INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Characterization and authentication of the honeydew honey from *Quercus pyrenaica* from Montesinho Natural Park

Rania SLAMA

Dissertation submitted to Escola Superior Agrária de Bragança to obtain the Degree of Master in Quality and food safety under the scope of the double diploma with ULT

Supervised by

Doctor Soraia Isabel Domingues Marcos Falcão

Professor Doctor Miguel José Rodrigues Vilas Boas

Bragança

2021/2022

Characterization and authentication of the honeydew honey from *Quercus pyrenaica* from Montesinho Natural Park

Trabalho financiado pelo Projeto ACORNDEW (MTS/SAS/0099/2020), financiado pela Fundação para a Ciência e Tecnologia (FCT), no âmbito Programa Internacional de Investigação sobre Montesinho "Montesinho International Research Awards". Este trabalho foi também parcialmente financiado pelo CIMO UID/AGR/00690/2020) e SusTEC (LA/P/0007/2021).

IMPULSO À SUSTENTABILIDADE
DA FLORESTA DE CARVALHOS
DO PARQUE NATURAL
DE MONTESINHO
ATRAVÉS DA INOVAÇÃO:
VALORIZAÇÃO
DA BOLOTA E DA MELADA
DO CARVALHO NEGRAL

ACKNOWLEDGMENTS

It fills my heart with joy to express my gratitude to everyone who contributed in the successful accomplishment of my thesis.

My intense gratification of my supervisors whom their contribution has raised the quality of this thesis. They have always supported me and have given me enthusiasm for research, they have patiently guided me. I'm also very grateful to the CIMO team for their great support and their kind care.

A huge thanks goes to my lovely and friendly supervisor **Dr. Soraia Isabel Domingues Marcos Falcão** for her guidance, understanding, patience and most importantly long time and tremendous effort to offer every possible help, she has provided positive encouragement and a warm spirit to finish this thesis with her constant warm smile. It has been a great pleasure and honor to have her as my supervisor.

Immeasurable appreciation and deepest gratitude for **Dr. Miguel José Rodrigues Vilas Boas** for his extended help and support, who in one way or another have contributed in making this study possible with his valuable comments and suggestion.

I am using this opportunity to acknowledge **Andreia Tomas** and **Mouffida Kheira** who supported me throughout this thesis. I am thankful for their invaluably constructive criticism and friendly advice during the project work. I am sincerely grateful to them for sharing her truthful and illuminating views on a number of issues related to the project.

The persons with the greatest indirect contribution to this work: my parents, their presence and their encouragement are for me the founding levels of what I am and what I do, I want to deeply thank them, my father "**Chiheb SLAMA**", my mother "**Houyem KACEM**" as well as my little sister "**Amira SLAMA**" for their constant encouragements.

I am also thankful to my friends, for being present. you will recognize yourselves!

Abstract

The commercial interest in honeydew honeys is increasing due to the differentiated nutritional, sensorial and therapeutic characteristics of this honey. In this context, this work aimed to characterize the production of honeydew honey from *Quercus pyrenaica* oak and identify marker compounds that discriminates the botanical origin of this honey. For that, 42 honey samples were collected in apiaries located in Montesinho Natural Park, Bragança, Portugal, during September of 2021. To achieved the honey characterization, the evaluation of the physicochemical parameters (colour, humidity, acidity, electrical conductivity, diastase index, HMF, proline), as well as, the chemical characterization though mineral analysis (by atomic absorption spectrophotometry), sugarcontent (through high-pressure liquid chromatography with a refraction index detector (HPLC-RI)), proteins (by the Kjeldahl method)) and phenolic compounds (by liquid chromatography coupled to mass spectrometry detector (LC-MS), were performed.

The results of the physio-chemical parameters analysis showed that the color ranged from 130 to 150 mm pfund, corresponding to a dark amber color, while the moisture ranged from 14.4 to 18.5 %. The electrical conductivity varied from 0.93 to 1.4 mS.cm^{-1} with ash levels between 0.45 to 0.74 g/100g. The amount of HMF range from 0 to 24 mg.kg^{-1}, while the diastase varied from 9 to 33 DN. The studied honey samples have pH values ranging between 3.73 and 4.94. The results for free acidity, determined at the equivalence point (pH 7) ranged from 0.19 to 1.026 meq.kg-1 and at the equivalence point (pH 8.3) ranged from 0.325 to 1.28 meq.kg-1. The lactone acidity values of the samples varied between 7.17 and 10.3 meq.kg-1 Finally, the proline content ranged from 0.90 to 8.1 mg.g^{-1}. The analysis of the sugar content showed fructose being the most abundant monosaccharide followed by glucose, ranging from 34 to 36% and 25 to 32%, respectively. The presence of erlose and melezitose was an indication of honeydew. Protein content ranged from 0.42 to 0.96 g/100g honey. Regarding phenolic compounds, 17 phenolic compounds were identified in the honeydew honey samples, where flavonoids were the most abundant compounds, specially chrysin and pinobanksin-5-methyl ether, followed by phenolic acids, specially, caffeic acid and ellagic acid.

Keywords: honeydew honey; black oak; chemical markers; *Apis mellifera*

Resumo

O interesse comercial pelos méis de melada tem vindo a crescer devido às características nutricionais, sensoriais e terapêuticas diferenciadoras deste mel. Neste contexto, este trabalho teve como objetivo caracterizar a produção de mel de melada de carvalho *Quercus pyrenaica* e identificar compostos marcadores que discriminam a origem botânica deste mel. Para tal, foram recolhidas 42 amostras de mel em apiários localizados no Parque Natural de Montesinho, Bragança, Portugal, durante o mês de setembro de 2021. Para a caracterização do mel procedeu-se à avaliação dos parâmetros físico-químicos (cor, humidade, acidez, condutividade elétrica, índice de diastase, HMF, prolina), bem como, à caracterização química através da análise dos minerais (por espectrofotometria de absorção atómica), teor de açúcares (por cromatografia líquida de alta pressão com detetor de índice de refração (HPLC-RI)), proteínas (pelo método Kjeldahl)) e compostos fenólicos (por cromatografia líquida acoplada a detetor de espectrometria de massas (LC-MS).

Os resultados da análise dos parâmetros físico-químicos mostraram que a cor variou de 130 a 150 mm pfund, correspondendo a uma cor âmbar escura, enquanto a humidade variou de 14,4 a 18,5 %. A condutividade elétrica variou de 0,93 a 1,4 $mS.cm^{-1}$, enquanto os teores em cinza entre 0,45 a 0,74g/100g. A quantidade de HMF variou de 0 a 24 $mg.kg^{-1}$, enquanto a diástase variou de 9 a 33 DN. As amostras de mel estudadas apresentam valores de pH variando entre 3,73 e 4,94. Os resultados de acidez livre, determinados no ponto de equivalência (pH 7) variaram de 0,19 a 1,026 $meq.kg^{-1}$ e no ponto de equivalência (pH 8,3) variaram de 0,325 a 1,28 $meq.kg^{-1}$. Os valores de acidez lactona das amostras variaram entre 7,17 e 10,3 $meq.kg^{-1}$. Por fim, o teor de prolina variou de 0,90 a 8,1 $mg.g^{-1}$. A análise do teor de açúcar mostrou que a frutose é o monossacarídeo mais abundante seguido da glicose, variando de 34 a 36% e 25 a 32%, respetivamente. A presença de erlose e melezitose é uma indicação de melada. O teor de proteína variou de 0,42 a 0,96 g/100g de mel. Em relação aos compostos fenólicos, 17 compostos fenólicos foram identificados nas amostras de mel de melada, onde os flavonoides foram os compostos mais abundantes, especialmente a crisina e a pinobanksin-5-metil éter, seguidos dos ácidos fenólicos, em especial o ácido cafeico e o ácido elágico.

Palavras-chave: Mel de melada; carvalho negral, marcadores químicos, *Apis mellifera*

Table of contents

Acknowledgements…………………………………………………………………...i

Abstract ii

Resumo iii

Table of contents iv

List of figures vii

List of table.. viii

Abbreviation list…………………………………………………………….....ix

Chapter I: Literature review 1

1. Production of honey in Europe..2

2. Honey bees (*Apis mellifera*)...3

3. General information about honey 4

3.1. Definition...4

3.2. Honey origin..4

3.3. Difference between nectar honey and honeydew honey 4

4. Honey composition...6

4.1. Sugars...6

4.2. Nitrogen compounds...7

4.2.1. Proteins 7

4.2.2. Amino acids...8

4.2.3. Enzymes 8

4.3. Vitamins 9

4.4. Volatile compounds..9

4.5. Phenolic compounds 10

4.6. Organic acids 11

4.7. Mineral contents 11

5. Melissopalynological analysis 12

6. Quality and physiochemical parameters of honeydew honey 12

6.1. Color...12

6.2. Moisture content 13

6.3. Ash content...14

6.4. Electrical conductivity 14

6.5. pH and free acidity..15

6.6. Hydroxymethylfurfural 15

6.7. Diastase activity 16

6.8. Antioxidant activity 17
6.9. Anti-inflammatory activity 18
7. Objectives 18
Chapter II : Materials and methods...20
1.Sampling 20
2. Honey analysis 22
3. Physico-chemical parameters of honeydew honeys 22
3.1 Moisture content 22
3.2 Color 22
3.3 Electrical conductivity 23
3.4. pH, free and lactonic acidity...23
3.5 Proline 24
3.6 Hydroxymethylfurfural (5-HMF) 25
3.7 Diastase activity...25
4. Chemical characterization..26
4.1 Sugars 25
4.2.Protein content...27
4.3 Ashes 27
4.4 Carbohydrates..28
4.5 Energy..28
4.6 Minerals...28
4.6.1 Sample digestion..28
4.6.2 Potassium and Sodium..28
4.6.3 Calcium and Magnesium..29
4.6.4 Iron...30
4.6.5 Manganese, Copper and Cadmium...30
4.7 Total phenolic content..31
4.7.1 Phenolic compound profile...31
ChapterIII: Physiochemical parameters...35
1.Color. 35
2. Moisture content. 37
3. Electrical conductivity...37
4. pH, free acidity and lactonic acidity
....38
5.Hydroxymethylfurfural. 40
6.Diastase activity..42
7.Proline 42
7.Ash content...43
8. Protein content...44
9. Total carbohydrates and energy..45

10. Sugars 45
11. Total phenolic compounds. 48
12.Mineral content………………………………………………………………….50
Chapter IV : CONCLUSION AND WORK PERSPECTIVE 54
Bibliographic References 57
Webography 59

List of figures

Figure 1 : Apis mellifera 3

Figure 2: Quercus ilex acorns showing honeydew secretions. 6

Figure 3: Structure of hydroxymethylfurfural 16

Figure 4: Geographical origin for honey samples 20

Figure 5: refractometer...22

Figure 6: Colorimeter..23

Figure 7: Conductivity meter...23

Figure 8: Automatic titrator..24

Figure 9 - UHPLC/DAD/ESI-MSN equipment…………………………………………...32

Figure 10 – Chromatographic profile at 280 nm of sample Rio de Fornos 101…………49

List of table

Table 1: Statistics about honey in Portugal 3

Table 2: Geographical origin and year of collection of honey samples 211

Table 3: Range of concentrations for each standard, and respective calibration equation 27

Table 4: Physical-chemical parameters: color, humidity, and conductivity...................................35

Table 5: pH and acidity of the honey samples..39

Table 6: Physio-chemical honey parameters: 5-HMF, diastase index, and proline.........................41

Table 7: Nutritional parameters: ashes, proteins, energy, and carbohydrates 43

Table 8: sugar profile, obtained by HPLC-RI, of the studied honey sample………………………46

Table 9: Averages of Sugar profile, obtained by HPLC-RI, of the studied honeydew honey

classified by apiary (values expressed in %) 46

Table 10: average results of F/G (fructose/glucose) and G/H (glucose/humidity) ratios.................48

Table 11: Phenolic and isoprenoid compounds identified by UHPLC/DAD/ESI-MS^n...................49

Table 12: Average of Phenolic compounds quantification in the honey samples classified by apiary

expressed in mg/kg 50

Table 13: Minerals contents, obtained by using flame atomic absorption

spectrophotometer………………………………………………………….…………….51

Abbreviation list

HMF- Hydroxymethylfurfural

DPPH- 2, 2-diphenyl-1-picrylhydrazyl-hydrate

EC- European Commission

EU- European Union

HPLC-RI- High-pressure Chromatography Coupled to a Refractive Index Detector.

IHC- International Honey Commission

GC-MS- Gas-Chromatography Coupled to Mass Spectrometry

LC-MS- Liquid Chromatography Coupled with Mass Spectrometry

Abs – Absorbance

GAE- Gallic acid equivalents

HPLC– High pressure liquid chromatography

LC- Liquid chromatography

T_R - Retention time

MS- Mass spectrometry

UPLC/DAD/ESI-MS^n - Ultra-pressure liquid chromatography with photodiode detection coupled to tandem mass spectrometry with electrospray ionization.

TPC – Total phenolic compounds

EC- Electrical conductivity

Chapter I: Literature review

Chapter I: Literature review

1. Production of honey in Europe

Europe is the world's second largest honey producer after China (Bicudo de Almeida-Muradian et al., 2020). Despite of this, European honey market give a demonstration of a structural imbalance between request and domestic production, as around 40 % of Europe's consumption needs are fulfilled by extra-European sources (Terrab et al., 2019), being only 60% self-sufficient in honey (Bicudo de Almeida-Muradian et al., 2020). In fact, honey imports have increase notably since 2011. It happened because of a reduce in the significance of the European beekeeping sector, modifications of climatic conditions, bees' diseases, and the insistent use of chemicals, which are lethal to bees, in agriculture. Germany is the main European importer and consumer of honey, representing around 26 % and 23 % of the volume of European honey imports and consumption, respectively (Terrab et al., 2019).

The Center and Northeast region of Portugal is traditionally known for good quality honey production, principally *Eucalyptus globulus* (produced in North Coast), *Erica* spp., *Castanea sativa* Mill., among other multifloral and honeydew honeys (Karabagias et al., 2018).

Portugal has a favorable climate for the beekeeping activity. Nonetheless, in the historical background, apiculture has been considered as a hobby or complemental activity to agriculture. Nowadays, in spite of a decreased number of beekeepers, the volume of honey bee farms and the total of full-time commercial beekeepers increased (from 4 % in 2013 to 10 % in 2015), as a result strengthening honey's production capacity(Ribeiro et al., 2019).

Portuguese honey production has grown significantly from 2000 to 2015, around 158%, producing 11521 tons of honey in 2015. In similar way, the purchase of honey for eating increased by almost 71 % from 2000 to 2013. The actual valorization of the sector's organization, the international market, the dynamics and investment into qualitative production of bee products are the principal reasons for the good conduct of domestic beekeeping sector (Ribeiro et al., 2019).

Table 1 represents statistics of the year 2020, about annual honey production, percentage of self-sufficiency of honey and human consumption of honey in total and per capita, in Portugal.

Table 1: Statistics about honey in Portugal(Instituto Nacional de Estatística- https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_main, accessed: 04/02/2022)

	Annual honey production (t)	Self sufficiency of honey (%)	Human consumption of honey per capita (kg/ inhab.)	Human consumption of honey (t)
Year 2020	9817	90,9	1,1	11000

2. Honey bee (*Apis mellifera*)

The honey bee is the most important eusocial insect maintaining the natural ecosystem and being straightly useful to mankind. They produce a plethora of bee products such as honey, propolis, beebread, bee pollen, bee venom, bee wax and royal jelly and provide pollination service for both wild and agricultural crops (Ribeiro et al., 2019). Honey bees can cover the distance of about 10 kilometers to assemble primary food resources such as nectar and pollen that are stored in their bee hives as honey and beebread (Ribeiro et al., 2019).

The natural lineage of *Apis mellifera* (figure 1) involves Africa and Eurasia. This species has been introduced by humans to all other continents except Antarctica (Ilyasov et al., 2020). The modern taxonomic pattern of honey bee *Apis mellifera* is: thirty-three distinct honey bee subspecies distributed across all Africa (11 subspecies), Western Asia and the Middle East (9 subspecies), and Europe (13 subspecies) (Mortensen et al.,).

Figure 1 : *Apis mellifera*(https://www.biodiversity4all.org/taxa/47219-Apis-mellifera, accessed in 05/01/2022)

3. General information about honey

3.1.Definition

Honey is one of the oldest products used by humans. It´s a natural sweet substance produced by honeybees from nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances or their own, deposit dehydrate, store and leave in the honey comb to ripen and mature (Codex Alimentarius). The appreciation of its quality changed during history based essentially on sensory characteristics, geographical and botanical origin because soil and climate characteristics are determinant for the melliferous flora (Delrazen lusic et al., 2007).

It has a complex composition with about 200 substances, mainly composed by carbohydrates (80% to 85%, specially fructose and glucose), water (15% to 17%), ash (0.2%), small amounts of amino acids (specially proline), proteins (0.1% to 0.4%), volatile compounds, enzymes, phenolic compounds, lipids, minerals (mostly K), organic acids, pigments, pollens, hormones, vitamins, essential oils, and waxes (Silva et al., 2020).

3.2.Honey origin

There is an innumerable variety of honeys, corresponding to the flowers and plants visited by the bees, as well as to the source harvested (nectar or honeydew). The evaluation of honey's botanical and geographical origin is very complex (Drazen lusic et al., 2007), resulting of a combination of microscopic, chemical and sensory analyses (Castro-Vázquez et al., 2006).

The confirmation of the botanical origin is more difficult in honeydew honeys, because determination of the honeydew type for a given honey can´t be revealed by palynological analysis. The search for compounds that differentiate these types of honey is of particular interest (Castro-Vázquez et al., 2006).

3.3.Difference between nectar honey and honeydew honey

Honey is a naturally sweet substance produced by bees (*Apis mellifera*), from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on

the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature (Codex Alimentarius). If the production is from the nectar of flowering plants is classified as nectar honey, in contrast if it is produced from plant secretions or excretions of plant-sucking insects on the living parts of plant as they feed on plant sap, classified as honeydew honey (Shantal Rodríguez Flores et al., 2015a). A wide variety of sucking insects are related to the production of honeydew, in particular the phytophagous Hemipterans, such as scale insects and aphids (Trencheva et al., 2009). Oak honeydew production from plant secretion was reported to be the main botanical source of honeydew honey in the Iberian Peninsula, but nothing is known about insect contribution or the effective productivity of sweet saps from *Q. pyrenaica* (Pita-Calvo & Vázquez, 2017a; Seijo et al., 2019; Shantal Rodríguez Flores et al., 2015a; Terrab et al., 2019).

Plants can also produce honeydew secretions (Figure 2), as a result of injury by insects, or simply through high phloem pressure, where large amounts of phloem sap exudates in the acorns of oak trees during the summer within mountain areas, which contain natural sugars and minerals that are ingested by bees and deposited in their hives as a dark honey (Terrab et al., 2019). In Spain, this latter secretion type is observed on oak forests during the summer, especially in mountain areas with moderate humidity. In this areas, different oak trees exude a large amount of phloem sap in its acorns. The breaking of the vessels that connect the cupule with the nut of the acorn due to high pressures of phloem result in the acorn drying out and being released from the cupule. This liberates sweet sap that contains natural sugars and minerals and is ingested by bees and deposited in their hives as a dark honey (Jerković & Marijanović, 2010).

Honeydew honeys have different physicochemical and biochemical properties comparing to nectar honeys, mainly depending on the botanical sources. They are darker than most nectar honeys and usually described as having higher values of several quality physicochemical parameters evaluated in honey, such as the electric conductivity, acidity, pH, and ash content (Pita-Calvo & Vázquez, 2018).

Differentiation between both types of honey is requested to avoid frauds and adulterations(Pita-Calvo & Vázquez, 2017a). Some physicochemical parameters such as pH, acidity, diastase, ash content, color, optical rotation, proline content, sugar profiles, melissopalynological analysis and electrical conductivity can be used to indicate the

presence of honeydew in honey to discriminate honeydew honey and nectar honey (Shantal Rodríguez Flores et al., 2015b).

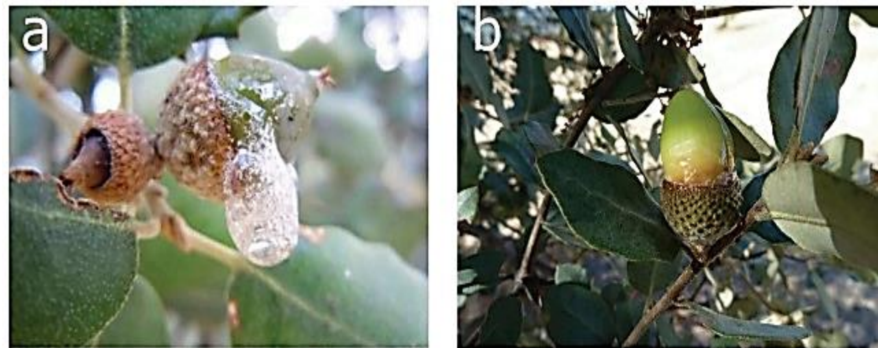


Figure 2: Quercus ilex acorns showing honeydew secretions. **A.** Dried acorn (left) and developing acorn secreting honeydew, that arise between the cupule and the nut of the acorn. **B.** Acorn secreting honeydew, that arise between the cupule and the nut. Photographs: P. López (A), and A. Gómez Pajuelo (B) (Terrab et al., 2019)

4. Honey composition

4.1.Sugars

Honey is a high saturated sugar solution, where carbohydrates are the principal components accounting for about 95% of dry matter. The most important physicochemical and nutritional properties of honey, such as viscosity, granulation, sweetness, hygroscopicity, specific rotation and energy value depend on sugars' composition. Furthermore, the osmotic pressure produced by high sugars concentration is a major honeys' antimicrobial factor (Machado De-Melo et al., 2018).

Honey, mainly incorporates the monosaccharides fructose (32-44%) and glucose (23-38%). More than 45 di-, tri- and other oligo- and polysaccharides have been detected in honey in small amounts (5–15%), like maltose, sucrose, turanose, trehalose, gentiobiose, isomaltose, lactose, kojibiose, raffinose, erlose, melezitose, maltotriose, panose, isomaltotriose and maltotetraose, among others. Honey oligosaccharides present potential prebiotic activity increasing the populations of bifidobacteria and lactobacilli in human gut (Pita-Calvo & Vázquez, 2017a).

Disaccharides, maltose, sucrose, trehalose and turanose, are principal sugars in blossom honey, while in honeydew honey there are a higher amount of oligosaccharides, mainly trisaccharides such as melezitose and raffinose, which are considered as characteristic for honeydew´s indicators (Vasić et al., 2019). Honeydew honeys have also been characterized

by significantly higher values of trehalose and isomaltose, and lower values of glucose, turanose , sucrose and melezitose, than blossom honeys. Authors have also reported a higher erlose concentration in honeydew honey (Pita-Calvo & Vázquez, 2017a).

4.2.Nitrogen compounds

Normally, the nitrogen content of honey is low, containing colloids, proteins, free amino acids and enzymes as the most important nitrogenous compounds. About 40 to 80% of the total honey nitrogen comes from the protein fraction, and most of the remainder resides in free amino acids

4.2.1. Proteins

The origin of protein in honey comes from both plants (nectar, honeydew and mainly pollen) and bees (salivary glands). About 20 different nonenzymatic proteins have been recognized in honey, being globulins, albumins, proteases and nucleoproteins the most common to all honeys (Machado De-Melo et al., 2018). Due to the low concentration of proteins in honeys and the difficulty of its extraction and characterization by conventional methods, few studies are found in the literature, especially related to honeydew honeys (Seraglio et al., 2019).

The use of proteins was also investigated to differentiate filtered and unfiltered honeys. In unfiltered forest honeydew honeys and blossom honeys from different geographical origins, two dominant protein bands of 40 and 65 kDa from sucrase enzyme fraction were found, whereas in filtered honeys the protein band of 65 kDa was almost vanished (Seraglio et al., 2019).

Protein content can vary with geographical origin, in pine honeydew honeys from Turkey and Spanish honeydew honeys (unknown origin), it was similar presenting 1.16 and 1.00 $g.100g^{-1}$, respectively. In forest honeydew honeys from Malaysia, the protein content ranged from 0.43 to 1.02 $g.100g^{-1}$. Whereas low protein content was reported in honeydew honeys from Brazil and Slovakia presenting mean values of $0.04g.100g^{-1}$, Lebanon honeydew honeys with values between 0.08 to 0.14 $g.100g^{-1}$ and Croatian honeydew honeys with values ranging from 0.03 and 0.10 $g.100g^{-1}$ (Seraglio et al., 2019).

4.2.2. Amino acids

About 35 to 60% of the total nitrogen in honeys is estimated belonging to free amino acids (Seraglio et al., 2019). The variation of the free amino acid's amount is related to the geographical and botanical origin of the honeydew honey (Seraglio et al., 2019), and it´s characterization is a promising indicator of adulteration or immaturity of honeys. Indeed, the profile and amount of free amino acids occurs a good differentiation between honeydew honeys and blossom honeys as well as between honeydew honeys from different geographical origins (Seraglio et al., 2019).

Up to 90% of the total free amino acid content is usually composed by the main amino acid present in honeydew honeys which is proline. The origin of proline is mainly from the honey bees and has been suggested as a maturity indicator of honey and sugar adulteration in some cases. It is suggested that pure honeys must have a minimum value of 180 $mg.kg^{-1}$ of proline, however, considerable variation in proline content is related to the type of honey and low values can be found even in ripeness and non-adulterated honeys (Seraglio et al., 2019).

Besides proline, at least 29 amino acids have been reported in honeydew honeys: aspartic acid, glutamic acid, asparagine, serine, glutamine, histidine, glycine, threonine, arginine, α and β-alanine, α and β-aminobutyric acid, tyrosine, valine, tryptophan, phenylalanine, isoleucine, leucine, ornithine, lysine, cysteine, methionine, hydroxyproline, α-aminoadipic acid, γ-aminobutyric acid, ethylamine, homoserine, and taurine (Seraglio et al., 2019).

4.2.3. Enzymes

Enzymes in honey are used as indicator of aging and/or overheating, since their activities decrease in these conditions, as they are thermolabile (Machado De-Melo et al., 2018). Natural honey contains small amounts of enzymes, the most important ones are diastase, invertase and glucose-oxidase. Other enzymes have been found in honey like acid phosphatase, catalase and β-glucosidase. Enzymes such as invertase or glucose oxidase have animal origin for the reason that they are mainly produced in the hypopharyngeal glands of the bees. Honey bees add these enzymes so that they fulfill the nectar to honey ripening process (Machado De-Melo et al., 2018).

Some enzymes have vegetal origin, from the nectar, honeydew or pollen, such as catalase and acid phosphatase. And finally, enzymes such as diastase have a double origin. Honey microorganisms could be other possible origins of enzymes and in the case of honeydew honey, some enzymes could come from the plant sucking insects (Machado De-Melo et al., 2018).

4.3.Vitamins

Honey contains vitamins that come majorly from the pollen of the flowers visited by bees, as well as from nectar or honeydew. Honey cannot be considered as a good source of vitamins since the amount present is small. Water-soluble vitamins are higher in quantity than fat-soluble vitamins, because honey hardly contains lipidic substances (Machado De-Melo et al., 2018).

In honeydew honeys, the vitamins generally found are the vitamins of B group (B1, B2, B3N, B3H, B5, B6) and the vitamin C (Seraglio et al., 2019). Some authors studied quantities of vitamin C in blossom honeys and honeydew honeys from Spain. Honeydew honeys presented a mean value of 7.70 $mg.kg^{-1}$ of vitamin C, very low value compared to blossom honey of thyme, which reached a mean value of 571.5 $mg.kg^{-1}$. Some factors that can cause the loss of vitamin C in honeys are the commercial filtration process and its oxidation by the hydrogen peroxide, which is naturally present in honeys (Seraglio et al., 2019). Concerning the B group, vitamin B1 was quantified in high concentration in honeydew honeys (mean value of 4.00 $mg.kg^{-1}$), while the vitamin B2 presented the highest concentration in this honey type (mean value of 0.16 $mg.kg^{-1}$) compared to the blossom honeys (Seraglio et al., 2019).

4.4.Volatile compounds

The volatile composition of honeys can differ from each other, which with no doubt will affect the sensory characteristics of individual honey types. Thus, they may in some cases be considered “floral markers” (Castro-Vázquez et al., 2006), since aroma depends on them (Soria et al., 2005).

The origins of volatile compounds in honey are numerous: from the plant, from the transformation of plant compounds by the metabolism of a bee, from heating or handling

during honey processing and storage, or from microbial or environmental contamination (Castro-Vázquez et al., 2006).

High levels of acetic acid were found in honeydew honeys (unknown origin) from Spain and from Brazil, which was proposed as indicative of the presence of honeydew in honeys (Seraglio et al., 2019). Also, 1-(2-Furanyl)-ethanone, butane-2,3-diol, 3-hydroxy-butan-2-one and 1-hydroxy-propan-2-one were suggested compounds for discrimination among nectar and honeydew honeys (Jerković & Marijanović, 2010). Thus, 3-hydroxy-2-butanone, butane-2,3-diol and 1-hydroxy-2- propanone were most positively correlated to honeydew honey and 1-(2-furanyl)-ethanone was most positively correlated to blossom honey (Pita-Calvo & Vázquez, 2017b). Other authors found that butane-2,3-diol was present only in honeydew honey and that the other compounds were more abundant in honeydew honey than in blossom honey (Pita-Calvo & Vázquez, 2017b).

4.5.Phenolic compounds

With nearly 10,000 compounds, phenolic compounds are a chemically heterogeneous class of substance. They can be grouped in phenolic acids and flavonoids, according to their basic chemical structure (Vasić et al., 2019). The healthy honey characteristics are linked to the presence of these two types (phenolic acids and flavonoids) Honey contains very low concentrations of phenolic compounds (Pita-Calvo & Vázquez, 2018), ranging from 5-1300 mg/kg (Machado De-Melo et al., 2018).

The plants are the main origin of the phenolic compounds in honeys. Therefore, they could be an interesting tool in elucidating the botanical origins of honeydew honeys (Pita-Calvo & Vázquez, 2018).

Due to the high correlation between antioxidant activity and the content of phenolic compounds, many studies have suggested that phenolic compounds are mostly responsible for the antioxidant activities (Pita-Calvo & Vázquez, 2018).

Dark honeys, including honeydew honey, tend to have higher amounts of phenolic compounds(Seraglio et al., 2016). Therefore, darker honeys such as honeydew honeys have higher antioxidant activities because of their higher total phenolic content (TPC)s (Pita-Calvo & Vázquez, 2018). Some authors affirm that values between 100 and 154.4 $mg.100g^{-1}$ were found in *Quercus pyrenaica* (Pyrenean oak) honeydew honey (Pita-Calvo & Vázquez, 2018).

Furthermore, *p*-aminobenzoic acid, luteolin, hesperidin, isorhamnetin, pinobanksin and conifer aldehyde have been identified and quantified for the first time in honeydew honeys, which represent a contribution to the characterization and quantification of phenolic compounds from honeydew honeys (Seraglio et al., 2016).

4.6.Organic acids

Less than 0.5% of total solids, is represented by honey organic acids, but these compounds are important for honey taste, aroma, color and honey preservation, making it difficult for microorganisms to grow. Honey organic acids are characteristic of the botanical origin (Machado De-Melo et al., 2018). They contribute to honey acidity and electrical conductivity. Some honeys' organic acids are originated directly from nectar or honeydew (citric, malic and oxalic acid), but the vast majority of them are produced from nectar and honeydew sugars by the action of enzymes secreted by bees during ripeness and storage (Machado De-Melo et al., 2018). Gluconic acid is the main organic acid, representing the 70–90% of the total. It is formed from glucose by the action of glucose oxidase (Machado De-Melo et al., 2018). Some acids such as acetic acid led by the transformation of honey sugar by the action of glucose oxidase during storage, can be a possible indicator of honey fermentation (Machado De-Melo et al., 2018).

4.7.Mineral contents

Mineral content in honey is generally low, ranging between 0.02 and 0.3% in blossom honeys, while in honeydew honeys can reach 1% of the total (Machado De-Melo et al., 2018). This parameter is influenced by soil and climatic conditions, as well as the chemical composition of nectar that varies relating to the different botanical sources involved in honey formation. Variations can also be accorded to harvesting, beekeeping techniques (such as extraction methods) and the material collected by the bees during foraging on flowers (Machado De-Melo et al., 2018).

The most important minerals found in honeys are potassium, sodium, calcium and magnesium. Less abundant elements are iron, copper, manganese, chlorine and in minor quantities trace elements such as boron, phosphorus, sulfur, silicon, bare and nickel, among others (Machado De-Melo et al., 2018).

5. Melissopalynological analysis

Pollen analysis of honey, or mellissopalynology, is very important for quality control. Honey always contains many pollen grains (mainly from plant species that bees forage) and honeydew elements (such as wax tubes, algae, and fungal spores), which together provide a good fingerprint of the environment where the honey came from. Therefore, pollen analysis can be used to determine and control the geographic and botanical origin of honey, even though sensory and physicochemical analysis is also required to properly diagnose botanical origin. In addition, pollen analysis provides some important information on honey extraction and filtration, fermentation, type of adulteration and hygienic aspects such as contamination with mineral dust, soot, or starch grains. The palynological characteristics of honeydew honey depend on the production area, season and meteorological conditions (Terrab et al., 2019).

Castanea sativa, Cytisus type and *Rubus* as well as different *Erica* species (*Erica umbellata, Erica australis, Erica arborea, Eric cinerea*) and to a lesser extent, pollen from *Eucalyptus, Trifolium, Campanula, Salix or Echium* are present in honey produced in the northern Spain and Portugal (Shantal Rodríguez Flores et al., 2015a).

A study has shown that spanish *Quercus* honey is characterized by medium high pollen content (average Pn = 286 134/10 g). Honeydew honey contains less pollen than nectar honey because the sugar source for bees is extra-floral (Shantal Rodríguez Flores et al., 2015a).

6. Quality and physiochemical parameters of honeydew honey

6.1.Color

The color of honey is mainly related to its botanical origin. It also depends on its ash content, temperature, time of storage (Pita-Calvo & Vázquez, 2017b), the flora involved and on associated pigment, polyphenols, flavonoids, and mineral content (Seijo et al., 2019).

Honey color can range from pale to dark (Tuberoso et al., n.d.), having an average value between 108–150 mm Pfund (Shantal Rodríguez Flores et al., 2015b). Generally, honeydew honeys are darker than the blossom honeys. A significant difference in the mean color value (mm Pfund) was found between both types of honey by several researchers (Pita-Calvo & Vázquez, 2017b), being dark or dark amber honeys, is frequently associated with honeydew

(Shantal Rodríguez Flores et al., 2015a). This difference can be used as a tool in the differentiation between honeydew honey from nectar honeys by color (Jara-Palacios et al., 2019).

6.2.Moisture content

Moisture content is related to different factors such as botanical and geographic origin, soil and climatic conditions, harvest season, nectar flux intensity, maturity, beekeeper operations during harvest, and extraction, processing, and storage conditions (Machado De-Melo et al., 2018). It is not recommended to harvest honey during rainy days or when relative humidity is high, as this may lead to an increase in the moisture content of the product (Bicudo de Almeida-Muradian et al., 2020). Council Directive 2001/110/EC (European Commission, 2001) and Brazilian legislation (Ministerio da Agricultura Pecuaria e Abastcimento, 2000) and Codex Alimentarius (Codex Alimentarius Commission, 2001) recommend a limit of 20% for the moisture content in honey sample (Bicudo de Almeida-Muradian et al., 2020). Typically, moisture in honey is between 13% and 25%, with an optimum value around 17% (Machado De-Melo et al., 2018), with studies showing that Portuguese honeys range from 15.23 ± 0.15 to 17.93 ± 0.06 (Karabagias et al., 2018).

Several researchers found no significant differences for moisture and water activity between honeydew and blossom honeys (Bicudo de Almeida-Muradian et al., 2020). However, high correlation between these two parameters was found for both types of honey (Bicudo de Almeida-Muradian et al., 2020). Actually, water activity should be considered as a better honey quality control criterion than moisture; because it indicates the free water content that eventually is used by microorganisms to cause fermentation (Machado De-Melo et al., 2018).

Honeys with very low moisture contents are difficult to handle and process. Conversely, honeys whose moisture is higher than 18% are prone to ferment, because sugar's osmotic pressure is not powerful enough to avoid osmophilic yeast proliferation (Machado De-Melo et al., 2018), resulting in the formation of ethyl alcohol and carbon dioxide, leading to its deterioration, sour taste, and consequently, loss of quality (Silva et al., 2020).

6.3.Ash content

In honey, ash is mainly composed of minerals such as K, Ca, Na, and Mg. High ash levels may indicate excess inorganic materials from external contaminants such as processing and equipment as well as environmental contamination. For these reasons, it is considered an important quality parameter in honey. Honeydew honey naturally has a high ash value, but is generally below 1.2 $g.100g^{-1}$ (Seraglio et al., 2019).

Ash content is often used to determine the botanical origin of honey (foral, mixed, or honeydew) (Jara-Palacios et al., 2019), and geographic origin (Silva et al., 2020). In terms of botanical origin, nectar honeys generally have lower ash content or conductivity than honeydew honeys (Machado De-Melo et al., 2018). Currently, the determination of ash content has been replaced by electrical conductivity measurement, mainly because it is more sensitive to small changes in mineral content than ash content (Seraglio et al., 2019). Ash gives a direct measure of inorganic residue and electric conductivity measures all ionizable organic and inorganic substances (Silva et al., 2020).

6.4.Electrical conductivity

Electrical conductivity (EC) refers to the ability of a material to conduct electrical current (Karabagias et al., 2018).This is a parameter widely used to distinguish nectar from honeydew honey (Kolayli et al., 2018). It is a conventional physicochemical parameter, associated with the botanical origin of honey, combined with melissopalynological analysis data for the identification of botanical origin of honey (Karabagias et al., 2018). Although it is closely related to ash and mineral content (Seraglio et al., 2019), conductivity can also be affected by ions, organic acids and proteins (Bicudo de Almeida-Muradian et al., 2020). Honeydew honey is more conductive than blossom honey, which is a good parameter to differentiate the two types of honey (Seraglio et al., 2019), and thus can be used to indicate the presence or absence of honeydew in honey (Shantal Rodríguez Flores et al., 2015a).

According to the European regulation (European Commission, 2001), and Codex Alimentarius (Commission, 2001) electrical conductivity of blossom honey must be < 0.8 mS/cm of EC, while electrical conductivity of honeydew honey and chestnut honey must be > 0.8 mS/cm (Bicudo de Almeida-Muradian et al., 2020).

Currently, the determination of ash content has been replaced by the measurement of electrical conductivity, mainly because it is more sensitive to small changes in the mineral levels than the ash content (Seraglio et al., 2019). Relatively to ash content and electrical conductivity, both depend on the mineral level of the honey. Ash gives a direct measure of inorganic residue after carbonization and electric conductivity measures all ionizable organic and inorganic substances (Silva et al., 2020).

6.5.pH and free acidity

The pH is related to the stability and honey conservation´s duration. It is a convenient indicator for possible microbial contamination. The ranges of honey pH values usually are between 3.5 and 5.5, due to the presence of organic acids, particularly gluconic acid, and inorganic ions, such as phosphate and chloride (Pita-Calvo & Vázquez, 2017b). In honeydew honey pH values are higher, because of their higher mineral contents, so it´s ranging from 4.5 to 6.5 (Machado De-Melo et al., 2018). The reduction of the pH value could be caused by the release of organic acids from the pollen during the honey processing (Stojković et al., 2021).

Honey pH is not directly related to the acidity, because some honey components have buffer capacity, among which salts and some mineral compounds (Machado De-Melo et al., 2018). Free acidity is related to organic acids (Karabagias et al., 2018), internal esters and inorganic ions, such as phosphates, chlorides, sulfates and nitrates, which could produce their corresponding acids (Machado De-Melo et al., 2018).

According to European regulation (European Commission, 2002a) and Codex alimetarius (Codex Alimentarius Commission, 2001), the honey free acidity must be 50 milli-equivalents/kg (Seraglio et al., 2019). Honeydew honeys showed a higher values of acidity than blossom honey (Pita-Calvo & Vázquez, 2017a). An increase in the acidity, may be indicative of honey fermentation of sugars by yeasts (Jara-Palacios et al., 2019), and transformation of alcohol into organic acid (Karabagias et al., 2018).

6.6.Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is a furanic compound (figure 3), produced by the degradation of sugars (hexoses decomposition such as fructose), from hexoses dehydration

in acidic medium and to a lesser extent, as an intermediate in the Maillard reactions (Machado De-Melo et al., 2018). It is formed slowly and naturally (Seraglio et al., 2019), and it is widely recognized as an indicator of honey freshness because the production of high levels of HMF are caused by excessive heating during honey manufacturing process or inadequate conditions of storage (Pita-Calvo & Vázquez, 2017b). This compound is absent or present in trace amounts in fresh honey (Silva et al., 2020). Higher amounts of HMF in honeydew honey than in blossom honey were found by several researchers (Pita-Calvo & Vázquez, 2017b). Also, high values of HMF are naturally present in honeys from warm climate areas, such as tropical and subtropical countries (Machado De-Melo et al., 2018).

According to European regulation (European Commission, 2002a) and codex Alimentarius (Codex Alimentarius Commission, 2001), an HMF content lower than 10.0 $mg.kg^{-1}$ is frequently found in honeydew honey, indicating acceptable freshness and proper handling of these honeys, since it is established a maximum value of 40 $mg.kg^{-1}$ of HMF for honeys in general and a maximum value of 80 $mg.kg^{-1}$ is accepted for honeys of locations with tropical climate and blends of these honeys (Seraglio et al., 2019), and 15 mg/kg for honey with low enzymatic level (Pita-Calvo & Vázquez, 2017b).

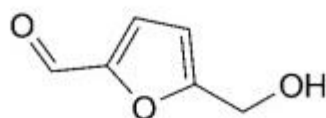


Figure 3: Structure of hydroxymethylfurfural*(Tuberoso et al., n.d.)*

6.7.Diastase activity

Enzymes are present in small amounts in honey, which mainly includes diastase, invertase and glucose-oxidase. Thus, diastase and invertase assume a significant role in quality evaluation and are used as markers for honey freshness.

Diastases which includes α- and β-amylase, have a double origin, animal and vegetal origin. Other possible origins could be honey microorganisms and in the case of honeydew honey, some enzymes could come from the plant sucking insects that produce honeydew (Machado De-Melo et al., 2018).

Honeys in general should present diastase activity of at least 8 Schade units, minimum value accepted by regulatory organizations (Codex Alimentarius Commission, 2001; European Commission, 2002a) (Seraglio et al., 2019).

According to literature, the values of diastase activity in honeydew honey can range from 9.0 to 30.0 diastase units, nonetheless values higher than 30.0 diastase units were also found for this type of honey (Seraglio et al., 2019). The wide range of diastase values suggests that different factors such as botanic origin, processing and storage conditions, secretion of bees and plant-sucking insects, and honey composition can influence the diastase activity of honeydew honeys (Seraglio et al., 2019).

6.8.Antioxidant activity

Honeydew honey usually presents higher content of bioactive compounds such as phenolics, proteins, and amino acids compared to blossom honeys. As a consequence, they present higher antimicrobial and antioxidant activity (Seraglio et al., 2019). Honey antioxidant activity depends on its botanical source, and moreover such factors as environmental and seasonal changes have a strong influence on this property (Machado De-Melo et al., 2018).

Honey had shown antioxidant activity that provides this food with nutritional and technological advantages (Machado De-Melo et al., 2018). Up-to-date research has highlighted that, because of its antioxidant activity, honey could play an interesting role in the management of oxidative stress-associated with chronic diseases (Machado De-Melo et al., 2018). Some components like free amino acids are responsible for some antioxidant properties, and also vitamin C, which has a strong antioxidant effect (Machado De-Melo et al., 2018). Also, flavonoids and other honey's phenolic compounds provide this food with functional properties such as antioxidant capacity (Machado De-Melo et al., 2018).

Because of the different purposes and responses obtained, studies in honeydew honeys have used different methods, such as ABTS (2,2- azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical), DPPH (2,2- diphenyl-picrylhydrazyl radical), FRAP (ferric reducing antioxidant power), and ORAC (oxygen radical absorbance capacity), among others tests (Seraglio et al., 2019).

6.9. Anti-inflammatory activity

Potential therapeutic honey properties have been attributed to bioactive compounds, which provide this food with antioxidant, antibacterial, and anti-inflammatory activities, among others (Machado De-Melo et al., 2018). Honey has shown that can reduce inflammation in several experiments carried out with laboratory animals (Machado De-Melo et al., 2018). In humans, honey ingestion revealed to be able to lower such inflammatory mediators as thromboxane and prostaglandins (Machado De-Melo et al., 2018).

Anti-inflammatory activity of honeys has been attributed to flavonoids, which could inhibit the delivery of proinflammatory cytokines, as well as the expressions of the inducible nitric oxide synthase and the production of reactive oxygen species (Machado De-Melo et al., 2018).

7. Objectives

This work aims to characterize the production of honeydew honey with origin in *Quercus pyrenaica* oak from Montesinho Natural Park and identify marker compounds that discriminates the botanical origin of this honey.

The main objectives of this work were:

-Evaluation of the physico-chemical parameters according to standard parameters such as: colour, humidity, acidity, electrical conductivity, diastase index, HMF, proline.

-Evaluation of *Quercus Pyrenaica* honeydew honey chemical characterization to find specificities of minerals (by atomic absorption spectrophotometry), sugars (through high-pressure liquid chromatography with a refraction index detector (HPLC-RI)), proteins (by the Kjeldahl method)), phenolic compounds (by liquid chromatography coupled to mass spectrometry detector (LC-MS).

-Identify Marker compounds that discriminates the botanical origin of the honey.

Chapter II : Materials and methods

Chapter II : Materials and methods

1.Sampling

This study was performed on 42 honey samples collected from different apiaries of *Apis mellifera iberiensis*, located in Montesinho Natural Park, Bragança, Portugal, during September of 2021. The apiaries were located in areas of Quercus pyrenaica forests. Each sample corresponded to the production of one beehive. The locations were the following: Paço (8 samples), Espinhosela (10 samples), Ciradelha (9 samples), Rio de Fornos (10 samples), Soeira (4 samples) and Zoio (1 sample), Figure 4.

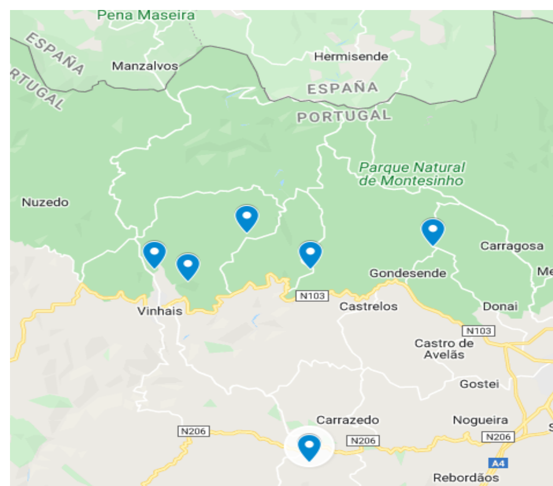


Figure 4: Geographical origin for honey samples

Table 2 shows the information on honey samples used throughout this work, in particular their geographical origin and month/year of collection. All honey samples were stored at room temperature, in the original packaging until they were analyzed.

Table 2: Geographical origin and year of collection of honey samples

sample code	Geographic origin
P82	Paço
P74	Paço
P89	Paço
P95	Paço
P134	Paço
P138	Paço
P152	Paço
P209	Paço
ESP 100	Espinhosela
ESP 153	Espinhosela
ESP 201	Espinhosela
ESP 202	Espinhosela
ESP 203	Espinhosela
ESP 204	Espinhosela
ESP 205	Espinhosela
ESP 206	Espinhosela
ESP 207	Espinhosela
ESP 208	Espinhosela
CIR 148	Ciradelha
CIR 17	Ciradelha
CIR 137	Ciradelha
CIR 136	Ciradelha
CIR 135	Ciradelha
CIR 130	Ciradelha
CIR 146	Ciradelha
CIR 145	Ciradelha
CIR 141	Ciradelha
Rdf 79	Rio de Fornos
Rdf 80	Rio de Fornos
Rdf 81	Rio de Fornos
Rdf 85	Rio de Fornos
Rdf 101	Rio de Fornos
Rdf 111	Rio de Fornos
Rdf 132	Rio de Fornos
Rdf 142	Rio de Fornos
Rdf 143	Rio de Fornos
Rdf 200	Rio de Fornos
SFM 1	Barreiros
SFM 2	Lamedo
SFM 3	Fonte indevacas
SFM 4	Fonte Indueiro
Z 1	Labanho cima

2. Honey analysis

The quality of honey was evaluated according to standard parameters such as: color, humidity, acidity, electrical conductivity, diastase index, HMF, proline.

Different chemical parameters were also evaluated in honeydew honey to find specificities: minerals (by atomic absorption spectrophotometry), sugars (through high pressure liquid chromatography with a refraction index detector (HPLC-RI)), proteins (by the Kjeldahl method)), and phenolic compounds (by liquid chromatography coupled to mass spectrometry detector (LC-MS)).

Also, the antioxidant activity was evaluated through the DPPH, ABTS and reducing power methods.

3. Physicochemical parameters of the honeydew honeys

3.1 Moisture content

The water content was determined, using a refractometer, figure 5 (Digit-5890, Ref: 8100.5890), expressing the result in percentage.

Figure 5: refractometer

3.2 Color

The color intensity of honey samples was measured according to the Pfund scale. Briefly, homogeneous honey samples were transferred into a cuvette with a 10 mm light path until the cuvette was approximately full. Then, the cuvette was inserted into a C221 colorimeter (Hanna Instruments, Woonsocket, RI, USA), (figure 6). Color grades were expressed in millimeter (mm) Pfund grades, compared to an analytical-grade glycerol standard.

Figure 6: Colorimeter

3.3 Electrical conductivity

After calculating the necessary honey amount, taking in account the humidity values, a honey solution was prepared by diluting 5 g of anhydrous honey in 25 mL of distilled water, and its electrical conductivity was measured by using a consort C868 conductivity meter, Figure 7, previously calibrated. The results were expressed in mS.cm-1

Figure7:Conductivity meter

3.4. pH, free and lactonic acidity

To evaluate the acid properties of honey, three different parameters were evaluated: pH value of the initial honey solution, free acidity, and lactonic acidity. Titration was performed with an automatic titrator, Figure 8, (Hanna instruments, pH 211 Microprocessor pH meter, Woonsocket, USA).

Figure 8: Automatic titrator

For the determination of free acidity, the procedure reported by the IHC was performed. Initially, a solution was prepared by dissolving 10 g of honey in 100 mL of deionized water. Then 25 mL of this solution was put into a beaker where the pH electrode was placed, recording the initial pH value, and then titration of the solution with sodium hydroxide (NaOH) 0.1 mol.dm^{-3}. The base volumes consumed to reach the equivalence point (pH 7) were recorded. The obtained value allows determining the free acidity which is measured by titration with sodium hydroxide (NaOH) up to the equivalence point (pH 7). Then we continue the titration with sodium hydroxide (NaOH) 0.1 mol.dm^{-3} until reaching another equivalence point value which is pH=8.3 and the values obtained allows the determination of the free acidity up to the equivalence point (pH 8.3).

To determine the lactonic acidity, after reaching the equivalence point, the base was added until reaching the final volume of 10 mL, then a re-titration of the excess base with sulphuric acid (H2SO4) 0.025 mol.dm^{-3} until reaching the equivalence point again (pH 7). The difference in NaOH consumed in the two titrations allows the calculating of the lactation acidity and the total acidity (free + lactonic). The results are expressed in meq.kg^{-1}.

3.5 Proline

Proline evaluation was performed by spectrophotometric methods using an aqueous honey solution obtained by dilution of 0.5 g of honey in 10 mL distilled water. For the analysis, 0.5 mL of the honey solution was placed in a test tube (sample), 0.5 mL of distilled water in a second tube (white), and 0.5 mL of standard proline solution (0. 032 mg.ml^{-1}) in triplicate in other tubes (standard), together with the same volume of water. To each of the

10 tubes, 1 mL of formic acid (98%) was added with 1 mL of ninhydrin solution (3%) and stirred vigorously for 15 minutes. After that time, the tubes were placed in a boiling water bath for 15 minutes, and then in another bath at 100 ºC for an additional 15 minutes. In the end, 5 mL of propan-2-ol was added to each test tube and after being closed, the tubes were cooled for 45 minutes and then reading the absorbance at 510 nm using a spectrophotometer (Specord 200 spectrophotometer, Analytikjena, Jena, Germany). The proline content was calculated using the following equation, and the results expressed in $mg.g^{-1}$.

Proline= ((Abs Sample/Abs standard) x (mass standard/mass Sample)) x 80/4 (Equation nº1)

3.6 Hydroxymethylfurfural (HMF)

For the 5-HMF quantification, 5 g of honey were weighted and dissolved in 25 mL of deionized water and transferred quantitatively into a 50 mL volumetric flask. Then, 0.5 mL Carrez solution I and Carrez solution II were added, completing the final volume of 50 mL with deionized water. The solution was filtered through Waltman paper, rejecting the first 10 mL of filtrate. The filtrate was pipetted into each of two test tubes. To one of the tubes, 5 mL of distilled water (sample solution) was added and to the other 5 mL of sodium bisulphite solution, NaHSO3, 0.2% (reference solution). The absorbance was measured at 284 nm and 336 nm in a spectrophotometer (Specord 200 spectrophotometer, Analytikjena, Jena, Germany), and the 5-HMF value was expressed in mg/kg and determined according to the following equation:

HMF= (Abs284-Abs336) ×149.7× (5/ (sample weight)) (Equation n° 2)

3.7 Diastase activity

The analysis of the diastase activity was performed by the Phadebas method (Bogdanov et al.,1997). This spectrophotometric method is performed by preparing an aqueous honey solution obtained by dilution of 0,25 g of honey in a 25 mL volumetric flask. After preparing the solution, 5 mL was transferred to a test tube and placed in a bath at 40 ºC for 5 minutes, together with a second tube (blank) containing 5 mL of acetate buffer solution 0.1 M (pH 5), each sample was put in 3 tubes (5 mL). The Phadebas tablets were then placed in the three

tubes at 40 ºC for 15 minutes. Subsequently, 1 mL of sodium hydroxide (NaOH) 0.5 M was added. Samples were put in centrifuge at 3700 rpm for 5 min. The absorbance was measured at 620 nm in a spectrophotometer. The result is obtained as a diastase index (DN) in Schade units, equivalent to the unit of diastase and the enzymatic activity of 1g of honey capable of hydrolyzing at an hour, 0.01 g of starch at 40 ºC. The equations used for the calculation of the value of DN were as follows:

$$DN = 28.2 * Abs620 + 2.64 \text{ if } DN > 8 \quad \text{(Equation nº 3)}$$

$$DN = 35.2 * Abs620 - 0.46 \text{ if } DN < 8 \quad \text{(Equation nº 4)}$$

4. Chemical characterization

4.1 Sugars

For sugars analysis, about 2.5 g of honey was mixed with 20 mL of deionized water and 12.5 mL of methanol and 1 mL of xylose (internal standard, 30mg/mL) and the resulting solution was diluted to a final volume of 50 mL with deionized water. Afterwards, the sample was passed through a 0.2 μm filter and analyzed by high performance liquid chromatography coupled to a refractive index detector (HPLC-RI). HPLC-RI was performed on an integrated Knauer system with pump (Smartline 1000), a degasser (Smartline 5000), a UV detector (Knauer Smartline 2300) and an autosampler (Jasco, AS-2057). Data acquisition and remote control of the HPLC system was done by Clarity-Chrom software (Knauer, Berlin, Germany). The chromatographic separation was achieved using a Eurospher 100-5 NH2 (4.6 × 250 mm, 5 mm, Knauer) column at 30 ˚C. The mobile phase was composed by acetonitrile/water, 80:20 (v/v) at a flow rate of 1.3 mL/min. The identification of sugars was obtained by comparing the retention times of the peaks of the samples with those of standards, namely fructose, glucose, sucrose, turanose, maltulose, maltose, trehalose, melezitose, raffinose, melibiose, erlose, isomaltose, and kojibiose. For each of these standards, a calibration line was established by the internal standard method, using a range of concentrations according to the expected levels for each sugar, Table n°3. The obtained values by the samples were calculated from the peak area and are presented in mg/g of honey. The analysis of the sugar profile was also considered in terms of fructose + glucose,

fructose/glucose and glucose/water ratio, to assess the tendency to crystallization of the honey

Table 3: Range of concentrations for each standard, and respective calibration equation

Sugar	Concentration range (mg.mL-1)	Calibration equation	R^2
Frutose	3,78 - 60,4	y= 115,53x - 45,464	0,9997
Glucose	2,8 - 45	y= 113,48x - 56,267	0,9995
Sucrose	0,9 - 15	y= 117,61x - 12,948	0,9995
Turanose	0,28 - 4,5	y= 105,55x - 1,1727	0,9997
Maltulose	0,27 - 4,4	y= 103,45x - 1,288	0,9995
Maltose	0,28 - 4,5	y= 103,7x - 9,7402	0,999
Trealose	0,28 - 4,5	y= 115,71x - 3,1328	0,9992
Melezitose	0,28 - 4,5	y= 106,19x - 0,8925	0,9992
rafinose	0,28 - 4,5	y= 97,037x - 7,7229	0,9991
Melibiose	0,3 - 5	y= 78,264x - 3,1532	0,9938
Isomaltose	0,5 - 8,1	y= 35,997x + 1,2782	0,9775
Kojibiose	0,084 - 1,35	y= 99,885x - 5,3913	0,9913
Erlose	0,41 - 6,7	y= 73,549x - 9,7642	0,9991

4.2. Protein content

For the determination of the protein content, the Kjeldahl method was applied, which consists of indirect determination based on the quantification of total organic nitrogen. This process began with the digestion of 1 g of honey by the addition of 15 mL sulfuric acid and a metallic catalyst that accelerates the oxidation process of organic matter in a digester at 400 ºC for 70 minutes. After the degradation of the sample and transformation of nitrogen into ammonium sulfate, a process of neutralization, distillation, and finally titration of released ammonia is followed. For the conversion of nitrogen content into total protein, a conversion factor of 6.25 was applied, expressing the results in g/100 g of honey.

4.3 Ashes

The ash content was determined in triplicate, indirectly through its calculation, according to what is defined in the literature (Sancho et al., 1992) from the following formula:

$\% Ashes = ((\ ondutivity\ /1000\ ) - 0{,}14)\ /\ 1{,}74$ (Equation nº 5)

4.4 Carbohydrates

The carbohydrate content of the honey samples was obtained by differential calculation considering the following expression defined in the literature:

% Carbohydrates = 100 - % moisture - (% ash + % protein + % lipids) (Equation nº 6)

The lipid content for our calculations, was considered as zero, since it´s absent in our honey samples.

4.5 Energy

The energy value expressed in kcal was calculated in 100 g of honey, using the following equation: Energy value (kcal/100g) = 4 x (% protein + % carbohydrates) + 9 x (% lipids) (Equation nº 7)

As mentioned above for the calculation of the carbohydrates, the lipid content was considered as zero for the equation nº 7 too, since it´s absent in our honey samples,.

4.6 Minerals

To check the minerals content, the following elements were assessed: magnesium (Mg), calcium (Ca), sodium (Na), potassium (K), and iron (Fe) via the spectrophotometer of flame atomic absorption (Pye Unicam PU9100X). The detection of manganese (Mn), copper (Cu) and cadmium was done using atomic absorption spectrophotometry thought graphite chamber via a Perkin Elmer PinAAcle 900 spectrophotometer (AOAC International, 2016).

4.6.1 Sample digestion

0.5g of sample was weight into a PTFE digestion tube, then 10 mL of concentrated nitric acid (HNO3) was added. The sample was digested in a microwave via the following temperature gradient sequencer: a power of 1200 W during 40 minutes until 200 ºC. After that, samples were left to cool and quantitatively transferred into a volumetric flask of 50 mL that we fulfilled the volume with distillated water.

4.6.2 Potassium and Sodium

For the quantification of the sodium and potassium elements, a cesium chloride buffer (10 g/L) and the preparation of different standard solutions were done according to the following requirement: solution 1: 10 mL of the potassium standard (1000 ppm) and 5 mL of sodium standard (1000 ppm) were pipetted into a flask of 20 mL and the volume completed with deionized water.

The calibration standards were done in the spectrophotometer resulted from the ten-fold dilution of these standards (5.0 mL solution of each standard and 5mL CsCl buffer in a the final volume of 50 mL). For the analysis of potassium in the supplement, a digested supplement solution of 5 mL, buffer solution of 1mL and 4 mL of deionized water were added. For the analysis of sodium in the supplement, 10 mL of the digested supplement solution, 1 mL of the buffer solution were added. The recording of the result was taken

according to the conditions suggested for the tools.

4.6.3 Calcium and Magnesium

For the detection and quantification of calcium and magnesium, a solution (10 g/L) of lanthanum was prepared by diluting 13.15 g of La $(NO_3)_2$ in 1 L of deionized water. Also, a Ca standard solution (1000 ppm, solution 2) and an Mg standard solution (1000 ppm, solution 3) was set in 10 mL of deionized water.

The standards applied in the spectrophotometer calibration to determine the content of Ca was done from the ten-fold dilution of these standards (5.0 mL solution of each standard and 5 mL of solution La $(NO_3)_2$ to a final volume of 50 mL). The standards applied in the spectrophotometer calibration to determine the content of Mg was done from the thirty-three-fold dilution of these standards (1.50 mL solution of each standard and 5 mL of solution La $(NO_3)_2$ to a final volume of 50 mL).

4.6.4 Iron

Matrix modifier: diluted 1.7 mL of magnesium nitrate solution, Mg $(NO_3)_2$, 10 g/L to 10 mL of solution with deionized water. Standard 1: diluted 0.50 mL of 1000 ppm standard solution to 50 mL with deionized water. Standard 2: diluted 0.50 mL of standard solution to 50 mL with deionized water.

4.6.5 Manganese,Copper and Cadmium

To determine the content of manganese, a modified matrix was applied by the dilution of 1.7 mL of a magnesium nitrate solution, Mg $(NO_3)_2$, 10 g/L, to a final volume of 10 mL with deionized water. Two standards solutions for manganese were prepared diluting 0.50 mL of standard solution (1000 ppm) to a final volume of 50 mL of deionized water and 0.20 mL of the previous solution to a final volume of 50 mL (standard 2). For copper, a

modified matrix resulted from the dilution of 1.0 mL of palladium solution, 10 g/L, and 0.1 mL of magnesium nitrate solution, Mg $(NO_3)_2$, to a final volume of 10 mL of solution in deionized water.

After that, the preparation of two copper standards was done by the dilution of 0.50 mL of the 1000 ppm standard solution (V_f = 50 mL deionized water, standard 1) and the dilution of 0.50 mL of the previous solution to a final volume of 50 mL (standard 2).

To determine the cadmium content, preparation of modified matrix was done by the dilution of 0.10 mL of magnesium nitrate solution, Mg $(NO_3)_2$, and 1.0 mL of 10% monobasic ammonium phosphate solution, $NH_4H_2PO_4$, in 10 mL of deionized water. The preparation of two standard solutions was then done, the first by the dilution of 0.25 mL of standard solution (1000 ppm) to 50 mL with deionized water (standard 1) and a second, the dilution of 0.10 mL of the above solution to 50 mL with deionized water (standard 2). The standards applied for the construction of the calibration curve resulted from diluting standard 2. To analyze all the samples, 20 µL of sample and 5µL of the modified matrix were pipetted with the application of the recommended instrumental conditions for each analysis.

4.7 Total phenolic content

Total phenolic content was determined according to a previously described method (Feás et al., 2010), with some modifications. Initially, a solution was prepared to weigh 1 g of honey in 10 mL of methanol. Then 0.5 mL of sample solution (or blank or standard) was mixed with 0.5 mL of Folin-Ciocalteu reagent. After 3 minutes, 1 mL of saturated sodium carbonate solution (Na_2CO_3) (10% w/v) and 3 mL of deionized water were added. The final solution was kept in the dark at room temperature for 1 hour. The absorbance was then read at 760 nm using a spectrophotometer. The total phenolic content expressed in milligram of gallic acid equivalent per gram of sample (mg GAE/g).

4.7.1 Phenolic compounds profile

Extraction

For the quantification and determination of the phenolic profile, honey samples were extracted in triplicate, weighing 25 g of honey in 125 mL of acidified water (pH 2, HCl). The solution was then filtered to remove any solid particles. The filtered solution was passed through an Amberlite® XAD®-2 column, which can selectively retain phenolic compounds. To remove sugars and other polar compounds, a wash was carried out with the passage of acidified water at pH 2 (50 mL). Subsequently with deionized water (150 mL). The phenolic fraction was eluted with methanol (150 mL) and taken to dryness under reduced pressure (40 ºC). The residue was redissolved in 5 mL of water and extracted with diethyl ether (5 mL). The ether extracts were combined, concentrated under reduced pressure, and re-dissolved in 0.5 mL of methanol for UPLC/DAD/ESI-MS^n analysis.

UPLC/DAD/ESI-MSn phenolic compounds analysis

UHPLC/DAD/ESI-MSn analysis was performed on a Dionex UPLC 3000 equipment (Thermo Scientific, USA), Figure 9, equipped with a photodiode array detector coupled to a mass detector. The chromatographic system consisted of a quaternary pump, an automatic sampler maintained at 5 ºC, a degassing, a photodiode array detector, and an automatic thermostatic column compartment. Chromatographic separation was performed with a UVDSpher PUR C18-E 100 mm x 2.0 mm, 1.8µm column, with a particle size of 1.8 µm (VDS Optilab, Germany) maintained at 30 ºC. The mobile phase was composed of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile, previously degassed and filtered using a [illegible] h 0.22 µm porosity.

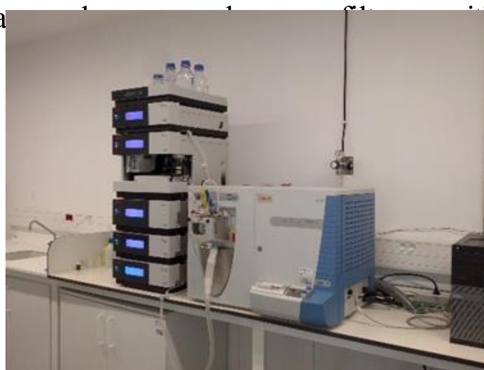
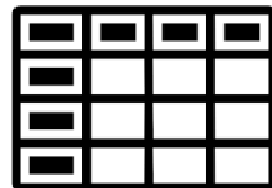


Figure 9 - UHPLC/DAD/ESI-MSN equipment.

For the analysis, a gradient with a flow rate of 0.3 mL.min^{-1} was used: 0.0- 1.0 min 20% B; 1.0-11.1 min 20-95% (B); 95% (B) for 2 min; 13.0-13.3 min 95- 20% (B); and 20% (B) for 5 min. The injection volume was 3 µL. Spectral data from all peaks were collected in the range of 190-600 nm. Each sample was filtered through a 0.2 µm (Whatman) nylon membrane. Mass analysis was performed on an LTQ XL mass spectrometer (Thermo Scientific, CA, USA), in negative mode, equipped with an ESI electrospray ionization source: spray voltage, 5 kV; capillary voltage, -20 V; capillary tube voltage, -65 V; capillary temperature, 325 °C; gas flow and auxiliary gas (N_2), 50 and 10 (arbitrary units), respectively. The mass spectra were acquired in the mass range of 100 - 1000 m/z. The collision energy used in the MS^n experiments was 35 (arbitrary units). Data acquisition was performed using Xcalibur software® (Thermo Scientific, CA, USA). Quantification was performed with standard substance calibration curves for caffeic acid (y = 2x107x+112587; R^2 = 0.9996), p-coumaric acid (y = 3x107x+233317; R^2 = 0,9995), quercetin (y =9x106x+59708; R^2= 0.9999), chrysin (y = 2x107x+571657; R^2= 0.9990), naringenin (y = 2x107x + 398504, R^2= 0.9991) and abscisic acid (y = 3x107x+854658; R^2= 0.9984). When standards were not available, the compounds were expressed by equivalents of the structurally more similar phenolic compound. The elucidation of the structure of phenolic compounds was carried out by comparing their chromatographic behaviour, UV spectra and mass profile with that obtained for commercial standards and with the information obtained in the literature, when these were not available.



Chapter III: Results and discussion

Chapter III: Physiochemical parameters

1.Color

The color of honey is mainly related to its botanical origin. It also depends on its ash content, temperature, time of storage (Pita-Calvo & Vázquez, 2017b), the flora involved and on associated pigment, polyphenols, flavonoids, and mineral content (Seijo et al., 2019).

The colorimetric analysis of the honey samples under study was performed using the Pfund scale by direct reading on the colorimeter.

The results showed values ranging from 130 to 150 mm pfund, this means that the color is classified as dark amber for all the samples, Table 4, which was higher than the previously published for honeydew honeys from Spain (Escuredo et al, 2019), where the colour varied from 108 to 150 mm Pfund.

Table 4: Physical-chemical parameters: color, humidity, and conductivity

Sample	Color (mmPfund)	Moisture (%)	Condutivity (mS/cm)
		Paço	
74	150 ± 0 (Dark Amber)	16 ± 0	1.14 ± 0.00
82	150 ± 0 (Dark Amber)	17 ± 0	1.24 ± 0.01
89	150 ± 0 (Dark Amber)	15 ± 0	1.19 ± 0.02
95	149 ± 0 (Dark Amber)	15 ±0	1.16 ± 0.01
134	150 ± 0 (Dark Amber)	17 ± 0	1.20 ± 0.01
138	150 ± 0 (Dark Amber)	17 ± 0	1.10 ± 0.01
152	148 ± 0 (Dark Amber)	18 ± 0	1.17 ± 0.00
209	150 ± 0 (Dark Amber)	16 ± 0	1.10 ± 0.01
		Espinhosela	
100	149 ± 0 (Dark Amber)	16 ± 0	1.31 ± 0.02
153	136 ± 0 (Dark Amber)	17 ± 0	1.31 ± 0.01
201	150 ± 0 (Dark Amber)	19 ± 0	1.32 ± 0.00
202	150 ± 0 (Dark Amber)	17 ± 0	1.38 ± 0.01
203	1390 ± 0 (Dark Amber)	16 ± 0	1.34 ± 0.00
204	142 ± 0 (Dark Amber)	17 ± 0	1.41 ± 0.02
205	150 ± 0 (Dark Amber)	17 ± 0	1.31 ± 0.01
206	150 ± 0	19 ± 0	1.31 ± 0.03

	(Dark Amber)		
207	138 ± 0 (Dark Amber)	17 ± 0	1.35 ± 0.01
208	146 ± 0 (Dark Amber)	15 ± 0	1.35 ± 0.01
Ciradelha			
17	142 ± 0 (Dark Amber)	15 ± 0	1.01 ± 0.01
30	123 ± 0 (Dark Amber)	16 ± 0	1.06 ± 0.01
135	136 ± 0 (Dark Amber)	16 ± 0	1.14 ± 0.01
136	147 ± 0 (Dark Amber)	15 ± 0	0.96 ± 0.00
137	144 ± 0 (Dark Amber)	15 ± 0	0.99 ± 0.01
141	131 ± 0 (Dark Amber)	15 ± 0	1.01 ± 0.01
145	130 ± 0 (Dark Amber)	16 ± 0	0.95 ± 0.00
146	137 ± 0 (Dark Amber)	15 ± 0	0.98 ± 0.01
148	148 ± 0 (Dark Amber)	15 ± 0	0.93 ± 0.01
Rio de Fornos			
79	136 ± 0 (Dark Amber)	16 ± 0	1.09 ± 0.01
80	150 ± 0 (Dark Amber)	16 ± 0	1.22 ± 0.03
81	130 ± 0 (Dark Amber)	16 ± 0	1.17 ± 0.00
85	150 ± 0 (Dark Amber)	15 ± 0	1.18 ± 0.01
101	150 ± 0 (Dark Amber)	15 ± 0	1.14 ± 0.01
111	150 ± 0 (Dark Amber)	15 ± 0	1.21 ± 0.02
132	150 ± 0 (Dark Amber)	16 ± 0	1.14 ± 0.01
142	142 ± 0 (Dark Amber)	14 ± 0	1.11 ± 0.02
143	150 ± 0 (Dark Amber)	16 ± 0	1.15 ± 0.01
200	130 ± 0 (Dark Amber)	15 ± 0	1.11 ± 0.01
Barreiros			
SFM1	145 ± 0 (Dark Amber)	15 ± 0	1.40 ±0.01
Lamedo			
SFM2	146 ± 0 (Dark Amber)	15 ± 0	1.42 ± 0.01
Fonte Indevacas			
SFM3	150 ± 0 (Dark Amber)	16 ± 0	1.33 ± 0.00
Fonte Indueiro			
SFM4	150 ± 0 (Dark Amber)	15 ± 0	1.43 ± 0.00
Labanho Cima			
Z1	150 ± 0 (Dark Amber)	16 ± 0	1.22 ± 0.01

2. Moisture content

The moisture content is a very significant feature in the honey analysis, being associated with many factors like the geographical and botanical origin of nectar, the soil, the climatic conditions, the intensity of nectar flow, the season of harvesting, the manipulation by beekeepers during harvesting, as well as the conditions of extraction, storage, processing, and the degree of maturation (Machado De-Melo et al., 2017). This parameter affects other features of honey, like viscosity and its tendency of crystallization, taste, color, conservation, and solubility.

According to Council Directive 2001/110/EC (European Commission, 2001) and Brazilian legislation (Ministerio da Agricultura Pecuaria e Abastcimento, 2000) and Codex Alimentarius (Codex Alimentarius Commission, 2001) recommend a limit of 20% for the moisture content in honey sample (Bicudo de Almeida-Muradian et al., 2020). Typically, moisture in honey is between 13% and 25%, with an optimum value around 17% (Machado De-Melo et al., 2018), with studies showing that Portuguese honeys range from 15.23 ± 0.15 to 17.93 ± 0.06 (Karabagias et al., 2018).

Honeys with very low moisture contents are difficult to handle and process. Conversely, honeys whose moisture is higher than 18% are prone to ferment, because sugar's osmotic pressure is not powerful enough to avoid osmophilic yeast proliferation (Machado De-Melo et al., 2018), resulting in the formation of ethyl alcohol and carbon dioxide, leading to its deterioration, sour taste, and consequently, loss of quality (Silva et al., 2020).

The results of moisture content analyze of our samples ranged from 14.4% to 19.3% as shown above in Table 4. All the samples are respecting the maximum value of 20% established with mean value of 15.9%, which was below the mean value reported for Spanish *Quercus pyrenaica* honeydew honey with mean value 17.4% (Shantal Rodríguez Flores et al.,2015a).

3. Electrical conductivity

Electrical conductivity (EC) is closely related to the concentration of mineral and organic

acids and shows great variability according to the floral origin. The sample with electrical conductivity values higher than 0.8 $mS.cm^{-1}$ are considered honeydew honeys. While those that express values below 0.8 $mS.cm^{-1}$ are considered nectar honey or mixtures of different nectars (Bogdanov, 2011)

All analyzed honeys presented values more than 0,8 $mS.cm^{-1}$,ranging from 0.96 to 1.43 $mS.cm^{-1}$, Table 4 , being considered as honeydew honey. The high mean value of EC, was for the sample SFM4 related to the Fonte Indueiro apiary with value of 1.43 $mS.cm^{-1}$.

The lowest mean value was registered for the sample CIR 136 related to Ciradelha apiary with value of 0,96 $mS.cm^{-1}$. Honey colour and electrical conductivity are correlated, with the darker honeys presenting higher values of electrical conductivity.

4. pH,free and lactonic acidity

Acidity is one of the most significant features of honey responsible for its conservation and stability and helps in the prevention of microorganism's development and correlated with its flavor.

The evaluation of the acid properties of honey is performed by three different parameters, the initial pH value, the free acidity and the lactonic acidity. Free acidity is the measure obtained from titration with sodium hydroxide until the equivalence point at pH=7. The lactonic acidity is obtained by adding an excess of sodium hydroxide that is titrated with sulphuric acid. The determination of total acidity is obtained by the sum of free acidity and lactation.

Although the Codex Alimentaruis (Codex et al. 2001) does not set a limit on the pH value in honey. The values should be between 3.2 and 4.5 to inhibit the majority of microorganisms (Doner et al., 2003).

In this study. all the samples have values of pH in the range between 3,73 and 4,94, presented respectively by CIR 130 and Z1, Table 5.

Free acidity gives information about the origin of honey and influencing its stability (Pataca et al., 2007). The values obtained for free acidity in our study were between 0,19 and 1,026 $meqkg^{-1}$ and at the equivalence points (pH=7) and for (pH=8.3) it was between

0,325 and 1,28 $meqkg^{-1}$ (Table 5).
All the honeys analyzed are within the required standard of the Codex Alimentarius (1998), which is 50 $meqkg^{-1}$, indicating an absence of unwanted fermentation in our samples.

Lactonic acidity is considered as an acidity reserve when honey becomes alkaline (Gonnet, 1982). The values obtained in our lactonic acidity study are between 7,173 and 10,286 $meqkg^{-1}$ (Table 5).

Total acidity is the sum of free and lactonic acidity, it is a quality criterion, and our results showed values between 8.073 and 10,606 $meqkg^{-1}$, and these results indicate that all the honeys analyzed comply with the standard required by the codex.

Table 5: pH and acidity of the honey samples

Sample Code	Initial pH	Free pH = 7 ($meqKg^{-1}$)	Free pH 8.3 ($meqKg^{-1}$)	Lactonic ($meqKg^{-1}$)	Total ($meqKg^{-1}$)
		Paço			
82	4,133	0,841	1,145	8,83	9,671
74	4,407	0,756	1,022	9,201	9,957
89	4,339	0,728	1,016	9,276	10,004
95	4,574	0,711	0,972	9,371	10,082
134	4,388	0,642	0,929	9,364	10,006
138	4,209	0,63	0,893	9,314	9,944
152	4,201	0,662	0,936	9,172	9,834
209	4,482	0,563	0,854	9,495	10,058
		Espinhosela			
100	4,458	0,683	0,996	9,054	9,737
153	4,832	0,678	1,002	9,07	9,748
201	4,277	0,785	1,096	8,7	9,485
202	4,48	0,587	0,86	9,161	9,748
203	4,467	0,664	0,921	9,155	9,819
204	4,4	0,686	0,949	9,052	9,738
205	4,28	0,819	1,088	8,71	9,529
206	4,62	0,629	0,976	9,03	9,659
207	4,616	0,598	0,873	9,21	9,808
208	4,799	0,538	0,788	9,364	9,902
		Ciradelha			
148	4,524	0,568	0,83	9,708	10,276
17	4,552	0,568	0,826	9,65	10,218
137	4,356	0,61	0,892	9,538	10,148
136	4,1	0,676	0,937	8,984	9,66
135	3,881	1,026	1,28	7,173	8,199
130	3,726	0,961	1,216	8,316	9,277
146	4,244	0,609	0,887	9,065	9,674
145	4,79	0,544	0,859	7,621	8,165
141	4,658	0,561	0,832	9,226	9,787
		Rio de Fornos			
79	4,347	0,667	0,917	8,635	9,302
80	4,559	0,694	1,048	9,196	9,89
81	4,612	0,742	0,509	9,864	10,606
85	4,646	0,712	0,473	9,746	10,458
101	4,582	0,608	0,854	9,587	10,195

111	4,368	0,681	0,964	9,38	10,061
132	4,867	0,598	0,855	9,559	10,157
142	4,66	0,474	0,733	9,806	10,28
143	4,54	0,566	0,828	7,507	8,073
200	4,34	0,514	0,74	9,91	10,424
Barreiros					
SFM 1	4,793	0,19	0,325	10,046	10,236
Lamedo					
SFM 2	4,744	0,2	0,338	10,073	10,273
Fonte Indevacas					
SFM 3	4,56	0,242	0,381	10,286	10,528
Fonte Indueiro					
SFM 4	4,731	0,198	0,35	10,064	10,262
Labanho Cima					
Z1	4,937	0,23	0,359	10,273	10,503

5.Hydroxymethylfurfural

The hydroxymethylfurfurfural (HMF) content is considered a quality indicator in honey, as its presence is indicative of its deterioration. Immediately after the extraction process, the 5-HMF is practically absent from honey. However. during processing and prolonged storage its concentration has tendency to increase gradually due to the degradation reactions. in acidic medium. of sugars. such as glucose and fructose (Castro-Vázquez et al. 2008) and Maillard reactions that occur between reducing sugars and some amino acid residues (Soares et al. 2017).

According to European Commission, 2002a and codex Alimentarius Commission, 2001, an HMF content lower than 10.0 $mg.kg^{-1}$ is frequently found in honeydew honey, indicating acceptable freshness, since it is established a maximum value of 40 $mg.kg^{-1}$ of HMF for honeys in general, with the exception for honeys from tropical countries or regions where the maximum value may reach 80 $mg.kg^{-1}$.

The results in this study, Table 6, are between 0 and 25.3 $mg.kg^{-1}$, being within the standard required by the European legislation, indicating acceptable freshness. storage and handling conditions. The high value of 5-HMF recorded in sample CIR 137 may be due to different factors. including poor sample storage or exposure to high temperatures. The results was higher to what was previously reported in Spanish *Quercus Pyrenaica* honeydew honey, with mean values of 0.1 mg/100 g (Shantal Rodríguez Flores et al., 2015a). Despite this, the results still indicates that the honey was fresh.

Table 6: Physio-chemical honey parameters: 5-HMF, diastase index, and proline

Sample	HMF ($mgKg^{-1}$)	Diastase index (DN)	Proline (mgg^{-1})
	Paço		
74	8.9 ± 2.3	29.8 ± 1.9	2.0 ± 0.2
82	11.0 ± 2.3	22.4 ± 0.4	2.3 ± 0.4
89	5.8 ± 0.7	30.7 ± 1.5	1.0 ± 0.1
95	7.7 ± 2.4	27.2 ± 0.8	2.6 ± 0.1
134	17.1 ± 2.9	19.2 ± 0.7	2.3 ± 0.8
138	0	26.7 ± 0.4	2.7 ± 0.5
152	0	30.8 ± 1.6	2.6 ± 0.3
209	6.4 ± 1.2	24.2 ± 0.8	3.3 ± 0.1
	Espinhosela		
100	0	14.5 ± 0.8	3.8 ± 0.3
153	0	16.4 ± 0.8	3.7 ± 0.1
201	0	10.0 ± 0.9	4.0 ± 0.3

202	0	21.7 ± 0.2	3.6 ± 0.2
203	0	31.0 ± 1.6	3.8 ± 0.1
204	0	30.2 ± 0.0	3.4 ± 0.2
205	0	22.3 ± 0.6	3.7 ± 0.2
206	3.4 ± 0.3	14.1 ± 0.4	4.0 ± 0.3
207	0	29.2 ± 1.2	3.3 ± 0.3
208	0	30.1 ± 0.2	3.8 ± 0.4
Ciradelha			
17	0	15.6 ± 0.9	3.2 ± 0.3
130	0	23.5 ± 1.8	3.3 ± 0.1
135	0	22.7 ± 0.9	3.9 ± 0.8
136	0	25.3 ± 1.4	3.8 ± 0.9
137	21.6 ± 3.7	21.4 ± 0.2	3.9 ± 0.5
141	3.1 ± 2.0	17.1 ± 0.4	3.6 ± 0.1
145	0	15.9 ± 0.6	4.0 ± 0.8
146	0	15.8 ± 0.5	3.6 ± 0.8
148	1.6 ± 0.2	26.2 ± 1.2	3.4 ± 0.1
Rio de Fornos			
79	0	23.1 ± 0.1	6.2 ± 0.1
80	0	16.7 ± 1.1	1.7 ± 0.3
81	0	24.0 ± 0.7	4.8 ± 0.1
85	0	21.1 ± 0.9	1.9 ± 0.1
101	0	19.7 ± 0.7	2.9 ± 0.2
111	6.0 ± 0.7	24.3 ± 0.6	3.4 ± 0.8
132	0	18.2 ± 0.9	1.5 ± 0.9
142	0	23.6 ± 0.6	3.3 ± 0.6
143	6.1 ± 1.0	16.2 ± 1.3	2.4 ± 0.7
200	0	23.0 ± 1.5	2.2 ± 0.3
Barreiros			
SFM1	0	16.3 ± 1.4	0.72 ± 0.00
Lamedo			
SFM2	0	15.1 ± 0.7	0.74 ± 0.01
Fonte Indevacas			
SFM3	0	14.9 ± 0.3	0.68 ± 0.00
Fonte Indueiro			
SFM4	0	15.5 ± 0.6	0.74 ± 0.00
Labanho Cima			
Z1	0	15.5 ± 0.4	0.62 ± 0.01

6. Diastase activity

Similarly to the hydroxymethylfurfural content. diastase activity is also used as an indicator of freshness (European Honey Directive. 2001). Diastase is composed by honey enzymes (α and β-amylase) secreted by the bee in honey. usually used as an indicator of honey aging since they have a high sensitivity to heat. Honeys in general should present diastase activity of at least 8 Schade units (DN), minimum value accepted by regulatory organizations (Codex Alimentarius Commission, 2001; European Commission, 2002a)

The results of our honeys were between 9,01 DN and 32,67 DN (Table 6).they were in accordance with the minimum of 8 DN established by the European Community Regulation.

The wide range of diastase values suggests that different factors such as botanic origin, processing and storage conditions, secretion of bees and plant-sucking insects, and honey composition can influence the diastase activity of honeydew honeys (Seraglio et al., 2019).

7. Proline

Proline is an important amino acid that originates mostly from the salivary secretions of *Apis mellifera* during the conversion of nectar into honey (Bergner and al, 1972). Proline content is an indication of honey ripeness and, in some cases, sugar adulteration. Some authors have reported that high concentrations of proline are also typical for honeydew honeys. Indirectly, proline levels also reflect botanical origin (Cotte and al, 2004).

Although the proline content is not present in the European Union's quality criteria. it is recognized that genuine honey should have proline contents greater than 0.18 $mg.g^{-1}$, lesser values could mean that the honey is possibly corrupted by sugar addition (Bogdanov. 2002).

In this study, it was observed that the values obtained for the proline content ranged between 0.9 $mg. g^{-1}$ and 8.13 $mg. g^{-1}$, Table 6. This results was higher than what was reported previously for Turkish *Quercus spp* honeydew honey with values between 0,43 $mg. g^{-1}$ and 1,24 $mg. g^{-1}$ (Kolayli et al., 2018).

The obtained values indicated a high proline content indicative of unadulterated honey and an excellent degree of maturation.

7. Ash content

The Codex Alimentarius (1999) provide values for ashes parameter and establishes that must have a maximum of 0.6 % for nectar honey and 1.2 % for honeydew honey or a mixture of honeydew honey with blossom honey or chestnut honey.

The obtained results in this study for the ash content were between 0.45 to 0.74%, Table 7. The results were similar to previous study of Turkish *Quercus spp* honeydew honey with reported values between 0.36% to 0.72%, and higher than in many nectar honeys (Kolayli et al., 2018).

Table 7: Nutritional parameters: ashes, proteins, energy, and carbohydrates

Sample	Ash (g/100g)	Proteins (g/100g)	Energy (Kcal)	Carbohydrates (g/100g)
Paço				
74	0.57 ± 0.01	0.57 ± 0.06	331.7	82.5
82	0.63 ± 0.00	0.96 ± 0.06	333.9	82.6
89	0.60 ± 0.01	0.66 ± 0.05	336.0	83.3
95	0.59 ± 0.00	0.79 ± 0.07	336.9	83.5
134	0.61 ± 0.01	0.58 ± 0.03	330.4	82.0
138	0.55 ± 0.01	0.54 ± 0.09	331.4	82.3
152	0.59 ± 0.00	0.60 ± 0.00	327.6	81.2
209	0.55 ± 0.00	0.48 ± 0.06	332.2	82.5
Espinhosela				
100	0.67 ± 0.01	0.64 ± 0.07	331.7	82.3
153	0.68 ± 0.01	0.54 ± 0.03	328.5	81.6
201	0.68 ± 0.00	0.57 ± 0.05	320.1	79.5
202	0.71 ± 0.00	0.54 ± 0.02	330.0	82.1
203	0.69 ± 0.00	0.53 ± 0.03	334.0	82.9
204	0.73 ± 0.01	0.58 ± 0.03	328.3	81.6
205	0.67 ± 0.01	0.61 ± 0.09	330.1	81.9
206	0.67 ± 0.02	0.59 ± 0.04	323.3	80.4
207	0.69 ± 0.00	0.52 ± 0.00	329.2	81.8
208	0.70 ± 0.01	0.51 ± 0.02	336.4	83.6
Ciradelha				
17	0.50 ± 0.01	0.44 ± 0.00	336.4	83.6

30	0.53 ± 0.00	0.51 ± 0.07	333.9	83.0
135	0.57 ± 0.01	0.39 ± 0.05	334.5	83.2
136	0.47 ± 0.00	0.42 ± 0.02	338.5	84.2
137	0.49 ± 0.01	0.44 ± 0.00	336.4	83.7
141	0.50 ± 0.01	0.45 ± 0.09	337.2	83.8
145	0.46 ± 0.00	0.48 ± 0.06	336.1	83.6
146	0.48 ± 0.01	0.50 ± 0.02	338.5	84.1
148	0.46 ± 0.00	0.52 ± 0.04	338.2	84.0
Rio de Fornos				
79	0.55 ± 0.00	0.57 ± 0.05	335.8	83.4
80	0.62 ± 0.02	0.49 ± 0.01	333.9	82.9
81	0.59 ± 0.00	0.71 ± 0.01	334.4	83.0
85	0.60 ± 0.00	0.56 ± 0.08	338.4	84.0
101	0.57 ± 0.00	0.51 ± 0.00	338.5	84.1
111	0.61 ± 0.00	0.53 ± 0.09	335.9	83.5
132	0.57 ± 0.01	0.64 ± 0.03	335.7	83.4
142	0.56 ± 0.01	0.71 ± 0.01	340.2	84.4
143	0.58 ± 0.01	0.45 ± 0.02	335.7	83.4
200	0.56 ± 0.00	0.55 ± 0.02	336.2	83.5
Barreiros				
SFM1	0.72 ± 0.00	0.69 ± 0.04	337.5	83.7
Lamedo				
SFM2	0.74 ± 0.01	0.57 ± 0.04	337.9	83.9
Fonte Indevacas				
SFM3	0.68 ± 0.00	0.68 ± 0.03	335.3	83.1
Fonte Indueiro				
SFM4	0.74 ± 0.00	0.75 ± 0.00	336.2	83.3
Labanho de Cima				
Z1	0.62 ± 0.01	0.50 ± 0.03	334.7	83.2

8. Protein content

Typically. the proteins present in honey are enzymes derived from nectar and pollen from the plant and from the secretions of the salivary glands of bees. The amount of total protein can contribute to defining the aroma that characterizes each type of honey due to the Maillard reactions that may occur.

The total protein content of samples ranged from 0,47 to 1,02 g/100g (Table 7) The obtained results showed remarkable similarity with the protein amounts recorded in forest honeydew honeys from Malaysia, the protein ranged from 0.43 to 1.02 $g.100g^{-1}$ (Seraglio et al., 2019). Whereas, high protein content was recorded comparing to what was reported in honeydew honeys from Brazil and Slovakia presenting mean values of $0.04g.100g^{-1}$, Lebanon honeydew honeys (0.08 to 0.14 $g.100g^{-1}$) and Croatian honeydew honeys (0.03 to 0.10 $g.100g^{-1}$) (Seraglio et al., 2019).

9. Total carbohydrates and energy

As with mineral and protein content. there is also no legislation regulating the limits for energy value and carbohydrate content present in different methods. The honey samples studied showed similar values of carbohydrates, ranging from 80.4 to 84.4 g/100g and of energy value with values between 320.1 and 340.2 Kcal, Table 7.

10. Sugars

Honey is a natural mixture prepared by honeybees from flower's nectar, classified as nectar honey, or from exudates of living parts of plants or excretions of sucking insects living in parts of plants, classified as honeydew honey. Honey is a concentrated aqueous solution of sugars, so, its physicochemical and nutritional properties depend on the sugar composition.

The analysis of the sugar content in the samples was performed by liquid chromatography coupled to a refraction index detector (HPLC-RI). All the samples displayed a similar sugar profile, with fructose being the most abundant monosaccharide followed by glucose, ranging from 34 to 36% and 25 to 32%, respectively, Table 9. The results are in accordance with the values established in the regulation for honeydew honeys, where the combined concentration of these two reducing sugars should be between 45-60% of the total sugar content present in honey samples. The analysis of the sugar profile of the honeydew honey samples showed that the main compounds present were fructose and glucose, which in total make up in average more than 80% of the sugars found, which was higher than previous results reported for Turkish *Quercus spp* honeydew honey with values between 58% and 68% (Kolayli et al., 2018).

Disaccharides such as turanose, maltulose, isomaltose, trehalose and melibiose, as well as trisaccharides such as erlose, and melezitose were also detected but quantified at low concentrations.

The presence of erlose and melezitose was an indication of the presence of honeydew in honey. Since these two compounds are absent in nectar honey, this can be a tool in order to differentiate honeydew honey from nectar honey, Table 8.

Table 8: Sugar profile, obtained by HPLC-RI, of the studied honey sample (values expressed in g/100g of honey)

Sample	Frutose	Glucose	Turanose	Maltulose	Isomaltose	Trealose	Melibiose	Erlose	Melezitose
Paço									
74	35.9	28.9	2.7	4.6	5.2	1.4	2.2	1.5	0.7
82	35.3	27.1	2.4	3.9	3.0	0.9	2.8	0	0.5
89	36.2	27.7	2.8	5.1	6.8	1.3	2.9	0	0.8
95	34.7	25	1.9	2.5	1.8	0.8	1.8	1.1	0.6
134	35.2	26.6	2.2	2.6	2.5	0.8	2.0	1	0.5
138	35	25.9	2.07	2.77	2.4	0.89	1.9	0	0.6
152	34.25	25.06	2.14	3.11	2.15	0.78	1.88	0	0.46
209	34.84	25.85	2.23	2.62	2.75	0.96	2.16	0	0.58
Espinhosela									
100	35.45	27.6	1.85	1.88	2.36	0.8	1.9	0.92	0.47
153	35.61	27.87	2.24	3.79	4.28	1.16	2.05	0	1.35
201	36.21	29.65	1.81	3.024	3.65	1.10	1.73	0	1.12
202	36.46	27.10	1.89	2.4	2.18	0.85	2.23	0.955	0.95
203	37.4	27.77	2.1	3.83	4.01	1.124	2.33	0.72	0.9
204	37.56	28.1	2.233	3.82	4.15	1.09	2.5	0	1.7
205	36.43	28.4	1.93	3.13	3.25	0.97	1.86	0	1.22
206	35.9	28.5	1.27	1.92	0.8	1.04	1.85	1.54	0.39
207	34.70	26.12	2.1	3.54	3.6	1.14	2.11	0	1.23
208	37.54	27.5	2.5	4.46	5.54	1.18	2.39	0	1.61

Table 9: Averages of Sugar profile, obtained by HPLC-RI, of the studied honeydew honey classified by apiary (values expressed in %)

Apiary	Frutose	Glucose	Turanose	Maltulose	Isomaltose	Trealose	Melibiose	Erlose	Melezitose
Paço	35,2	26,5	2,3	3,4	3,3	1,0	2,2	0,4	0,6
Espinhosela	36,3	27,9	2,0	3,2	3,4	1,0	2,1	0,4	1,1
Ciradelha	35,4	27,8	2,5	3,8	4,6	1,2	2,2	0,5	0,9
Rio de Fornos	37,1	29,2	2,7	4,5	6,2	1,4	2,4	0,7	0,8
Barreiros	40,6	29,2	2,5	4,7	6,1	1,1	2,5	0,6	0,4
Lamedo	38,4	28,0	2,2	4,6	5,4	1,1	2,3	0,7	0,7
Fonte indevacas	38,1	28,5	2,2	4,3	5,3	1,2	2,1	0,6	0,5
Fonte Indueiro	38,6	28,2	2,3	4,3	5,8	1,3	2,3	0,6	0,4
Labanho cima	37,6	29,8	2,4	4,3	5,5	1,2	2,2	0,7	0,7

Crystallization is a process that happens naturally in honey and is associated with its content in sugars, moisture, and honey type. The F/G (fructose/glucose) and G/H (glucose/humidity) ratios show evidence of how long a honey sample takes to crystallize. The ratio of fructose and glucose (F/G) is related largely to the source of nectar

The F/G (fructose/glucose) and G/H (glucose/humidity) ratios show evidence of how long a honey sample takes to crystallize, according to literature: When F/G is less than 1.1 crystallization is fast, and when it's between 1.1 and 1.5 the crystallization is slow. Above 1.5, it means no crystallization (Escuredo et al., 2014)

The speed at which glucose crystallization happens is also related to the G/H ratio. When G/H is less than 1.7, the crystallization is slow or null , and when G/H is superior to 2.2, the crystallization is fast (Escuredo et al.,2014).

Results showed that the F/G mean values ranged between 1.3 and 1.4. This value demonstrate that all samples showed slow crystallization. The G/H mean values range from 1.6 to 2.0 means that it's also slow but with propensity for crystallization for some samples (Table10).

The analysis of the sugar profile indicated that in general honeydew honey samples had an average of slow tendency for crystallization.

Table 10: average results of F/G (fructose/glucose) and G/H (glucose/humidity) ratios

Apiaries	F/G	G/H
Paço	1.3	1.6
Espinhosela	1.3	1.6
Ciradelha	1.3	1.8
Rio de Fornos	1.3	1.9
Barreiros	1.4	2
Lamedo	1.4	1.9
Fonte indevacas	1.3	1.8
Fonte Indueiro	1.4	1.9
Labanho cima	1.3	1.9

11. Phenolic compoundsprofile

Nowadays, new analytical methodologies, such as the analysis of the phenolic compounds profile, are used in the characterization and evaluation of the authenticity of honey associated with botanical origins (Soares et al., ,2017). The phenolic compound profile of samples was evaluated by UPLC/DAD/ESI-MS^n. The analysis allowed the elucidation of phenolic compounds by comparing their chromatographic profile, UV spectrum, and mass spectrometry information, with reference compounds. When patterns were not available, structural information was confirmed with the combination of UV data and MS fragmentations described in the literature.

In the analysis of ESI-MS^n, the negative mode was used due to the great sensitivity that this mode represents in the measurement of different classes of phenolic compounds (Falcão et al.,2013).

In this study, it was possible to identify 17 phenolic compounds in the honeydew honey samples, where flavonoids were the most abundant compounds, specially chrysin and pinobanksin-5-methyl ether, followed by phenolic acids, specially, caffeic acid and ellagic acid, Figure 10 and Table 11.

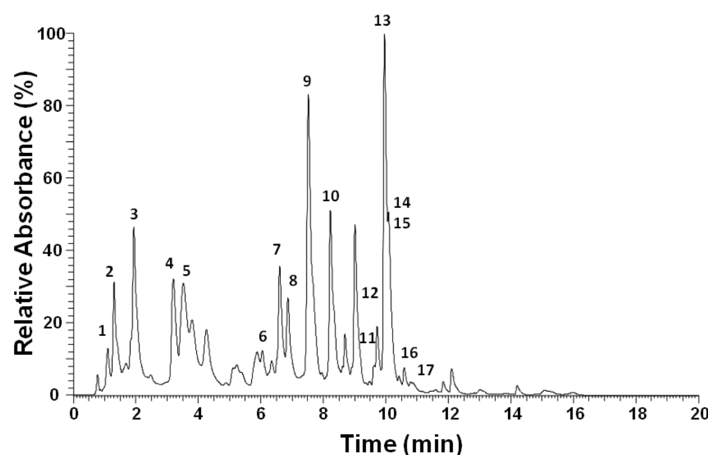


Figure 10 – Chromatographic profile at 280 nm of sample Rio de Fornos 101.

Table 11: Phenolic and isoprenoid compounds identified by UHPLC/DAD/ESI-MS^n in the analyzed honeydew honey samples.

Nº	Proposed compound	t_R (min)	λ_{max} (nm)	$[M-H]^-$	$[M-H]^{-2}$
1	Gallic acid[a]	1.08	277	169	125
2	Protocatechuic acid[a]	1.24	253	153	109
3	Caffeic acid[a]	1.82	292, 322	179	135
4	*p*-Coumaric acid[a]	3.03	310	163	119
5	Ellagic acid[a]	3.32	253, 367	301	301
6	Salicylic acid[a]	5.96	256	137	93
7	Camphoric acid[b]	6.52	221	199	155 *(100)*, 137 *(20)*
8	*cis,trans*-Abscisic acid[b,c2]	6.79	265	263	153 *(69)*, 154 *(100)*, 220 *(36)*
9	Pinobanksin-5-methyl-ether[b,c1]	7.47	287	285	267 *(100)*, 252 *(13)*, 239 *(29)*
10	Pinobanksin[b,c1]	8.18	292	271	253 *(100)*, 225 *(19)*, 151 *(10)*
11	Quercetin-dimethyl-ether[b,c1]	9.50	256, 367	329	314
12	Caffeic acid isoprenyl ester[b,c1]	9.69	298, 325	247	179 *(100)*, 135 *(100)*
13	Chrysin[a]	9.93	268	253	253 *(100)*, 225 *(17)*, 209 *(49)*, 151 *(1)*
14	Pinocembrin[a]	10.08	290	255	255 *(16)*, 213 *(100)*, 211 *(40)*, 151 *(26)*
15	Galangin[a]		265, 300sh, 358	269	269 *(100)*, 241 *(61)*, 227 *(20)*, 197 *(22)*, 151 *(20)*
16	Acacetin[b,c1]	10.55	268, 331	283	268
17	Caffeic acid cinnamyl ester[b,c1]	10.75	295, 324	295	178 *(100)*, 134 *(80)*

[a]Confirmed with standard; [b]Confirmed with MS^n fragmentation; Confirmed with references: [c1]Falcão et al., 2013; [c2]Bertoncelj et al., 2011.

Table 12: Average of phenolic compounds quantification in the honey samples classified by apiary expressed in mg/kg

Tr (min)	Proposed compound	Espinoselha (mg/kg)	Paço (mg/kg)	Rio de fornos (mg/kg)	Ciradelha (mg/kg)
1.08	Gallic acid	0.90	0.56	1.58	1.17
1.24	Protocatechuic acid	4.23	2.80	5.59	4.55
1.82	Caffeic acid	11.26	8.66	17.05	16.09
3.03	p-Coumaric acid	2.02	2.68	6.82	5.20
3.32	Ellagic acid	15.06	6.87	15.51	14.45
5.96	Salycilic acid	1.18	1.39	3.07	1.95
6.52	Camphoric acid	2.45	2.27	6.87	5.08
6.79	cis,trans-Abscisic acid	5.70	2.77	4.61	4.68
7.47	Pinobanksin-5-methyl-ether	22.18	14.07	28.68	30.51
8.18	Pinobanksin	10.51	7.37	15.06	18.45
9.5	Quercetin-dimethyl-ether	1.24	0.47	1.92	1.39
9.69	Caffeic acid isoprenyl ester	1.47	0.53	5.38	1.81
9.93	Chrysin	9.34	6.81	20.64	17.68
10.08	Pinocembrin	6.69	3.91	7.22	10.54
	galangin	7.79	6.08	8.19	15.35
10.75	Caffeic acid cinnamyl ester	0.29	0.21	0.38	0.77

12. Mineral Content

Honey contains diversified amounts of mineral substances, ranging from 0.02 to 1.03g/100g (White, 1975). Potassium, with an average of about one third of the total, is the main mineral element (Feller-Demalsy et al., 1989; Gonzalez-Miret et al., 2005). The amount of different minerals in honey is largely dependent on the soil composition, as well as various types of floral plants (Anklam 1998). In addition to these factors, the beekeeping practices, environmental pollution, and honey processing may also contribute to the diversified mineral content present in honey (Pohl, 2009).

Sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca) were determined using a flame ionization atomic absorption spectrophotometer, while the other minerals such as copper (Cu), manganese (Mn), iron (Fe) and cadmium (Cd) were determined using a graphite chamber atomic absorption spectrophotometry The contents of each mineral found in our honeys expressed in mg/kg are shown in Table 13.

Table 13: Minerals contents, obtained by using flame atomic absorption spectrophotometer

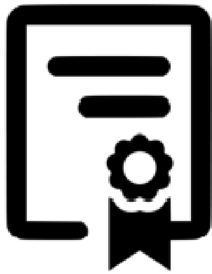
Sample	[K]/(mg/Kg)	[Na]/(mg/Kg)	[Ca]/(mg/Kg)	[Mg]/(mg/Kg)	[Fe]/(mg/Kg)	[Mn]/(mg/Kg)	[Cu]/(mg/Kg)	[Zn]/(mg/Kg)
RdF 85	1690,84	376,36	139,09	4043,30	0,74	33,50	1,197	10,13
RdF 132	1859,59	456,31	131,74	4263,14	0,27	28,25	1,391	11,04
P 209	2648,88	694,91	100,61	3760,34	0,93	24,86	1,420	15,06
RdF 81	1863,73	329,06	114,44	4225,21	0,58	29,70	1,019	11,88
RdF 143	2201,29	417,00	116,39	4611,90	1,27	31,16	1,022	9,96
P 138	2188,10	279,58	146,15	3871,81	2,26	19,65	1,237	7,83
P 134	1491,89	137,28	144,67	3924,94	0,71	15,42	1,039	13,03
P 82	2637,21	481,18	155,66	4669,86	0,75	18,45	1,088	10,57
P 74	1774,08	310,68	138,60	4245,69	0,79	16,38	1,140	12,22
RdF 80	2439,42	275,26	159,97	4952,57	0,87	37,22	1,309	11,50
ESP 202	2574,31	300,20	114,35	3555,10	0,77	24,20	0,974	8,56
SFM 2	2374,19	294,41	113,39	4036,75	0,85	27,12	1,112	10,63
SFM 1	2841,38	349,09	131,06	4331,60	0,86	25,68	1,124	10,06
P 152	2046,96	306,50	133,74	3825,70	0,87	19,87	1,343	11,50
RdF 142	2278,32	338,16	123,49	4407,86	0,88	33,08	1,115	14,59
RdF 200	2234,27	352,53	121,01	4231,36	1,01	31,58	1,232	13,00
RdF 111	2082,91	594,54	119,33	3864,59	0,90	33,52	1,360	12,99
SFM 3	2900,12	612,26	139,47	4801,36	0,91	27,56	1,008	10,69
ESP 207	2661,37	374,07	120,36	4246,71	0,24	26,68	1,444	11,80
SFM 4	3000,94	397,30	123,30	3946,46	1,06	31,60	1,095	10,49
ESP 100	2740,82	333,60	124,50	3730,50	0,89	29,05	1,542	11,87
P 89	2146,57	330,66	143,81	4250,66	0,98	16,63	0,972	13,53
ESP 205	2588,67	305,73	129,83	4240,12	0,70	27,62	0,980	12,58
ESP 204	2753,82	363,46	131,23	4061,00	0,65	23,50	1,224	11,89
RdF 79	2242,54	414,92	143,99	4393,36	1,00	28,41	1,112	14,09
ESP 201	2540,77	310,56	126,33	4161,60	0,05	28,35	1,070	11,78
CIR 141	2104,01	495,02	123,53	5207,69	1,00	33,34	1,067	14,16
CIR 136	1657,44	300,10	114,21	3572,73	0,51	38,50	1,213	12,91
CIR 135	1989,93	300,15	118,96	4027,91	1,02	36,13	1,432	14,94
Z 1	2807,41	400,47	122,77	4286,00	0,66	47,97	1,031	10,36
RdF 101	2132,95	272,08	117,87	3686,60	0,58	34,34	1,360	13,28
ESP 206	3010,43	409,52	139,65	4558,03	0,34	25,55	1,290	11,12
ESP 208	2562,90	374,13	153,05	3910,20	0,79	24,11	0,868	12,53
CIR 130	2327,69	434,71	127,25	4354,33	0,57	37,96	1,163	12,10
CIR 145	2101,83	418,33	127,58	4727,86	1,14	38,61	1,443	13,23
P 95	2082,08	225,10	145,83	4025,86	0,28	19,34	1,456	12,69
CIR 146	2001,47	457,22	126,20	4778,36	0,52	37,70	1,040	11,48
ESP 203	2863,92	438,59	127,21	4958,57	0,56	25,24	1,044	11,06
CIR 148	2004,47	405,43	155,26	4469,76	1,44	40,23	1,111	13,16
CIR 137	1784,27	294,05	117,32	3897,10	1,00	36,49	1,254	11,83
ESP 153	2591,96	267,25	116,90	3523,71	0,95	25,83	1,707	10,52
CIR 17	1723,21	229,20	139,54	4228,85	1,22	36,73	0,983	12,82

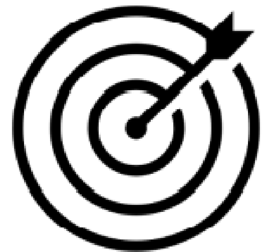
The magnesium was quantitatively the most important mineral, 59.24% of total minerals quantified, having an average content 4126.92 mg/kg, followed by potassium, 33.07% with average content 2303.58 mg/kg.

Sodium and calcium were present in moderate amounts in the honeys (5.32% and 1.74% of total minerals, respectively), while cadmium, iron and zinc were below the

detection limit, with values 0.02% for copper and iron and 0.17% for zinc. The iron content had average value of 1.48 mg/kg which is less than the maximum limit set by the codex Alimentarius 15 mg/kg and copper was above the limit 5mg/kg with average value of 1.21 mg/kg.

Moreover, Cadmium is considered bioindicator for honey contamination (Licata et al. 2004). The regulations establish a maximum level of 300 μg/kg, recommended by FAO/WHO/1984 (Al-Eed et al. 2002) while for Cd the European legislation and the Codex Alimentarius, 2001 fixed a maximum of 0.05 mg/kg, nevertheless our results did not reveal its presence.





Chapter IV: CONCLUSION AND WORK PERSPECTIVE

Chapter IV: CONCLUSION AND WORK PERSPECTIVE

1. Conclusion

The commercial interest in honeydew honeys is increasing due to the differentiated nutritional, sensorial and possible therapeutic characteristics of this honey. In this context, this work aimed to characterize the production of honeydew honey from *Quercus pyrenaica* oak and identify marker compounds that discriminates the botanical origin of this honey.

The samples presented for color analysis values ranging from 130 to 150 mm pfund, this means that the color was classified as dark amber. Then results of moisture content of the samples ranged from 14.4% to 19.3%, which was in accordance with the maximum value established of 20%, and safe to avoid fermentation. In addition, all analyzed honeys presented for electrical conductivity values more than 0,8 $mS.cm^{-1}$, ranging from 0.96 to 1.43 $mS.cm^{-1}$, being considered as honeydew honey. Regarding the acidity, in this study, all the samples had values in the range between 3,73 and 4,94, presented respectively by CIR 130 and Z1. The values obtained for free acidity were between 0,19 and 1,026 $meqkg^{-1}$ and at the equivalence points (pH=7) and for (pH=8.3) it was between 0,325 and 1,28 $meqkg^{-1}$. All the honeys analyzed are within the required standard of the Codex Alimentarius (1998), which is 50 $meqkg^{-1}$. The values obtained for lactonic acidity study were between 7,173 and 10,286 $meqkg^{-1}$. Total acidity showed values between 8.073 and 10,606 $meqkg^{-1}$. For HMF, the results, were between 0 and 24.25 $mg.kg^{-1}$, being within the standard required by the European legislation, indicating acceptable freshness. storage and handling conditions. The high value of HMF recorded in sample CIR 137 may be due to different factors, including poor sample storage or exposure to high temperatures. Furthermore, the diastase results of our honey were between 9,01 DN and 32,67 DN. They were in accordance with the minimum of 8 DN established by the European Community Regulation. Regarding proline, it was observed that the values obtained for the proline content ranged between 0.9 $mg.g^{-1}$ and 8.13 $mg.g^{-1}$. The obtained values indicated a high proline content indicative of unadulterated honey and an excellent degree of maturation. Ash content were between 0.45 to 0.74%, while total protein content of samples ranged from 0.47 to 1.02 g/100g. Total carbohydates and energy, showed similar values of carbohydrates, ranging from 80.4 to 84.4 g/100g and of energy value with values between 320.1 and 340.2

Kcal.

The analysis of the sugar content in the samples was performed by liquid chromatography coupled to a refraction index detector (HPLC-RI). All the samples displayed a similar sugar profile, with fructose being the most abundant monosaccharide followed by glucose, ranging from 34 to 36% and 25 to 32%, respectively The presence of erlose and melezitose was an indication of the presence of honeydew in honey. Since these two compounds are absent in nectar honey, this can be a tool in order to differentiate honeydew honey from nectar honey. Results showed that the F/G mean values ranged between 1.3 and 1.4. This value demonstrate that all samples showed slow crystallization. The G/H mean values range from 1.6 to 2.0 means that it's also slow but with propensity for crystallization for some samples.

Regarding the phenolic compounds, 17 substances were identified in the honeydew honey samples, where flavonoids were the most abundant compounds, specially chrysin and pinobanksin-5-methyl ether, followed by phenolic acids, specially, caffeic acid and ellagic acid.

2.Future Work perspective

This work aims to characterize the production of honeydew honey with origin in *Quercus pyrenaica* oak from Montesinho Natural Park and identify marker compounds that discriminates the botanical origin of this honey.

-In terms of future perspectives, a statistical analysis must be applied to obtain a differentiation between honeydew honeys using the chemical parameters analysed in this work in order to obtain standard values for each honeydew honey.

-The study of the honeydew honeys from Montesinho Natural Park, North Portugal can be extended to more regions of Portugal, to confirm the pattern of results observed, as well as, new botanical origins different than *Quercus pyrenaica* can be included in future studies.

Bibliographic References

Bicudo de Almeida-Muradian, L., Monika Barth, O., Dietemann, V., Eyer, M., Freitas, A. da S. de, Martel, A. C., Marcazzan, G. L., Marchese, C. M., Mucignat-Caretta, C., Pascual-Maté, A., Reybroeck, W., Sancho, M. T., & Gasparotto Sattler, J. A. (2020). Standard methods for Apis mellifera honey research. In *Journal of Apicultural Research* (Vol. 59, Issue 3, pp. 1–62). Taylor and Francis Ltd. https://doi.org/10.1080/00218839.2020.1738135

Castro-Vázquez, L., Díaz-Maroto, M. C., & Pérez-Coello, M. S. (2006). Volatile Composition and contribution to the aroma of spanish honeydew honeys. Identification of a new chemical marker. *Journal of Agricultural and Food Chemistry*, *54*(13), 4809–4813. https://doi.org/10.1021/jf0604384

drazen lusic, olivera koprivnjak, duska curic, anna G.sabatini, & lanfranco S:conte. (2007). *honeydew croatia[288]*. 156–165.

Ilyasov, R. A., Lee, M. lyeol, Takahashi, J. ichi, Kwon, H. W., & Nikolenko, A. G. (2020). A revision of subspecies structure of western honey bee Apis mellifera. In *Saudi Journal of Biological Sciences* (Vol. 27, Issue 12, pp. 3615–3621). Elsevier B.V. https://doi.org/10.1016/j.sjbs.2020.08.001

Jara-Palacios, M. J., Ávila, F. J., Escudero-Gilete, M. L., Gómez Pajuelo, A., Heredia, F. J., Hernanz, D., & Terrab, A. (2019). Physicochemical properties, colour, chemical composition, and antioxidant activity of Spanish Quercus honeydew honeys. *European Food Research and Technology*, *245*(9), 2017–2026. https://doi.org/10.1007/s00217-019-03316-x

Jerković, I., & Marijanović, Z. (2010). Oak (quercus frainetto ten.) honeydew honey-Approach to screening of volatile organic composition and antioxidant capacity (DPPH and FRAP assay). *Molecules*, *15*(5), 3744–3756. https://doi.org/10.3390/molecules15053744

Karabagias, I. K., Maia, M., Karabagias, V. K., Gatzias, I., & Badeka, A. v. (2018). Characterization of eucalyptus, chestnut and heather honeys from Portugal using multi-parameter analysis and chemo-calculus. *Foods*, *7*(12). https://doi.org/10.3390/foods7120194

Kolayli, S., Can, Z., Cakir, H. E., Okan, O. T., & Yildiz, O. (2018). An investigation on Trakya region Oak (Quercus spp.) honeys of Turkey: Their physico-chemical, antioxidant and phenolic compounds properties. *Turkish Journal of Biochemistry*, *43*(4), 362–374. https://doi.org/10.1515/tjb-2017-0174

Machado De-Melo, A. A., Almeida-Muradian, L. B. de, Sancho, M. T., & Pascual-Maté, A. (2018). Composición y propiedades de la miel de Apis mellifera: una revisión. In *Journal of Apicultural Research* (Vol. 57, Issue 1, pp. 5–37). Taylor and Francis Ltd. https://doi.org/10.1080/00218839.2017.1338444

Mortensen, A. N., Schmehl, D. R., & Ellis, J. (n.d.). *European Honey Bee Apis mellifera Linnaeus and subspecies (Insecta: Hymenoptera: Apidae) 1*. http://entnemdept.ifas.ufl.edu/creatures/.

Pita-Calvo, C., & Vázquez, M. (2017a). Differences between honeydew and blossom honeys: A review. In *Trends in Food Science and Technology* (Vol. 59, pp. 79–87). Elsevier Ltd. https://doi.org/10.1016/j.tifs.2016.11.015

Pita-Calvo, C., & Vázquez, M. (2017b). Differences between honeydew and blossom honeys: A review. In *Trends in Food Science and Technology* (Vol. 59, pp. 79–87). Elsevier Ltd. https://doi.org/10.1016/j.tifs.2016.11.015

Pita-Calvo, C., & Vázquez, M. (2018). Honeydew Honeys: A Review on the Characterization and Authentication of Botanical and Geographical Origins. In *Journal of Agricultural and Food Chemistry* (Vol. 66, Issue 11, pp. 2523–2537). American Chemical Society. https://doi.org/10.1021/acs.jafc.7b05807

Ribeiro, M. I., Fernandes, A. J., Cabo, P. S., & Diniz, F. J. (2019). Trends in honey purchase and consumption in Trás-os-Montes Region, Portugal. *Economy of Region*, *15*(3), 822–833. https://doi.org/10.17059/2019-3-15

Seijo, M. C., Escuredo, O., & Rodríguez-Flores, M. S. (2019). Physicochemical properties and pollen profile of oak honeydew and evergreen oak honeydew honeys from Spain: A comparative study. *Foods*, *8*(4). https://doi.org/10.3390/foods8040126

Seraglio, S. K. T., Silva, B., Bergamo, G., Brugnerotto, P., Gonzaga, L. V., Fett, R., & Costa, A. C. O. (2019). An overview of physicochemical characteristics and health-promoting properties of honeydew honey. In *Food Research International* (Vol. 119, pp. 44–66). Elsevier Ltd. https://doi.org/10.1016/j.foodres.2019.01.028

Seraglio, S. K. T., Valese, A. C., Daguer, H., Bergamo, G., Azevedo, M. S., Gonzaga, L. V., Fett, R., & Costa, A. C. O. (2016). Development and validation of a LC-ESI-MS/MS method for the determination of phenolic compounds in honeydew honeys with the diluted-and-shoot approach. *Food Research International*, *87*, 60–67. https://doi.org/10.1016/j.foodres.2016.06.019

Shantal Rodríguez Flores, M., Escuredo, O., & Carmen Seijo, M. (2015a). Assessment of physicochemical and antioxidant characteristics of Quercus pyrenaica honeydew honeys. *Food Chemistry*, *166*, 101–106. https://doi.org/10.1016/j.foodchem.2014.06.005

Shantal Rodríguez Flores, M., Escuredo, O., & Carmen Seijo, M. (2015b). Assessment of physicochemical and antioxidant characteristics of Quercus pyrenaica honeydew honeys. *Food Chemistry*, *166*, 101–106. https://doi.org/10.1016/j.foodchem.2014.06.005

Silva, L. R., Gonçalves, A. C., Nunes, A. R., & Alves, G. (2020). Authentication of honeys from Caramulo region (Portugal): Pollen spectrum, physicochemical characteristics, mineral content, and phenolic profile. *Journal of Food Science*, *85*(2), 374–385. https://doi.org/10.1111/1750-3841.15023

Soria, A. C., González, M., de Lorenzo, C., Martínez-Castro, I., & Sanz, J. (2005). Estimation of the honeydew ratio in honey samples from their physicochemical data and from their volatile composition obtained by SPME and GC-MS. *Journal of the Science of Food and Agriculture*, *85*(5), 817–824. https://doi.org/10.1002/jsfa.1890

Stojković, M., Cvetković, D., Savić, A., Topalić-Trivunović, L., Velemir, A., Papuga, S., & Žabić, M. (2021). Changes in the physicochemical, antioxidant and antibacterial properties of honeydew honey subjected to heat and ultrasound pretreatments. *Journal of Food Science and Technology*, *58*(7), 2555–2566. https://doi.org/10.1007/s13197-020-04762-2

Terrab, A., Berjano, R., Sanchez, J. A., Gómez Pajuelo, A., & Díez, M. J. (2019). Palynological and geographical characterisation of Spanish oak honeydew honeys. *Grana*, *58*(1), 63–77. https://doi.org/10.1080/00173134.2018.1509124

Trencheva, K., Gounari, S., Trenchev, G., & Kapaxidi, E. (2009). The coccoidea on quercus (Fagaceae) in Bulgaria and Greece, with particular reference to their importance as honeydew-producing insects. *Entomological News*, *120*(2), 216–223. https://doi.org/10.3157/021.120.0215

Tuberoso, C. I. G., Jerkovic´b, I., Jerkovic´b, J., Bifulco, E., Marijanovic´c, Z., & Marijanovic´c, M. (n.d.). *Biodiversity of Salix spp. Honeydew and Nectar Honeys Determined by RP-HPLC and Evaluation of Their Antioxidant Capacity*.

Vasić, V., Gašić, U., Stanković, D., Lušić, D., Vukić-Lušić, D., Milojković-Opsenica, D., Tešić, Ž., & Trifković, J. (2019). Towards better quality criteria of European honeydew honey: Phenolic profile and antioxidant capacity. *Food Chemistry*, *274*, 629–641. https://doi.org/10.1016/j.foodchem.2018.09.045

Webography

https://www.biodiversity4all.org/taxa/47219-Apis-mellifera (seen: 05-01-2022)

https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_main (seen: 04-02-2022)