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Exploring geographical influences on physicochemical characteristics of honey: the Montesinho Natural Park scenario

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Abstract

Objectives: In recent years, there has been a substantial increase in the global consumption of honey, driven by the high demand for natural products that offer health benefits. Consequently, consumers show a preference for honey, recognised for its superior quality, namely honey with a Protected Designation of Origin (PDO) or honey produced in protected areas, as it is associated with superior attributes and characteristics. Portugal is one of the leading countries in the production of PDO honey, with Montesinho Natural Park (MNP) being an excellent example of high-quality Portuguese honey, known for its distinctive attributes. However, environmental threats pose a double challenge, leading to a decline in honey production and compromising its overall quality. This study aimed to assess the specific physicochemical parameters and nutritional characteristics of MNP honey and investigate their correlation with the different locations of apiaries.

Materials and Methods: MNP honey samples (n=13) were obtained from local producers or purchased at supermarkets. Standard physicochemical parameters, such as 5-hydroxymethylfurfural, diastase activity, moisture and ash content, free acidity, electrical conductivity and pH, were determined according to honey legislation. Additionally, colour parameters, protein, low-molecular-weight carbohydrates (mono- and disaccharides), and mineral content were determined.

Results: The results obtained were consistent with the requirements outlined in the legislation and those described in the literature. The results suggest that geographical factors within the park and boundaries do not contribute to variations in the analysed parameters.

Conclusions: A significant level of homogeneity was evident in all parameters evaluated among the MNP honey samples. This is the first comprehensive study of the physicochemical properties of honey from various apiaries within the MNP.

Keywords: Honey; MNP; physicochemical properties; honey quality; geographical influences.

Introduction

The Montesinho Natural Park (MNP) is one of the largest natural parks of the 12 in Portugal. It is located in the Trás-os-Montes region, in the extreme north-east of the country. This park comprises a vast expanse of mountains, fields and pastures with a rich diversity of vegetation, including endemic and rare plant species, due to its great geological and climatic variability (Castro *et al.*, 2010). Activities related to the park are an essential source of socioeconomic development in the region, with honey production representing an important contribution. According to consumers, honey produced at MNP is considered to have unique organoleptic and health-related attributes, probably conferred by the park's special and unique vegetation and climatic conditions. Moreover, it has been a product with a Protected Designation of Origin (PDO) since 1994, representing a major boost in its economic appreciation. Nowadays, PDO certification is recognised by consumers, who associate these products with valued nutritional properties (Soares *et al.*, 2017b). In regard to honey consumption, nutritional interests are aligned with its health-related properties. In turn, the properties and quality of honey depend greatly on botanical and geographical origin, which corroborates consumer interest in honey produced under biological and PDO standards or produced in specific environments, such as natural parks (Soares *et al.*, 2017b). However, the quality of honey can be compromised during its production, as bees and the plants that provide nectar are subject to various environmental threats. Climatic change and its

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Figure 1. Montesinho Natural Park (MNP) map and location of apiaries corresponding to the honey samples analysed. 1, Lindolfo; 2, Pinela; 3, Merize; 4, Cabanelas; 5, Vale de Cavage; 6, Nuzedo de Cima; 7, Quiraz; 8, Montesinho; 9, Guadramil. M1 and M2, Vila Verde (Vinhais). C1 and C2, commercial honey samples originating from MNP.

consequences for animal and plant kingdoms affect honey production and the presence of honeybees. In recent years, honey production has declined due to a lack of vegetation and the collapse of honeybee colonies, which has led to an increase in fraudulent practices in honey production and the import of inferior quality honey. Preserving the delicate balance between honey production, the environment, and the honeybee population is a critical aspect of ensuring the continued availability of high-quality honey (Villalba *et al.*, 2020).

Thus, the evaluation of the physicochemical parameters of honey has become an important contribution to guaranteeing fair competition between producers, the commercial quality of products and the interests of consumers.

Honey is mainly composed of sugars, water and small amounts of other compounds such as proteins (including enzymes), amino acids and organic acids, phenolic compounds, vitamins, and minerals (Soares *et al.*, 2017b). The honey composition can be strongly influenced by botanical and geographical origin, as well as by climate, season, harvest time, processing, and storage conditions (Crăciun *et al.*, 2020). According to honey-related standards, moisture, ash, 5-hydroxymethylfurfural (HMF), sugar content, electrical conductivity (EC), diastase activity (DA), free acidity (FA), and pH are the recommended parameters to evaluate the honey quality (Codex Alimentarius, 2001; EU Commission, 2002). Other analytical techniques targeting honey compounds are used to investigate the authenticity of honey. In recent years, several studies have evaluated the characteristics of honey from different origins showing differences in parameters depending on the honey production region (Soares *et al.*, 2017a). Honey labelled and marketed as PDO from MNP is a composite product sourced from multiple apiaries located within the region. These apiaries are collectively managed by a beekeeping association, which enforces standardized protocols to ensure the consistent quality and production of honey. To determine the overall quality of MNP honey, it is essential to evaluate the specific contributions of each apiary; nonetheless, there is a glaring absence of data. Thus, this study aimed to provide the first comprehensive characterization of the main physicochemical properties of honey harvested at different apiaries in the MNP. For this purpose, honey samples from independent apiaries of MNP were evaluated for regimented parameters (Codex Alimentarius, 2001; EU Commission, 2002), as well as for colour, low weight carbohydrates, protein content and mineral and heavy metal contents.

Materials and Methods

MNP honey sampling

A total of 13 honeys were included in this study (Figure 1). Nine honey samples were obtained directly from local producers in 2021 in apiaries at different locations within the MNP (H1–H9). The preselection of apiaries was meticulously conducted to encompass a diverse spectrum of floral sources, environmental conditions, and a wide range of territories within the MNP. Two honey samples produced within the boundary limits of the MNP (M1 and M2) were acquired directly from producers and two honey samples commercially available and labelled as produced in MNP were purchased in local supermarkets (C1 and C2). All the samples were immediately transported to the laboratory and stored at room temperature in the dark.

The HMF content, DA, moisture, ash, FA, pH, and EC of the honey samples were determined according to the methods described in the International Honey Commission (IHC) harmonised methods (Bogdanov *et al.*, 2002).

5-Hydroxymethylfurfural

HMF content was determined by high-pressure liquid chromatography (HPLC) with diode array detection (HPLC-DAD) as previously described by Soares *et al.* (2017a), with minor changes. Briefly, 1 g of honey was diluted in 10 mL ultrapure water and then filtered through a 0.22-µm nylon syringe filter. An aliquot was injected (20 µL) into the Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an LC-20AD Prominence pump, a DGU-20A5 Prominence degasser, a CTO-10AS VP column oven, a SIL-20A HT Prominence autosampler, and an SPD-M20A photodiode array detector. The total run time was 15 min, using a Gemini C_{18} column (250 mm×4.6 mm, 5 µm; Phenomenex, Alcobendas, Spain) conditioned at 30 °C and eluted with isocratic of acetonitrile and water, at a ratio of 10/90 at a flow rate of 1.0 mL/min. HMF peak area was

acquired at 284 nm and peak identification in the samples was made by comparing retention time with the standard peak area acquired at 284 nm. The external calibration curve method (25 µg/L to 20 mg/L) was used for quantification of HMF in the samples. Results were expressed as mg/kg honey.

Diastase activity

Diastase enzyme activity in the honey samples was evaluated through a photometric method using commercial reagent tablets (Phadebas® Honey Diastase Test, Phadebas AB, Kristianstad, Sweden; Bogdanov *et al.*, 2002).

Moisture

The water content was determined by the refractometric method, using an Abbé refractometer (Bogdanov *et al.*, 2002). Briefly, the sample was heated to 50 °C to dissolve the sugar crystals and placed on the prism to fill it after cooling to room temperature. Readings (refractive index) were taken in triplicate at 20 °C. The corresponding moisture content was obtained from a table available in the literature (Bogdanov *et al.*, 2002).

Ash content

For the determination of total ash content, 5 g of honey was placed in previously calcined ceramic pots and preheated on an electric cooker until dark, to prevent the honey from foaming. Then, the samples were incinerated at 550 °C in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) for 5 h. After cooling at room temperature in a desiccator, the ash obtained was weighed. All determinations were performed in triplicate. The total ash content was the result of the difference between the weight of the ceramic pot+ash and the weight of the ceramic pot (weighed after calcination), considering the weight of the honey sample taken. Results were expressed as percentages (%) (Bogdanov *et al.*, 2002).

Free acidity and pH

The pH was measured using a pH meter, followed by titration to pH 8.3 to determine FA. Approximately 10 g of honey was homogenized in 75 mL of CO_2-free distilled water and the pH was potentiometrically measured at 20 °C using a Crison micropH 2002 (Crison Instruments, Barcelona, Spain). To determine FA, the same solution was titrated with 0.1 mol/L NaOH to pH 8.3 and the volume of NaOH spent was recorded. Both pH and FA determinations were performed in triplicate. FA results were expressed as milliequivalents of acid per kilogram of honey (Bogdanov *et al.*, 2002).

Electrical conductivity

A solution of 20 g of honey dry matter in 100 mL of distilled water was prepared and the EC was measured directly at 20 °C with a Crison Basic 30 conductivity meter (Crison Instruments). The cell constant K was calculated using the following formula: K=11.691×1/G, where G is the electrical conductance constant (mS). The EC of the honey samples was calculated using the formula S_H=KG, where K is the cell constant (cm^{-1}), and G is the conductance (mS), measured for each sample. The EC of the honey sample was expressed in mS/cm (Bogdanov *et al.*, 2002). All determinations were performed in triplicate.

Colour

Colour measurements were performed according to Soares *et al.* (2017a). A colorimeter (Chroma Meter CR 410, Konica Minolta, Tokyo, Japan), previously calibrated with a white standard tile, was used. A 5.5-cm diameter Petri plate was filled with gently homogenised honey samples prior to measurements. The colour indices L^*, a^*, and b^* were provided directly by the colorimeter, where L^* refers to the lightness component, a^* refers to intensity of red (+) and green (–), and b^* refers to intensity of yellow (+) and blue (–). The colour data corresponded to the average of 5 measurements in each honey sample.

Low-weight carbohydrate determination

The low-molecular-weight carbohydrates (mono- and disaccharides) were determined by HPLC with refractive index (RI) detection, as described in the IHC harmonised methods (Bogdanov *et al.*, 2002). The chromatographic analyses were performed using the HPLC (Jasco, Tokyo, Japan) apparatus under the conditions described in Santos *et al.* (2016). Sucrose, glucose and fructose in honey samples were identified by comparing peak retention times with those obtained from a standard mixture of sugars. For quantification, the external calibration curve ranging from 0.25 to 10 mg/L was used, and results were expressed as mg/kg. Unknown peaks of disaccharides were expressed as sucrose equivalents. D(+)-sucrose (≥99%-HPLC), D(+)-glucose (≥99.5%-GC), and D(-)-fructose (≥99%-HPLC) were obtained from Honeywell Fluka (Seelze, Germany).

Protein content

The protein content was determined by the Bradford method, using bovine serum albumin (BSA) as the standard (Chen *et al.*, 2019; Mureșan *et al.*, 2022). Aliquots (50 µL) of previously prepared honey/water solutions of the samples were transferred to wells on a 96-well plate containing 200 µL of Bradford reagent (Amresco, Solon, OH, USA). In a few minutes, the red colour of the mixture changed to blue due to the protein–dye reagent binding. The absorbance at 595 nm was measured on a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). Protein concentration was calculated using a calibration curve obtained with BSA at 0, 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL. The determination was performed in triplicate for each sample (Mureșan *et al.*, 2022).

Mineral and trace element content

Honey samples were mineralized by closed-vessel microwave-assisted acid digestion in an ETHOS EASY microwave oven (Milestone, Sorisole, Italy) equipped with an SK-15 easyTEMP high-pressure rotor. A sample mass of approximately 0.5 g was weighed directly into microwave oven modified polytetrafluorethylene (PTFE-TFM) vessels and 8 mL high-purity HNO_3 (≥69%, Puriss, Honeywell Fluka, Seelze, Germany) and 1 mL high purity H_2O_2 (30%, volume fraction; Suprapur®, Supelco, Steinheim, Germany) were added. Sample digestion was performed using the following microwave oven programme: a gradual increase in temperature for 20 min to 210 °C, followed by 15 min at 210 °C. After cooling to room temperature, the PTFE-TFM vessels were carefully opened and 0.5 mL high-purity

HCl (≥30%, TraceSELECT™, Honeywell Fluka, Steinheim, Germany) was added to the sample solutions before transferred in 50-mL polypropylene tubes. The final volume (25 mL) was adjusted with ultrapure water (>18.2 MΩ·cm at 25 °C), obtained with a Sartorius Arium® Pro water purification system (Gottingen, Germany). Sample blanks were prepared using the same procedure. The obtained solutions were stored at 4 °C until analysis. Each sample was processed in duplicate and at least one digestion blank was run in each digestion batch.

Determination of trace elements was carried out using an iCAP™ Q (Thermo Fisher Scientific, Waltham, MA, USA) inductively coupled plasma mass spectrometer (ICP-MS). Blanks and sample solutions were diluted with a diluent solution containing 2% (volume fraction) HNO_3, 0.5% (volume fraction) HCl, 500 µg/L Au (Gold Standard for ICP, 1000 mg/L; Honeywell Fluka, Steinheim, Germany), and 10 µg/L internal standards (Internal Standard Mix 1—SCP-IS7, 10 mg/L; SCP Science, Quebec, Canada). The elemental isotopes ^{7}Li, ^{9}Be, ^{23}Na, ^{25}Mg, ^{39}K, ^{43}Ca, ^{51}V, ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{87}Rb, ^{88}Sr, ^{98}Mo, ^{111}Cd, ^{121}Sb, ^{133}Cs, ^{137}Ba, ^{202}Hg, ^{205}Tl, ^{206}Pb, ^{207}Pb, and ^{208}Pb were measured for analytical determination and the elemental isotopes ^{6}Li, ^{73}Ge, ^{89}Y, ^{115}In, ^{159}Tb, and ^{209}Bi were monitored as internal standards.

For analytical quality control purposes, two certified reference materials (CRM) were used: hay powder (BCR-129) and bladderwrack (*Fucus vesiculosus*) powder (ERM-CD200), both from the European Commission's Joint Research Centre. The CRM were subjected to the same pretreatment and analytical procedure as the sample.

Statistical analysis

Data were reported as mean±standard deviation of at least duplicate experiments. Statistical analysis was performed using the SPSS 28.0 (SPSS Inc., Chicago, IL, USA) software. The normality of the data was assessed using the Shapiro–Wilk test and the homogeneity of variance was assessed using Levene's test. One-way analysis of variance (ANOVA) was employed to investigate potential differences among honey samples concerning the analysed parameters. Post-hoc comparisons were performed using Tukey's HSD test. Statistical significance was considered for $p<0.05$. Principal component analysis (PCA) was used as an unsupervised classification method for pattern recognition.

Results and Discussion

The European Commission has established limits for the physicochemical parameters defined as relevant for assessing the quality of different types of honey (EU Commission, 2002). Additionally, the development of studies over time has made it possible to establish relationships between their values and various factors such as botanical and geographical origin, harvest time and climatic conditions.

Different honey samples from MNP were analysed to assess their physicochemical characteristics. This study aimed to investigate how the distinctive geographical location of the park and the specific flora characteristics impact the quality and composition of honey. The results obtained for HMF, DA, moisture, ash, FA, pH, EC, and colour are summarized in Table 1.

Hydroxymethylfurfural and diastase activity

A large variation in HMF content was observed with values ranging from 0.67 to 4.79 mg/kg (mean: 2.09 mg/kg). Statistically significant differences were observed among the samples, with sample C2 (commercial) showing the highest content (4.79 mg/kg). All samples complied with the limit value (40 mg/kg) defined in current legislation. Regarding the DA, the results varied between 5.42 Gothe units and 27.22 Gothe units, indicating significant variability, with a mean value of approximately 18.83 Gothe degrees. Notably, the sample exhibiting the highest HMF content also exhibited the lowest DA activity (Sample C2—HMF: 4.79 mg/kg and DA—5.42 Gothe units). A higher HMF content and a lower DA can be the result of different harvest times and, consequently, the different climatic conditions to which the honeycombs were subjected. The relationship between harvest time and climatic conditions with HMF content and DA was observed by Pasias *et al.* (2018). According to their results, when comparing honey samples harvested in the same region but during different periods, HMF content values were higher in honey samples that have been exposed to meteorological conditions with temperatures above 32 °C for more than 3 months. On the other hand, the results showed that late harvest did not appear to affect DA (Pasias *et al.*, 2018).

Ash content

In the context of this study, the analysis of the honey samples revealed ash content values ranging from 0.55 to 1.27 g/100 g, indicating notable differences between the samples, with a mean value of approximately 0.81 g/100 g, as detailed in Table 1. The ash content in honey serves as an indicator closely associated with the presence of minerals and trace elements, as it signifies the inorganic residue remaining following the complete combustion of organic matter within the honey. The results obtained from assessing mineral and trace element content (outlined below) distinctly show that elements like potassium (K), which constitute approximately roughly 86% of the elements found in honey samples, exhibit a pattern that aligns consistently with the ash content. A higher ash content can suggest the potential presence of impurities or contaminants, whereas a lower ash content generally points to a more natural and pure product. Notably, upon comparing these findings with previous analyses of honey samples in Portugal (Corbella and Cozzolino, 2006), the obtained results exhibit similarities in ash content. However, compared with honey samples from different countries (da Silva *et al.*, 2016; Khan *et al.*, 2021; Al-Shehri *et al.*, 2022), the values obtained are notably lower. These outcomes provide compelling evidence supporting the purity and quality of the analysed honey samples.

Moisture

The moisture content of the honey samples varied between 15% and 17%, with an average value of 16%. No statistically significant differences were observed for this parameter. The values obtained are below the 20% limit defined in current legislation (EU Commission, 2002). Moisture content is an important quality parameter related to honey stability, as it influences crystallization, viscosity, palatability, specific weight, preservation and flavour (Rajindran *et al.*, 2022). A high water content in honey contributes to the formation of alcohol through fermentation, increasing the acidity of the

Table 1. Physicochemical characterization of honey samples from MNP (mean±standard deviation, *n*=2)

Sample	HMF content (mg/kg honey)	DA (Gothe degree)	Ash content (g/100 g)	Moisture (%)	Free acidity (mmol acid/kg honey)	pH	Electrical conductivity (mS/cm)	Colour		
								*L**	*a**	*b**
H1	1.87±0.08^{e}	20.72±0.51^{e}	0.59±0.04^{a}	15.30±0.90^{a}	33.5±0.7^{a}	4.67±0.01^{c,d}	0.121±0.01^{d}	24.77±0.01^{e}	–0.61±0.04^{b,c}	6.40±0.01^{f,g}
H2	0.69±0.00^{a,b}	14.79±0.23^{c}	0.75±0.04^{a,b,c}	15.40±0.20^{a}	32.5±2.8^{a}	5.18±0.04^{g,h}	0.147±0.00^{g}	23.22±0.01^{b}	–1.25±0.06^{a}	4.04±0.01^{a,b}
H3	0.67±0.01^{a}	17.68±0.07^{d}	0.98±0.21^{a,c,d}	15.20±0.40^{a,b}	38.0±5.6^{a,b}	5.21±0.04^{h}	0.151±0.00^{h}	23.41±0.03^{b,c,d}	–1.10±0.22^{a,b,c}	4.74±0.05^{b,d}
H4	3.27±0.02^{h}	27.22±0.18^{i}	0.57±0.04^{a}	16.50±0.10^{a}	35.0±1.4^{a,b}	4.53±0.04^{b}	0.129±0.00^{e}	24.23±0.02^{e,f}	2.91±0.08^{e,f}	5.93±0.02^{e,f}
H5	1.15±0.01^{c,d}	21.77±0.07^{e,f}	0.61±0.02^{a,b}	15.50±0.10^{a}	30.0±2.8^{a}	5.10±0.00^{g}	0.138±0.001^{f}	23.51±0.06^{c,d,e}	2.81±0.06^{e,f}	4.56±0.00^{b,c}
H6	2.67±0.00 ^{g}	25.31±0.39^{h}	0.55±0.01^{a}	15.80±0.20^{a}	30.7±1.1^{a}	4.82±0.02^{e}	0.120±0.00^{d}	24.34±0.01^{e}	5.79±0.04^{h}	7.61±0.05^{h}
H7	2.73±0.06^{g}	23.00±0.20^{f,g}	0.82±0.05^{a,b,c}	16.20±0.00^{a}	34.5±8.5^{a,b}	4.58±0.00^{b,c}	0.129±0.001^{e}	23.64±0.01^{d,e}	2.71±0.17^{e}	5.55±0.41^{d,e}
H8	1.07±0.01^{c}	13.12±0.32^{b}	0.66±0.00^{a,b,c}	16.20±0.20^{a}	32.5±0.7^{a}	4.98±0.01^{f}	0.127±0.001^{e}	22.75±0.04^{a}	–0.47±0.03^{c}	3.40±0.33^{a}
H9	1.02±0.01^{b,c}	18.67±0.01^{d}	nd^{a,b,c}	15.40±0.40^{a}	39.0±2.8^{a,b}	4.32±0.03^{a}	0.089±0.001^{a}	27.71±0.01^{g}	7.88±0.03^{j}	11.20±0.00^{i}
M1	1.45±0.06^{d}	23.47±0.21^{g}	1.07±0.18^{d}	16.60±0.40^{a}	43.2±0.3^{a,b}	4.76±0.01^{d,e}	0.139±0.00^{f}	23.12±0.01^{b}	1.78±0.05^{d}	3.98±0.01^{a,b}
M2	3.60±0.13^{h}	18.04±0.14^{d}	0.76±0.03^{c,d}	17.00±0.60^{a,b}	nd^{a,b}	nd^{d,e}	0.117±0.00^{c}	23.46±0.01^{c,d}	1.92±0.05^{d}	4.30±0.04^{b}
C1	2.22±0.06^{f}	15.60±0.21^{c}	1.27±0.10^{c,d}	15.90±0.10^{a}	49.0±1.4^{c}	4.40±0.00^{a}	0.113±0.001^{b}	24.36±0.04^{b,e}	3.33±0.09^{f}	5.31±0.06^{c,d,e}
C2	4.79±0.10^{i}	5.42±0.04^{a}	1.05±0.10^{a,b,c}	15.80±0.00^{a}	35.7±7.4^{a,b}	4.37±0.04^{a}	0.088±0.00^{a}	23.31±0.17^{b,c}	5.07±0.10^{g}	7.08±0.03^{g,h}
Mean	2.09	18.83	0.81	15.91	36.1	4.74	0.124	23.99	2.37	5.70
Min	0.67	5.42	0.55	15.20	30.0	4.32	0.088	22.75	(–)1.25	3.40
Max	4.79	27.22	1.27	17.00	49.0	5.21	0.151	27.71	7.88	11.20

Different letters in the same column indicate significant differences (P<0.05) between the samples for a specific parameter.

nd: not determined.

honey and, consequently, the acidic taste (Rajindran *et al.*, 2022). Therefore, honey is expected to have low moisture content to ensure its resistance to deterioration by fermentation and granulation during storage, thus increasing its shelf life (El Sohaimy *et al.*, 2015). The moisture values obtained in this work are similar to each other, which corroborates previous studies that proved the dependence of this parameter on the harvest time and the degree of maturity reached in the beehive (Bogdanov *et al.*, 1999; Rodríguez *et al.*, 2004; Karabagias *et al.*, 2018), as well as the temperature, relative humidity and floral origin of the environment surrounding the beehives during honey production (Corbella and Cozzolino, 2006; Khan *et al.*, 2021; Al-Shehri *et al.*, 2022).

Free acidity and pH

In the present work, FA was below the limits of Codex Alimentarius and European regulations (Codex Alimentarius, 2001; EU Commission, 2002) (50 mEq/kg) in all tested samples, indicating the absence of undesirable fermentation. The values obtained varied between 30.0 mmol of acid/kg of honey and 49.0 mmol of acid/kg of honey, showing considerable variability in free acidity among the samples, with a mean value of approximately 36.1 mmol of acid/kg of honey. The pH values obtained in the analysed honey samples varied between 4.32 and 5.21, indicating high values compared with the literature (da Silva *et al.*, 2016; Silva *et al.*, 2017).

FA and pH in honey are related to botanical origin, resulting in the natural presence of organic acids and amino acids (Oroian, 2012; Majewska and Wolosiak, 2019). Therefore, it is expected to observe some variability of these parameters in honey with different botanical origins. The relationship between acidity and botanical origin was evaluated by Majewska and colleagues (Majewska and Wolosiak, 2019). According to their work, light honey had a considerably lower content of organic acids than dark honey (Majewska and Wolosiak, 2019). Although limit values for pH are not established in honey legislation, low pH values are preferred, as they prevent microbiological contamination and may indicate high mineral content (El Sohaimy *et al.*, 2015).

Electrical conductivity

The EC values in the analysed samples ranged from 0.088 to 0.151 mS/cm, which were under the maximum limit set by legislation (0.8 mS/cm) for all samples (EU Commission, 2002). The results show some variability, with a mean value of approximately 0.124 mS/cm. In a study conducted by Alves *et al.* (2013) using Portuguese honey, the EC results fell within the legislation, ranging from 0.1 to 0.8 mS/cm. The authors noted that monofloral honey from heather exhibited the highest EC values, while monofloral honey from rosemary displayed the lowest values. Thus, the results obtained in our current research align with expectations, as honey produced in the MNP is a multifloral honey and contains components from both plant species. Furthermore, Alves *et al.* (2013) observed higher EC values in honey samples rich in organic acids and inorganic matter, suggesting a relationship between these parameters. Similarly, the findings in this study regarding ash content (inorganic matter) mirrored the trends observed in EC. Within the MNP honey samples, H3 exhibited the highest ash content and EC values, measuring at 0.98 g/100 g and 1.151 mS/cm, respectively. Conversely, the H6 honey sample had the lowest ash content and the second lowest EC, at 0.55 g/100 g and 0.120 mS/cm, respectively (the lowest EC value corresponded to a honey sample that did not yield enough material for ash content assessment).

Colour

The colour results obtained in this work are presented in Table 1. The colour measurements of the honey samples ranged from 22.75 to 27.71 for L^*, from −1.25 to 7.88 for a^*, and from 3.40 to 11.20 for b^*. L^* corresponds to luminosity; for a^*, (+) values correspond to red and (−) values to green; for b^*, (+) values correspond to yellow and (−) values to blue (Syahriati *et al.*, 2021). According to Syahriati *et al.* (2021), for values of $L^*<50$ with red and yellow components, honey samples are considered dark honey.

Along with the physicochemical parameters, colour is an important organoleptic parameter of honey, being one of the main characteristics responsible for consumer choice and being highly related to the price of honey on the world market (da Silva *et al.*, 2016). The importance of evaluating this parameter is due to its relationship with the composition of the honey. Previous studies have indicated a close correlation between honey colour and different composition parameters, such as EC and mineral, ash and pollen content (González-Miret *et al.*, 2005; Iglesias *et al.*, 2012; Habib *et al.*, 2014; da Silva *et al.*, 2016), highlighting the contribution of botanical and geographical origins to honey colour. On the other hand, the colour of honey also depends on external factors, such as temperature and storage time (da Silva *et al.*, 2016).

Low-weight carbohydrates

The content of the main sugars was determined in the 13 honey samples studied in this work. The results are presented in Table 2. The fructose content ranged from 36.6 to 42.2 g/100 g and glucose content ranged from 20.2 to 27.6 g/100 g. Honey legislation establishes minimum limits for the sum of these two main sugars: 60 g/100 g for nectar honey and 45 g/100 g for honeydew honey or a mixture of both. Based on the results obtained, all honey samples complied with the legislation for honey from nectar. The fructose/glucose ratio (F/G) is another important factor related to the quality of honey. F/G is a predictor of the tendency for honey to crystallize and should range from 0.9 to 1.35. Once the ratio exceeds 1.0, the crystallization process becomes increasingly slower, while a lower ratio leads to faster crystallization (Alghamdi *et al.*, 2020). According to the results, the F/G ranged from 1.46 to 1.99, indicating that the probability of the studied honey samples crystallizing was is reduced. Additionally, the addition of fructose or glucose is reflected in the F/G (usually 1–1.2 in pure honey) and a significant deviation from this ratio may indicate that the honey has been adulterated (Siddiqui *et al.*, 2017). Although the F/G values exceeded the limit for all analysed samples, the similar values observed between them imply that this deviation is likely due to regional factors and not adulteration. Thus, the F/G values obtained can be considered characteristic of this region.

Protein content

Although proteins are present in small quantities in honey, they play an essential role in honey's health properties (Erban *et al.*, 2019). The total protein content of honey samples ranged from 0.21 to 0.36 mg/g (Table 2), which is in agreement with previous studies (Mureșan *et al.*, 2022).

Table 2. Low-weight carbohydrates (unit: g/100 g) and protein content of honey samples from the MNP (mean±standard deviation, *n*=2)

Sample	Fructose (g/100 g)	Glucose (g/100 g)	Disaccharides (g/100 g)	F+G (g/100 g)	F/G	Protein content (mg/g honey)
H1	37.0±1.1^{a}	25.4±0.2^{a,b}	10.9±0.7^{a,b}	62.4±1.3^{a}	1.46±0.03^{a}	0.28±0.02^{e,f}
H2	38.1±0.2^{a}	25.2±0.4^{a,b}	10.8±0.03^{a,b}	63.3±0.6^{a}	1.52±0.01^{a}	0.21±0.01^{a}
H3	38.7±0.7^{a}	25.2±0.3^{a,b}	11.0±0.1^{a,b}	63.8±1.0^{a}	1.54±0.01^{a,b}	0.25±0.00^{b}
H4	39.5±0.4^{a}	23.3±0.4^{a,b}	10.8±0.6^{a,b}	62.8±0.03^{a}	1.70±0.05^{a,b}	0.36±0.01^{h}
H5	36.6±0.9^{a}	23.0±0.4^{a,b}	11.6±0.05^{b,c}	59.6±1.3^{a}	1.59±0.01^{a,b}	0.26±0.01^{b,c,d}
H6	41.5±1.5^{a}	22.7±0.6^{a,b}	13.9±0.2^{c,d}	64.2±2.1^{a}	1.82±0.02^{a,b}	0.28±0.01^{d,e,f}
H7	38.7±2.1^{a}	24.4±1.3^{a,b}	11.1±0.3^{a,b}	63.1±3.4^{a}	1.58±0.01^{a,b}	0.34±0.03^{g,h}
H8	39.5±1.9^{a}	25.2±1.2^{a,b}	11.2±1.1^{a,b}	64.6±3.1^{a}	1.57±0.00^{a,b}	0.32±0.03^{g}
H9	40.1±1.9^{a}	27.1±1.5^{b}	8.9±0.1^{a}	67.2±3.4^{a}	1.48±0.01^{a}	0.26 ± 0.00^{b,c}
M1	38.7±1.7^{a}	24.8±0.1^{a,b}	11.3±0.6^{a,b}	63.5±1.8^{a}	1.56±0.04^{a,b}	0.27±0.01^{e,f}
M2	39.1±0.4^{a}	22.7±0.4^{a,b}	14.4±0.01^{d}	61.8±0.8^{a}	1.72±0.01^{a,b}	0.30±0.03^{c,d,e}
C1	42.2±0.1^{a}	27.6±0.7^{b}	9.2±0.03^{a,b}	69.7±0.8^{a}	1.53±0.04^{a,b}	0.28±0.01^{b,c,d,e}
C2	39.4±0.1^{a}	20.2±2.9^{a}	14.3±0.1^{d}	59.6±3.0^{a}	1.99±0.28^{b}	0.27±0.01^{f}
Mean	39.1	24.4	11.5	63.5	1.62	0.28
Min	36.6	20.2	8.9	59.6	1.46	0.21
Max	42.2	27.6	14.4	69.7	1.99	0.36

Different letters in the same column indicate significant differences (P<0.05) between samples for a certain parameter.

As expected, the results showed similar values for all samples, which is related to the location of the apiaries and the same predominance of botanical species around the beehives. Additionally, the MNP has only one predominant bee species, *Apis mellifera iberiensis*, contributing to the similarity between values. However, significant differences were observed between sample H2 and the remaining samples, presenting the lowest protein content (0.21 mg/g).

Mineral and trace element content

The results obtained in the determination of minerals and trace elements are summarised in Supplementary Table 1. Mineral K was the most abundant element with a mean content of 3027 μg/g, followed by Mg (mean: 256.4 μg/g) and Ca (150.5 μg/g). Na and Mn were present with mean contents of 34.1 μg/g and 27.3 μg/g, respectively. All other elements presented contents below 10 μg/g. Statistically significant differences were observed for all analysed elements. Notably, sample H2, which is rich (P<0.05) in Mg, Ca, Mn, Sr, and Ba, and sample C1 presented the highest content (P<0.05) of Co, Zn, and Cu. In general, the mean contents of K (3027 μg/g), Mg (256 μg/g), Ca (150 μg/g), Na (34.1 μg/g), Mn (27.3 μg/g), and Cu (1.08 μg/g) were higher than those reported for honey from other regions of Portugal (K (292.7 μg/g; 701.9 μg/g), Na (13.1 μg/g; 31.0 μg/g), Ca (20.1 μg/g; 28.4 μg/g), Mg (13.3 μg/g; 74.0 μg/g), Mn (2.78 μg/g), and Cu (0.65 μg/g; Alves *et al.*, 2013; Silva *et al.*, 2017). The element content was found to vary widely and in agreement with previous studies on the mineral composition of honey (Alves *et al.*, 2013). The well-recognized toxic elements Pb and Cd were undetectable. These findings strongly confirm the high quality and safety of the analysed honey samples.

Statistical analysis

PCA was performed to explore the impact of the geographical distribution of apiaries on the studied parameters (Figure 2). This statistical method was used to identify hidden patterns in the data set and to identify correlated variables. The 15 parameters under study were selected because all of them displayed a correlation coefficient above 0.5 and/or below –0.5 (the level at which the variables were considered important) in the first two components (PC1 and PC2). Both Bartlett's test for sphericity (P<0.001) and Kaiser–Meyer–Olkin (KMO) test (0.681) results indicate that a dimension-reduction technique such as PCA can be applied to the obtained data set. The results showed distinct relationships between variables and geographical locations of the apiaries. The first two components (PC1 and PC2) retained 73% of the total variance of the whole data set. The biplot of the 15 selected parameters is shown in Figure 2. Principal component 1 (PC1, which explains 56% of the total variance) was positively loaded by EC, pH, Mg, Ca, K, Mn, Sr, Cu, and Ba and negatively loaded by a^*, b^*, L^*, and HMF. PC2 (which explains 17% of the total variance) was positively loaded by b^*, L^*, and Ba and negatively loaded by moisture and protein.

Three main clusters were clearly observed, one composed of samples H9 and C2 (primarily driven by their similarities in $L^*a^*b^*$ parameters and HMF content, as indicated by their positions in the PCA biplot and the loadings of these parameters on the relevant principal components), another composed of samples H2 and H3 (honey rich in Mg, Ca, K, Mn, Sr, Cu, and Ba) and the last was composed of all other samples (H1; H4–H8; C1; M1 and M2). The contents of alkali-earth elements (Mg, Ca, Sr, and Ba) as well as those of Cu and Mn seem to be good predictors for honey classification according to their origin. In addition, pH, EC and colour parameters are also very important parameters when trying to discriminate honey samples according to their origin. The relationship between honey physicochemical parameters and its botanical and geographical origin has been explored by several authors (Alves *et al.*, 2013; El Sohaimy *et al.*, 2015; Siddiqui *et al.*, 2017; Pasias *et al.*, 2018; Majewska and Wolosiak, 2019;

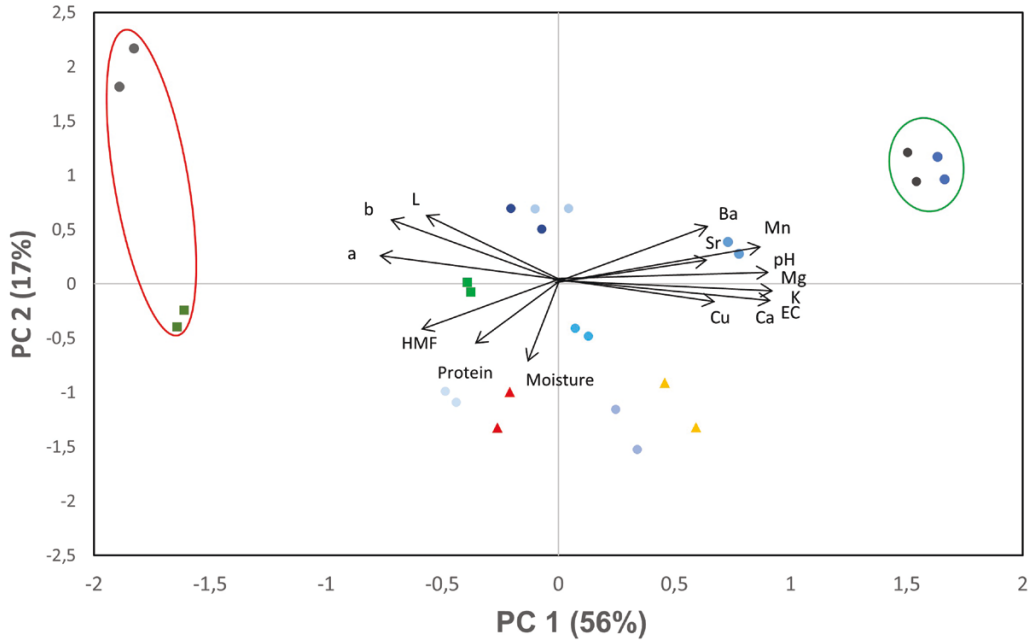

Figure 2. PCA biplot for the 15 honey parameters under study. Circles represent honey samples from the MNP, triangles represent honey samples from the boundary's limits of the MNP (red triangles, M2; yellow triangles, M1), and squares represent commercial honey samples (dark green triangles, C2; soft green triangles, C1).

Crăciun *et al.*, 2020). Composition parameters are highly dependent on plants visited by honeybees and the surrounding environment of honeycombs, allowing conclusions to be made about honey origin in addition to its quality (El Sohaimy *et al.*, 2015; Majewska and Wolosiak, 2019).

In recent years, both plants and honeybees have faced numerous environmental threats, significantly impacting honey production and the survival of honeybee colonies. Climate fluctuations have led to a scarcity of plants essential for honeybee nutrition, while pesticide usage near apiaries has not only contributed to a decline in honeybee colonies but also compromised the quality of honey (Soares *et al.*, 2017b; Villalba *et al.*, 2020). Therefore, selecting natural and protected regions for beekeeping has emerged as a potential strategy. The MNP stands as one such area, demonstrating the production of high-quality honey, as supported by the findings of this study.

Conclusions

Honey labelled and marketed as PDO from MNP is a natural product derived from different apiaries located within the park. To evaluate the specific contributions of the geographic location of each apiary in MNP honey, a comprehensive evaluation of physicochemical parameters was carried out on honey samples collected from different apiaries within the park. The study also included commercially available honey samples labelled as originating from the MNP, along with honey samples from apiaries located along the park boundaries. Notably, this is the first comprehensive study aiming to characterise honey from different apiaries within the MNP.

The results are in accordance with current legislation and within the values reported in the literature; therefore, proving the quality of the honey produced in the MNP. The results from PCA showed that the parameters colour, HMF and Mg, Ca, K, Mn, Sr, Cu, and Ba contents contributed to the clustering of honey samples, showing that the geographical location of the apiaries could contribute to the differences observed in the honey samples. However, these differences do not appear to compromise the quality of the final product (honey commercialized as PDO from MNP). In fact, when assessed individually, each apiary's honey parameters fall within the expected range for high-quality honey. Nevertheless, further investigation is needed to better understand the relationship between the geographical origin of MNP honey and its quality parameters. Furthermore, investigating quality parameters under different environmental conditions within the MNP could enhance the understanding of this premium product and offer crucial insights for maintaining beekeeping in the region.

Supplementary Material

Supplementary material is available at *Food Quality and Safety* online.

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Author Contributions

Conceptualization: all authors; Data curation: Sónia Soares; Investigation, Sónia Soares, Rui Azevedo, and Olga Viegas; Methodology: Sónia Soares, Leandro Magalhães, Manuela M. Moreira, Diana Rede, Virgínia Cruz Fernandes, Olga Viegas, Edgar Pinto, and Rui Azevedo; Formal analysis: Sónia Soares, Leandro Magalhães, Manuela M. Moreira, Diana Rede, Olga Viegas, Edgar Pinto, and Rui Azevedo; Writing original draft preparation: Sónia Soares; Review and editing: all authors; Supervision and validation: Cristina Delerue-Matos; Project administration: Cristina Delerue-Matos; Funding acquisition: Cristina Delerue-Matos; Resources: Cristina Delerue-Matos. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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