipids (%) was determined according to the Soxhlet intermittent method as described by Nogueira et al.

(2012) and de Arruda et al. (2017), based on lipid extraction by diethyl ether. Briefly, two grams (2 g) of BP were macerated in a mortar with anhydrous Na_2SO_4. This mixture was then extracted with n-hexane for about 4 h in the Soxhlet apparatus. At the end of extraction, the flask was placed in an oven at 102 °C (for extract evaporation) and dried until reaching a constant weight.

Ash content

The ash content (%) was determined following the procedures described by Nogueira et al. (2012) and Bárbara et al. (2015) after ignition at 550 ± 15 °C, by gravimeter. The following equation was applied:

$$\text{Ash content}\,(\%) = \left[(z-x)/(y-x)\right] \times 100,$$

where x represents the weight of the empty crucible; y represents the weight of the crucible containing the sample prior to ashing; and z is the weight of the crucible containing the ash (Thiex et al., 2012).

Fiber content

Fiber content (%) was determined using the method recommended by AOAC 991.43 (Lee et al., 1992). This method consists of the enzymatic removal of starch and protein from the bee pollen samples and further separated into soluble and insoluble fractions by filtration. Total dietary fiber was calculated as the sum of soluble and insoluble fractions.

Carbohydrates

The content of carbohydrates (%) was calculated using the following formula (Nogueira et al., 2012):

$$\text{Carbohydrates} = [100 - (\text{ash} + \text{proteins} + \text{lipids})](\%).$$

Energy

The energy value was estimated using the Atwater general factor system, which is based on the heats of combustion of protein, fat, and carbohydrate, corrected for losses in digestion, absorption, and urea excretion. The energy values are 4.0 kcal/g for protein and carbohydrates and 9.0 kcal/g, for fat, as follows (Nogueira et al., 2012):

$$\text{Energy}\ (\text{kcal}) = 4 \times (\text{protein} + \text{carbohydrate}) + 9 \times (\text{lipid}).$$

Phenolic compounds

Bee pollen samples preparation

The extraction was performed as described in Pascoal et al. (2014), by mixing the BP with methanol (1:2) (w/v). After maceration, the extract was evaporated in a vacuum evaporator. The dried BP extract was kept in the dark at room temperature until further analysis.

Total phenolic content

Phenolic content was determined using the Folin-Ciocalteau method as described by Pascoal et al. (2014) and Dias et al. (2016). Briefly, 0.5 mL of each bee pollen sample was diluted in MeOH (MeOH-bee pollen; 500 µL of 1:10 g/mL) and mixed with 500 µL of Folin–Ciocalteu reagent and 500 µL of Na_2CO_3 (10% w/v). Following incubation in the dark at room temperature for 1 h the absorbance at 700 nm of the mixture was determined against the blank (the same mixture without the MeOH + sample) using a Unicam Helios Alpha UV–visible spectrometer (Thermo Spectronic, Cambridge, UK). Gallic acid standard solutions (0.01×10^{-3} to 0.08×10^{-3} M) were used for constructing the calibration curve ($y = 1.9965x + 0.0018$; $R^2 = 0.9997$). Results were expressed as mg of gallic acid equivalent per g (GAE/g) of BP dry weight.

Total flavonoids content

The aluminum chloride method also was used as described by Pascoal et al. (2014)and Dias et al. (2016). Briefly, MeOH-bee pollen (250 µL) was mixed with 1.25 mL of distilled H_2O and 75 µL of a 5% $NaNO_2$ solution. After 5 min 150 µL of a 10% $AlCl_3$ H_2O solution was added. After 6 min 500 µL of 1 M NaOH and 275 µL of distilled H_2O were added to the mixture and vortexed. The solution was mixed well, and the intensity of the pink color was measured at 510 nm. Catechin standard solutions (0.022×10^{-3} to 0.34×10^{-3} M) were used for constructing the calibration curve ($y = 1.0421x - 0.0093$; $R^2 = 0.9918$). Results were expressed as mg of quercetin equivalent per g (QE) of BP dry weight.

Antioxidant activity

Antioxidant activity was evaluated by two analytical methods: β-carotene bleaching (BCB) and free radical scavenging assay (DPPH). According to Morais et al. (2011) and Aličić et al. (2014) the antioxidant capacity of BP must be approached using more than one methodology to avoid possible interferences due to the complexity and heterogeneity of the matrix.

β-Carotene bleaching assay

The antioxidant activity was determined following the methodology described by Dias et al. (2016). Four milliliters (4 mL) of a solution (5 mg of β-carotene + 25 mL of $CHCl_3$) were pipetted into a 100 mL round-bottom flask and $CHCl_3$ was removed under vacuum. After that, 80 mg of linoleic acid, 800 mg of

Table 1. Summary of pollinic analysis of the BP samples.

Pollen type	Mogadouro ($n = 19$)					Vimioso ($n = 50$)				
Genus (abbreviation), family	Number[a] samples detected	Low	High	$\bar{x} \pm SD^b$	Median*	Number[a] samples detected	Low	High	$\bar{x} \pm SD^b$	Median
Cistus (Cis), Cistaceae	8 T, 2 S, 2 IM, 4 M	0.00	22.85	3.70 ± 7.15	0.00*	38 T, 1 P, 16 S, 17 IM, 4 M	0.00	65.14	13.14 ± 13.21	12.44
Quercus (Que), Cistaceae	8 T, 1 P, 2 S, 5 IM	0.00	56.50	7.48 ± 14.02	0.00	17 T, 1 P, 5 S, 10 IM, 1 M	0.00	50.99	5.45 ± 11.53	0.00
Rubus (Rub), Rosaceae	7 T, 1 P, 1 S, 4 IM, 1 M	0.00	76.14	6.86 ± 17.58	0.00	21 T, 2 P, 5 S, 12 IM, 2 M	0.00	57.37	6.86 ± 12.62	0.00
Eucalyptus (Euc), Myrtaceae	7 T, 1 S, 5 IM, 1 M	0.00	17.98	3.72 ± 6.16	0.00	24 T, 6 S, 12 IM, 6 M	0.00	35.88	5.21 ± 8.81	0.00
Thymus (Thy), Lamiaceae	7 T, 1 S, 2 IM, 4 M	0.00	30.95	3.29 ± 7.81	0.00	7 T, 1 S, 2 IM, 4 M	0.00	23.35	0.77 ± 3.43	0.00
Castanea (Cas), Fagaceae	0	0.00	0.00	0.00	0.00*	34 T, 3 P, 12 S, 15 IM, 4 M	0.00	66.59	13.14 ± 16.47	6.25
Echium (Ech), Boraginaceae	7 T, 1 P, 1 S, 5 IM	0.00	48.74	5.69 ± 11.79	0.00*	46 T, 18 P, 21 S, 6 IM, 1 M	0.00	83.77	34.05 ± 20.76	34.42
Malva (Mal), Malvaceae	2 T, 1 IM, 1 M	0.00	9.72	0.53 ± 2.23	0.00	8 T, 4 IM, 4 M	0.00	15.65	0.79 ± 2.69	0.00
Carduus (Car), Asteraceae	2 T, 2 IM	0.00	12.95	0.93 ± 3.11	0.00	10 T, 5 IM, 5 M	0.00	12.28	0.76 ± 2.20	0.00
Prunus (Pru), Rosaceae	7 T, 1 S, 4 IM, 2 M	0.00	25.82	3.29 ± 6.72	0.00	10 T, 2 S, 2 IM, 6 M	0.00	24.57	1.19 ± 4.21	0.00
Erica (Eri), Ericaceae	14 T, 8 P, 2 S, 3 IM, 1 M	0.00	67.86	29.63 ± 27.58	23.90*	6 T, 1 S, 4 IM, 1 M	0.00	19.20	1.04 ± 3.67	0.00
Vicia (Vic), Fabaceae	2 T, 1 S, 1 M	0.00	28.54	1.63 ± 6.54	0.00	6 T, 5 IM, 1 M	0.00	10.82	0.82 ± 2.46	0.00
Cytisus (Cys), Fabaceae	13 T, 3 P, 7 S, 3 IM	0.00	59.53	21.07 ± 19.28	16.46*	19 T, 5 S, 12 IM, 2 M	0.00	24.23	4.30 ± 6.63	0.00
Leontodon (Leo), Asteraceae	0	0.00	0.00	0.00	0.00	4 T, 3 IM, 1 M	0.00	5.07	0.30 ± 1.13	0.00
Lavandula (Lav), Lavandula	2 T, 2 IM	0.00	10.75	0.89 ± 2.78	0.00	19 T, 15 IM, 4 M	0.00	12.89	2.30 ± 3.52	0.00
Taraxacum (Tar), Asteraceae	3 T, 1 S, 1 IM, 1 M	0.00	16.83	1.67 ± 4.63	0.00	11 T, 1 S, 9 IM, 1 M	0.00	16.07	1.78 ± 3.81	0.00
Salix fragilis (Sal), Salicaceae	9 T, 5 S, 3 IM, 1 M	0.00	34.19	8.81 ± 12.18	0.00	24 T, 13 S, 8 IM, 3 M	0.00	35.60	8.13 ± 11.55	0.00

[a]T, total samples; P, predominant pollen (>45%); S, secondary pollen (16–45%); IM, important minor pollen (3–15%); M, minor pollen (<3%).
[b]Mean and standard deviation (SD).
*Median in the same row differs according to the Mann–Whitney test ($p < 0.05$).

Tween 80 emulsifier, and 200 mL of distilled water were added. Next, 4.8 mL of this mixture were transferred into test tubes containing 200 μL of different concentrations of the pollen extracts; the tubes were shaken and incubated in a water bath at 50 °C. The absorbance at 470 nm was measured immediately after incubation (zero time) and then recorded at 20-min intervals until the color of the control sample had changed. A blank (without β-carotene) was used for background subtraction. Lipid peroxidation (LPO) inhibition was calculated using the equation:

$$\text{LPO inhibition} = (\beta - \text{carotene content after 2h} / \text{initial } \beta - \text{carotene content}) \times 100.$$

The extract concentration providing 50% of antioxidant activity (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. Butylated hydroxytoluene (BHT) at a concentration of 40 mg/mL was used as a standard antioxidant.

Free radical scavenging assay

Antioxidant activity was also evaluated using the free radical scavenging assay which was previously described by Pascoal et al. (2014). In brief, different extracts concentrations (300 µL) were mixed with 2.7 mL of a solution containing MeOH and DPPH radicals (6×10^{-5} mol/L); this mixture was left in the dark until stable absorption values were obtained. Thereafter, DPPH radical's reduction was measured by continuously monitoring the decrease of absorption at 517 nm. The radical-scavenging activity was calculated as a percentage of DPPH discoloration using the equation:

$$\text{RSA } (\%) = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100,$$

where A_S is the absorbance of the solution containing BP extract at different concentrations and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radical scavenging activity (RSA), EC_{50}, was calculated by interpolation from the graph of RSA percentage against extract concentration. Butyl hydroxyanisole (BHA) and α-tocopherol were used as standard antioxidants.

Statistical analysis

All experiments were carried out in triplicate and data was analyzed using R Statistical Software, version 3.5.0. After testing data normality with the Shapiro–Wilk test, the Mann–Whitney test was used to compare the results obtained for the pollinic profile of the two BP types (nonparametric data). On the other hand, the physicochemical parameters and antioxidant activity were compared by multivariate analysis of variance (MANOVA).A significance level of

Table 2. Physicochemical parameters of the BP samples under study.

Parameters	Mogadouro (mean ± SD, $n = 19$)	Vimioso (mean ± SD, $n = 50$)	U-statistic (Wilks' λ)	Z	p-value*
Moisture (%)	3.99 ± 0.70	4.61 ± 0.84	0.89	8.21	0.006*
pH	4.60 ± 0.79	4.61 ± 0.58	1.00	0.008	0.931
a_w	0.41 ± 0.11	0.36 ± 0.07	0.93	4.87	0.031*
Reducing sugar (%)	37.64 ± 4.62	35.19 ± 4.81	0.95	3.66	0.060
Protein (%)	26.09 ± 2.86	20.58 ± 3.02	0.59	46.99	0.000*
Lipids (%)	4.07 ± 0.68	4.94 ± 0.94	0.83	13.50	0.000*
Ash (%)	2.51 ± 0.49	3.28 ± 0.93	0.85	11.52	0.001*
Fiber (%)	2.86 ± 0.77	3.55 ± 1.11	0.92	6.01	0.017*
Carbohydrates (%)	63.35 ± 2.79	66.60 ± 3.52	0.84	12.96	0.001*
Energy (kcal)	394.37 ± 4.63	393.13 ± 6.49	0.99	0.58	0.451
Total phenols (GAE/g)	26.71 ± 6.19	22.75 ± 5.04	0.90	7.84	0.008*
Flavonoids (QE/g)	4.60 ± 1.53	4.37 ± 1.48	0.99	0.35	0.559
BCB (EC_{50} mg/mL)	3.35 ± 1.17	4.05 ± 1.31	0.94	4.05	0.048*
DPPH (EC_{50} mg/mL)	2.98 ± 0.63	3.90 ± 1.22	0.87	9.74	0.003*

*Significant differences amongst BP harvested in different regions (Wilks' lambda test, $p < 0.05$).

5% ($p < 0.05$) was adopted. Fisher's linear discriminant was used for the classification of heterofloral BP.

Results

Pollinic identification

Pollen grains of 15 genera were identified in the samples harvested in Mogadouro ($n = 19$) and 17 genera were found in samples from Vimioso ($n = 50$) (Table 1). Of the recognized pollen types, 15 were present on samples from both regions while only *Castanea* spp. (*Cas*) and *Leontodon* spp. (*Leo*) were exclusive from Vimioso. The median percentages obtained for *Cistus* spp. (*Cis*), *Castanea* spp. (*Cas*), *Echium* spp. (*Ech*), *Erica* spp. (*Eri*), and *Cytisus* spp. (*Cys*)differed significantly ($p < 0.05$) between the two regions.

Physicochemical properties

The results obtained for the physicochemical parameters are presented in Table 2.

Moisture

The moisture varied between 3.99% and 4.61%and significant differences *(p = 0.006)* were observed between the two regions. These values are slightly higher than the maximum allowed by both Brazilian (Brazil, 2001) and Argentinean (Krell, 1996) regulations (4%). On the other hand, the French and Swiss regulations establish a moisture maximum of 6% (Bogdanov, 2004).

pH values

The pH determined in the studied BP samples was acidic (4.60–4.61). This parameter did not differ significantly among samples.

Water activity

In this study a_w varied between 0.36 ± 0.07 and 0.41 ± 0.11 for Vimioso and Mogadouro regions, respectively. Statistically significant differences ($p = 0.031$) were observed in both groups of BP samples.

Reducing sugars

In this study, the content of reducing sugars varied from 35.19 ± 4.81% to 37.64 ± 4.62%, in Mogadouro and Vimioso regions, respectively. No significant differences were observed between BP samples from these regions.

Protein content

The protein content varied between 20.58 ± 3.02% and 26.09 ± 2.86% (Table 2). Significant differences ($p = 0.000$) were observed between both regions.

Lipids

The lipid content ranged between 4.07 ± 0.68% and 4.94 ± 0.94% and significant differences ($p = 0.000$) were observed between the two regions.

Ash content

The amount of ash obtained in this study ranged from 2.51 ± 0.49% to 3.28 ± 0.93 and was significantly different between the two regions under analysis ($p = 0.001$).

Fiber content

The content of fiber obtained in this study varied from 2.86 ± 0.77 to 3.55 ± 1.11% in Mogadouro and Vimioso regions, respectively. Significantly differences ($p = 0.000$) were observed between these regions.

Carbohydrates

Higher carbohydrate content was observed in pollen from Vimioso (66.60 ± 3.52%) in comparison to samples from Mogadouro (63.35 ± 2.79%). Again, statistically

Table 3. Classification function coefficients (Fisher's linear discriminant functions).

Parameters	Mogadouro	Vimioso
Moisture (%)	12.06	12.37
pH	13.56	14.12
a_w	97.15	93.38
Reducing sugar (%)	1.95	1.82
Protein (%)	1.98	1.26
Lipids (%)	1.44	2.40
Ash (%)	4.51	5.56
Fiber (%)	−1.97	−1.21
Total phenols (GAE/g)	0.71	0.70
Flavonoids (QE/g)	3.00	3.15
β-carotene bleaching (EC_{50} mg/mL)	3.75	3.47
DPPH (EC_{50} mg/mL)	1.16	2.60
Constant	−169.30	−163.08

significant differences ($p = 0.001$) were observed between regions.

Energy

In this study, the energy values varied from 393.13 ± 6.49 to 394.37 ± 4.63 kcal. No significant differences were observed between samples from the two geographic proveniences.

Phenolic compounds

Total phenolic content

The total phenols content of the pollen samples was 22.75 ± 5.04 GAE/g (Vimioso) and 26.71 ± 6.19 GAE/g (Mogadouro). Significant differences ($p = 0.008$) among these two regions were observed.

Total flavonoids content

The total flavonoid content (mean ± standard deviation) of the pollen samples was 4.37 ± 1.48 and 4.60 ± 1.53 QE/g for Vimioso and Mogadouro regions, respectively.

Antioxidant activity

The antioxidant capacity of BP is influenced by many factors (Aličić et al., 2014; Dias et al., 2016). To avoid possible interferences due to the complexity and heterogeneity of the matrix this biological property was assessed using two methodologies.

β-Carotene bleaching assay

The mechanism of β-carotene bleaching is a free radical-mediated phenomenon, resulting from the hydroperoxides formed from linoleic acid. The antioxidant activity of BP, measured by the BCB assay in EC_{50}, varied from 3.35 to 4.05 mg/mL for Mogadouro and Vimioso regions, respectively. Significant differences between the two regions were observed ($p = 0.048$). The same pattern was verified when the DPPH method was used. In spite of that, the protection of the β-carotene bleaching by the samples was lower than that provided by the DPPH assay.

Table 4. Results of the classification of BP samples from Portugal.

Number of samples classified in to regions					
			Predicted group membership		
		Group	Mogadouro	Vimioso	Total
Original[a]	Count	Mogadouro	17	2	19
		Vimioso	1	49	50
	%	Mogadouro	89.5	10.5	100
		Vimioso	2.0	98.0	100

The 95.7% of original grouped cases correctly classified.

Free radical scavenging assay

In general, the BP extracts had low EC_{50} values (ranging from 2.98 ± 0.63 to 3.90 ± 1.22 mg/mL of extract), suggesting a strong antioxidant activity. Significant differences ($p = 0.003$) were observed between both regions.

Overall comparison

Significantly higher ($p < 0.05$) amounts of a_w, protein, total phenols, and antioxidant activity were observed in samples collected in Mogadouro compared to those harvested in Vimioso. On the other hand, samples harvested in Vimioso had significantly higher moisture, lipids, ash, fiber, and carbohydrates. The pH, reducing sugars, energy, and flavonoids did not differ amongst regions ($p \geq 0.05$), having little discriminant power. Within-group covariance matrices suggest that the values obtained for the two groups differ to some extent, yet according to Box's test for equality of covariance these differences are not statistically significant ($F = 1.271$, p-value = 0.06), which is appropriate for the use of Fisher's linear discriminant function. The first discriminant function was significant (Wilks' λ 0.38, p-value = 0.000), which represented 100% of the total variance explained and canonical correlation coefficient of 0.788, indicating a high degree of association between the discriminant function and the groups.

The coefficients obtained using Fisher's linear discriminant functions are shown in Table 3. In Table 4, it can be seen that 95.7% of the BP samples are correctly classified. Therefore, the discriminant functions accurately classified samples as coming from Mogadouro or Vimioso.

Discussion

Pollinic identification

The diversity of the detected pollen spectrum confirms that both regions have a wide range of botanical species supplying pollen grains to *Apis mellifera* bees. Even though, in general, the two regions have great botanical similarities, the median percentage of BP for each botanical genus differed amongst the two regions. This botanical diversity has been reported to be strictly related to BP biochemical particularities. A BP sample is classified as unifloral whenever a particular botanical species contributes to over 45% of the total grains counted (Feás et al., 2012). In this study, 50% of the samples harvested in Vimioso and 74% from Mogadouro were unifloral. The most common predominant pollen in those samples was *Echium* sp. (in 18 samples from Vimioso) and *Erica* sp. (in 8 samples harvested in Mogadouro); these two botanical species are very representative of the flora present in the northeast of Portugal.

Physicochemical properties

Moisture

The results obtained in this study were similar to those reported by Carpes et al. (2009) in BP harvested on the Southern region of Brazil (from 3.45% to 4.85%); while Melo and Almeida-Muradian (2011) reported moisture levels of 4.67% and 3.96%, using the infrared and lyophilization methodologies. More recently Gonçalves et al. (2018) found a mean of 4.92% in Portuguese BP. Higher values (7.7 g/100 g) were also reported by Fuenmayor et al. (2014) in Colombian BP and Gabriele et al. (2015), who reported 10.75% to 12.03% of moisture contents in BP samples from Tuscany (Italy).

pH values

The results observed in this parameter are in agreement with those reported by Feás et al. (2012) (mean pH value of 4.8) in BP from Portugal, Fuenmayor et al. (2014) in Colombian BP (mean pH of 4.6), and those recently reported by Gonçalves et al. (2018) (4.68 ± 0.62).

Water activity

Free water plays an important role in influencing the "shelf lifetime" and organoleptic characteristics of BP (Estevinho et al., 2012; Feás et al., 2012). High a_w values may potentially promote microbial contamination, mainly by molds and yeasts (Morgano et al., 2011). The results obtained in this study are within the range reported by Estevinho et al., (2012), Nogueira et al. (2012), and Gonçalves et al., (2018), who studied Portuguese BP samples.

Reducing sugars

The content of reducing sugar in BP is associated with the amount of honey or nectar present in the product. The results were similar to those reported by previous studies assessing BP from Portugal (Gonçalves et al., 2018; Nogueira et al., 2012). On the other hand, the results obtained in this study are much higher than those obtained by Bárbara et al. (2015) (12 ± 0.02%), in BP from Bahia (Brazil). Also, these results are slightly above the percentage obtained by Campos et al. (2011) (31.82 ± 2.25%), in a work that aimed to propose international directives for BP quality control.

Protein content

For protein content, these results fit within the range (10–40%) established by Bogdanov (2016) and Campos et al. (2011) suggesting that this product is of good quality. Similar results were also obtained by de Arruda et al. (2013b) (23.38%) and Negrão et al. (2014) (18.66–24.39%) in Brazilian BP, Kostić et al. (2015) (14.81–27.25%) in Serbian BP and Fuenmayor et al. (2014) (mean value 23.8 ± 3.2%) in Colombian BP. On the other hand, the results obtained in this study are higher than those reported by Bárbara et al. (2015) (21%) and de-Melo et al. (2016) (8.3–11.4%) in Brazilian samples. Differently, Yang et al. (2013) (14.26–28.95%) and Gabriele et al. (2015) (25.87–28.42%) found results slightly higher than the reported in this study in BP samples from China and Italy, respectively.

Lipids

Lipids (fatty acids, sterols, and triglycerides) are essential for the adequate development of the pollen grains (Salazar-González & Díaz-Moreno, 2016; Villanueva et al., 2002), being also crucial in the reproduction, development, and growth of honey bees (Yang et al., 2013). The results obtained for this parameter fit into the range established by Bogdanov (2016) and Campos et al. (2011) (1–13 g/100g) and were similar to those reported in BP from Spain (Villanueva et al., 2002) and Serbia (Kostić et al., 2015).

Ash content

The ash content may be strongly influenced by geographical origin, soil type, and floral species (Campos et al., 2016). Campos et al. (2011) established the range from 2 to 6 g/100g as a possible international directive for quality control of BP. The results of the present study were similar to those reported in samples from other countries, like Brazil (de Arruda et al., 2013b; Rebelo et al., 2016), China (Yang et al., 2013), Portugal (Gonçalves et al., 2018), and Italia (Gabriele et al., 2015).

Fiber content

Fiber has been reported as one of the major components of BP with concentrations ranging between 0.3% and 20% (Villanueva et al., 2002), depending on the botanical origin but also on the methodology used for determination (Bogdanov, 2016; Taha, 2015). The Brazilian regulation (Brazil, 2001) establishes the minimum concentration of 2%.

The concentration of fiber obtained in this study was similar to that found by Gonçalves et al. (2018) (between 1.23% and 5.45%), in other Portuguese BP samples, but lower than those reported in samples from Colombia (7.8–18.1%, Fuenmayor et al., 2014), Brazil (9.30–13.65%, Rebelo et al., 2016), and France (9.2–14.4% Du Sert, 2009).

Carbohydrates

Carbohydrates (fructose, glucose, sucrose, fibers) are the main constituents of BP accounting for about 2/3 of the total weight. However, its specific composition strongly depends on the geographical location, climatic conditions, soil type, plant source, growth conditions, the harvesting season, bee species, and factors like beekeeper activities and the conservation methods (Estevinho et al., 2012; Feás et al., 2012; Villanueva et al., 2002). The concentration of carbohydrates was similar to that reported by Estevinho et al. (2012) (60.82–70.76%) and Feás et al. (2012) (61.2–70.6%) in Portuguese pollen; higher than those reported by Rebelo et al. (2016) (25.66–44.27%) in Brazilian samples, Du Sert (2009) (40.97–64.5%) in French samples and Gabriele et al. (2015) (54.84–57.98%) in Italian samples. Our results are above the range established by Bogdanov (2016) and Campos et al. (2011) (from 13 to 55 g per 100 g), who aimed to propose a new international directive for BP quality control.

Energy

The range of values obtained in the present study was slightly lower than that reported by Feás et al. (2012) (396.4–411.1 kcal) and Nogueira et al. (2012) (400.70–411.75 kcal) in Portuguese and Spanish BP, respectively. On the other hand, lower energy values were reported by Rebelo et al. (2016) (331.33–350.47 kcal) in Brazilian BP, Du Sert (2009) (316–354 kcal) in French BP, and Kostić et al. (2015) (350.65–395.6 kcal) in BP collected in Serbia. These differences corroborate the importance of characterizing BP from different origins, prior to their application as food supplements or components.

Phenolic compounds

Total phenolic content

Phenolic compounds are bioactive substances that are not synthesized by the human body and must, therefore, be provided by the diet. These compounds have been pointed to exert physiological actions, such as antimicrobial, anti-carcinogenic, anti-aging, anti-thrombotic, anti-diabetic, anti-hyperlipidaemic, anti-inflammatory activities, and antioxidant (Ares et al., 2018). The concentration of total phenolic was above the proposed as an international directive for quality control of BP (0.23 ± 0.02%) (Campos et al., 2011). Similar results were observed in Portuguese BP from other regions (Pascoal et al., 2014) (18.55–32.15 GAE/g), while higher values were obtained by Kalaycı oğlu et al. (2017) (509.0–1746.0 GAE/g) in Turkish honey BP and Freire et al. (2012) (41.5–213.2 GAE/g) in Brazilian BP. However, a lower concentration of phenolic compounds was obtained by Vasconcelos et al. (2017) (7.57–25.85 GAE/g) and de Arruda et al. (2013a) (13.76–24.60 GAE/g) in Brazilian BP, Morais et al. (2011) (10.5–16.8 GAE/g) and Dias et al. (2016) (12.75–35.05 GAE/g) in Portuguese BP, Gabriele et al. (2015) (13.53–24.75) in Italian BP, Fatrcová-Šramková et al. (2013) (16.4–4.4 mg/GAE/g) in Slovakian BP, and Sun et al. (2017) (0.81–11.76 mg/GAE/g) in Chinese BP.

Total flavonoids content

Flavonoids have been reported as the most common group of polyphenols in the human diet. Several in vitro pharmacological activities have been attributed to these compounds, including antimicrobial, anti-inflammatory, antioxidant, anti-allergic, anti-cancer, and anti-diarrheal (Sun et al., 2017). The total flavonoid content was similar to those proposed by the international directive for the quality control of BP (4.64 ± 0.07 mg/g) (Campos et al., 2011). The concentration of phenols was significantly higher in samples harvested in Mogadouro region, being expectable that the same would occur for flavonoids. Our results are within the range described by de Arruda et al. (2013a) (2.52–6.90 QE/g) and were higher than those found by Bárbara et al. (2015) (1.0 ± 0.2 mg CAE/g) in Brazilian BP. On the other hand, the concentration was lower than that reported by Pascoal et al. (2014) (3.71–10.14 QE/g), Dias et al. (2016) (12.75–35.05 QE/g), Gonçalves et al. (2018) (2.05–10.78 mg QE/g pollen) in other Portuguese BP samples.

Antioxidant activity

β-Carotene bleaching assay

Higher antioxidant activity was observed in the samples harvested in Mogadouro; which also presented higher content of phenolic compounds and flavonoids. The mutual and significant dependencies between phenolic and flavonoids compounds that contribute directly to the antioxidant capacity, as well as the importance of botanical and geographical origins have been established (Aličić et al., 2014; de-Melo et al., 2016; Kaškonienė et al., 2015).

Globally, our results were lower than that obtained by Dias et al. (2016) (2.96–6.99 mg/g pollen) and Morais et al. (2011) (3.11–6.52) in dried BP from Portugal but were higher than those reported by de Arruda et al. (2013a) (61.63–83.90%) and de Florio Almeida et al. (2017) (91.93 ± 0.22%) in Brazilian BP. On another hand, the values of β-carotene bleaching found in this study are within the limits described by Campos et al. (2010) (1–20 mg/100 g).

Free radical scavenging assay

As above, significantly lower EC_{50} concentrations were obtained from samples harvested in Mogadouro (2.98 ± 0.63). According to Campos et al. (2003), BP with the highest activity of DPPH is generally that containing the highest levels of flavonoids and phenolic acid derivatives. However, the presence and the concentration of particular flavonoids and phenolic compounds may be the reason for increasing the antioxidant activity and not only the quantity of total these compounds (de-Melo et al., 2016). The results obtained using assay were similar to those reported by Gonçalves et al.,(2018) (0.60–6.71 EC_{50} mg/g), Morais et al. (2011) (2.16–5.87), Pascoal et al.,(2014) (2.98–6.69) in Portuguese BP, de Arruda et al. (2013a) (1.30–8.08), Araújo et al. (2017) (1.25–7.99), and Kalaycıoğlu et al. (2017) (12.30–33.84) in Turkish BP.

Conclusions

This study performed a comparative study of BP harvested in two regions of the northeast of Portugal; higher values of water activity, protein, total phenols, and superior antioxidant activity were found in the samples harvested in Mogadouro while moisture, lipids, ash, fiber, and carbohydrates were higher in samples collected in Vimioso. Moisture, pH, protein content, lipids, fiber, ash, flavonoids, and β-carotene bleaching were within the established by the European regulations. On the other hand, reducing sugars, water activity, carbohydrates, and phenols did not fit into the proposed international recommendations. This mismatch suggests that drying conditions must be improved to satisfy the requirements and ensure safety and quality. Portuguese BP is an important source of total phenols and possesses good antioxidant properties which may differ according to the specific composition. However, it remains difficult to predict the potential therapeutic application of samples without a detailed chemical analysis.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Ananias Pascoal would like to thank Foundation for Science and Technology (FCT, Portugal), Programa Operacional Potencial Humano (POPH), and European Union (EU) for his Postdoctoral grant SFRH/BPD/91380/2012. The Research Support Foundation of the State of Amazonas (Fundação de Amparo à Pesquisa do Estado do Amazonas - FAPEAM) for the financial support to the project coordinated by Emerson Dechechi Chambó entitled "Physicochemical, microbiological characteristics and bioactive properties of honey from meliponines from Alto Solimões, Amazonas" (Notice 004/2017 - PPP).

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