جامعة سيدي محمد بن عبد الله بفاس
UNIVERSITE SIDI MOHAMED BEN ABDALLAH DE FES
كلية العلوم ظهر المهراز فاس
FACULTE DES SCIENCES DHAR EL MEHRAZ FES

Formation Doctorale : **Molécules Bioactives, Santé et Biotechnologie**

Discipline : **Biologie**

Spécialité : **Physiologie et Pharmacologie**
Laboratoire : **Physiologie, Pharmacologie et Santé Environnementale**

THESE DE DOCTORAT

Présentée par

Youssef Elamine

Bupleurum spinosum honey characterisation: from the routinely assessed parameters to chemometrics and e-tongue technology, propolis phytochemical analysis and pharmacological perspectives.

Soutenue le 08/02/2020 devant le jury composé de :

Pr. Abdallah Souleimani	***Faculté des Sciences Dhar-Mahraz, Fès***	***Président***
Pr. Maria da Graça Costa Miguel	***Universidade do Algarve, Portugal***	***Rapporteur***
Pr. Javier Vioque Peña	***Instituto de la Grasa, CSIC, Spain***	***Rapporteur***
Pr. ElHoussine Derwich	***Faculté des Sciences Dhar-Mahraz, Fès***	***Rapporteur***
Pr. Henrique Leonel Gomes	***Universidade do Algarve, Portugal***	***Examinateur***
Pr. Fatima Lamchouri	***Faculté Polydisciplinaire de Taza***	**Examinateur**
Pr. Ilham ElArabi	***Faculté des Sciences Dhar-Mahraz, Fès***	**Examinateur**
Pr. Ismail Boumhidi	***Faculté des Sciences Dhar-Mahraz, Fès***	**Examinateur**
Pr. Toufik Hamid	***Faculté Polydisciplinaire de Taza***	**Examinateur**
Mr. Mezzour Mohammed	***Direction provinciale de l'Agriculture, Sefrou***	**Invité**
Pr. Badiaa Lyoussi	***Faculté des Sciences Dhar-Mahraz, Fès***	**Directeur de thèse**

Année universitaire : 2019-2020

PhD dissertation
Youssef Elamine
FSDM © 2020

Acknowledgments

Foremost, I would like to express my sincere gratitude to my advisor Pr. Badiaa Lyoussi for accepting me as a PhD student in her Laboratory, for her continuous support, patience, motivation, enthusiasm and orientation during those years of my PhD study and research. Her availability helped me to go through the thesis problematics and by providing international collaborations environment to make the thesis in the form and quality that one can see in the present manuscript.

Besides my advisor, I'm thankful to Pr. Maria da Graça Costa Miguel and Pr. Henrique Leonel Gomes from Algarve university (Portugal) and to Pr. Javier Vioque Peña from the Fat institute (Spain), for providing laboratory facilities, full guidance and knowledge during my stay in their institutions.

My sincere thanks go also to Pr. Leticia Estevinho (Instituto Politécnico de Bragança-Portugal), Pr. Ofélia Anjos (Instituto Politécnico de Castelo Branco-Portugal), Pr. Ana Cristina Figueiredo (Faculdade de Ciências da Universidade de Lisboa- Portugal), Pr. Manuel Alaiz (Instituto de la Grasa (C.S.I.C.)- Spain) and Pr. Julio Girón-Calle (Instituto de la Grasa (C.S.I.C.)-Spain) for their collaboration and their active contributions for composition determination, data analysis or articles writing.

I would like also to thank my dear colleagues and friends Dr. Smail Aazza, Pedro Inácio and Dr. Hamada Imtara for their active contribution during the elaboration of the works of this thesis staring form experiments to data analysis and articles writing. My thanks go also to all my colleagues from physiology, pharmacology and environmental health laboratory (FSDM), Chemistry of natural products laboratory (Algarve university) and Organic electronic laboratory (Algarve university), for the good moments we spent together, and the research environments we developed during the stays.

My kind gratitude and thanks to Pr. Abdallah Souleimani (FSDM - Fez), Pr. Maria da Graça Costa Miguel (Algarve university - Portugal), Pr. Javier Vioque Peña (SCIS - Spain), Pr. ElHoussine Derwich (FSDM - Fez), Pr. Henrique Leonel Gomes, Pr. Fatima Lamchouri (FPT – Taza), Pr. Ilham ElArabi (FSDM - Fez), Pr. Ismail Boumhidi (FSDM - Fez), Pr Toufik Hamid (FSDM - Fez), and Mr. Mezzour Mohammed (DPA - Sefrou) for their kind and honourable acceptance to judge and evaluate my thesis.

My acknowledgement go also to National centre of scientific and technical research (Morocco), Erasmus+ Program (European Union), Organic electronic Lab (FCT - Portugal) and Superior council of scientific investigations (SCIC - Spain) for providing scholarships and financial grants that helped the accomplishment of international internships in good conditions.

This work is dedicated

To my parents for their endless love, support and encouragement

To my supervisor, Pr. Badiaa Lyoussi

To my Sisters Laila, Najat and Hanane

To my nephews Abdelilah, Mohemed, Maryam and Mouad

To my Love Asmae Bellamine and her kind family

To all my family members

To all my collaborators, friends and colleagues

To you (the readers)

Contents

List of figures....10
List of tables....13
Abbreviations....15
Abstract....16
نبذة مختصرة....18
Résumé....19
Preface....21
General introduction: State of the art and main issues in honey research....22
1. Introduction....23
2. Honey characterization and floral origin determination....25
2.1. Honey composition and holistic view of the quality control parameters....25
2.2. Multivariate analysis for botanical origin determination of honey....29
2.3. Non-specific and reliable tools for botanical determination of honey: the electronic tongue (e-tongue) technology....32
3. Biological activities of honey....35
3.1. The effect of heat processing on honey biological activities....35
3.2. Honey antimicrobial activity and the effect of physicochemical properties....38
3.3. Cell-based antioxidant and antiproliferative activities of honey....39
4. Propolis: characterization and its use as a source of antiproliferative and cell-based antioxidant agents....40
References....41
Part I: *B. spinosum* honey characterisation....47
Chapter I. Preliminary characterisation of *Bupleurum spinosum* honey....48
Abstract....49
1. Introduction....49
2. Material and methods....51
2.1. Melissopalynological analysis....52
2.2. Microbiological evaluation....52
2.3. Physico-chemical analysis....52
2.4. Honey colour and melanoidins estimations....53
2.5. Sugar content....53
2.6. Total content of polyphenols....53

2.7. Total flavonoid content 53
2.8. Antioxidant activities 53
2.9. Statistical analysis 55
3. Results and discussion 55
3.1. Melissopalynological analysis 55
3.2. Microbiological evaluation 56
3.3. Physico-chemical analyses 58
3.4. Sugar content 63
3.5. Antioxidant activity 64
3.6. Variance explained by principal component analysis 66
4. Conclusion 67
5. References 68
Chapter II: Zantaz honey monoflorality Chemometric applied to the routinely assessed parameters 71
Abstract 72
1. Introduction 72
2. Materials and Methods 74
2.1. Sample Preparation 74
2.2. Pollen analysis 74
2.3. Acidity, pH, conductivity, moisture, color, proline, diastase activity 74
2.4. Colour and melanoidins 74
2.5. Sugars 74
2.6. Minerals 75
2.7. Volatile composition: extraction and analysis 75
2.8. Statistical tools 76
3. Results and Discussion 77
3.1. Preliminary descriptive characterization 77
3.2. Multivariate analysis 84
4. Conclusion 91
5. References 92
Chapter III: Insight into the sensing mechanism of an impedance based electronic tongue for honey botanic origin discrimination 94

Contents

Abstract....95
1. Introduction....95
2. Material and methods....98
2.1. Honey samples....98
2.2. Experimental set-up and statistics....98
2.3. Results and discussion....99
3. Conclusions....111
4. References....112
Part II. Pharmacological properties of *B. spinosum* honey....116
Chapter I. Effect of extreme heat processing on Zantaz' honey antioxidant activities. 117
Abstract....118
1. Introduction....118
2. Materials and Methods....120
2.1. Reagents and instruments....120
2.2. Sample preparation....120
2.3. Preliminary characterization....121
2.4. Colour, and melanoidins estimation....121
2.5. Polyphenols....121
2.6. Flavonoids....121
2.7. Total antioxidant activity by ammonium molybdate reduction method....122
2.8. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity....122
2.9. $ABTS^{\bullet+}$ (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging activity....122
2.10. Reducing power....122
2.11. Chelating metal ions....123
2.12. Nitric oxide (NO) scavenging activity....123
2.13. Superoxide anion radical (O2•-) scavenging activity (non-enzymatic method)....123
2.14. FTIR-ATR (Fourier-transform infrared-attenuated total reflection) analysis....124
2.15. Statistical analysis....124
3. Results and discussion....124
3.1. Effect of heat processing on the physicochemical parameters....124
3.2. Effect of thermal treatment on the antioxidant activities....126

3.3. Effect of heating evaluated by FTIR-ATR analysis....128
4. Conclusions....131
5. References....132
Chapter II. Antibacterial activity of Zantaz honey and influence of its physicochemical properties....134
Abstract....135
1. Introduction....135
2. Material and methods....137
2.1. Sampling....137
2.2. Physico-chemical characterization....137
2.3. Antimicrobial activity....138
2.4. Statistical analysis....139
3. Results and discussion....139
4. Conclusion....143
5. References....144
Chapter III. Physicochemical and functional characteristics of a Moroccan Zantaz honey rich in methyl syringate....146
Abstract....147
1. Introduction....147
2. Material and methods....149
2.1. Honey samples and physicochemical characterisation....149
2.2. Determination of sugar composition....149
2.3. Determination of phenolic composition....149
2.4. Cell culture....149
2.5. Determination of the antiproliferative activity of Zantaz honey....150
2.6. Caco-2 based antioxidant activity....150
2.7. Statistical analysis....151
3. Results and discussion....151
3.1. Preliminary characterization: sugars, phenolic compounds, minerals and other physicochemical properties....151
3.2. Honey interferences in the MTT assay....155
3.3. Antiproliferative and antioxidant activities of Zantaz honey....157
3.4. Correlations and multivariate analysis....159

4. Conclusion 161

5. References 162

Part III. Phytochemical and functional characterization of propolis samples 165

Chapter I. Phytochemical characterization and antiproliferative activity of propolis samples obtained from hives in the surrounding area of *Bupleurum spinosum* melliferous plant 166

Abstract 167

1. Introduction 167

2. Material and methods 170

2.1. Samples preparation. 170

2.2. Phenolic composition 170

2.3. Cell culture and antiproliferative activity of propolis. 171

2.4. Statistical analysis. 171

3. Results and discussion 172

4. Conclusion 175

5. References 175

General conclusion: Discussion and perspectives 178

1. Introduction 179

2. Zantaz honey: Quality control and monoflorality declaration. 179

2.1. Preliminary characterization 179

2.2. Chemometrics for monoflorality determination 181

2.3. Electronic tongue technology 181

3. Honey biological activities. 183

3.1. Effect of heat processing. 183

3.2. Antimicrobial activity. 184

3.3. Antiproliferative and antioxidant activities. 184

4. Propolis phytochemical characterization and pharmacological activities. 186

5. Perspectives 186

6. References 187

Annexes 190

List of figures

General introduction: State of the art and main issues in honey research

Figure 1 Honey chemical composition…….27

Figure 2. Radar chart comparing European monofloral honeys regarding their glucose and fructose contents, color, conductivity, free acidity and diastase activity. ……30

Figure 3. Description of multivariate analysis applied to honey research……31

Figure 4. Main steps for performing analysis with an e-tongue……33

Figure 5. Experimental setup and the equivalent circuit of impedance analysis of a honey solution with an e-tongue. ……34

Figure 6. Changes in honey chemical composition upon heat processing……36
Figure 7. Diagram of Cell based antioxidant assay. (1) extracellular antioxidant activity. (2) intracellular antioxidant activity. OSI: oxidative stress inducer. AOx: Antioxidant. DCFH-DA: 2′,7′-Dichlorodihydrofluorescein diacetate..39

I- Chapter I. Preliminary characterization of a Moroccan honey with a predominance of *Bupleurum spinosum* pollen

Figure 1. (a) Flowers of B. spinosum plant; (b) B. spinosum pollen under microscope (400×)……51
Figure 2. Principal component analysis with all measured parameter: projection of loadings and honey samples in the same system of vectors……67

I- Chapter II. Zantaz honey monoflorality Chemometric applied to the routinely assessed parameters

Figure 1. PCA of the pollen analysis and the physicochemical parameters assessed in the 30 Zantaz honey sample. (a) Plot using the first and the second component. (b) Plot using the first and the third components. (c) Plot using the first and the second component. (d) Plot using the first and the third components……..85

Figure 2. PCA of the pollen analysis and the mineral content of the 18 Zantaz honey samples. (a) Plot using the first and the second component. (b) Plot using the first and the third components……..87

Figure 3. The discrimination amongst the 18 samples of the present work using a PCA integrating the information of all assessed parameters (a), and the possible effect of the most abundant characterized pollen species comparing the samples discriminated by the first PC using student t test (b). the onset of the figure (a) is the variable plot, and the red lines are plotted as facultative. (b) Comparison between the means of the groups discriminated in Fig. 3 (a) considering the main identified pollen species * $P < 0.05$……..88

Figure 4. Dendrogram obtained by cluster analysis of the percentage composition of the volatiles isolated from the *Zantaz* honey, based on correlation and using unweighted pair-group method with arithmetic average (UPGMA)…90

Figure 5. Score plot of the PCA using all identified volatile compounds. Bar plot comparing the means of *Bupleurum spinosum* pollen based on the clusters obtained in fig. 3. Student t test was used. *($p<0.05$)……..91

I- Chapter III. Insight into the sensing mechanism of an impedance based electronic tongue for honey botanic origin discrimination

Figure. 1. (a) Photograph of an e-tongue, (b) schematic of the e-tongue showing the spatial distribution of the electrodes and electrical connections. Gold, ITO and n+- silicon electrodes have an individual active area of 1 cm2. The carbon electrode has an area of 0.39 cm2……..99
Figure. 2. Frequency dependence of the gold/honey/platinum impedance. (a) Frequency dependence of the capacitance (CP) and loss=1/(ωRP). (b) Frequency dependence of the tan δ (c) Equivalent circuit description of the electrodes immersed into the honey solution……100
Figure. 3. E-tongue electrical stability and reproducibility. (a) Time dependence of the capacitance for all the sensing electrodes measured at 100 Hz. (b) the repeatability assay for all the sensors. Results are expressed as tan δ±standard deviation. (c) CP dependence on the honey dilution factor……103
Figure. 4. PCA discrimination patterns for etongue. (a) Gold sensor. (b) n+-Si sensor. (c) ITO sensor. (d) Carbon sensor. Blue circles represent samples with lavender pollen prevalence; black squares samples with Bupleurum pollen

prevalence. Empty black squares and empty blue circles represent odd samples purchased, as belonging to a specific floral origin but this origin was not confirmed using melissopalynology (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)……..…..104
Figure. 5. The discrimination power of e-tongue in comparison with and the physicochemical parameters. (a) PCA biplot using the overall data (tan δ). (b) PCA biplot using the resistance (RP) and capacitance (CP) data. (c) PCA biplot using normalized physicochemical characterizations (d) PCA biplot using both pollen data and physicochemical parameters. Black squares represent honey samples with Bupleurum pollen prevalence and blue circles represent lavender floral origin (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)……...…107
Figure. 6. Dendrogram of the scores resulted from the PCA in Fig. 4. (a) Dendrogram of Lavandula honey; The three clusters were labelled as L1, L2 and L3. (b): Dendrogram of Bupleurum honey; The three obtained clusters were labelled as B1, B2 and B3……108
Figure. 7. Electrical conductivity and colour of the two honey subgroups L1, L2 and L3 (Fig. 6 (a)) for Lavandula honey and B1, B2 and B3 for Bupleurum honey (Fig. 6 (b)). (a) electrical conductivity and (b) colour for the two groups. The values are plotted as mean ± SD, difference was accepted to be significant with a α=0.05. *p < 0.05; ***p < 0.001. ……...…110

II- Chapter I. Effect of extreme heat processing on Zantaz' honey antioxidant activities

Figure 1. FTIR-ATR spectra for honey samples with and without heating. A) Average spectra obtained by FTIR-ATR, for honey with and without heating. B) Processed FTIR-ATR (All spectra). C) Processed FTIR-ATR (Region used in the analysis). ……...129

Figure 2. Score plot of two principal components from raw spectral data acquire with FTIR-ATR data of honey based on: a) marks without fill correspond to honey samples without heat processing, b) marks filled belong to samples after heat processing from 800 to 1250 cm^{-1}. ……...…130

Figure 3. Plot of two principal components from all analysed parameter (A) and Scores (B) processing. Each rectangle refers to one honey sample before and after heat processing. ……...…130

II- Chapter II. Antibacterial activity of Zantaz honey and the influence of its physicochemical properties

Figure 1: Heat map illustrating the correlation between the physiochemical properties and the assessed antimicrobial activities. ……142

Figure 2: (a) sugars heatmap; (b) minerals heatmap. ……...…143

II- Chapter III. Physicochemical and functional characteristics of a Moroccan Zantaz honey rich in methyl syringate

Figure 1. interference of honey with the MTT assay. Comparison between raw and corrected data with corresponding visual inspections of THP-1 cells after 24h of incubation (a) and after 72 hours (b). ** p<0.01; ***p<0.001. Sugars contribution to the interference in comparison to the whole honey after 24 hours (c) and 72 hours (d). Samples or sugars solutions flagged with the same alphabet are not significantly different (p<0.05)……....156

Figure 2. Antiproliferative activity of Zantaz honey samples after 24 hours incubation with THP-1 cells……..158

Figure 3. Antioxidant activity of Zantaz honey samples in Caco-2 cells expressed as cellular antioxidant activity (CAA) quercetin equivalence mg/g of honey……..158

Figure 4. Heat map of the evaluated correlation r value supported with the clustering ability of dendrogram presentation. Significant correlation at p<0.05 is flagged by a star "*"……161
III- Chapter I. Phytochemical characterization and antiproliferative activity of propolis samples obtained from hives in the surrounding area of *Bupleurum spinosum* melliferous plant

Figure 1. Preparation procedure of propolis samples. ……170

Figure 2. HPLC-DAD profile of one propolis sample with the available standards. ……..172

Figure 3. Antiproliferative activity of propolis samples against THP-1 cells. (a) Cell viability in function of propolis concentration after 24h of incubation. (b) Cell viability at 1.5 µg/mL of propolis after 24h of incubation. (c) Cell

viability in function of propolis concentration after 72h of incubation. (d) Cell viability at 1.5 µg/ml of propolis after 72h of incubation. # IC_{50} determination was not possible because of the low activity…….174

List of tables

General introduction: State of the art and main issues in honey research

Table 1. Physico-chemical parameters used for honey quality control, and their standard limits as revised by the international honey commission [3]. ……..…28

Table 2. Main antioxidant assays, their principles and principal targeted groups……...36

I- Chapter I. Preliminary characterization of a Moroccan honey with a predominance of *Bupleurum spinosum* pollen

Table 1. Sample code, places of collection, year of production and the most predominant pollen of twelve *B. spinosum* honey samples from Morocco. ……..…57

Table 2. Some physicochemical parameters and microbial analysis of twelve *B. spinosum* honey samples from Morocco. …….……59

Table 3. Some physicochemical parameters of twelve *B. spinosum* honey samples from Morocco……....61

Table 4. Sugar content (g/100 g) of twelve *B. spinosum* unheated honey samples from Morocco……64

Table 5. Antioxidant activity and capacity for scavenging free radicals of twelve *B. spinosum* honey samples from Morocco. ……..65

I- Chapter II. Zantaz honey monoflorality Chemometric applied to the routinely assessed parameters

Table 1. Retention time, limit of quantification of the analysed sugars……75

Table 2. Pollen profiling of the studied honey samples……78

Table 3. Physicochemical characterization and sugar profile of the analysed honey samples……...80

Table 4. Mineral content of honey samples…….……..83

Table 5. Minimum and maximum percentage range of main components (≥1%) identified in the volatiles isolated by hydrodistillation from different *Zantaz* honey samples……89

I- Chapter III. Insight into the sensing mechanism of an impedance based electronic tongue for honey botanic origin discrimination

Table 1. Melissopalenological, and physico-chemical characterization of the honey samples. Results are presented as Mean±SD. ……..…110

II- Chapter I. Effect of extreme heat processing on Zantaz' honey antioxidant activities

Table 1. The physicochemical characterization and the antioxidant activities of honey sample before and after heat processing……..125

Table 2. Correlation matrix between baseline values and the percentage of change following heat processing……128

II-Chapter II. Antibacterial activity of Zantaz honey and the influence of its physicochemical properties

Table 1. Physico-chemical features of the analysed honey samples……..140

Table 2. Antimicrobial activity of Zantaz honey samples against Gram-positive and Gram-negative bacteria……141

II- Chapter III. Physicochemical and functional characteristics of a Moroccan Zantaz honey rich in methyl syringate

Table 1. Physicochemical characterisation of Zantaz honey. results are the average ± sd of six different samples……152

Table 2. Phenolic composition of studied *Zantaz* honey samples……154

III- Chapter I. Phytochemical characterization and antiproliferative activity of propolis samples obtained from hives in the surrounding area of *Bupleurum spinosum* melliferous plant

Table 1. Detected compounds in propolis samples and their respective areas obtained by HPLC-DAD……...172

Table 2. Pearson's correlation between the areas of the detected phenolic compounds and antiproliferative activity of propolis samples. ……174

Abbreviations

ABTS: 2,2'-Azino-Bis (3-ethylbenzoThiazoline-6-Sulfonic acid).

ANOVA: Analysis of Variance.

Caco-2: Epithelial cells of human Colorectal adenocarcinoma

DAD: Diode Array Detection.

DPPH: 2,2-DiPhenyl-1-PicrylHydrazyl.

E-tongue: Electronic Tongue.

EC: Electrical Conductivity.

EDL: Electrical Double Layer.

FTIR: Fourier Transformation of Infrared Spectrum.

HMF: Hydroxymethylfurfural

HPLC: High Performance Liquid Chromatography.

IHC: International Honey Commission.

LC-MS: Liquide Chromatography coupled to Mass Spectrometry.

NO: Nitric Oxide.

OH: hydroxyl.

PCA: Principal Component Analysis.

THP-1: Monocytes from acute monocytic leukaemia.

Abstract

Zantaz honey is produced mainly from *Bupleurum spinosum* that is a melliferous plant characterizing the high Moroccan Atlas Mountains. Samples were collected from different regions during different beekeeping seasons. A first step in the present thesis was to characterize the collected samples following the harmonized methods of the international honey commission. The obtained parameters were in accordance with the internationally recognized quality control limits. Furthermore, the matrix of the data has been used as an input for chemometric analysis aiming monoflorality threshold determination using pollen data as reference. This last procedure showed that Zantaz honey obey at least to the general monoflorality rule stating that a honey can be labelled as monofloral when the frequency of its main pollen exceeds 45%.

Considering the high cost and time consumption of the above techniques, a contribution was made to develop an impedance based electronic tongue. The e-tongue has been formed by four sensing units (gold, carbon, ITO, Silica) surrounding a reference circular platinum electrode inside of a vessel used as container of honey solution. The e-tongue showed to be promising for botanical origin discrimination as good sensitivity and repeatability was obtained for most of the used electrodes. In addition, the e-tongue was able to distinguish subgroups amongst honey samples belonging to the same floral origin with more sensitivity toward electrical conductivity as low as 100 $mS.cm^{-1}$.

The second section of the present has been considered for the evaluation of biological activities of Zantaz honey and propolis samples collected in the surrounding areas of the same plant. An assessment of the effect of an extreme heat processing on the antioxidant activities of Zantaz honey has been made and showed different behaviours depending on the used radical, which questions the use of heating processing for industrial purposes. Furthermore, the effect of Zantaz honey physico-chemical features has been investigated on its antimicrobial activity against gram+ and gram- bacterial strains. The polyphenolic composition revealed the abundance of methyl syringate at concentrations above 200 µg/100g, and similar to those used to confirm the monoflorality of Manuka honey. This observation may question the mentioned use of methyl syringate unless it is combined to other tools. The antiproliferative activity of Zantaz honey samples against THP-1 cell line and their antioxidant activity on Caco-2 cells were evaluated and had presented positive correlation with the identified phenolic compounds.

For propolis samples collected from hives in the surrounding areas of *B. spinosum*, HPLC-DAD analysis showed the presence of hydrophobic phenolic compounds. The antiproliferative activity was tested against THP-1 cells, a phenotype of leukaemia. The IC_{50} was as low as

0.60±0.07 µg/mL. it is worth mentioning that samples with the lowest phenolic content had the lowest antiproliferative activity and vice versa, although the correlation was not statistically significant.

Key words

Honey, propolis, *Bupleurum spinosum*, harmonized standards, quality control, chemometrics, electronic tongue, antiproliferative activity, antioxidant activity, THP-1, Caco-2.

نبذة مختصرة.

يتم إنتاج عسل الزنتاز بشكل أساسي من نبات البوبلوروم سبينوسوم (*Bupleurum spinosum*) وهو نبات زهري يميز جبال الأطلس المغربية العالية. أُخذت عَيّنات هذه الدراسة من مناطق مختلفة خلال عدة مواسم، ثم حُللت وفقًا للطرق المُنسّقة للجنة العسل الدولية كخطوة أولى في هذه الأطروحة. قورنت المعلومات المحصل عليها مع حدود مراقبة الجودة المعترف بها دوليا. بالإضافة إلى ذلك، استخدمت مصفوفة البيانات كقاعدة للتحليل الكيميائي (chemometrics) لتحديد عتبة تعيين عسل زنتاز كعسل أحادي الأصل الزهري باستخدام بيانات حبوب اللقاح كمرجع. أظهر هذا الإجراء الأخير أن عسل الزنتاز يخضع على الأقل للقاعدة العامة لأحادية الأصل الزهري التي تحدد عتبة 45٪ من حبوب اللقاح الرئيسية كحد أدنى.

بالنظر إلى التكلفة العالية والوقت المستهلك خلال الطرق التحليلية أعلاه، قُدِّمَتْ، كخطوة ثانية، مساهمةٌ لتطوير لِسَانٍ إلكتروني قائم على المعاوقة الكهربائية (electrical impedance). يتكون اللسان الإلكتروني من أربع وحدات للكشف (الذهب، والكربون، وITO، والسيليكا) تحيط بإلكترود دائري من البلاتين كمرجع، وصفت داخل أنبوب اختبار يستخدم كحاوية لمحلول العسل المراد تحليله. أظهرت نتائج استعمال اللسان الإلكتروني أنه قد يكون وسيلة واعدة لتمييز الأصل الزهري للعسل، حيث أظهر حساسية وقابلية تكرار جيدين لمعظم الأقطاب الكهربائية المستخدمة. بالإضافة إلى ذلك، كان اللسان الإلكتروني قادرا على التمييز بين مجموعات فرعية داخل عينات عسل تنتمي لنفس الأصل الزهري، مع حساسية أكبر للتوصيل الكهربائي تصل إلى $100\ \mu S.cm^{-1}$.

خُصِّص القسم الثاني من هذه الأطروحة لتقييم الأنشطة البيولوجية لعينات من عسل نبتة الزنتاز وعكبر جُمِعَت عيناته من خلايا النحل في المناطق المحيطة بنفس النبات. أجريت الدراسة الأولى على تأثير المعالجة الحرارية، التي تستعمل للتخلص من تبلور العسل، على الأنشطة مضادات الأكسدة لعسل الزنتاز. أظهر النتائج سلوكيات مختلفة بحسب الجذور الحرة المستعملة، مما يثير التساؤل حول استخدام تسخين العسل للأغراض الصناعية. بالإضافة إلى ذلك، دُرِسَ تأثير الخواص الفيزيائية والكيميائية لعسل زانتاز على نشاطه المضاد للميكروبات ضد سلالات بكتيرية موجبة الغرام وسالبة الغرام. كشفت التركيبة الفينولية عن وفرة الميثيل سيرينجات بتركيز يزيد عن 200 ميكروغرام / 100 غرام ، وهي نسبة مماثلة لتلك المستخدمة لتأكيد أحادية الأصل الزهري لعسل مانوكا. هذه الملاحظة قد تسائل الاستخدام المذكور لميثيل سيرينجات، ما لم يتم إقرانه مع وسائل إثبات أخرى. بعد ذلك، قمنا بقياس النشاط المضاد لتكاثر الخلايا السرطانية لعينات عسل زانتاز، بالتحديد ضد خلايا THP-1 ونشاطها المضاد للأكسدة على خلايا Caco-2، حيث أظهرت النتائج مفعولا إيجابيا ذي علاقة إيجابية مع محتوى العيانات من المركبات الفينولية.

بالنسبة لعينات العكبر المأخوذة من خلايا النحل بالقرب من نبتة الزنتاز (*B. spinosum*) ، أظهر تحليل HPLC-DAD وجود مركبات فينولية كارهة للماء. أُخْتُبِرَ أيضا نشاط العيانات المضاد لتكاثر خلايا THP-1 السرطانية المشتقة من سرطان الدم. وكانت IC_{50}، أو التركيز الذي يكبح تكاثر 50 % من الخلايا، منخفضا جدا 0.60 ± 0.07 ميكروغرام / مل، وهي قيمة تنبؤ بإمكانية استخراج مواد فعالة من هذا العكبر، ودفع الدراسة إلى مستويات متقدمة. تجدر الإشارة إلى أن العينات التي تحتوي على أقل محتوى من الفينول كان لديها نشاط قليل ضد تكاثر الخلايا المذكورة والعكس بالعكس، على الرغم من أن العلاقة لم تكن معبرة إحصائيا.

كلمات مفتاحية:

عسل، عكبر، بيبلوروم سبينوزوم، معايير منسقة، مراقبة جودة، تحليل كيميائ، لسان إلكتروني، نشاط مضاد للتكاثر، نشاط مضاد أكسدة، THP-1، Caco-2.

Résumé

Le miel de Zantaz est produit principalement à partir de *Bupleurum spinosum* qui est une plante mellifère caractérisant les hautes montagnes des Atlas Marocains. Des échantillons ont été collecté dans différentes régions au cours de différentes saisons apicoles. Une première étape de la présente thèse était de caractériser les échantillons collectés selon les méthodes harmonisées de la commission internationale du miel. Les paramètres ont prouvé la conformité des échantillons analysés en comparaison aux limites de contrôle de qualité internationalement reconnues. En outre, la matrice de données a été utilisée comme *input* pour l'analyse chimiométrique visant à déterminer le seuil de monofloralité en utilisant les données de pollen comme référence. Cette dernière procédure a montré que le miel de Zantaz obéit au moins la règle générale de monofloralité qui précise le seuil de 45% du pollen principal pour qu'un miel peut être étiqueté comme monofloral.

Compte tenu du coût élevé et de la consommation de temps, une contribution a été apportée pour développer une langue électronique basée sur l'impédance. La langue électronique a été formée par quatre unités de détection (or, carbone, ITO, silice) entourant une électrode de platine circulaire de référence à l'intérieur d'un récipient utilisé comme conteneur de solution de miel. La langue électronique s'est révélée prometteuse pour la discrimination de l'origine botanique car une bonne sensibilité et répétabilité ont été obtenues pour la plupart des électrodes utilisées. De plus, la langue électronique a pu distinguer des sous-groupes à l'intérieur parmi des échantillons de miel appartenant à la même origine florale avec plus de sensibilité envers la conductivité électrique qui était aussi faible que 100 $\mu S.cm^{-1}$.

La deuxième section de la présente thèse a été consacré à l'évaluation des activités biologiques des échantillons de miel Zantaz et de propolis collectée dans les ruches des zones environnantes de la même plante. Une évaluation de l'effet d'un traitement thermique extrême sur les activités antioxydantes du miel de Zantaz a été faite et a montré différents comportements dépendant du radical utilisé, ce qui remet en question l'utilisation du chauffage du miel à des fins industrielles. En outre, l'effet des caractéristiques physico-chimiques du miel de Zantaz a été évalué sur son activité antimicrobienne contre des souches bactériennes gram + et gram -. La composition phénolique a révélé l'abondance du méthyle syringate à une concentration supérieure à 200 µg / 100g, et similaire à celles utilisées pour confirmer la monofloralité du miel de Manuka. Cette observation peut remettre en question l'utilisation mentionnée du méthyle syringate à moins qu'il ne soit combiné à d'autres outils. L'activité antiproliférative des échantillons de miel de Zantaz contre la lignée cellulaire THP-1 et leur activité antioxydante sur les cellules Caco-2 ont été

évaluées et ont présenté une corrélation positive avec le contenu en composés phénoliques identifiés.

Pour les échantillons de propolis collectés dans les ruches des environs de *B. spinosum*, l'analyse HPLC-DAD a montré la présence de composés phénoliques hydrophobes. L'activité antiproliférative a été testée dans des cellules THP-1, un phénotype de leucémie. La IC_{50} était aussi faible que 0,60 ± 0,07 µg / mL. Il est à mentionner que les échantillons ayant la plus faible teneur en phénols avaient la plus faible activité antiproliférative et vice versa, bien que la corrélation n'était pas statistiquement significative.

Mots clés :

Miel, propolis, *Bupleurum spinosum*, standards harmonisés, contrôle de qualité, chimiométrie, langue électronique, activité antiproliférative, activité antioxydante, THP-1, Caco-2.

Preface

When approaching an uncharacterised honey, a crucial step is to make a quality control screening of its characterizing properties, to insure its suitability for human consumption or health uses. The first aim of the present thesis was to investigate Zantaz honey produced in Morocco, mainly from *Bupleurum spinosum* considering its characteristics following internationally established standards. Secondly, a contribution has been made to determine its monoflorality threshold using the evaluated properties and pollen analysis as a guiding tool. In addition, an attempt has been made to propose alternatives to the costly and time-consuming technics by introducing a simple and reliable technology, namely an impedance-based e-tongue for the discrimination of honey botanical origins. Furthermore, biological activities of Zantaz honey and propolis collected from hives in the surrounding areas of *B. spinosum* were investigated, alongside with their phytochemical characterization.

The thesis will be introduced by a state-of-the-art chapter which will introduce the main problematics of the honey research giving insights into the importance of characterizing and valorising Zantaz honey. The core of the thesis will be divided into three parts. The first will be made of three chapters aiming the characterization of Zantaz honey, the chemometric application and the e-tongue technology. The second part will focus on evaluating pharmacological properties of Zantaz honey and will be made of three chapters: heat processing effect on Zantaz honey antioxidant activities, Zantaz honey antibacterial and antioxidant and antiproliferative activities using cell-based assays. The last part will be formed by one chapter focusing on propolis phytochemical analysis and its potential use as chemopreventive anticancer drug. The thesis will be concluded by a discussion and perspectives chapter.

To share the achieved results with the scientific community, the three chapter of the first part were published in peer reviewed journals, the first chapter of the second part is accepted for publication, and the remaining three chapters are being considered for submission. Hence, information repetition in several parts (introductions and material and methods sections) of the thesis was unavoidable.

General introduction: State of the art and main issues in honey research

1. Introduction

Honey is "the natural sweet substance produced by *Apis mellifera* bees 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. The collected product is then transformed by honeybees by combination with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature"[1]. Several points can be considered in honey research:

First, since honey is a product used as food and medicine, it is of the highest importance to establish a set of standardized features that make it suitable for both uses. This includes assessing the presence of undesirable microorganisms, that can accelerate honey deterioration or, cause infectious diseases [2]. In addition, the characterization of physico-chemical properties helps the control of counterfeiting, and quality decline by aging, processing or environmental conditions such as humidity and temperature [3].

A second aspect is the assessment of honey origin by determining if it is a blossom honey or a honeydew honey or a mixture of both and identify all the participating plant species [3]. This is a very important point in honey research, since the botanical origin of honey determines its nutritional, therapeutic and sensory characteristics [4]. This task is achieved , mainly with melissopalynology or the identification and count of pollen grains in the honey brought by bees during collection of nectar and/or honeydew [5]. Hence, a monofloral honey is defined as a honey for which the physico-chemical and sensory properties are determined by the features of one nectar or honeydew. Generally, a honey sample is accepted to be monofloral when the pollen of the predominant pollen specie represent more than 45% of the sum of all identified pollens [6]. Several exceptions to this rule have been previously reported. For example, in *Lavandula latifolia* honey, only 15% of the main pollen is enough for monoflorality declaration [7]. In opposition, *Castanea sativa* honey needs, at least, 90% pollen representability to be considered as monofloral [5]. This divergence comes from the fact that *L. latifolia* produces little amount of pollen grain, while *C. sativa* have high pollen production. These observations leaded to the introduction of over-represented pollen (the case of *C. sativa*) and under-represented pollen (the case of *L. latifolia*) concepts [5].

To declare the pollen influences of each plant species in honey characteristics, it is very important to know the relationships between physico-chemical and sensorial features of honey on one side and the pollen counts of all identified plant species on the other [7]. This can be further complicated by the presence in the honey of pollen of nectar less plants such as Gramineae (wild grasses, cereals, maize) and conifers (*Abies*, *Pinus*, *Picea*, *Juniperus*, *Larix*) [5].

Overall, characterizing new honey, with melissopalynology alone, is not the best option, especially when the relationship nectar-pollen of identified plant species is ignored. It is then imperative to determine also the physico-chemical panel of parameters (e. g. the international honey commission), such as sugars, polyphenols, volatile composition and sensorial characteristics, amongst others [6]. When combined with multivariate analysis, a process known as chemometric, relationships can be established between parameters and the identified pollen species and help the understanding of the pollen/nectar proportions. Authors have followed this procedure to determine the floral [7] and even the geographical origins of honeys [8].

Since the above-mentioned methods are time consuming and requires high expertise and technical support, an increasing attention has been paid to the development of low cost, fast and reliable techniques. The electronic tongue is an example of this that will be described in this thesis. This technology relies on the use of sensors monitoring nonspecific information of given properties of the analysed substance and providing an output, that when analysed, allow discriminating samples presenting different features [9]. In the honey field it is used to discriminate the botanical and/or geographical origins of honey samples as well as for determining any counterfeiting or adulteration of the honey [10].

Another important point in honey research is related to its biological activities. The variety of its botanical origins makes it a good source of different bioactive compounds (plant secondary metabolites), and several health benefits have been reported for honey, from simple antimicrobial activity, and cicatrisation to antiproliferative properties [11]. Considering that honey is characterized by several physico-chemical properties, a relationship between them and the biological activity needs to be established [12,13]. Such task will provide a guiding tool when the selection of honey samples for a given activity or health benefits is desired.

The selected honey for the present thesis is the Moroccan Zantaz honey. Zantaz honey is produced mainly from the melliferous plant Zantaz (*Bupleurum spinosum* Gouan) (*Apiaceae*). This species grows mostly in the siliceous high peaks of eastern Anti-Atlas, generally above 2,000 m a.s.l. [14]. Some authors also reported the presence of this species at higher altitudes in the High Atlas (above 2900 m a.s.l.) and in some mountains of the middle Atlas [15] where the material for this study was mainly collected. The presence of this plant is also reported in other countries of the Mediterranean region like Algeria [16] and Spain [17]**.**

The flowering period of *B. spinosum* is between August and September [18]. In this period is also produced a dark coloured honey, with specific flavour and no tendency to crystallization, known as *Zantaz*, *Zandaz*, *Aguerbaz* or *Airbaz* honey. It is one of the most appreciated honeys in the region where it is produced. According to the regional agriculture direction of Fez region, the

production of this honey is growing due to the transition from traditional to modern beekeeping and also to the gained attention from national beekeepers. To the best of our knowledge, no previous work aimed the analysis of this honey, which make it an interesting material to address some issues in honey research. So far, the published contributions within this thesis are the first serious works aiming the characterization and the valorisation of Zantaz honey.

2. Honey characterization and floral origin determination.

2.1. Honey composition and holistic view of the quality control parameters.

Before approaching the composition or the quality control of honey by the standard physico-chemical parameters, screening the existence of undesirable microorganisms is necessary. The concentration of sugars in honey makes it not appropriate for bacterial growth [19]. However, in some cases, honey may be contaminated during post-harvest handling (i.e., yeasts and spore-forming bacteria) producing sanitary and commercial quality deterioration of the honey [20]. The most frequently evoked bacteria is *Clostridium* spp. because of the undesirable effects of its toxin causing botulism [2]. The microbiological quality control may be extended to some aerobic mesophilic bacteria and *Enterobacteriaceae* [20]. The presence of osmotolerant yeast is usually revealed by their direct identification and count or by monitoring the metabolites of their fermentation process. To control the undesirable honey fermentation, limits were established for yeast counts, glycerol and ethanol contents, and the maximums allowed values are 50,000 per gram, 300 mg/kg and 150 mg/kg, respectively [21].

The variability of honey botanical origins makes it a rich complex mixture of about 200 compounds [22]. Carbohydrates are the most abundant constituent of honey, reaching 95 % in some cases. They come from the nectar or honeydew collected by the bees and are mainly glucose and fructose [21]. Some of them are brought to the hive as larger saccharides (mainly sucrose) that are broken down by bee enzymes into monosaccharides [23]. A minor fraction of sugars are oligosaccharides. Those sugars (mainly melezitose and raffinose), although are not important for floral origin determination, are useful to discriminate between blossom and honeydew honeys [21].

While the origin of sugars in honey is limited to the nectar and/or honeydew, proteins and amino acids originate both from vegetal sources (nectar and honeydews) and honeybee salivary secretions. [22]. Proline is the main amino acid (50% to 85%) that is produced mainly by the honeybee [22]. Proline is considered indicative of honey maturity and absence of adulteration by sugar syrups. [21]. The international standards establish that honey needs to have a minimum of 180 mg/Kg proline to be considered authentic [3].

Carbohydrates and amino acids provide two techniques to monitor the aging process and storage conditions of honey. Hydroxymethylfurfural (HMF), the decomposition product of fructose is one of the best known Amadori compounds, produced in carbohydrate-containing foods as result of Maillard reactions [22]. The international freshness limit of HMF for honey was fixed below 40 mg/kg [3]. In opposition, the diastase activity, that hydrolysed polysaccharides in honey, decreases with the age of honey [21]. Hence, honey needs to have a minimum of 8.0 Shade units/g diastase activity, according to the European legislation [21].

Also, organic acids, even at their low concentrations in honey (up to 0.57%), are important for honey characterization because they are responsible for the colour, flavour, and other physical and chemical characteristics (described later). They are produced from sugars by bees secreted enzymes when transforming nectar to honey, although some are brought directly with the nectar [24]. The most abundant organic acid in honey is gluconic acid, followed by citric acid. The concentration of both organic acids is useful to discriminate floral honey from honeydew [22].

Another important fraction in determining the honey aroma is the volatile fraction. It is determined by several factors, such as the origin of nectar, storage conditions and undesirable fermentation. This fraction may be useful for different objectives [22]. For examples, monofloral honey is governed by the nectar of a single plant, hence it can be characterized by a specific aroma, or even by the presence of a unique compound such as methyl anthranilate in *Citrus* honey [25].

Phenolic compounds can be divided into several groups including phenolic acids, tannins and flavonoids (flavones, flavonols, flavanones, flavanols, anthocyanidin, isoflavones and chalcones) [11]. Flavonoids represent the main functional compounds in honey and are mostly responsible for the honey antioxidant capacity, in addition to other biological activities (detailed in the section of honey biological activities) [26]. Phenolic profiling in honey can be used as fingerprint for the determination of the honey botanical origin. The presence of quercetin and kaempferol is considered as a biomarker of sunflower and rosemary honeys [4]. Also, a significant decrease in the concentration of some phenolic compounds (galangin, kaempferol, myricetin and p-coumaric acid) may occur after heat treatment [27].

Honey contains also minerals (0.04% in light honeys to 0.2% in dark honeys) such as K, Mg, Ca, Fe, P, Na, Mn, I and Zn amongst others [4]. While organic compounds from plants indicate the botanical origin of the honey, trace elements depend, mainly on the type of soil of the apiary area and hence are a good indicator of honey geographical origins [25]. In addition, some mineral elements may indicate also the botanical origin of a specific honey [25]. Potassium (K) is the most abundant element in honey, reaching up to 33% (one third) of total minerals [4].

Figure 1. shows main compounds identified in honey. Values are illustrative and reflect the general cases of honey.

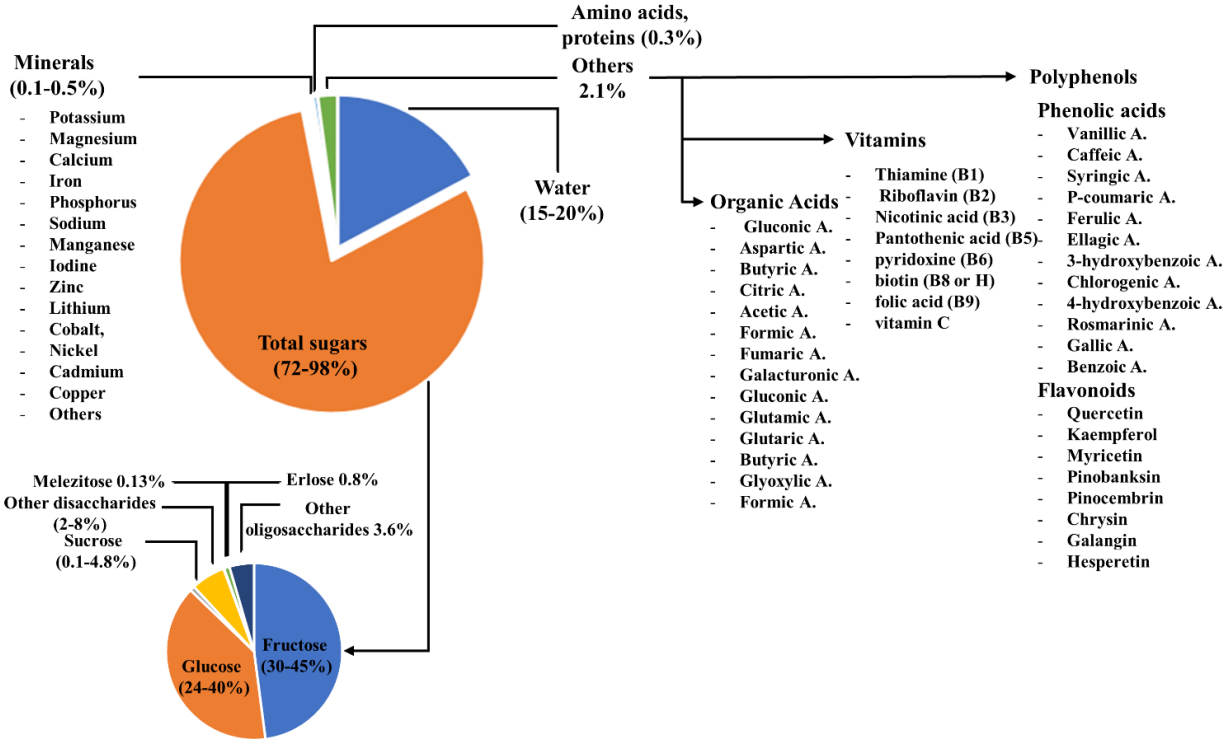

Figure 1 Honey chemical composition.

Honey can be also characterized by other physicochemical properties, that without being specific to a given compound, are useful for its characterization, and are easy to evaluate. For example, ash content is an important parameter for estimating the mineral content of honey and is useful for both botanic and geographical origins discrimination [28,29]. Determination of the electrical conductivity of honey is easier than the ash content and provide almost the same information, explaining the positive correlation between minerals content, ash content and electrical conductivity [29]. In addition, electrical conductivity is also determined by the presence of organic acids. Both parameters are used for honey counterfeiting detection and the international legislation established the maximum in honey to be 0.6% for ash content and 800 µS/cm, for electrical conductivity [1]. Also, it should be mentioned that honeydew honey is characterized by relatively high electrical conductivity in comparison with blossom honey [29].

Honey pH and acidity are other important parameters for its characterization. The low pH of honey, generally around 3.9 is due to the presence of formic, citric and gluconic acids (2,3,4,5,6-pentahydroxyhexanoic acid), that are the main organic acids in honey [23]. Gluconic acid is produced by glucose oxidase from bee secretions acting on glucose [28]. Other organic acids identified in honey include acetic, butyric, lactic, maleic, malic, oxalic, and succinic acids [23,28].

The organic acid composition defines the acidity characteristics of honey that the international standards limit to a maximum of 50 mEq/kg [29].

Another important factor determining the quality of honey is the moisture content. This parameter does not have a direct effect on honey quality but, when exceeding 20 %, maximum value, allowed by the European legislation, it is a factor that favor the growth of undesirable microorganisms [28]. The water content indicates also the maturity level of honey [29]. Hence, when honey is prematurely harvested it is more likely to present high water contents.

Table 1 summarizes the above-mentioned information, with illustrative values of measured parameters and/or their international standards if available.

Table 1. Physico-chemical parameters used for honey quality control, and their standard limits as revised by the international honey commission [3].

Parameter	Specification	Codex Alimentarius	EU legislation
Moisture content	General	≤21 g/100g	≤21 g/100g
	Heather, clover	≤23 g/100g	≤23 g/100g
	Industrial or bakery honey		
Free acidity		≤50 meq/kg	≤40meq/kg
Ash content	General	≤0.6 g/100 g	≤0.6g/100g
	Honeydew or blends of honeydew and blossom honey or chestnut honey	≤1.2 g/100 g	≤1.2 g/100 g
Electrical conductivity	General	≤ 0.8 mS/cm	≤0.8mS/cm
	Honeydew, chestnut	≥ 0.8 mS/cm	≥ 0.8 mS/cm
	Exceptions: *Arbutus*, *Banksia*, *Erica*, *Eucalyptus*, *Eucryphia*, *Leptospermum*, *Melaleuca*, *Tilia*		
Proline content		≤ 180 mg/kg	≤180mg/kg
Diastase activity (Schade scale)	General	≥3	≥3
	After processing	≥8	≥8

HMF	General	-	≤40mg/kg
	After processing	≤60 mg/kg	-
Apparent reducing sugars content	Honeys not listed below	>65 g /100 g	>65 g/100g
	Honeydew honey or blends of honeydew honey and blossom honey	>45 g /100 g	>45 g/100g
	Xanthorrhoea	>53 g /100 g	>53 g/100g
Apparent sucrose content	Honeys not listed below	<5 g/100 g	<5 g/100 g
	Robinia, Lavandula, Hedysarum, Trifolium, Citrus, Medicago, Eucalyptus cam., Eucryphia luc.	<10 g/100g	<10g/100g
	Banksia menz., Rosemarinus Calothamnus san., Eucalyptus scab., Banksia gr., Xanthorrhoea pr., honeydew honey and blends of blossom with honeydew honey	<15 g/100g	-

2.2. Multivariate analysis for botanical origin determination of honey

Honey chemical composition described in previous section has several applications, including simple quality control, botanical origin determination and geographical label confirmation or assessment of honey bioactivity. Choosing the suitable set of parameters depends on the objective as they may provide different information about honey, and with differences in accuracy. Although, more accuracy is achieved when the maximum of parameters is considered.

Botanical origin determination of the honeys is not a trivial task, since different honeys may present same values for given parameters. In addition, honeys of same botanical origin may show differences in the parameters conditioning their classification. This phenomenon is shown in figure 2. The data of the figure was collected from a review analyzing main European honey types (Persano Oddo & Piro, 2004).

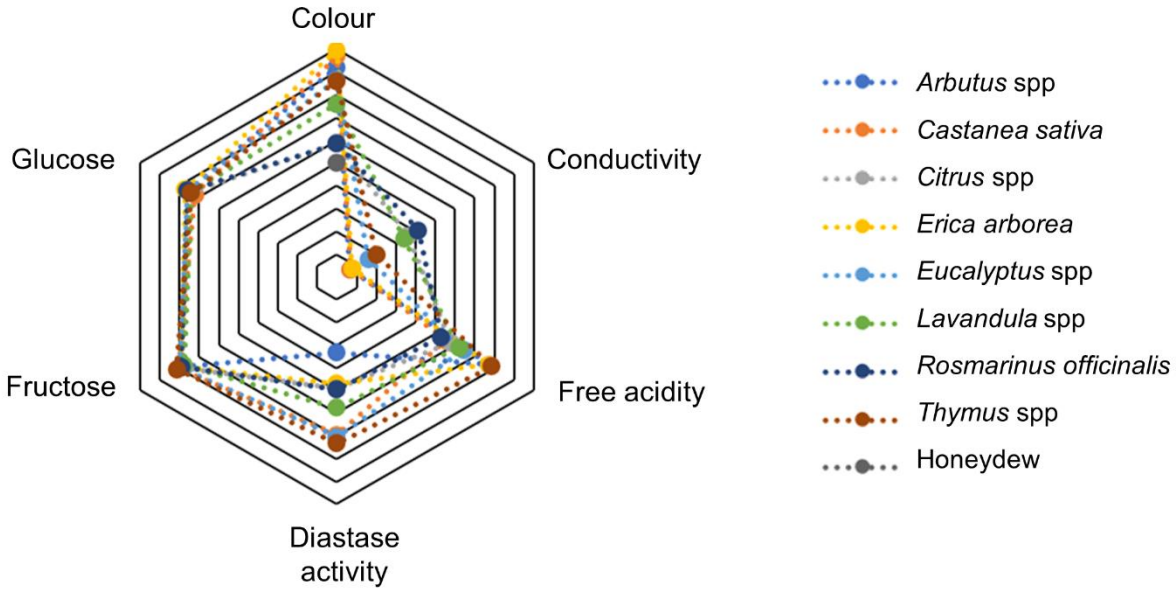

Figure 2. Radar chart comparing European monofloral honeys regarding their glucose and fructose contents, color, conductivity, free acidity and diastase activity.

For instance, *Thymus* and *Erica* honeys showed roughly the same values considering glucose and fructose contents, and free acidity, which if considered for botanical origin discrimination will be misleading. Both honeys would be only distinguishable when the diastase activity, the conductivity, and the colour intensity were also considered. Hence screening as much as possible parameters is crucial for a correct characterization of a honey sample and better discrimination from other types.

Hence, for a correct characterization of honeys more sophisticated tools that integrates variables of multivariate matrices and generate new and easily interpretable information are needed. First attempts in the application of mathematical tools for the classification of blossom and honeydew honeys date to 1960 [31]. These authors discriminate honey samples by integrating pH, ash and sugar contents in discriminant functions.

Multivariate data analysis (also known as chemometrics) is the statistical analysis of a collection of random variables. It is designed to deal with the big amount of data generated worldwide and analysed by scientists for extraction of useful information hidden in these large data matrices [32]. Multivariate data analysis is based on concepts from the computer science and artificial intelligence fields, allowing the performance of exploratory or predictive data analysis in complex data sets [33]. The focus in the present thesis will be on the exploratory use, as the main objective is to determine which variables are more likely to be linked to the presence of determined pollen species.

Principal component analysis (PCA) is one of the most powerful tools to solve classification issues. PCA is a dimension-reduction tool that is used to reduce a large set of variables into a small set of new variables named principal components, but without losing most of the input information [32]. PCA allows to figure out the overall "shape" of data, identifying the similarity between samples. This enable the identification of groups of samples that are more similar and determine which variables differentiate one group from another. In other words, PCA is a mathematical procedure that transforms several (possibly) correlated variables into a (smaller) set of uncorrelated variables or the principal components (PCs) [34].

When it comes to results presentation, both the initial variables, and samples (scores) can be plotted (biplot when they are plotted at the same time) in new obtained PCs (those with higher accumulated variance). These allows clustering the samples into groups (clusters) distinguished by a set of variables [35]. The representation of the variables allows determining affinities between them and distinguishing variables with discriminatory power from others that possess low or no effect [35,36]. The figure bellow shows the most important steps in data analysis using multivariate analysis, namely PCA (figure 3).

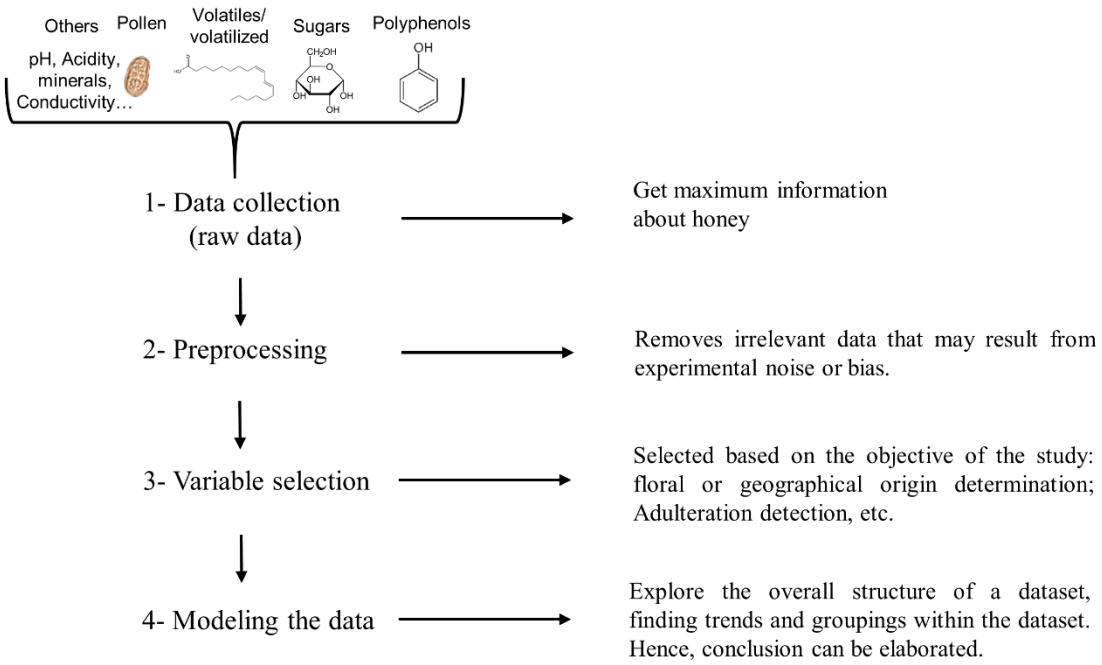

Figure 3. Description of multivariate analysis applied to honey research.

In honey research, the first objective of PCA is to determine the capacity of a set of variables to discriminate the botanical origins of honey samples. When the output of the PCA is obtained, the influence of other parameters can be determined. An example is reported by Estevinho et al (2016) with lavender honey [6]. These authors, after discriminating honey samples based on physicochemical parameters, studied the influence of pollen grains (not used for PCA input) by

comparing their contents in the obtained groups of samples using mean comparison statistical tools. In the present thesis, both above mentioned methods were employed in the chapter “Zantaz honey: chemometric applied to the routinely assessed parameters". The main aim was to study the strength of mellissopalynology and several physicochemical tools to discriminate Zantaz honey samples. A second objective was to assess how the presence of the most abundant pollen species influences and shapes the physico-chemical parameters. The last objective was an attempt to determine the monoflorality threshold (the percentage of the main pollen above which Zantaz honey can be considered monofloral) by integrating all obtained data.

2.3. Non-specific and reliable tools for botanical determination of honey: the electronic tongue (e-tongue) technology

For the determination or discrimination of honey origins, even the benefits of associating the analysis of physical and chemical parameters from one side, and the chemometric tools from the other, the whole process is costly and time consuming. In addition, highly qualified professionals are needed for some experiments and for data processing and interpretation. Also, because of the complex nature of honey and the different types of adulteration, its authentication has been challenging and has prompted the development of several advanced analytical approaches [37]. Theoretically, the best tool needs to be fast, cheap, reliable, and retain maximum information that will allow the determination and/or confirmation of honey botanical and geographical origins, the detection of adulteration and the control of counterfeiting practices [9].

Electronic tongue technology is one of the best examples of those tools. “The electronic tongue is defined as an analytical instrument comprising an array of nonspecific, low-selective, chemical sensors with high stability and cross-sensitivity to different species in solution and an appropriate method of multivariate calibration for data processing”[9]. From this definition an electronic tongue is formed by a sensing unit, a measurement instrumentation able to detect changes occurring at the environment of the sensing units, and an algorithm to transform the collected raw data to interpretable information (figure 4)**.** Several reviews gives an overview on e-tongues and other technologies that relay on the same principles [9,38,39].

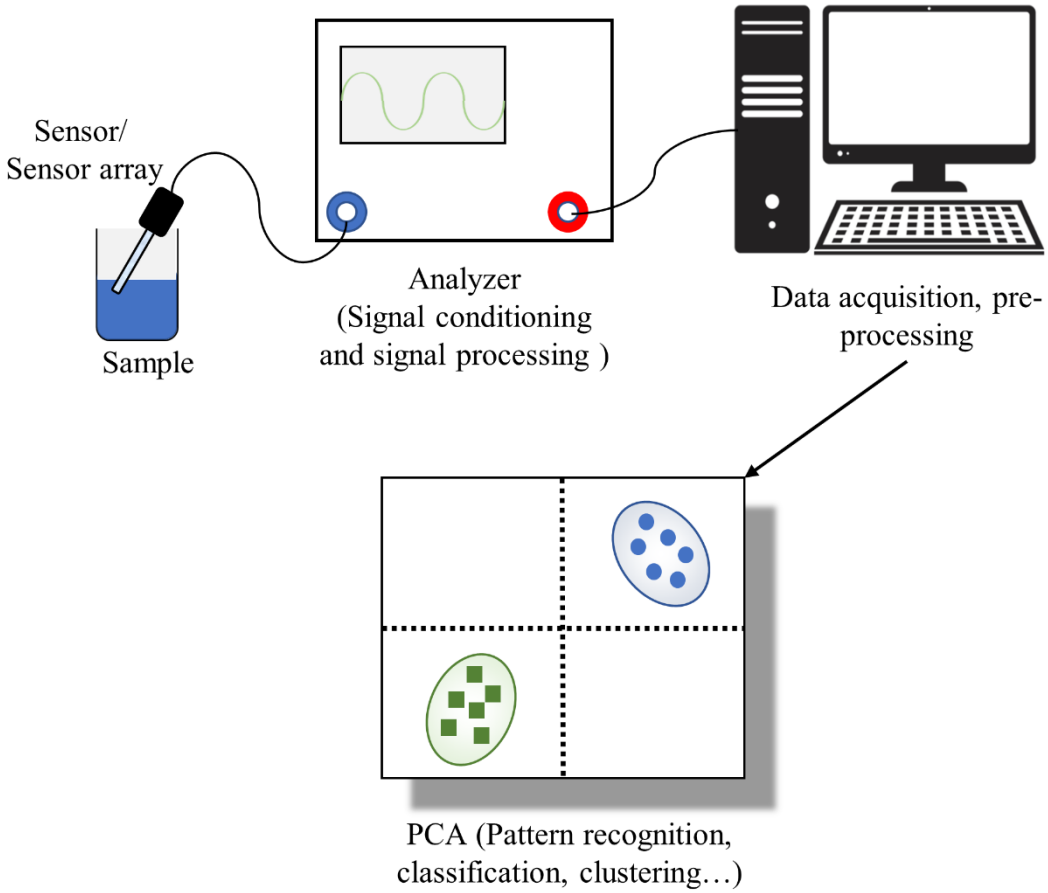

Figure 4. Main steps for performing analysis with an e-tongue

Several materials can be considered for building e-tongues, and different techniques are employed for measurement, ranging from electrochemical (potentiometric, amperometric, voltammetric, impedimetric, conductometric) through gravimetric and optical (absorbance, luminescence, reflectance, etc.) [39]. In the present thesis, an impedance-based e-tongue was developed, characterized and applied to honey botanical origin determination.

Impedance measurements are usually applied to characterize materials or circuits used in the field of electronic engineering [40]. Its high sensibility may detect minor changes in the chemical composition of a solution/material and even monitor the tiny changes caused by bounding of proteins (antibodies) on a conductive surface [41]. Innovative uses include the application of impedance measurements to monitor changes in the number and structure of living cells, activities like the wound healing, and cell migration [42].

If we consider a conductive or semi-conductive material submersed in a chemical solution, there will be a development of a specific interface between the material and the chemical solution known as electrical double layer (EDL) [43]. The sensing mechanism of an impedimetric tongue relies on measuring the impedance of the EDL established at the electrode/electrolyte interface. Figure 5 shows an example of the used setup of a simple model impedance-based e-tongue. It is

made of a sensing gold electrode and a counter platinum electrode submersed in a chemical solution. The impedance will measure the resistance and capacitance properties of used materials via application of a sinusoidal alternative current (AC) excitation signal [41]. The variation of the frequency will result in a series of capacitance and resistance values used for further calculations.

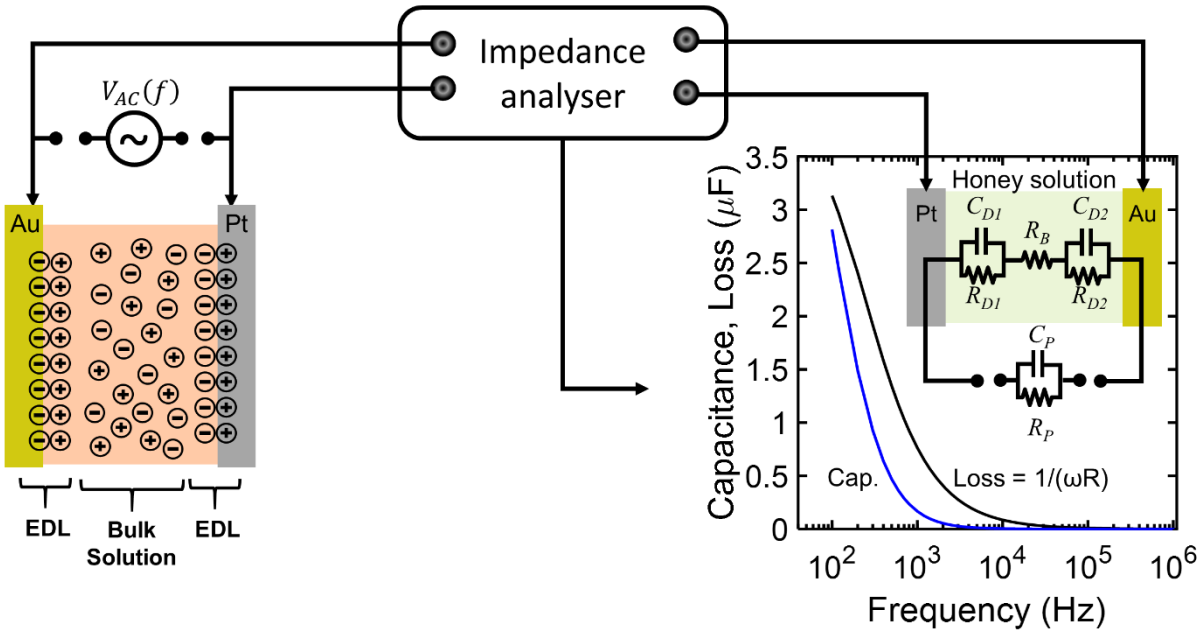

Figure 5. Experimental setup and the equivalent circuit of impedance analysis of a honey solution with an e-tongue.

From a physical point of view, an effective system to probe the properties of honey needs to be able to measure the EDL and not only the bulk solution. Considering that the developed double layer is dependent on the chemical composition of the solution and the electrical features of the material, the perfect impedance-based e-tongue is a multisensory system that can form different electrical double-layers. Each of the developed EDLs will sense a given property of the solution and help extracting maximum information about it. [9]. At the output of the impedance analyser, a matrix made of resistance and capacitance at several frequencies of each sensor is built and processed with appropriate statistical tools to interpret the provided information [44]. Multivariate analysis will be needed here also to analyse the obtained data and extract the useful information. In the present thesis, the PCA will be a corner stone as it will reduce the big scales of the obtained data matrices and turns it into few numbers of new variables known as principal components.

At the end of the process, and as the e-tongue technology is still under development, a validation step is required [44]. For validation, the obtained clusters and accuracy levels can be determined in comparison with the other already established procedures. As stated before, in the honey research pollen analysis is the most important, alongside with physicochemical parameters that are botanical origins dependant.

3. Biological activities of honey

Besides their importance in botanical origin determination, honey compounds present important biological activities, which explain their definition as bioactive compounds [4,26]. These compounds present different biological activities when applied as raw honey, or when extracted using specific extraction methods [11]. Honey is applied even at clinical level, even if such use need screening many of the honey features [45]. The second part of this introducing chapter will highlight the main issues in relation with the health benefits of honey and will be limited to those treated in the present thesis.

3.1. The effect of heat processing on honey biological activities

Honey is a food and therefore is subjected to customer preferences [26] . Flavor, color, viscosity, tendency to crystallization, and shelf-life, are some of the parameters considered by honey consumers [28]. Honey producers pay great attention to those parameters and try to make them meet customers' demands. For example, to prevent or delay crystallization, honey producers used to submit it to heat processing. Such practice, besides making honey less viscous, eliminates potential developed microorganisms that through by fermentation may low honey quality and reduces its shelf-life [2].

However, when heat processing is inadequately applied, affects negatively the quality of honey as does the inadequate storage and/or the age. the deterioration of honey with the heating is kinetic dependent on temperature and exposure time [2]. The most important degradation product of honey is hydroxymethylfurfural (HMF), formed by hexose dehydration at low pH, or by Maillard reaction. Hence the international trade fixed the value of 40 mg/kg of HMF, as the maximum allowed amount in honey samples to be declared as fresh [46]. Heat processing is linked also with the increase of other parameters such as color intensity and melanoidins contents, although there are no established limits because both parameters are highly influenced by the botanical origin of honey [28]. While the above-mentioned parameters increase following heat processing, vitamins and some amino acids may decrease.

The changes caused by heat processing modify the functional groups of honey, resulting in altered biological activities [28]. For example, formed browning pigments and Maillard reaction products are responsible for enhanced honey antioxidant activities assessed using DPPH scavenging activity assay [47]. These changes are more marked in light coloured honey than in dark ones. In contrast, Nagai, Sakai, Inoue, Inoue, & Suzuki, (2001) linked the heat processing to reduced honey antioxidant activity when employing a linoleic acid-based lipid peroxidation assay [48]. Hence, conclusions on the antioxidant depend on the selected free radical species and on the honey composition (functional groups). Figure 6 shows possible interactions occurring in honey following thermal processing.

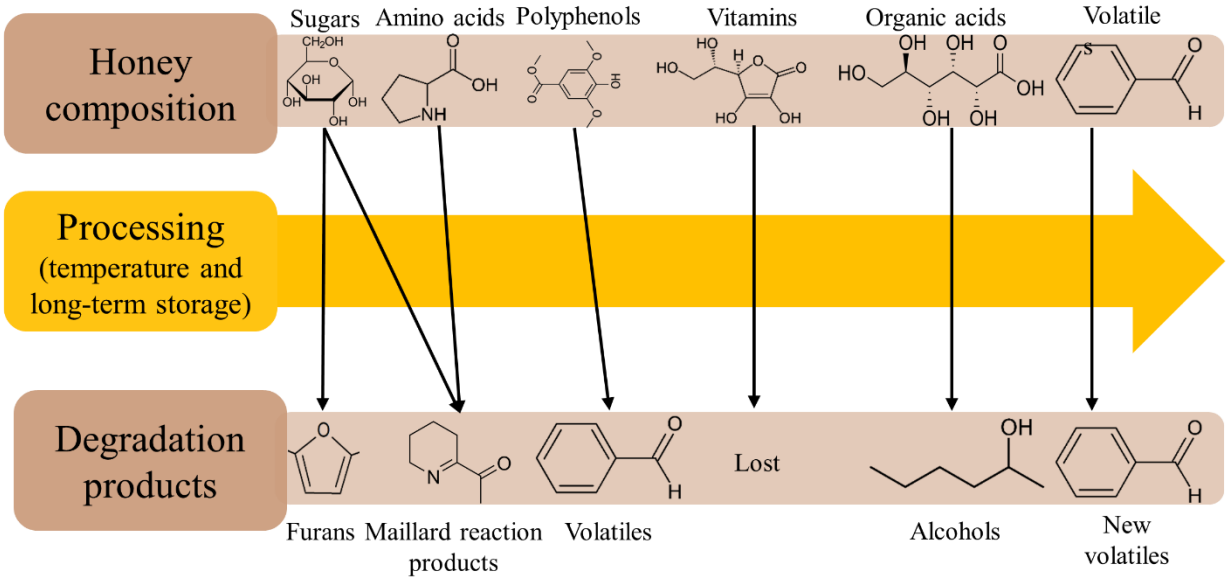

Figure 6. Changes in honey chemical composition upon heat processing.

In vivo, during normal metabolism, the organism produces a variety of free radicals, and at the same time possesses effective strategies for scavenging these radicals [49]. An unbalance between production and scavenging of free radicals, internally, by an impairment of the antioxidant tools or an excessive production of free radicals, or externally, by exogenous stresses, causes oxidative stress [49]. Oxidative stress is linked to different diseases, such as diabetes and cancer. [11].

Different assays are available to assess the ability of antioxidants to scavenge free radicals. [45]. The employed free radicals can be synthetic such as DPPH and ABTS. Others are similar to those produced at the *in vivo*, and are more specific such as NO, OH [50]. Table 2 resumes the most commonly used assays with a short description of their principles, and targeted group of molecules.

Table 2. Main antioxidant assays, their principles and principal targeted groups

Antioxidant assay	Principle	Targeted chemical groups in honey
DPPH	DPPH radicals get reduced (H/e-transferring ability) after receiving hydrogen atom from an antioxidant and lowers the violet colour [50]	Phenolic/polyphenolic entities [51]

ABTS	Reduction of the radical cation $ABTS^{\bullet+}$ The antioxidant reduces it to ABTS and decolorize it [50].	Phenolic/polyphenolic entities [51]
Chelating power	Measures the ability of antioxidants to compete with ferrozine which can quantitatively form complexes with Fe^{+2}. In the presence of chelating agents, the complex formation is disrupted, leading to a decrease in the red color of ferrous ion and ferrozine complex [52]	Organic acids and phenolic compounds [53]
Hydroxyl scavenging capacity	2-Deoxyribose is oxidized by OH• that is produced by the Fenton reaction and degraded to malondialdehyde. Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radicals [54]	Flavonoids substrate action [55]
Superoxide scavenging activity	In the PMS–NADH–NBT system, the superoxide anion derived from dissolved oxygen through the PMS–NADH coupling reaction reduces the NBT. This step is inhibited by the presence of antioxidant compounds inducing a decrease in the color intensity of the reaction [52]	Flavonoids substrate action [55]

A strong positive correlation has been established between the antioxidant activity of honey and its contents in bioactive compounds (vitamins, polyphenols, etc) [11]. As the thermal processing affects the composition of honey in different ways depending on their nature, it is expected that the antioxidant activities will also behave differently in heated honey. This hypothesis will be the corner stone of our approach when assessing the effect of heat processing on Zantaz honey.

3.2. Honey antimicrobial activity and the effect of physicochemical properties

The antimicrobial activity of honey was demonstrated for the first time by Van Ketel in 1892, [19]. Since then, may studies have been published dealing with the antimicrobial activity of honey of different botanical origins, and the mechanisms underlined [11,56,57]. In fact, the medicinal use of honey is older. Islamic traditions, ancient Egyptians, ancient Greek, Chinese and other civilizations have used honey as a remedy for many diseases [19]. Furthermore, the power of honey to treat wound healing, an activity linked to antibacterial activity have also been reported [11]. Nowadays, the emergence of multi-resistant bacteria has stimulated the use of natural products, including honey, as antibacterial compounds [45].

The low pH of honey is one of the critical parameters contributing to its antimicrobial activity. The minimum pH values for growth inhibition of some common pathogenic bacteria are pH 4.3 (*Escherichia coli*), pH 4.0 (*Salmonella* spp), pH 4.4 (*Pseudomonas aeruginosa*) and pH 4.5 (*Streptococcus pyogenes*) [56]**.** The production of hydrogen peroxide as a result of the bee glucose oxidase activity and the osmotic effect of the high contents of sugars in honey are also important factors in its antibacterial activity [2].

From the above information if we consider one honey sample, its ability to induce a bactericide or bacteriostatic effect will depend on the chosen bacterial strain. Similarly, if we consider one bacterial or fungi species, several sensitivity levels will be presented toward different honey types. Hence, it is critical to establish predicting tools that will allow the selection of the adequate honey sample for a given microbial species. Keeping in mind that honey is characterized by specific patterns of physicochemical parameters highly dependent on its botanical origin, it is very important to establish specific predicting tools for each honey type. This issue will be the guiding idea in the present thesis. Specific attention will be payed to the influence of honey physicochemical features, and to their possible use as predictive tools when it is to choose the adequate Zantaz honey sample.

Supporting this hypothesis, is the work of laallam et al (2015) that evoked the concept notion of modelling the synergetic antibacterial effect of features of Algerian Sahara honey [12]. They concluded that antibacterial effect increases with increasing water contents and reducing sugars in honey, while decreases with increasing electrical conductivity. In addition, honeys of Fabaceae floral origin have a higher detrimental effect on bacteria compared to other spontaneous Saharan species. Other insight came from a study of Hamada et al. when studying synergic effect after mixing honey samples from Morocco and Palestine, with essential oils. Their results revealed the importance of physicochemical parameters in determining the antibacterial effect of honey and its ability of developing synergic effect with essential oils [13].

3.3. Cell-based antioxidant and antiproliferative activities of honey.

It is well known that a substance with antioxidant activity against free radicals in a chemical solution, may not necessarily be effective in a cell model. Cells represent a more complex model due to cell membranes and metabolism that introduce the notion of bioavailability of bioactive substances [58]. Hence, the use of cell-based assays is very important and an intermediate step between *in vitro* and *in vivo* models [59]. Figure 7 illustrate main steps of cell antioxidant assay (CAA) and highlights the importance of the bioavailability concept.

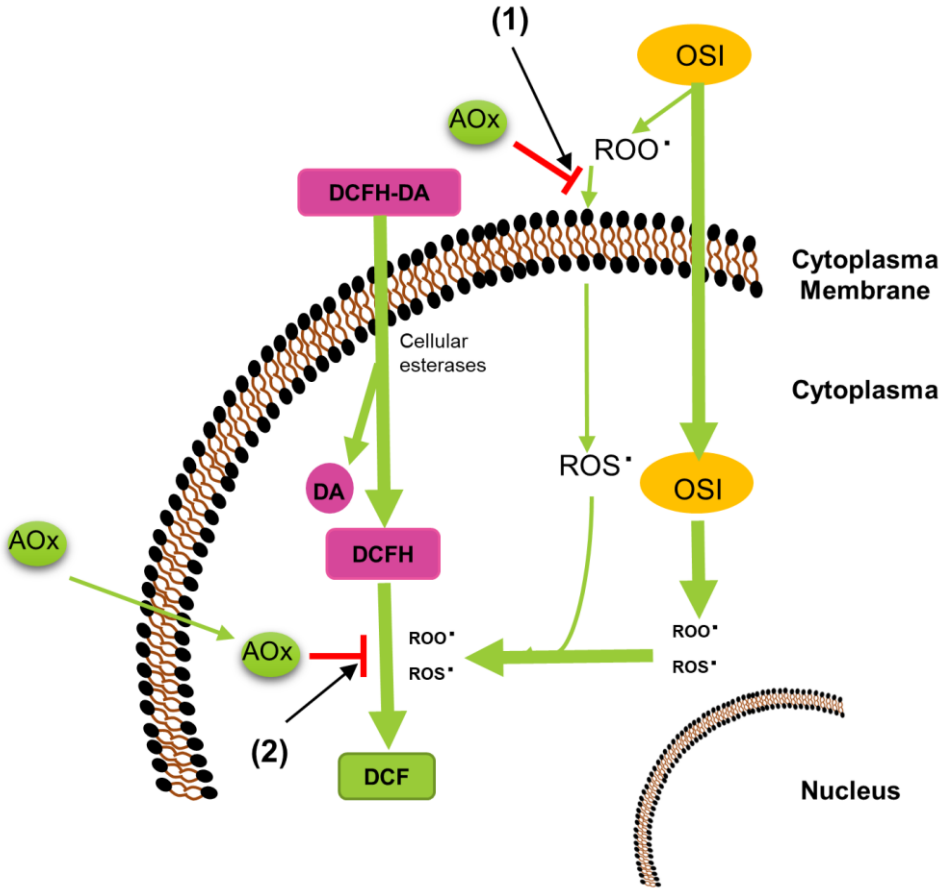

Figure 7. Diagram of Cell based antioxidant assay. (1) extracellular antioxidant activity. (2) intracellular antioxidant activity. OSI: oxidative stress inducer. AOx: Antioxidant. DCFH-DA: 2′,7′-Dichlorodihydrofluorescein diacetate.

Cell-based results may differ depending on the cell line used. When CAA was employed to study phenolic antioxidants using hepatocarcinoma cells (HepG2), quercetin showed antioxidant activity while (+)-catechin, bestowed virtually no activity [60]. In contrast when Caco-2 cells were employed, more antioxidant activity was observed with both phenolic compounds. These results highlight the importance of cell model on accessing the antioxidant activity of polyphenols or phenolic rich products. Both phenolic compounds are known to possess a high antioxidant activity toward many free radicals, although their bioavailability was dependent on the cell model used. Phenolic compounds are the most antioxidant compounds in honey [11,28]. As stated before, their

profile in honey is highly determined by its botanical origins. Hence, when choosing a honey type for the cell-based assay, the samples needs to be characterized for their phenolic contents.

The antiproliferative activity is another cell-based important assay for the study of the possible effect of bioactive compounds on cancer cells. The use of honey, or any other natural product for the anticancer activity evaluation brings to discussion the chemoprevention concept, which is defined as the use of natural or synthetic compounds to reduce the risk of developing or reoccurring cancer [61]. Increasing evidence suggests honey as a potential chemopreventive agent [60].

The antiproliferative activity of honey depends on the presence of plant secondary metabolites such as phenolic acids, and flavonoids. The involved mechanism of action may vary depending on the structure of the compounds and their concentration in honey [4]. These mechanisms of action may include cell cycle arrest, induction of apoptosis, modulation of oxidative stress and immuno-modulation [26]. However, the chemopreventive and therapeutic activity of honey is still not fully understood and constitute nowadays a challenging research [60].

The fact that oxidative stress is a key factor in cancer initiation and development, suggest that honey may act firstly through its antioxidant activity, especially when rich in compounds present high bioavailability. Among most bioactive honeys is Manuka honey. This honey shows a strong anti-proliferative activity, in a time and dose-dependent manner, on different cancer cell lines, including B16.F1 (murine melanoma), CT26 (colorectal carcinoma) and MCF-7 (human breast cancer) [57]. The activation of mitochondrial apoptotic pathways, the induction of apoptosis, the induction of DNA fragmentation and the loss of Bcl-2 expression is among the mechanisms reported for this honey. In addition, it has been shown that this honey attenuated the symptoms of several cancers *in vivo* [57].

4. Propolis: characterization and its use as a source of antiproliferative and cell-based antioxidant agents

Considering bee products and their biological activities, honey is not the only interesting one. Hence, propolis is also highly concentrated in bioactive compounds. Propolis, from the Greek ‘pro’=‘in front’ or ‘in defence’ and ‘polis’=‘the city’, meaning ‘defence of the hive’, is a strongly adhesive natural product manufactured by honey bees from the buds or exudates of plants near their hives [62]**.** Propolis is used by bees to seal holes in hives, smooth out the internal walls, making the hive defensible, and as a barrier against external invaders, diseases, thermal insulation, humidity and wind. [63]

When bee workers collect the raw resinous material to make propolis, unintentionally introduce also pollen grains. Those grains come from flowers visited by the bees for nectar and

pollen collection and from wind pollinated plants [64]. Even if the pollen does not come from the same plant delivering the exudates, the identification of plant species whose pollen occurs in propolis samples, allows a characterization of the vegetation surrounding the beehive and also the geographical origin of the propolis [65].

The early study of D'Albore (1979), who analyzed 56 samples of propolis from different countries and continents, showed that in most cases, it was possible to determine their phytogeographical origin by pollen analysis. Later on, Barth (1998), focused on analysing the famous Brazilian propolis and emphasized the importance of pollen grains from anemophilous plants in characterizing the geographical origin of this propolis [65].

From a compositional point of view, propolis is much more concentrated in bioactive compounds than honey, making it more suitable for both cell-based antioxidant and antiproliferative activities studies**.** Furthermore, the chemoprotective concept is also valid in the case of propolis, and authors reported its uses in attenuating the side effect, such as cytopenia, of radiation or chemotherapy in animal models [62]. In addition, propolis, or its isolated compounds, also showed good antiproliferative activities *in vitro*, using cancer cell lines, and *in vivo* using animal models

In Morocco, several studies aimed the characterization and some biological activities of propolis [66–69], However no work has been reported on propolis samples originating from hives in surrounding areas of *B. spinosum* populations. The present thesis intends to unravel the biological properties of these propolis samples not yet studied so far

References

[1] S. Bogdanov and P. Martin, Honey Authenticity : a Review, Mitt. Lebensm. Hyg. 93 (2002) 232–254.

[2] N.T. Grabowski, G. Klein, Microbiology and foodborne pathogens in honey, Crit. Rev. Food Sci. Nutr. 57 (2017) 1852–1862. doi:10.1080/10408398.2015.1029041.

[3] S. Bogdanov, C. Lüllmann, P. Martin, W. von der Ohe, H. Russmann, G. Vorwohl, L.P. Oddo, A.G. Sabatini, G.L. Marcazzan, R. Piro, C. Flamini, M. Morlot, J. Lhéritier, R. Borneck, P. Marioleas, A. Tsigouri, J. Kerkvliet, A. Ortiz, T. Ivanov, B. D'Arcy, B. Mossel, P. Vit, Honey quality and international regulatory standards: Review by the international honey commission, Bee World. 80 (1999) 61–68. doi:10.1080/0005772x.1999.11099428.

[4] M.G. Miguel, M.D. Antunes, M.L. Faleiro, Honey as a complementary medicine, Integr. Med. Insights. 12 (2017) 1–15. doi:10.1177/1178633717702869.

[5] J. Louveaux, A. Maurizio, G. Vorwohl, Methods of melissopalynology, Bee World. 5 (1978) 139–153. doi:10.1080/0005772X.1978.11097714.

[6] L.M. Estevinho, E.D. Chambó, A.P.R. Pereira, C.A.L. De Carvalho, V. De Alencar Arnaut De Toledo, Characterization of Lavandula spp. honey using multivariate techniques, PLoS One. 11 (2016) 1–15.

doi:10.1371/journal.pone.0162206.

[7] L. Castro-Vázquez, V. Leon-Ruiz, M.E. Alañon, M.S. Pérez-Coello, A. V. González-Porto, Floral origin markers for authenticating Lavandin honey (Lavandula angustifolia x latifolia). Discrimination from Lavender honey (Lavandula latifolia), Food Control. 37 (2014) 362–370. doi:10.1016/j.foodcont.2013.09.003.

[8] I.K. Karabagias, E.Z. Halatsi, S. Karabournioti, S. Kontakos, M.G. Kontominas, Impact of physicochemical parameters, pollen grains, and phenolic compounds on the correct geographical differentiation of fir honeys produced in Greece as assessed by multivariate analyses, Int. J. Food Prop. 20 (2017) S520–S533. doi:10.1080/10942912.2017.1300811.

[9] M. Podrazka, E. Báczyńska, M. Kundys, P.S. Jeleń, E.W. Nery, Electronic tongue-A tool for all tastes?, Biosensors. 8 (2017) 1–24. doi:10.3390/bios8010003.

[10] Z. Gan, Y. Yang, J. Li, X. Wen, M. Zhu, Y. Jiang, Y. Ni, Using sensor and spectral analysis to classify botanical origin and determine adulteration of raw honey, J. Food Eng. 178 (2015) 151–158. doi:10.1016/j.jfoodeng.2016.01.016.

[11] D. Cianciosi, T. Yuliett, S. Afrin, M. Gasparrini, P. Reboredo-rodriguez, P.P. Manna, J. Zhang, L.B. Lamas, S. Mart, P.A. Toyos, J.L. Quiles, F. Giampieri, M. Battino, Phenolic compounds in honey and their associated health benefits : A review, (2018) 1–20. doi:10.3390/molecules23092322.

[12] H. Laallam, L. Boughediri, S. Bissati, T. Menasria, M.S. Mouzaoui, S. Hadjadj, R. Hammoudi, H. Chenchouni, Modeling the synergistic antibacterial effects of honey characteristics of different botanical origins from the Sahara Desert of Algeria, Front. Microbiol. 6 (2015) 1–12. doi:10.3389/fmicb.2015.01239.

[13] H. Imtara, Y. Elamine, B. Lyoussi, Honey antibacterial effect boosting using *Origanum vulgare* L. essential oil, Evidence-Based Complement. Altern. Med. 2018 (2018). doi:10.1155/2018/7842583.

[14] J. Peltier, Ecologie De Quelques Especes Climaciques Dans Le Sous (Maroc Occidental), Doc. Cartogr. Ecol. XXVI (1983) 61–82.

[15] F. Nassif., B. El Amiri., Goat production systems in a mountainous community of the Middle Atlas, Morocco, Options Méditerranéennes. Séries A. Mediterr. Semin. 100 (2011) 199–203.

[16] R. Bencheraiet, A. Kabouche, Z. Kabouche, R. Touzani, M. Jay, Flavonol 3-O-glycosides from three algerian Bupleurum species, Rec. Nat. Prod. 6 (2012) 171–174.

[17] P.L. Ortiz, Contribucion al conocimiento de la flora apicola Gaditana, 16 (1990) 199–210.

[18] A. Khabbach, A. Libiad, M. Ennabili, Melliferous flora and apiculture in the pre-rif of the province of Taza (north of Morocco), Luna Azul. (2013) 240–256. doi:10.17151/luaz.2015.40.14.

[19] J.H. Dustmann, Antibacterial effect of honey, Apiacta. (1979) 7–11. doi:10.1038/ncomms3838.5.

[20] J.A. Snowdon, D. Cliverb, Microorganisms in honey, Int. J. Food Microbiol. 31 (1996) 1–26.

[21] S. Bogdanov, Honey composition, Honey B. (2017) 1–10. doi:10.1016/j.sjbs.2016.12.010.

[22] P.M. Da Silva, C. Gauche, L.V. Gonzaga, A.C.O. Costa, R. Fett, Honey: Chemical composition, stability and authenticity, Food Chem. 196 (2016) 309–323. doi:10.1016/j.foodchem.2015.09.051.

[23] D.W. Ball, The chemical composition of honey, J. Chem. Educ. 84 (2007) 1643–1646. doi:10.1021/ed084p1643.

[24] J.L. Fredrick, I.M. Kayla, and I.L.G.N. James, B. McKinlay, Differential carbohydrate utilization and organic acid production by honey bee symbionts, FEMS Microbiol Ecol. (2018). doi:10.1093/femsec/fiy113.

[25] V. Kaškonienė, P.R. Venskutonis, Floral markers in honey of various botanical and geographic Origins: A review, Compr. Rev. Food Sci. Food Saf. 9 (2010) 620–634. doi:10.1111/j.1541-4337.2010.00130.x.

[26] J.M. Alvarez-Suarez, S. Tulipani, S. Romandini, E. Bertoli, M. Battino, Contribution of honey in nutrition and human health: A review, Med. J. Nutrition Metab. 3 (2010) 15–23. doi:10.1007/s12349-009-0051-6.

[27] I. Escriche, M. Kadar, M. Juan-Borrás, E. Domenech, Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment, Food Chem. 142 (2014) 135–143. doi:10.1016/j.foodchem.2013.07.033.

[28] P. Missio, C. Gauche, L.V. Gonzaga, A. Carolina, O. Costa, Honey : Chemical composition , stability and authenticity honey : Chemical composition , stability and authenticity, FOOD Chem. 196 (2016) 309–323. doi:10.1016/j.foodchem.2015.09.051.

[29] S. Bogdanov, P. Martin, C. Lüllmann, R. Borneck, C. Ch. Flamini, M. Morlot, J. Heretier, G. Vorwohl, H. Russmann, L. Persano-Oddo, A.. Sabatini, G.. Marcazzan, P. Marioleas, K. Tsigouri, J. Kerkvliet, A. Ortiz, T. Ivanov, HARMONISED METHODS OF THE INTERNATIONAL IHC responsible for the methods :, (1997) 1–59.

[30] L. PERSANO ODDO, R. PIRO, Main European unifloral honeys: descriptive sheets, Apidologie. 35 (2004) 38–81. doi:10.1051/apido:2004049.

[31] K. Ruoff, W. Luginbühl, R. Künzli, M.T. Iglesias, S. Bogdanov, J.O. Bosset, K. Von Der Ohe, W. Von Der Ohe, R. Amadò, Authentication of the botanical and geographical origin of honey by mid-infrared spectroscopy, J. Agric. Food Chem. 54 (2006) 6873–6880. doi:10.1021/jf060838r.

[32] C. Maione, F. Barbosa, R.M. Barbosa, Predicting the botanical and geographical origin of honey with multivariate data analysis and machine learning techniques: A review, Comput. Electron. Agric. 157 (2019) 436–446. doi:10.1016/j.compag.2019.01.020.

[33] O. Anjos, C. Iglesias, F. Peres, J. Martínez, Á. García, J. Taboada, Neural networks applied to discriminate botanical origin of honeys, 175 (2015) 128–136. doi:10.1016/j.foodchem.2014.11.121.

[34] J. Lever, M. Krzywinski, N. Altman, Points of Significance: Principal component analysis, Nat. Methods. 14 (2017) 641–642. doi:10.1038/nmeth.4346.

[35] I. Escriche, M. Kadar, E. Domenech, L. Gil-Sánchez, A potentiometric electronic tongue for the discrimination of honey according to the botanical origin. Comparison with traditional methodologies: Physicochemical parameters and volatile profile, J. Food Eng. 109 (2012) 449–456. doi:10.1016/j.jfoodeng.2011.10.036.

[36] P.M. Cometto, P.F. Faye, M. Caccavari, M. V. Baroni, M.A.J. Aldao, Relationship between interannual variation of amino acid profile and pollen content in honey from a small Argentinian region, J. Agric. Food

Chem. 54 (2006) 9458–9464. doi:10.1021/jf061325n.

[37] J.S. Amaral, M.B.P.P. Oliveira, I. Mafra, A Comprehensive Review on the Main Honey Authentication Issues : Production and Origin, 00 (2017). doi:10.1111/1541-4337.12278.

[38] Y. Tahara, K. Toko, Electronic Tongues – A Review, 13 (2013), IEEE Sensors J. 3001–3011.

[39] P. Ciosek, W. Wróblewski, Sensor arrays for liquid sensing - Electronic tongue systems, Analyst. 132 (2007) 963–978. doi:10.1039/b705107g.

[40] G. Scandurra, G. Tripodi, A. Verzera, Impedance spectroscopy for rapid determination of honey floral origin, J. Food Eng. 119 (2013) 738–743. doi:10.1016/j.jfoodeng.2013.06.042.

[41] K.C. Honeychurch, Printed thick-film biosensors, Print. Film. Mater. Sci. Appl. Sensors, Electron. Photonics. (2012) 366–409. doi:10.1533/9780857096210.2.366.

[42] A.L.G. Mestre, P.M.C. Inácio, Y. Elamine, S. Asgarifar, A.S. Lourenço, M.L.S. Cristiano, P. Aguiar, M.C.R. Medeiros, I.M. Araújo, J. Ventura, H.L. Gomes, Extracellular electrophysiological measurements of cooperative signals in astrocytes populations, Front. Neural Circuits. 11 (2017). doi:10.3389/fncir.2017.00080.

[43] E. Katz, I. Willner, Probing biomolecular interactions at conductive and semiconductive surfaces by impedance spectroscopy: routes to impedimetric immunosensors, DNA-sensors, and enzyme biosensors, (2003) 913–947.

[44] P.A. Ulloa, R. Guerra, A.M. Cavaco, A.M. Rosa da Costa, A.C. Figueira, A.F. Brigas, Determination of the botanical origin of honey by sensor fusion of impedance e-tongue and optical spectroscopy, Comput. Electron. Agric. 94 (2013) 1–11. doi:10.1016/j.compag.2013.03.001.

[45] P. Combarros-Fuertes, L.M. Estevinho, L.G. Dias, J.M. Castro, F.A. Tomás-Barberán, M.E. Tornadijo, J.M. Fresno-Baro, Bioactive components and antioxidant and antibacterial activities of different varieties of honey: A screening prior to clinical application, J. Agric. Food Chem. 67 (2019) 688–698. doi:10.1021/acs.jafc.8b05436.

[46] I. Turhan, N. Tetik, M. Karhan, F. Gurel, H. Reyhan Tavukcuoglu, Quality of honeys influenced by thermal treatment, LWT - Food Sci. Technol. 41 (2008) 1396–1399. doi:10.1016/j.lwt.2007.09.008.

[47] N. Turkmen, F. Sari, E.S. Poyrazoglu, Y.S. Velioglu, Effects of prolonged heating on antioxidant activity and colour of honey, Food Chem. 95 (2006) 653–657. doi:10.1016/j.foodchem.2005.02.004.

[48] T. Nagai, M. Sakai, R. Inoue, H. Inoue, N. Suzuki, Antioxidative activities of some commercially honeys, royal jelly, and propolis, Food Chem. 75 (2001) 237–240. doi:10.1016/S0308-8146(01)00193-5.

[49] A.M. Pisoschi, A. Pop, The role of antioxidants in the chemistry of oxidative stress: A review, Eur. J. Med. Chem. 97 (2015) 55–74. doi:10.1016/j.ejmech.2015.04.040.

[50] M.N. Alam, N.J. Bristi, M. Rafiquzzaman, Review on in vivo and in vitro methods evaluation of antioxidant activity, Saudi Pharm. J. 21 (2013) 143–152. doi:10.1016/j.jsps.2012.05.002.

[51] G. Beretta, P. Granata, M. Ferrero, M. Orioli, R.M. Facino, Standardization of antioxidant properties of honey

by a combination of spectrophotometric/fluorimetric assays and chemometrics, Anal. Chim. Acta. 533 (2005) 185–191. doi:10.1016/j.aca.2004.11.010.

[52] A. Güder, H. Korkmaz, Evaluation of in-vitro antioxidant properties of hydroalcoholic solution extracts *Urtica dioica* L., *Malva neglecta* Wallr. and their mixture, Iran. J. Pharm. Res. 11 (2012) 913–923. doi:10.22037/ijpr.2012.1159.

[53] L.S. Chua, N.L.A. Rahaman, N.A. Adnan, T.T. Eddie Tan, Antioxidant activity of three honey samples in relation with their biochemical components, J. Anal. Methods Chem. 2013 (2013). doi:10.1155/2013/313798.

[54] M. V. Reshma, S. Shyma, T.M. George, A. V. Rishin, K.C. Ravi, L. Shilu, Study on the physicochemical parameters, phenolic profile and antioxidant properties of Indian honey samples from extrafloral sources and multi floral sources, Int. Food Res. J. 23 (2016) 2021–2028.

[55] M. Lewoyehu, M. Amare, Comparatice evaluation of analytical methods for determining the antioxidant activities of honey: a review, Cogent Food Agric. (2019).

[56] M. Aurongzeb, K. Azim, Antimicrobial properties of natural honey : a review of literature Antimicrobial properties of natural honey : a review of literature, (2016).

[57] J. Alvarez-Suarez, M. Gasparrini, T. Forbes-Hernández, L. Mazzoni, F. Giampieri, The composition and biological activity of honey: A focus on manuka honey, Foods. 3 (2014) 420–432. doi:10.3390/foods3030420.

[58] J. Deng, R. Liu, Q. Lu, P. Hao, A. Xu, J. Zhang, J. Tan, Biochemical properties, antibacterial and cellular antioxidant activities of buckwheat honey in comparison to manuka honey, Food Chem. 252 (2018) 243–249. doi:10.1016/j.foodchem.2018.01.115.

[59] H. Zhang, M. Yin, L. Huang, J. Wang, L. Gong, J. Liu, B. Sun, Evaluation of the cellular and animal models for the study of antioxidant activity: A review, J. Food Sci. 82 (2017) 278–288. doi:10.1111/1750-3841.13605.

[60] M.E. Kellett, P. Greenspan, R.B. Pegg, Modification of the cellular antioxidant activity (CAA) assay to study phenolic antioxidants in a Caco-2 cell line, Food Chem. 244 (2018) 359–363. doi:10.1016/j.foodchem.2017.10.035.

[61] M. López-Lázaro, Dual role of hydrogen peroxide in cancer: Possible relevance to cancer chemoprevention and therapy, Cancer Lett. 252 (2007) 1–8. doi:10.1016/j.canlet.2006.10.029.

[62] S. El-Guendouz, B. Lyoussi, M.G. Miguel, Insight on propolis from Mediterranean countries: chemical composition, biological activities and application fields, Chem. Biodivers. (2019). doi:10.1002/cbdv.201900094.

[63] A.F.N. Ramos, J.L. Miranda, PROPOLIS: A REVIEW OF ITS ANTI-INFLAMMATORY AND HEALING ACTIONS, J. Venom. Anim. Toxins Incl. Trop. Dis. 13 (2007) 697–710.

[64] G. RICCIARDELLI d'ALBORE, L'ORIGINE GÉOGRAPHIQUE DE LA PROPOLIS, Apidologie. 10 (1979) 241–267. doi:10.1051/apido:19790303.

[65] O.M. Barth, Pollen analysis of Brazilian propolis, Grana. 37 (1998) 97–101. doi:10.1080/00173139809362650.

[66] N. El Menyiy, N. Al Waili, M. Bakour, H. Al-Waili, B. Lyoussi, Protective effect of propolis in proteinuria, crystaluria, nephrotoxicity and hepatotoxicity induced by ethylene glycol ingestion, Arch. Med. Res. 47 (2016) 526–534. doi:10.1016/j.arcmed.2016.12.010.

[67] S. El-guendouz, L. Badiaa, Iranian Journal of Basic Medical Sciences Potential therapeutic effect of Moroccan propolis in hyperglycemia , dyslipidemia , and hepatorenal dysfunction in diabetic rats, (2019). doi:10.22038/ijbms.2019.33549.8004.

[68] S. El-Guendouz, S. Aazza, B. Lyoussi, V. Bankova, M. Popova, L. Neto, M.L. Faleiro, M. Da Graça Miguel, Moroccan Propolis: A natural antioxidant, antibacterial, and antibiofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure, Evidence-Based Complement. Altern. Med. 2018 (2018). doi:10.1155/2018/9759240.

[69] S. El-Guendouz, S. Aazza, B. Lyoussi, M.D. Antunes, M.L. Faleiro, M.G. Miguel, Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis, Int. J. Food Sci. Technol. 51 (2016) 1762–1773. doi:10.1111/ijfs.13133.

Part I: *B. spinosum* honey characterisation

Chapter I. Preliminary characterisation of *Bupleurum spinosum* honey

Chapter I. Preliminary characterization of a Moroccan honey with a predominance of *Bupleurum spinosum* pollen[*]

Abstract

Honey with *Bupleurum spinosum* (Zandaz) as a main pollen source has not been the subject of previous detailed study. Therefore, twelve Moroccan samples of this honey were subjected to melissopalynological, physicochemical and microbiological quality characterization, as well as antioxidant activity assessment. From a quality point of view, almost all samples were within the limits established by Codex Alimentarius, and/or the European legislation. All samples presented predominance of B. spinosum pollen (more than 48%). Relatively high levels of trehalose (1.3–4.0 g/100 g) and melezitose (1.5–2.8 g/100 g) were detected. Those sugars, not common in monofloral honeys, could be used as an important factor to discriminate zandaz honey.

Flavonoid content correlated positively with the honey color, melanoidin and polyphenol content, and negatively with the IC_{50} values of scavenging ABTS (2,2´-azino-bis(3-thylbenzothiazoline- 6-sulfonic acid) free radicals, while proline amount correlated negatively with IC_{50} values of nitric oxide scavenging activity and chelating power. This correlation supports the use of antioxidant activities as important variables for PCA (principal component analysis). Both components explained 70% from the given data and showed certain homogeneity upon analyzed samples independent of the region, suggesting the importance of *B. spinosum* nectar in the resulting honey characteristics.

1. Introduction

The floral origin determination of honey is very important in the field of apicultural research, due to its direct link with customer demand. For the purpose of consumer protection, the honey floral origin labelling has been required by the European Community legislation, i.e., Directive EEC/74/409 amended by the proposal COM/95/0722 (1996) [1]. Melissopalynolgical, sensory and physico-chemical analyses together are needed for establishing the botanical denomination of a honey, because when considered individually, each one has its own limitations. Regarding melissopalynolgical analysis, whereas some honey

[*] Youssef Elamine, Smail Aazza, Badiâa Lyoussi, Maria Dulce Antunes, Letícia M Estevinho, Ofélia Anjos, Mafalda Resende, Maria Leonor Faleiro & Maria Graça Miguel (2017): Preliminary characterization of a Moroccan honey with a predominance of *Bupleurum spinosum* pollen, Journal of Apicultural Research

types need high percentage of pollen to be considered as monofloral (90% for chestnut honey), for others only 15% is sufficient to declare their botanical origin (e.g., *Lavandula* spp.) [2].

Physico-chemical parameters are also important in the determination of the botanical origin of honey and are widely used in routine honey analysis. Persano Oddo et al. (2004) reported that colour, electrical conductivity, specific rotation, diastase, acidity, fructose and glucose content have the most important value in discriminating honey origins [3]. In addition, the physicochemical analysis: sugars [4] volatile compounds [5]; phenols [6]; and minerals [7] amongst others, is largely influenced by honey variability (honey seasonal, geographical variability, among other factors) [3]. The use of one cannot be sufficient in itself, and the discriminatory power only increases with the number of parameters used [3].

The whole image of the botanical origin can be established only, when the sensory correspondence is evaluated [3]; because this tool can exclude some samples from being characterized as monofloral, based on the other parameters, if some botanic components altering the sensory characteristics are detected [8]. Nevertheless, sensory evaluation is always linked to inherent subjective factors. The main goal of the present work is to establish a first screening of melissopalynological and physicochemical characterization and, at same time some, to determine the in vitro antioxidant activity of 12 samples of a Moroccan honey with *Bupleurum spinosum* as the main pollen source. In Morocco, this honey is called *zandaz*, *zentaz*, *aguerbaz* or *airbaz* honey, referring to the berbere name of *B. spinosum* Gouan (Syn. *Bupleurum fruticescens* subsp. *spinosum* (Gouan) O. Bolos & Vigo) (Figure 1(a)). This species can be found in the siliceous high peaks of eastern Anti-Atlas, generally above 2000 m a.s.l. [9]. Some authors also reported the presence of this species at high altitudes of the High Atlas (above approximately 2900 m a.s.l.) and in some mountains of the middle Atlas [10] where the samples of this study were mainly collected. The flowering period occurs during August–September [11].

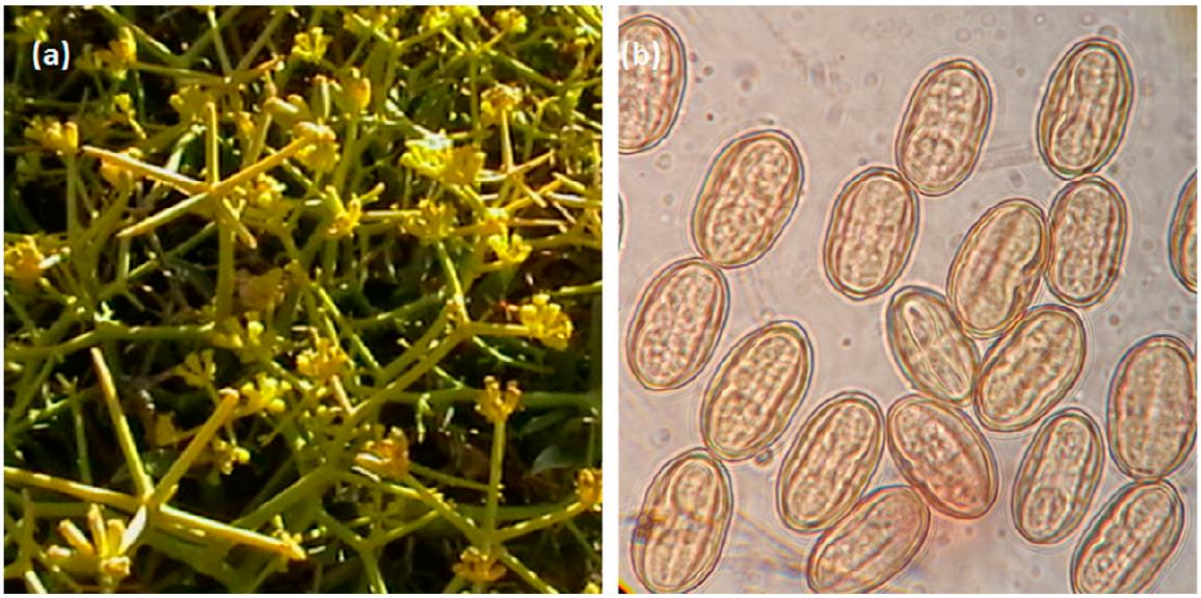

Figure 1. (a) Flowers of B. spinosum plant; (b) B. spinosum pollen under microscope (400×).

2. Material and methods

The twelve honey samples (n = 12) were directly purchased from beekeepers and centrifuged upon receipt at the laboratory. In Table 1, the sample codes, production years, places of collection and their most predominant pollen types are presented.

1.1. Chemicals, reagents and equipment

Culture media were purchased from Biokar, (Paris, France) or Oxoid (Basingstoke, Hampshire, UK); peptone water (Oxoid; Basingstoke, Hampshire, UK); NaOH, Na_2CO_3, sodium phosphate were purchased from Pronalab, Madalena, Portugal; HCl was purchased from Fisher Scientific UK Ltd.; Loughborough, UK; KCl was purchased from BHD Prolabo; Leuven, Belgium; acetic acid, ascorbic acid, Folin–Ciocalteu, fuchsin, KOH, methanol, 2-propanol were purchased from Merck KGaA; Darmstadt, Germany; 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ferrozine, $FeSO_4$, gallic acid, NBT, ninhydrine, PMS, proline were purchased from Acros Organics; New Jersey, USA; AlCl3 and anhydride acetic were purchased from Panreac Quimica, Montcada i Reixac; Barcelona, Spain; catechin was purchased from Fluka. Biochemika. Sigma Aldrich; Steinheim, Germany; glucose, fructose, sucrose, maltose, turanose, trehalose, melezitose, were purchased from Sigma Aldrich Chimie GmbH, Riedstr; Steinheim, Germany; H_2SO_4, sodium acetate, starch, potassium iodide, I_2, sodium nitroprusside (SNP), were purchased from Riedel-de-Haen; Germany; acetic acid was purchased from CHEM-Lab; Belgium; optic micro-scope (Leitz Messtechnik GmbH; Wetzlar, Germany); HPLC: Dionex ICS3000 equipment; microplate

reader (Tecan Infinite M200; Tecan, Austria); potentiometer combined with glass electrode (Thermo Electron Corporation, Orion 3 STAR; USA); analytical balance (Shimadzu, Aux 220; Philippines); electric furnace (Cassel; Portugal); conductivity meter (Thermo Electron Corporation, Orion 3 STAR; USA); Abbe Refractometer (HANNA, HI968601; Romania); HPLC (Hitachi, LaChrom Elite; Japan).

2.1. Melissopalynological analysis

The analysis of the honey samples' pollen qualitative and quantitative spectrum was performed according to the International Commission for Bee Botany (ICBB), as previously described in detail [12]. Pollen identification and count were carried out using a light microscope (Leitz Messtechnik GmbH; Wetzlar, Germany) with 400× and 1000× objectives, the latter being used when greater detail was required for the morphological identification. For each honey sample, we counted and analysed a minimum of 1000 pollen grains. Frequency classes were determined twice for each sample and designated as dominant pollen (>45% of a specific pollen type), secondary pollen (16–45%), important minor pollen (3–15%) and minor pollen (<3%).

2.2. Microbiological evaluation

The microbiological quality of the honey samples was examined by determining the counts of aerobic mesophilic bacteria, *Enterobacteriaceae* and sulfitereducing Clostridium spp. The counts of aerobic mesophilic bacteria were done using the Plate Count Agar medium as described in the standard NP-4405:2002. The determination of Enterobacteriaceae and sulfite-reducing Clostridium spp. were performed according to the standards ISO 21528-2:2004 and ISO 15213:2003, respectively (ISO 21528-2:2004, 2004; ISO 15213:2003, 2003). Homogenates of 10 g of each honey sample with 90 ml of peptone water (Oxoid) were subject to decimal dilutions that were inoculated in appropriate medium. The viability counts were done in triplicate.

2.3. Physico-chemical analysis

All measurements described in this section were performed in triplicate, except the sugar content that was performed in duplicate. Results were then expressed as mean ± SD. The parameters pH, free acidity, lactonic acidity, total acidity, ash content, electrical conductivity, moisture, proline content, diastase activity and HMF content, were assessed following the Harmonized methods of the International Honey Commission [13].

2.4. Honey colour and melanoidins estimations

Colour was determined by measuring the absorbance of aqueous solutions (10 g of honey in 20 ml of distilled water) at 635 nm (A635) in a Shimadzu spectrophotometer [14]. The mm Pfund values and honey colour were obtained using the following algorithm: mm Pfund = -38.7 + 371.39 × A635. Additionally, honey colour was determined by spectrophotometry by calculating net absorbance (A560 − A720). Melanoidin content was estimated based on the browning index (net absorbance = A450 – A720) [15]. Spectrophotometric measurements were performed in a 1 cm quartz cell; results were expressed as absorbance units (AU).

2.5. Sugar content

The method used was in accordance with that reported by Anjos et al [16].

2.6. Total content of polyphenols

The total polyphenol content was determined in honey solutions by the Folin–Ciocalteau method [17]. To test sugar interference, a sugar solution was made taking into account the sugar profile of each sample and two further dilutions were prepared for each one. The prepared sugar solutions were in accordance with the sugar content in the 50% honey solution prepared for pholyenols' estimation. Experiments were made in triplicates, and the results were expressed as mean ± SD mg equivalent of gallic acid/100 g honey, after the elimination of sugar interference.

2.7. Total flavonoid content

The total flavonoid content (TFC) of different honey samples was determined using the aluminum chloride assay according to Samatha et al [18]. To study a possible interference of sugars in this assay, the sugar solution made for polyphenol content estimation was also used. To avoid possible interaction of sample colour with the reaction's reagents, the blank was constituted by water (substituting the other reaction's components) and the sample as reported by Sancho et al. [19], and the resulting absorbance was subtracted from the whole reaction absorbance before TFC estimation. The TFC was expressed in mg of chatechin equivalents (CE) per 100 g of honey as the mean of three triplicate ± SD.

2.8. Antioxidant activities

All measurements described in this section were performed in triplicate, and the results were expressed as the mean ± SD.

2.8.1. Capacity for scavenging 2,2´-azino-bis(3- ethylbenzothiazoline-6-sulphonic acid)

The determination of ABTS radical scavenging capacity was carried out as reported previously [20]. Briefly, the ABTS radical was generated by the reaction of ABTS aqueous solution (7 mM) with Potassium persulfate ($K_2S_2O_8$) (2.45 mM) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.7 at room temperature. The samples (25 µl) were added to 275 µl ABTS and the absorbance at 734 nm was read after 6 min. Several concentrations of samples were used, and the percentage inhibition calculated by applying the formula: [(A_0 – A_1/ A_0) × 100], plotted against sample concentration, and IC_{50}, or half maximal inhibitory concentration was determined. A_0 is the absorbance of the negative control (blank sample containing the same amount of water and ABTS solution); A_1 is the absorbance of the sample.

2.8.2. Nitric oxide scavenging capacity

The nitric oxide (NO) scavenging activity of samples was measured according to the method described by [21]. NO was generated from SNP and was measured by using the Griess reagent (N-(1-Naphthyl) ethylenediamine). In this method, 50 µl of serially diluted honey sample were added to 50 µl of 10 mM SNP ($Na_2[Fe(CN)_5NO]$) in phosphate buffer saline (PBS) into a 96-well plate, which was then incubated at room temperature for 90 min. Finally, an equal volume of Griess reagent was added to each well and the absorbance was read at 546 nm. Several concentrations of samples were made and the percentage inhibition calculated using the formula: $[1 - (A_{sample} - A_{sample\ blank})/(A_{control} - A_{control\ blank})] \times 100$, where ($A_{sample}$ - $A_{sample\ blank}$) is the difference in the absorbance of a sample, with or without 10 mM SNP, and ($A_{control} - A_{control\ blank}$) is the difference in the absorbance of the PBS control, with or without 10 mM SNP. The inhibition percentage was plotted against sample concentration and IC_{50} was determined (concentration of sample able to scavenge 50% of NO free radical).

2.8.3. Chelating metal ions

The degree of chelating of ferrous ions by honey samples was evaluated according to Miguel et al. (2010) [20]. Briefly, samples were incubated with 0.05 ml of Iron (II) chloride, $FeCl_2 \cdot 4H_2O$ (2 mM). The addition of 0.2 ml of 5 mM ferrozine initiated the reaction, and after 10 min, the absorbance at 562 nm was measured. An untreated sample served as control. The percentage of chelating ability was determined according to the following formula: [(A_0 - A_1)/A_0 × 100], in which A_0 is the absorbance of the control and A_1 the absorbance of honey sample. The values of IC_{50} were determined as reported above for the previous assays.

2.8.4. Superoxide anion radical ($O_2^{\cdot-}$) scavenging activity (non-enzymatic method)

Measurements of $O_2^{\cdot-}$ scavenging activity of honey samples were based on Soares (1996) method [22]. $O_2^{\cdot-}$ were generated in a PMS-NADH system based on the oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). The sample's IC_{50} was then determined in triplicate for each sample.

2.9. Statistical analysis

One-way ANOVA was performed to determine significant differences among samples with a Scheffe post hoc test (95% confidence level). The results were also subjected to a multivariate analysis (principal component analysis). All experimental data were analyzed using StatSoft Statistics (version 7).

3. Results and discussion

3.1. Melissopalynological analysis

The main question facing the analysis of pollen profile for monofloral honey determination is: at which level of a pollen species is necessary to reach for declaring the discriminating point between monofloral and multifloral honey. In all the analysed samples of the present study, pollen grains of B. spinosum (Figure 1(b)) were predominant (Table 1), with percentages ranging from 48.8%, in the sample S9, to 83.0%, in the sample S5. Regarding the previously mentioned limitations of melissopalynology, and the variability in the remaining pollens of each analysed sample, the conclusion will not be final, and further detailed study will be needed. From the results of the present work it is possible to see that *Thymus* pollens could be detected in six samples, being the secondary pollen in two of them with percentages of 19.8% (S2) and 21.3% (S6), while *Cardus* spp. was secondary in S8 (25.3%), S9 (24.5%), S10 (22.2%), and S11 (30.8%). For the remaining samples the secondary pollen was *Olea* spp. (S9), *Cactus* spp. (S12), *Opuntia* spp. (S1 and S3) (Table 1). The occurrence of other pollens was also seen, namely: *Acacia* spp.; *Epilobium* spp.; *Eragrostis* spp.; *Eucalyptus spp*.; and *Phoenix* spp., although with percentages below 15% (important minor pollen). Pollen qualitative analysis provides important data for honey characterization, regarding the occurrence of nectariferous plants, harvest season, honey extraction and filtration, the occurrence of fermentations, as well the geographical origin [23]. Keeping in mind the absence of any previous reference of this honey, the last indication is, in fact, one of the limitations of the present study to declare the "monoflorality" of this honey: honey production in different

countries leads to the diffusion of different secondary plant nectars, and so changes the discriminatory percentage of the dominant pollen. This makes the 'ideal reference model' of monofloral type different from one analyst to another. This fact can be clearly seen in the variability of the same European monofloral honey, in terms of their pollen profile, from country to country [24]. More discussions about pollen profile will be addressed in the following sections of this work, as well as its possible influence on other honey parameters.

3.2. Microbiological evaluation

Microbial counts in organic honey samples are shown in Table 2. All honey samples had aerobic mesophilic bacteria, although the bacterial load was variable, with the highest level was recorded in sample S7. All samples were negative for Enterobacteriaceae and sulfite-reducing *Clostridium spp*. The counts of aerobic mesophilic bacteria in the samples S6, S7 and S12 suggest that the hygiene conditions of the honey production must be improved. The low numbers of aerobic mesophilic bacteria in the majority of the honey samples and the absence of Enterobacteriaceae and sulfite-reducing Clostridium spp. do, however, indicate the safe consumption of this honey.

Table 1. Sample code, places of collection, year of production and the most predominant pollen of twelve *B. spinosum* honey samples from Morocco.

Sample code	*B. spinosum* (PP)	SP Species (%)	IMP	MP	Region name	Harvest year
S1	58.9	*Opuntia* spp. (17.7)	*Epilobium* spp. (13.8) + *Eragrostis* spp. (3.5) + *Thymus* spp. (3.0) + Others (3.0)		Ait Bazza	2011
S2	63.0	*Thymus* spp. (19.8)	*Opuntia* spp. (8.6) + *Phoenix* spp. (8.6)		Ait Bazza	2013
S3	62.9	*Opuntia* spp. (18.8)	*Olea* spp. (9.2) + *Thymus* spp. (4.3) + Others (4.9)		Ait Bazza	2013
S4	53.7		*Cactus* spp (4.4) + *Epilobium* spp. (13.0) + *Eragrostis* spp. (5.9) + *Opuntia* spp. (11.2) + *Thymus* spp. (7.5) + Others (4.4)		Ait Bouilloul	2013
S5	83.0		*Olea* spp. (7.1) + *Opuntia* spp. (9.9)		Ait Bouilloul	2013
S6	62.4	*Thymus* spp. (21.3)	*Eragrostis* spp. (6.3) + *Phoenix* spp. (6.3) + Others (3.7)		Ait Ali	2013
S7	68.9		*Cardus* spp. (8.0) + *Eucalyptus* spp. (4.4) + *Olea* spp. (10.4) + *Thymus* spp. (8.0)		Ait ali	2012
S8	55.6	*Cardus* spp. (25.3)	*Phoenix* spp. (3.1) + *Thymus* spp. (11.4) + Others (4.7)		Ait bourais	2011
S9	48.8	*Cardus* spp. (24.5) + *Olea* spp. (17.4)	*Phoenix* spp. (6.5)	Others (2.8)	Boulemane	2011
S10	71.1	*Cardus* spp. (22.2)	*Acacia* spp. (3.9) + *Phoenix* spp. (3.9)		Bouiblane	2013
S11	65.0	*Cardus* spp. (30.8)	+ Others (4.2)		Bouiblane	2013
S12	56.8	*Cactus* spp (24.2)	*Acacia* spp. (7.0) + *Phoenix* spp. (12.0)		Bouiblane	2013

PP – Predominant pollen (>45%); SP – Secondary pollen (16-45%); IMP – Important minor pollen (3-15%); MP – Minor pollen (<3%)

3.3. Physico-chemical analyses

Tables 2 and 3 present the results of the physico-chemical parameters analysed in the twelve samples of honey. From a quality point of view, the analysed samples were, in most cases, within European limits [1] or the Codex Alimentarius [25], and showed no alarming values. However, a significant variability was observed.

In all the analysed samples, pH values, with 3.82 ± 0.16 as a mean did not show any outset value, to be in accordance with previous work [26 27], who analysed Moroccan honeys from different floral origin. The same observation made for lactonic acidity, free acidity, and the total acidity, for which the mean values were, respectively, 16.58 ± 1.40, 25.70 ± 6.46 and 42.28 ± 6.76 meq/kg. It has to be mentioned that EU Council, 2002 fixed honey free acidity to values bellow 50 meq/kg, which is important for avoiding undesirable fermentations [13].

Another determinant parameter in a possible fermentation process [28] is the moisture percentage, that ranged from 17.3% (S7) to 20.5% (S11) (Table 3), exceeding so 20%, or the maximum allowed by the Codex Alimentarius, and the EC directive [1]. Furthermore, sample S4, S5 were also above the required limit.

According to the European Unit, the values of electrical conductivity and ash must not exceed 800 μS/cm and 0.6%, respectively. Amongst the studied samples (Table 2), only S12 had an electrical conductivity higher than the maximum recommended value, which was coincident with the highest percentage of ash (0.37%). Indeed, the electrical conductivity depends on the ash and acid content, which, per se, reflects the presence of ions and organic acids; the higher their content, the higher the resulting conductivity [29].

It is well known that honey diastase activity is one of the indicators telling about storage conditions and/or processing, and it is used for honey freshness confirmation in routine analysis because of its sensitivity to heat or inadequate storage condition [7]. Nevertheless, its level also depends on the geographic and floral origins of samples [30]. All honey samples studied in the present work had higher diastase activity than the minimum required (8.0 Shade units/g) by European legislation [1]. Nevertheless, it is noteworthy to refer that a great variability was found among samples (11.3 in S7 to 30.3 Shade units/g in S4) (Table 2).

Table 2. Some physicochemical parameters and microbial analysis of twelve *B. spinosum* honey samples from Morocco.

Samples	AMB (CFU/g)*	pH	Ash (%)	Electrical conductivity (µS/cm)	Free acidity (meq/kg)	Lactonic acidity (meq/kg)	Total acidity (meq/kg)	Diastase activity (Shade units/g)
S1	<20	4.16±0.01^{c}	0.28±0.01^{bc}	635.67±1.15^{f}	14.15±2.85^{a}	16±3.77^{ab}	30.15±0.96^{a}	18.65±0.38^{b}
S2	<10	3.787±0.04^{ab}	0.19±0.02^{a}	443.33±5.03^{b}	27.85±4.89^{bc}	15.5±1.5^{ab}	43.35±4.2^{bc}	20.71±1.3^{bc}
S3	30±0.0	3.62±0.05^{a}	0.16±0.01^{a}	405.33±3.05^{a}	29.7±2.06^{bc}	18.5±3^{b}	48.2±1.04^{c}	23.91±1.2^{c}
S4	<10	3.88±0ab^{c}	0.18±0.01^{a}	453±1^{b}	28.25±3.57^{bc}	18±3.77^{b}	46.25±7.09^{bc}	30.3±1.86^{d}
S5	<10	3.61±0.58^{a}	0.2±0.01^{ab}	437.67±2.08^{b}	25.6±0.22^{c}	16.5±1.73^{ab}	42.1±1.91^{bc}	23.24±1.01^{c}
S6	425±35.4	3.9±0.05^{abc}	0.19±0.02^{a}	495.67±4.16^{cd}	18.5±1.06^{a}	18.5±1.49^{b}	37±2.12^{ab}	17.53±0.89^{b}
S7	2.4x10^{3}±183	3.9±0.04^{abc}	0.18±0.03^{a}	486±4.35^{c}	15.75±1.13^{a}	15±2.29^{ab}	30.75±1.25^{a}	11.33±0.05^{a}
S8	55.0±7.1	3.79±0.02^{ab}	0.23±0^{abc}	573.67±7.23^{e}	28.75±2.21^{bc}	17.5±0.86^{ab}	46.25±2.86^{bc}	27.71±1.07^{d}
S9	50.0±14.1	3.95±0.01^{bc}	0.3±0^{cd}	639±8.28^{f}	24.75±0.12^{c}	16.5±0.7^{ab}	41.25±0.6^{ab}	21.01±0.27^{bc}
S10	<10	3.67±0.05^{ab}	0.22±0.03^{abc}	508±6.55^{d}	28.55±1.91^{bc}	16±2.29^{ab}	44.55±1.36^{bc}	29±0.27^{d}
S11	<10	3.7±0.01^{ab}	0.18±0.01^{a}	442±2.64^{b}	30.55±1.13^{bc}	14±1.5^{a}	44.55±0.91^{bc}	28.57±0.12^{d}
S12	225±63.6	3.84±0.02^{ab}	0.37±0.01^{c}	833±7.54^{g}	36±1.98^{bc}	17±2.59^{ab}	53±0.74^{c}	21.6±0.39^{c}
Means±SD		3.82±0.16	0.22±0.06	529.4±122.7	25.70±6.46	16.58±1.40	42.28±6.76	22.80±5.55
Min- Max		3.61-4.16	0.16-0.37	405.3-833.0	14.15-36.00	14.00-18.50	30.15-53.00	11.33-30.30

AMB - Aerobic mesophilic bacteria ; *Data represent the mean ± standard deviation of duplicate replicates of each honey sample and are expressed as Colony Forming Units per gram (CFU/g)

Hydroxymethylfurfural (HMF) is one of the most known Amadori compounds, formed during the thermal treatments of carbohydrate-containing foods as a result of Maillard reaction, becoming another honey freshness indicator. This compound is found only in trace amount in fresh honey, with values bellow 40 mg/kg, the maximal value allowed by Codex Alimentarius [25] and EU Council [1]. Based on this parameter, all the analysed samples showed freshness signs, with values ranging from 1.1 mg/kg in S2 to 16.0 mg/kg in S7, collected in 2013 and 2011, respectively (Table 3).

In the present work, proline evaluation was followed according to the IHC method, although other methods can be used, as previously reported by Truzzi et al. [31]. All honey samples contained more than the minimum acceptable proline concentration, i.e., 200 mg/kg [32], nevertheless its amounts varied greatly among samples (710.0 mg/kg in S6 and 1202.5 mg/kg in S11) (Table 3).

Honey colour is an indicator of the presence of compounds, such as polyphenols, terpenes and carotenoids [14]. In the analysed honey samples a great variability of colour, determined at A635, was observed: from extra white (28.5 mm Pfund) in S7 sample to dark amber (116.9 mm Pfund) in S5 (Table 3). A strong correlation was observed between colour and melanoidin content ($r = 0.948$; $p < 0.01$) (Figure 1), confirming their participation in the resulting honey colour [33], and explaining the variability seen amongst the analysed samples (Table 3). Melanoidins, or Maillard reaction products, estimated as a difference between two wavelengths (A560 − A720), are multi-component polymers consisting of protein-polyphenol-oligosaccharide complexes, which along with phenols are responsible for the antioxidant activity of honey [15,34].

Table 3. Some physicochemical parameters of twelve *B. spinosum* honey samples from Morocco.

Samples	Moisture %	HMF mg/Kg	Proline mg/Kg	Melanoidins	Polyphenols (mg GAE/100 g)	Flavonoids (mg QE/100 g)	Colour Pfund scale (mm)
S1	19.53±0.23^{cd}	5±0.25^{e}	763.06±16.43^{a}	0.63±0.01^{b}	61.36±10.85^{a}	13.53±1.52^{bc}	42.37±2.24^{a} Light extra amber
S2	19.8±0.12^{d}	1.07±0.07^{a}	926.77±18.14^{b}	1.25±0.02^{d}	101.86±11.78^{cd}	20.87±1.31^{c}	72.48±1.9^{a} Light amber
S3	19.73±0.23^{cd}	10.9±0.64^{e}	970.43±44.4^{b}	0.93±0.01^{c}	85.03±9.32^{b}	20.65±2.1^{c}	63.46±1.19^{a} Light amber
S4	20.4±0.12^{e}	6.45±0.21^{d}	1007.49±7.58^{bc}	1.38±0.01^{e}	123.65±3.58^{d}	18.79±2.59^{c}	89.49±0.81^{a} Amber
S5	20.27±0.11^{e}	1.18±0.06^{a}	800.85±40.78^{a}	1.55±0.015^{f}	116.85±7.75^{cd}	20.3±2.59^{c}	116.91±6.56^{b} Dark amber
S6	19.07±0.11^{c}	5.35±0.1^{cd}	709.95±36.85^{a}	0.92±0.01^{c}	91.77±5.86^{b}	14.73±0.93^{c}	61.34±1.43^{b} Light amber
S7	17.27±0.11^{a}	15.97±0.09^{f}	717.8±114.24^{a}	0.56±0.02^{a}	59.32±2.93^{a}	5.11±1.36^{ab}	28.46±2.28^{c} Extra white
S8	17.93±0.3^{ab}	4.89±0.09^{c}	847.32±22.3^{ab}	0.61±0.01^{b}	61.08±5.96^{a}	9.86±1.14^{c}	32.21±1.23^{cd} White
S9	17.47±0.09^{a}	14.7±0.63^{f}	763.59±7.18^{a}	0.96±0.01^{c}	89.46±2.19^{bc}	4.87±3.85^{a}	44.24±0.95^{d} Light extra amber
S10	18.27±0.11^{b}	2.98±0.11^{b}	1005.35±39.07^{bc}	0.68±0.02^{b}	66.58±7.15^{a}	2.44±1.21^{a}	28.44±0.18^{d} White
S11	20.53±0.11^{e}	2.76±0.08^{b}	1202.53±42^{d}	0.97±0.01^{c}	99.04±3.83^{bc}	21.5±2.77^{c}	72.46±0.73^{e} Light amber
S12	19.2±0^{cd}	5.38±0.3^{cd}	1179.98±18.68^{cd}	0.67±0.01^{b}	72.72±2.86^{a}	1.74±2.11^{a}	34.46±0.89^{f} Light extra amber
Means±SD	19.12±1.14	6.97±5.16	907.93±169.70	0.93±0.32	85.73±22.01	12.86±7.73	57.20±27.49
Min- Max	17.27-20.53	1.07-15.97	709.95-1202.53	0.56-1.55	59.32-123.65	1.74-21.5	28.44-116.91

As the Folin-Ciocalteau reagents can not only oxidize phenolic molecules, but also non-phenolic organic compounds [35], , such as reducing sugars, sugar effect was studied. For this, the assay reaction was applied on sugar solutions for each sample based on its sugar profiling, and the values were subtracted from total polyphenol estimation, to obtain the results illustrated in Table 3. The effect of dilution on sugar interference was also evaluated, being the assay initiated with solutions with sugar content equivalent to that of 50% honey solution, and 12.5%. This concentration was also that used for the estimation of polyphenols in honey samples. The results showed a clear interference of sugars when they were in concentration of 50%, with values between 16.83 ± 0.67, for S10, and 23.67 ± 2.35 mg GA equivalent/100 g honey, for S1. In fact, polyphenol estimation was not possible in 50% honey solution, and a 1/4 dilution was used, so the same was done on sugar solutions. A clear decrease in sugar interference was observed with dilution and the values passed from 19.26 ± 2.43 mg GAE/100 g as the mean of all sugar solutions 50% to 7.28 ± 3.13 mg GAE/ 100 g in the 1/4 dilution.

Without sugar effect elimination, polyphenol content ranged from 66.15 mg GAE/100 g (S1) to a maximum of 130.04 mg GAE/100 g (S4) data not shown). These values were significantly lower than that reported for other Moroccan honey types [36]. In addition, and taking into account the sugar interference, the respective values were even lower because they ranged from 61.36 ± 10.85 mg GAE/100 g (S1) to 123.65 ± 3.58 mg GAE/100 g (S4).

Several methods are used for the quantification of total flavonoids, in spite of the interferences of some reagents with some flavonoid classes, being therefore a limitation of the method in unknown samples analysis [35,37]. The same authors proved that rutin, luteolin and catechins seem to be more accurate with the sodium nitrite ($NaNO_2$) used in some methods for flavonoid determination. For this reason, in the present work catechin was used as standard. As expected from the polyphenol content results, flavonoid content was also reduced compared to other Moroccan honeys, and the values ranged from 1.74 to 20.87 mg CE/100 g of honey. The effect of sugar on flavonoid estimation in honey samples was also studied and no interference was detected, independent of the dilution (data not shown).

Positive correlations were obtained between colour, melanoidin, phenol and flavonoid contents (Figure 2). These results reveal that polyphenols and melanoidins play a role in honey colour. Correlations between phenols, melanoidins and flavonoids may be attributed to the fact that all these compounds absorb light in the visible range, as previously reported by Aazza et al. (2014) for Moroccan honeys of diverse floral origin [36]. The samples S2, S4, S5 and S11 from Ait Bazza, Ait Bouilloul and Bouiblane respectively, presented the highest values of

phenols, melanoidins and flavonoids (Figure 2). Additionally, the colour and the electrical conductivity give an indication of the botanical origin of honey.

3.4. Sugar content

Fructose and glucose are the major monosaccharides in honey, and their proportion depends on nectar delivering plant [7]. Their sum in the analysed honey samples ranged from 56.9% in S1 to 69.9% in S8, (Table 4) which were in accordance with those reported by other authors that analysed honey from different origin [16,38]. The concentration of fructose ranged from 34.4 g/100 g in S1 sample to 42.3 g/100 g in S3 and could be found 5 homogenous groups with statistic differences from the 12 analysed samples (Table 4). Sample S1 had also the lowest concentration of glucose (22.2 g/100 g), while S8 had the highest amount of this monosaccharide (31.0 g/100 g).

Practically all samples had sucrose lower that the quantification limit (<0.2 g/100) except S12 that presented 4.3 g/100 g of sucrose, but still lower than the limit of 5% allowed by the Codex Alimentarius for this sugar [25]. Its structural isomer, turanose, as well as maltose were not detected also, except for sample S4 which had 1.8 g/100 g of maltose.

Trehalose was the most important disaccharide present in the zandaz honey samples, for which the occurrence with such relative high levels is, generally, not usual in honeys [16] and could be a discriminant characteristic of zandaz honey.

Another relevant characteristic in this honey type was the occurrence of melezitose, which is absent in unifloral honeys with the exceptions of heather, chestnut and cardoon [39,40], but is very common, even with higher levels, in honeydew [3]. In the present work, and independent of the region where the samples were collected, high levels of this trisaccharide were detected (1.5–2.8 g/100 g).

From a discriminatory point of view, and as expected from pollen spectrum, even with a predominance of *B. spinosum* pollen, the results of physico-chemical characterization showed variability. This makes the task to declare the monoflorality or not of the so called zandaz honey more complicated.

It is well established that the above studied parameters, when recorded at extreme low or high values, can be used as a discriminatory pattern to support the melissopalynological results. Low values of G/W ratio and high values of colour, electric conductivity, enzymes, pH and F/G ratio, when associated with more than 90% of chestnut pollen, constitute a discriminatory characterization for this monofloral honey [24]. In contrast, and in the same reference, the

physico-chemical pattern of lavender unifloral honey is characterized by low values of electrical conductivity, slightly low F + G and high values of sucrose. Focusing on the means of some analysed parameters, lead to the easy misclassification of the samples with other honey types. The electrical conductivity and colour are similar to those of dandelion, and eucalyptus honeys [24].

Table 4. Sugar content (g/100 g) of twelve *B. spinosum* unheated honey samples from Morocco.

Samples	Fructose	Glucose	Melezitose	Trehalose	Fructose+ glucose	Fructose/ glucose
S1	34.7±1.8^{a}	22.2±1.0^{a}	2.8±0.1^{b}	2.1±0.1^{ab}	56.9±2.8^{cd}	1.56±0.01^{a}
S2	40.0±0.6^{d}	23.4±0.4^{b}	1.7±0.1^{a}	4.7±0.1^{b}	63.4±1.0^{de}	1.71±0.00^{ab}
S3	42.3±0.2^{e}	24.4±0.1^{b}	1.5±0.1^{a}	4.1±0.1^{ab}	66.7±0.4^{e}	1.73±0.00^{b}
S4	39.8±0.4^{d}	24.6±0.2^{b}	2.2±0.3^{ab}	4.0±0.1^{ab}	64.4±0.6^{cd}	1.62±0.01^{ab}
S5	38.7±0.0^{cd}	24.2±0.0^{b}	2.0±0.1^{ab}	2.2±1.7^{ab}	62.9±0.1^{cd}	1.60±0.00^{ab}
S6	38.1±0.3^{cd}	26.8±0.3^{d}	1.7±0.0^{a}	3.1±0.0^{ab}	64.9±0.5^{b}	1.42±0.00^{b}
S7	36.5±0.8^{b}	28.5±0.2^{d}	2.0±0.1^{ab}	1.6±0.0^{a}	65.0±1.0^{a}	1.28±0.02^{b}
S8	38.9±0.6^{cd}	31.0±0.3^{e}	1.8±0.2^{a}	1.3±0.0^{a}	69.9±1.0^{a}	1.25±0.01^{b}
S9	39.1±0.5^{cd}	27.0±0.2^{d}	2.0±0.1^{ab}	2.1±0.0^{ab}	66.1±0.4^{b}	1.45±0.01^{b}
S10	36.5±0.2^{b}	28.7±0.0^{d}	1.9±0.1^{a}	2.0±0.0^{ab}	65.2±0.2^{a}	1.27±0.01^{b}
S11	38.4±1.8^{cd}	25.4±1.1^{c}	1.9±0.0^{a}	4.0±0.26^{ab}	63.8±2.8^{bc}	1.51±0.01^{ab}
S12	37.6±1.9^{bc}	28.9±0.3^{d}	2.0±0.2^{a}	1.1±0.0^{a}	66.5±2.1^{a}	1.30±0.05^{b}
Mean± SD	38.4±2.0	26.3±2.7	2.0±0.3	2.7±1.2	64.6±3.1	1.48±0.17
Min- max	34.7-42.3	22.2-31.0	1.5-2.8	1.1-4.7	56.9-69.9	1.25-1.74

3.5. Antioxidant activity

The antioxidant activities of the honey samples were measured using four different methods: three assays assessed the capacity for scavenging free radicals (ABTS, superoxide, and NO) and one assay to assess samples' capacity for chelating metal ions. The results are shown in Table 4. Sample S2 was the best for scavenging ABTS free radicals. The sample S4 was also a good scavenger of ABTS free radicals, along with S5. Samples S1, S2 and S6 were those honey samples that presented the best capacity for scavenging the superoxide anion radicals, whereas S9, S10 and S12 where those which had the best capacity for scavenging NO radicals. The capacity for chelating metal ions was higher in samples S1, S2 and S6 (Table 4).

The capacity for scavenging ABTS free radicals by samples, measuring the absorbance at 734 nm, changes proportionally with incubation time, nevertheless Sancho et al. (2016) observed that there were no differences between activity measuring the absorbance at 60 min and calculated activity at 60 min, by measuring absorbance at 6 min, independent on the type

of honey and extract [19]. The activity in the present work was followed by measuring the absorbance after 6 min of reaction.

The antioxidant activity of honey samples depends on the honey floral origin, because of the differences in the content of plant secondary metabolites such as polyphenolics, and enzyme activities [41,42]. However, the same monofloral honey may present diverse antioxidant activities as found in the present work, since the polyphenol content may vary. Such findings were already reported by Aazza et al. (2014) for the same monofloral honeys (thyme and jujube) from Morocco of different geographic origins [36]. The results of the present work also show that the capacity for scavenging free radicals depends on the type of radicals used, whereby it is important to discriminate the type of radicals that honey samples are able to scavenge.

Table 5. Antioxidant activity and capacity for scavenging free radicals of twelve *B. spinosum* honey samples from Morocco.

Samples	ABTS IC_{50} (mg/mL)	Superoxide IC_{50} (mg/mL)	Nitric oxide IC_{50} (mg/mL)	Iron chelating power IC_{50} (mg/mL)
S1	7.06 ± 0.26^{b}	9.92 ± 0.05^{a}	167.03 ± 16.78^{c}	9.92 ± 0.05^{a}
S2	4.94 ± 0.13^{a}	7.97 ± 0.1^{a}	121.25 ± 0.71^{ab}	7.97 ± 0.1^{a}
S3	7.40 ± 0.09^{b}	86.8 ± 1.68^{c}	135.39 ± 1.86^{b}	86.80 ± 1.68^{c}
S4	4.62 ± 0.08^{a}	75.45 ± 6.23^{c}	95.54 ± 0.99^{ab}	75.45 ± 6.23^{c}
S5	4.20 ± 0.03^{a}	132.19 ± 2.94^{d}	93.82 ± 15.67^{ab}	132.19 ± 2.94^{d}
S6	6.44 ± 0.15^{b}	10.06 ± 0.26^{a}	143.11 ± 0.34^{bc}	10.06 ± 0.26^{a}
S7	10.06 ± 0.86^{c}	51.64 ± 2.75^{bc}	189.60 ± 0.43^{c}	51.64 ± 2.75^{bc}
S8	12.18 ± 0.2^{d}	49.74 ± 3.24^{bc}	137.42 ± 5.22^{bc}	49.74 ± 3.24^{bc}
S9	6.50 ± 0.16^{b}	31.88 ± 0.47^{ab}	81.98 ± 3.44^{a}	31.88 ± 0.47^{ab}
S10	9.03 ± 0.3^{c}	173.20 ± 33.84^{e}	75.75 ± 1.9^{a}	173.20 ± 33.84^{e}
S11	6.36 ± 0.16^{b}	80.94 ± 2^{c}	110.12 ± 2.86^{ab}	80.94 ± 2^{c}
S12	9.56 ± 0.25^{c}	101.97 ± 3.61^{d}	69.60 ± 3.06^{a}	101.97 ± 3.61^{d}
Means± SD	7.37±2.42	51.31±25.65	118.38±37.45	67.65±51.48
Min- Max	4.20-12.18	15.02-81.96	69.60-189.60	7.98-173.21

Flavonoid content correlated positively with the honey color, melanoidins and phenol content but negatively with the IC_{50} values of the scavenging capacity of ABTS free radicals (Figure 2). Proline amount positively correlated with the IC50 values of the capacity for chelating metal ions and scavenging superoxide free radicals. The negative correlation between HMF content and antioxidant activity (e.g., IC_{50} values for ABTS assay) was not as evident as

observed for phenols and antioxidant activity. The weak correlation between those two factors was already reported [43] for honey samples, being the breakdown products of HMF the most probable contributors for the antioxidant activity of honey samples.

The negative correlation between IC_{50} values of NO scavenging activity and proline content may reveal an important role of this amino acid on the capacity for scavenging that free radical. Antioxidant activity of some amino acids (histidine, taurine, glycine, alanine, proline) has been reported [44,45]. Such property of proline may indicate some anti-inflammatory activity of these honeys since NO appears at higher concentrations during inflammatory reactions. Compounds with the capacity for scavenging NO can be therefore considered as possessing antioxidant and anti-inflammatory properties. Colour was strongly correlated with the capacity of samples to scavenge ABTS free radicals (Figure 2). The same happened with the capacity for scavenging these free radicals and melanoidins as reported before. The correlation of color and antioxidant activity as well as between melanoidin and antioxidant activity were also previously reported by Aazza et al. (2014), in Moroccan honeys of different floral origins [36].

3.6. Variance explained by principal component analysis

A principal component analysis was performed with all analytical data of B. spinosum honey from the 12 sites (Figure 2) to establish the differences among samples. For the PCA, two replicates of the physicochemical parameters were used (minimum and maximum) since for the sugar content, only two duplicate values of each analysis were available. Three samples (variables) were projected as supplementary variable because they do not contribute significantly to variance explanation (ash content, lactonic Acidity, and melizetose) in the first two components.

Factor 1 could be identified as the phenol content component, includes a series of properties, which are significantly correlated amongst themselves: phenols content; melanoidins; flavonoids; colour; F/G ratio as well trehalose, fructose and water content. ABTS IC_{50} and glucose content are also correlated with the previously mentioned group of properties, but negatively. The second component could be identified as the component related to the honey acidity. This factor correlated well iron chelating power IC50, superoxide IC50 and nitric oxide IC50 with pH, diastase activity, proline, free acidity and total acidity.

The principal component analyses explain 70.3% of the total variance observed (Figure 2). Moreover some samples are very similar, namely S10, S12, S8 and other group composed by the samples S2, S3, S4, S5, S11, the separation are not so large reason by which it was not

possible to group the samples belonging to different geographical regions (Figure 2). The separation between the previously groups are given basically by the sugar content and total flavonoids and polyphenol content, that could be explained by the different composition in the secondary floral resources. Given this result it is possible conclude that the zandaz honey from the different regions under study possesses similar properties

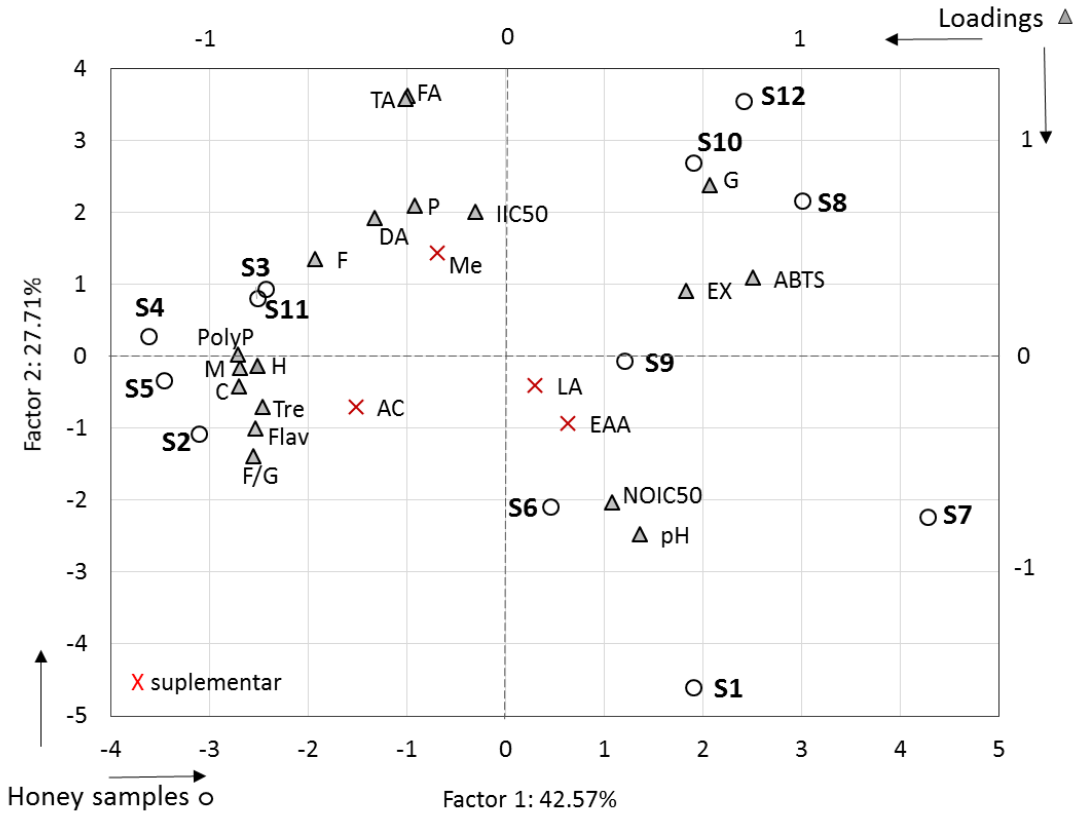

Figure 2 – Principal component analysis with all measured parameter: projection of loadings and honey samples in the same system of vectors.

Legend: FA - Free Acidity (mEq/Kg); LA - Lactonic Acidity (mEq/Kg); TA - Total Acidity (mEq/Kg); EX - electrical conductivity mS/cm; AC -Ash Content %; H - water Content %; C - Colour (Pfund); M – Melanoidines; DA - Diastase activity (shade number); P - proline mg/kg; PolyP - Phenol (mg GAE/100 g); Flav - flavonoides mg QE/100 g; EAA – total antioxidante activity mg EAA/g honey; ABTS - ABTS IC_{50} (mg/mL); IIC50 - Iron chelating Power IC_{50} (mg/mL); NOIC50 - Nitric oxide IC_{50} (mg/mL); F - fructose (g/100 g); G - glucose (g/100 g); Me – melezitose (g/100 g); Tre – trehalose (g/100 g); F/G -fFructose/glucose ratio.

4. Conclusion

While this is the first study reporting a preliminary investigation of some features of zandaz honey, characterized by the domination of *B. spinosum* pollen, and regarding the

variability that can occur in the same type of honey, it was not easy to claim determinant conclusions. In all analysed samples pollen count of B. spinosum exceeded 45%, suggesting the monofloraty of this honey. Other relevant feature that can be used for zandaz honey characterization is the occurrence of trehalose and melizetose, which is unusual in almost honey types.

The use of PCA allowed also seeing homogeneity in the analysed samples, a reason that did not allow grouping them into geographical groups, suggesting that belonging, or not, to a given region, zandaz honey conserved similar properties. To support the conclusions of the present study about this honey, large size samples will be needed, and subjected to the same analysed parameters, with others evolving, mainly, sensory investigation and composition profiling.

5. References

[1] COUNCIL DIRECTIVE 2001/110/EC of 20 December 2001 relating to honey, Off. J. Eur. Communities. (2002) 47–52.

[2] S. Gomes, L.G. Dias, L.L. Moreira, P. Rodrigues, L. Estevinho, Physicochemical , microbiological and antimicrobial properties of commercial honeys from Portugal, Food Chem. Toxicol. 48 (2010) 544–548. doi:10.1016/j.fct.2009.11.029.

[3] S. BOGDANOV, K. RUOFF, L.P. ODDO, Physico-chemical methods for the characterisation of unifloral honeys : a review, Apidologie 35. 35 (2004) 4–17. doi:10.1051/apido.

[4] E. De La Fuente, A.I. Ruiz-Matute, R.M. Valencia-Barrera, J. Sanz, I. Martínez Castro, Carbohydrate composition of Spanish unifloral honeys, Food Chem. 129 (2011) 1483–1489. doi:10.1016/j.foodchem.2011.05.121.

[5] A. Bouseta, V. Scheirman, S. Collin, Flavor and free amino acid composition of Lavender and Eucalyptus honeys, J. Food Sci. 61 (1996) 683–687. doi:10.1111/j.1365-2621.1996.tb12181.x.

[6] I. Escriche, M. Kadar, M. Juan-Borrás, E. Domenech, Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment, Food Chem. 142 (2014) 135–143. doi:10.1016/j.foodchem.2013.07.033.

[7] E. Anklam, A review of the analytical methods to determine the geographical and botanical origin of honey, Food Chem. 63 (1998) 549–562. doi:10.1016/S0308-8146(98)00057-0.

[8] M. Piana, L. Oddo, A. Bentabol, E. Bruneau, C. Declerck, M. Piana, L. Oddo, A. Bentabol, E. Bruneau, S. Bogdanov, E.B. Runeauc, S.B. Ogdanovd, C.G.U.D. Eclerckc, Sensory analysis applied to honey : state of the art To cite this version : HAL Id : hal-00891892 Sensory analysis applied to honey : state of the art 1, Apidologie. (2004) 26–37. doi:10.1051/apido:2004048.

[9] J. Peltier, Ecologie De Quelques Especes Climaciques Dans Le Sous (Maroc Occidental), Doc. Cartogr. Ecol. XXVI (1983) 61–82.

[10] F. Nassif., B. El Amiri., Goat production systems in a mountainous community of the Middle Atlas, Morocco, Options Méditerranéennes. Séries A. Mediterr. Semin. 100 (2011) 199–203. http://om.ciheam.org/om/pdf/a100/00801505.pdf.

[11] A. Khabbach, A. Libiad, M. Ennabili, Melliferous flora and apiculture in the pre-rif of the province of Taza (north of Morocco), Luna Azul. (2013) 240–256. doi:10.17151/luaz.2015.40.14.

[12] J. Louveaux, A. Maurizio, G. Vorwohl, Methods of melissopalynology, Bee World. 5 (1978) 139–153. doi:10.1080/0005772X.1978.11097714.

[13] S. Bogdanov, P. Martin, C. Lüllmann, R. Borneck, C. Ch. Flamini, M. Morlot, J. Heretier, G. Vorwohl, H. Russmann, L. Persano-Oddo, A.. Sabatini, G.. Marcazzan, P. Marioleas, K. Tsigouri, J. Kerkvliet, A. Ortiz, T. Ivanov, HARMONISED METHODS OF THE INTERNATIONAL HONEY COMMISSION, (2002). http://www.terezinka.cz/vcely/Med/IHCmethods_e.pdf.

[14] O.A. Naab, M.A. Tamame, M.A. Caccavari, Palynological and physicochemical characteristics of three unifloral honey types from central Argentina, Spanish J. Agric. Res. 6 (2008) 566–576.

[15] K. Brudzynski, D. Miotto, The recognition of high molecular weight melanoidins as the main components responsible for radical-scavenging capacity of unheated and heat-treated Canadian honeys, Food Chem. 125 (2011) 570–575. doi:10.1016/j.foodchem.2010.09.049.

[16] O. Anjos, M.G. Campos, P.C. Ruiz, P. Antunes, Application of FTIR-ATR spectroscopy to the quantification of sugar in honey, Food Chem. 169 (2015) 218–223. doi:10.1016/j.foodchem.2014.07.138.

[17] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (1965) 144 LP – 158. http://www.ajevonline.org/content/16/3/144.abstract.

[18] T. Samatha, R. Shyamsundarachary, P. Srinivas, N.R. Swamy, Quantification of total phenolic and total flavonoid contents in extracts of Oroxylum indicum L.Kurz, Asian J. Pharm. Clin. Res. 5 (2012) 177–179.

[19] M.T. Sancho, A. Pascual-Maté, E.G. Rodríguez-Morales, S.M. Osés, I. Escriche, Á. Periche, M.A. Fernández-Muiño, Critical assessment of antioxidant-related parameters of honey, Int. J. Food Sci. Technol. 51 (2016) 30–36. doi:10.1111/ijfs.12988.

[20] M.G. Miguel, S. Nunes, S.A. Dandlen, A.M. Cavaco, M.D. Antunes, Phenols and antioxidant activity of hydro-alcoholic extracts of propolis from Algarve, South of Portugal, Food Chem. Toxicol. 48 (2010) 3418–3423. doi:10.1016/j.fct.2010.09.014.

[21] S.C. Ho, Y.L. Tang, S.M. Lin, Y.F. Liew, Evaluation of peroxynitrite-scavenging capacities of several commonly used fresh spices, Food Chem. 119 (2010) 1102–1107. doi:10.1016/j.foodchem.2009.08.020.

[22] J.R. Soarey, T.C.P. Dinis, A.P. Cunha, L. Almeida, Antioxidant activities of some extracts of Thymus zygis, Free Radic. Res. 26 (1997) 469–478.

[23] M.M. Orlotc, P.M. Artind, Harmonized methods of melissopalynology, Apidology 35 (2004) 18–25. doi:10.1051/apido.

[24] L.P. Dddo, R. Piro, P.I. Czech, C.F. Lamini, J.L. Heritier, M.M.O. France, H.R. Ussmann, W.V.O.N.D.E.R.O. He, K.V.D.O.H.E. Germany, P.G. Otsiou, S.K. Arabournioti, P.K. Efalas, M.P.A. Atrali, A.T. Hrasyvoulou, G.L.M. Arcazzan, M.L.P. Iana, M.G.P. Iazza, A. Gloria, A.B. Entabol, A.O. Rtiz, S.B. Ogdanov, Main European unifloral honeys : descriptive sheets 1, Apidologie. 35 (2004) 38–81. doi:10.1051/apido.

[25] Codex Alimentarius Commission, Codex Alimentarius Commission Standards, Codex Stan 12-1981. (2001) 1–8. doi:10.1007/978-3-540-88242-8.

[26] M. Naman, M. Faid, C. EL Adlouni, Microbiological and physico-chemical properties of Moroccan honey, International Journal of Agriculture & Biology (2005).

[27] D. Hernanz, A. Marconi, F.J. Heredia, A. Terrab, Characterisation of Moroccan Spurge (*Euphorbia*) honeys by their physicochemical characteristics, mineral contents and colour, Arabian Journal of Chemistry (2015). doi:10.1016/j.arabjc.2015.01.003.

[28] E. Mendes, E.B. Proenc, I.M.P.L.V.O. Ferreira, M.A. Ferreira, Quality evaluation of Portuguese honey, Carbohydrate Polymers 37 (1998) 219–223.

[29] X. Feás, J. Pires, M.L. Estevinho, A. Iglesias, J.P.P. Araujo, Palynological and physicochemical data characterisation of honeys produced in the Entre - Douro e Minho region of Portugal Original article Palynological and physicochemical data characterisation of honeys produced in the Entre-Douro e Minho region of Por, Int. J. Food Sci. Technol. 45 (2010) 1255–1262. doi:10.1111/j.1365-2621.2010.02268.x.

[30] B. Fallico, E. Arena, A. Verzera, M. Zappalà, The European food legislation and its impact on honey sector, Accredit. Qual. Assur. (2006) 49–54. doi:10.1007/s00769-006-0128-6.

[31] C. Truzzi, A. Annibaldi, S. Illuminati, C. Finale, G. Scarponi, Determination of proline in honey: Comparison between official methods, optimization and validation of the analytical methodology, Food Chem. 150 (2014) 477–481. doi:10.1016/j.foodchem.2013.11.003.

[32] I. Hermosín, R.M. Chicón, M. Dolores Cabezudo, Free amino acid composition and botanical origin of honey, Food Chem. 83 (2003) 263–268. doi:10.1016/S0308-8146(03)00089-X.

[33] K. Brudzynski, L. Maldonado-Alvarez, Polyphenol-protein complexes and their consequences for the redox activity, structure and function of honey. A current view and new hypothesis – a Review, Pol. J. Food Nutr. Sci 65 (2015) 71–80. doi:10.1515/pjfns-2015-0030.

[34] A. Moussa, A. Saad, D. Noureddine, How Honey Acts as an Antioxidant ?, Medicinal & Aromatic Plants 1 (2012) 5–6. doi:10.4172/2167-0412.

[35] P. Anna, P. Krystyna, Evaluation of aluminium complexation reaction for flavonoid content assay, Food Anal. Methods. (2014) 1776–1782. doi:10.1007/s12161-014-9814-x.

[36] S. Aazza, B. Lyoussi, D. Antunes, M.G. Miguel, Physicochemical characterization and antioxidant activity of 17 commercial Moroccan honeys., Int. J. Food Sci. Nutr. 65 (2014) 449–457. doi:10.3109/09637486.2013.873888.

[37] D. Mammen, M. Daniel, A critical evaluation on the reliability of two aluminum chloride chelation methods for quantification of flavonoids, Food Chem. 135 (2012) 1365–1368. doi:10.1016/j.foodchem.2012.05.109.

[38] H. Shin, Z. Ustunol, Carbohydrate composition of honey from different floral sources and their influence on growth of selected intestinal bacteria : An in vitro comparison, Food Research International. 38 (2005) 721–728. doi:10.1016/j.foodres.2005.01.007.

[39] S. Aazza, B. Lyoussi, D. Antunes, M.G. Miguel, Physicochemical characterization and antioxidant activity of commercial Portuguese honeys, J. Food Sci. 78 (2013). doi:10.1111/1750-3841.12201.

[40] R. Mateo, F. Bosch-reig, Sugar profiles of Spanish unifloral honeys, Food Chemisrry. 60 (1997) 33–41.

[41] J.M. Alvarez-suarez, F. Giampieri, M. Battino, Honey as a aource of dietary antioxidants : structures , bioavailability and evidence of protective effects against human chronic diseases, Curr. Med. Chem. 2013 (2013) 621–638.

[42] M.J. Sousa, E.L. De Souza, G. Marques, B. Meireles, T.Â.D.M. Cordeiro, B. Gullón, M.M. Pintado, M. Magnani, Polyphenolic pro fi le and antioxidant and antibacterial activities of mono fl oral honeys produced by Meliponini in the Brazilian semiarid region, Food Res. Int. J. 84 (2016) 61–68. doi:10.1016/j.foodres.2016.03.012.

[43] N. Gheldof, X.-H. Wang, N.J. Engeseth, Identification and quantification of antioxidant components of honeys from various floral sources, J. Agric. Food Chem. 2002 (2002) 5870–5877.

[44] H. Wu, C. Shiau, H. Chen, T. Chiou, Antioxidant Activities of Carnosine , Anserine , Some Free Amino Acids and Their Combination, J. Food Drug Anal. 11 (2003) 148–153.

[45] A. Meda, C. Euloge, M. Romito, J. Millogo, O. Germaine, Food Chemistry Determination of the total phenolic , flavonoid and proline contents in Burkina Fasan honey , as well as their radical scavenging activity, Food Chem. 91 (2005) 571–577. doi:10.1016/j.foodchem.2004.10.006.

Chapter II: Zantaz honey monoflorality Chemometric applied to the routinely assessed parameters

Chapter II. Zantaz honey monoflorality Chemometric applied to the routinely assessed parameters*

Abstract

The comparison of Zantaz honey samples harvested in Morocco in two different years was performed through chemometric analysis of routinely assessed parameters. The objective was to study how the pollen profile of this newly reported honey shapes its physicochemical characteristics as well as to determine its monoflorality threshold, which has not yet been defined. The predominance of *B. spinosum* pollen was confirmed in the majority of samples reaching 45%, generally requested for monoflorality declaration. The principal component analysis (PCA) was used for clustering and variables correlations. Pollen qualitative and quantitative differences could discriminate the samples belonging to both seasons when combined with the sugar analysis (59.44%) better than the combination with physicochemical parameters (pH, acidity, ash content, electrical conductivity and colour) (60.62%). Positive correlation between the presence of *B. spinosum* pollen and melanoidins, colour, fructose, moisture, trehalose, melezitose, iron, manganese and calcium could be seen. Integrating all the parameters except the pollen data allowed distinguishing two groups with significant differences ($P < 0.05$) in B. spinosum representability ($58 \pm 11.24\%$ against $40 \pm 15.98\%$). This may suggest that 40% is the monoflorality threshold of the Moroccan Zantaz honey, although a confirmation with sensorial analysis is required.

1. Introduction

Chemometrics application to food chemistry parameters is attracting more attention from the scientific community due to its power on the elaboration of conclusions about the traceability [1] and the classification/clustering problems [2,3]. These techniques have been widely used and applied for the analysis of many matrixes, among which coffee [4] or milk [5]. Honey does not make an exception; indeed the chemometric tools were not only used as a discriminatory tool between honeys with different geographical [6,7] or botanical origins [8–10] but also as an effective way to detect honeys' adulteration [11]. Several tools can be employed depending on the study aim, implicating the filtration of the parameters. This parameters' filtration helps choosing the adequate parameters/variables carrying the adequate information as well as the elimination of the "noising" parameters [12].

* Y. Elamine, B. Lyoussi, O. Anjos, L.M. Estevinho, S. Aazza, J.D. Carlier, M.C. Costa, M.G. Miguel, Zantaz honey "monoflorality": Chemometric applied to the routinely assessed parameters, LWT - Food Sci. Technol. 106 (2019) 29–36. doi:10.1016/j.lwt.2019.02.039.

Honey monoflorality determination can be done using the combination of pollen spectrum analysis, physicochemical parameters and sensorial qualities [13]. This last step is highly expensive, requiring panels of assessors which go through controlled experimental protocols. The results need to be statistically reproducible to be considered [14]. In fact, a predictive relationship has been established between physicochemical characteristics and sensory properties [15], although this should be interpreted cautiously [7]. This relationship can be highlighted by the fact that both sensorial and physicochemical properties are determined by the nectar of the visited melliferous plants. As there is no way to evaluate the nectar delivered by each plant, researchers used to estimate it by using the pollen profiling, or mellissopalynology. The monoflorality threshold, considering a pollen species, is then the percentage above which honey samples becomes governed by the physicochemical and sensorial properties of the corresponding nectar.

Generally, a honey sample is considered to be monofloral, or governed by the physicochemical and sensorial properties of the main nectar, when the pollen of its main originating melliferous plant exceed 45% [16]. Two exceptions to this rule can be mentioned: lavender honey, for which the presence of 15% of lavender pollen is enough to be labelled as monofloral [7], and chestnut honey which is labelled only when presenting more than 90% of chestnut pollen [16]. Those exceptions highlight the importance of establishing a specific threshold for a given honey type, especially when it is newly introduced to the scientific community, which is the case of the Moroccan Zantaz honey. A holistic view needs to be given not only about its characterizing parameters, but on how the pollen profiling shapes them. In other words, at which level the main pollen representability needs to reach to consider a Zantaz honey sample as monofloral.

In fact, establishing monoflorality threshold for a given honey is not a trivial task. The variability of the contribution of the participating melliferous plants belonging to two different seasons, or geographical regions, is highly influential in shaping the final characteristics of honey samples [7]. The present contribution aimed a second characterization of a new set of the Moroccan Zantaz honey samples known by the prevalence of *Bupleurum spinosum* pollen exceeding 45%, as a sequence of the previously published study [17]. Furthermore, the data of both studies will be integrated using the appropriate multivariate analysis to assess the effect of seasonal variability on the identified pollen species, and how the pollen spectrum correlate the acquired physicochemical characteristics of Zantaz honey samples. In addition, PCA will be applied using all analysed parameters. The pollen profile of the output clusters will be statistically compared in an attempt to determine how the homogeneity of the samples forming each cluster is determined by the presence of *B. spinosum* pollen.

2. Materials and Methods

2.1. Sample Preparation

Honey samples (n=18) were purchased from beekeepers, centrifuged and kept under ambient temperature until their use. To strengthen the conclusions of the multivariate analysis section of the present work, the data of the samples (N=12) published in the previous study [17] was also used.

2.2. Pollen analysis

The qualitative and quantitative analysis of pollen spectrum was accomplished following the International Commission for Bee Botany (ICBB) method, as described previously [16]. An optic microscope (Leitz Messtechnik GmbH, Wetzlar, Germany) with 400 x and 1000 x objectives, was used for pollen identification and count. One thousand pollen grains were counted for each sample, and the frequent classes were determined twice. A pollen species was considered dominant, when it represents more than 45% from the pollen spectrum, secondary with percentage between 16% and 45%, important minor pollen from 3 to 15% and minor pollen when it is less than 3%.

2.3. Acidity, pH, conductivity, moisture, color, proline, diastase activity

Free acidity, lactonic acidity, total acidity, pH, ash content, electrical conductivity, moisture, diastase activity, were assessed following the Harmonized Methods of the International Honey Commission [18].

2.4. Colour and melanoidins

Colour was determined by measuring the absorbance of aqueous honey solutions (10g in 20mL H_2O) at 635 nm (A_{635}) in a Shimadzu spectrophotometer [19]. The mm Pfund values and honey colour were obtained using the following algorithm, mm Pfund=-38.7+371.39 x A_{635}. Additionally, honey colour was determined by spectrophotometry by calculating net absorbance (A_{560}-A_{720}). Melanoidin content was estimated based on the browning index (net absorbance =A_{450}-A_{720}) [20]. Spectrophotometric measurements were performed in a 1 cm quartz cell.

2.5. Sugars

The sugar contents were analysed by High Performance Liquid Chromatography (HPLC) with refraction index (IR) detector according the methodology proposed by [21]. The HPLC analysis was performed on analytical stainless-steel column, Purospher® STAR – NH2 with 4 mm in diameter, 250 mm length and with 5 mm particle size, using a flow rate of 1.3 mL/min; mobile phase: acetonitrile: water (80:20, v/v); column and detector temperature: 30ºC; and sample volume injection of 10 mL.

The different sugars (fructose, glucose, sucrose, melezitose, turanose, maltose, xilose, rhamnose, arabinose, melibiose, trehalose and erlose) were identified and quantified by comparison of the retention times and the peak areas of the honey sugars with those of calibration curves of standard sugars. The values expressed in g/100g of honey. Retention time (RT), limit of quantification (LQ) of the analysed sugars are illustrated in Table1.

Table 1. Retention time, limit of quantification of the analysed sugars.

Analyte	N	RT (min)	LQ (g/100 g)
Ramnose	3	3.83	0.17
Xylose	1	4.24	0.87
Arabinose	6	4.96	0.13
Fructose	31	5.46	0.77
Glucose	31	6.42	1.61
Sucrose	7	9.45	0.23
Turanose	31	10.45	0.06
Maltose	31	11.55	0.66
Melibiose	31	12.69	0.12
Trehalose	31	15.27	0.16
Melezitose	13	18.32	0.08
Erlose	6	22.60	0.16

2.6. Minerals

Sample preparation is initiated by calcination of 5g honey at 550°C. After cooling, 5mL of 0.1 M nitric acid was added to the ash and the mixture was stirred on a heating plate to almost complete dryness. Then, 10 mL of the same nitric acid solution was added, and the mixture was brought up to a final volume of 25 mL with distilled water. Afterwards, Ca, Mg, Mn, Zn, Cu, and Fe were measured by flame atomic absorption, while Na, K by emission spectrometry (air-acetylene) using a novAA 350 (Analytik Jena, Germany). After the determination of mineral concentrations in the prepared solutions, their values were extrapolated to be expressed as mg/kg honey.

2.7. Volatile composition: extraction and analysis

The isolation of honey volatiles was made by hydrodistillation for 3 h using a Clevenger-type apparatus. They were recovered from Clevenger tube with distilled n-pentane (n-pentane ≥ 99%

purity, HPLC grade) and allowed to settle for about 10–15 min. The mixture (volatiles and n-pentane) was then concentrated to a minimum volume of about 100 µl, at room temperature under nitrogen flux. The honey volatile samples were stored at −20°C in the dark until analysis.

Gas chromatography (GC)

Gas chromatographic analyses were performed using a Perkin Elmer Clarus 400 gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Inc., Rancho Cordova, CA) and a DB- 17HT fused-silica column ((50% phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.15 µm; J&W Scientific, Inc.). Oven temperature was programmed, 45–175°C, at 3°C/min, subsequently at 15° C/min up to 300°C and then held isothermal for 10 min; injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a n-pentane essential oil solution (1:1). The percentage composition of the volatiles was computed, by the normalization method from the GC peak areas, calculated as mean values of two injections, from each sample, without using the response factors.

Gas chromatography–mass spectrometry (GC-MS)

The GC-MS unit consisted on a Perkin Elmer Clarus 600 gas chromatograph, equipped with DB-1 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Inc.), and interfaced with a Perkin Elmer 600-T mass spectrometer (software version 5.4.2.1617; Perkin Elmer, Shelton, CT). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40–300 u; and scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C9–C30 n-alkane indices and GC-MS spectra from a laboratory- made library, created with reference essential oils, laboratory-synthesized components, laboratory isolated compounds and commercially available standards.

2.8. Statistical tools

To estimate the differences amongst the analysed samples regarding each of the assessed parameters, ANOVA, followed by a post hoc Tukey test was used and the means were considered to be significantly different when $p < 0.05$.

The PCA was used, as a powerful multivariate analysis for scales reduction, to integrate the information provided by several parameters in few principal components (PC). Prior to each PCA running, the data was normalized by subtracting the mean value and dividing by the standard deviation of each of the given values. To run the PCA a MultBiplot64 was used on MATLAB R2017a.

3. Results and Discussion

3.1. Preliminary descriptive characterization

Firstly, a descriptive approach was adopted for a preliminary characterization of the 18 honey samples. Table 2 shows their pollen analysis spectrum. *Bupleurum spinosum* pollen was the predominant species, exceeding 45%, except for S6, S7, S14 and S17, presenting values of 37.18%, 30.02%, 18.97% and 28.54%, respectively. Regardless being secondary pollen (16–45%) in the four samples, *Bupleurum spinosum* was the most abundant except for S14, where *Cytisus* sp., was the predominant species (45.77%), and S17 where it was third after *Artocarpus altilis* (28.54%) and *Eragrostis pilosa* (26.33%). Besides being predominant pollen in sample S14, the *Cytisus* sp. was the secondary pollen in 6 samples with values between 22.95% and 38.28% in S8 and S11, respectively. Other secondary pollen species were: *Eragrostis pilosa* (S3), *Populus* sp. (S5), and *Artocarpus altilis* (S15 and S17).

Table 2. Pollen profiling of the studied honey samples

Sample code	Predominant pollen (>45%)	Secondary pollen (16–45%)	important minor pollen (3–15%)	Minor pollen (<3%)
S1	*Bupleurum spinosum* (Apiaceae) (60.9)		*Artocarpus altilis* (Moraceae) (10.47), *Eragrostis pilosa* (Poaceae) (12.8), *Populus* sp (Salicaceae) (10.15), *Prunus* sp (Rosaceae) (3.08)	*Celtis* sp (2.6)
S2	*Bupleurum spinosum* (Apiaceae) (50.75)		*Artocarpus altilis* (Moraceae) (11.34), *Celtis* sp (6.95), *Eragrostis pilosa* (Poaceae) (11.9), *Populus* sp (Salicaceae) (8.05), *Prunus* sp (Rosaceae) (3.96), *Quercus* sp (Fagaceae) (5.175)	*Pinus* sp (Pinaceae) (1.875)
S3	*Bupleurum spinosum* (Apiaceae) (49.84)	*Artocarpus altilis* (Moraceae) (18.335), *Eragrostis pilosa* (Poaceae) (19.94)	*Populus* sp (Salicaceae) (11.885)	
S4	*Bupleurum spinosum* (Apiaceae) (79.795)		*Artocarpus altilis* (Moraceae) (4.165), *Eragrostis pilosa* (Poaceae) (5.935), *Populus* sp (Salicaceae) (8.21)	*Mimosa* sp (Fabaceae) (1.895), *Quercus* sp (Fagaceae) (2)
S5	*Bupleurum spinosum* (Apiaceae) (76.77)	*Populus* sp (Salicaceae) (19.4)		*Artocarpus altilis* (Moraceae) (2.715), *Eucalyptus* sp (Myrtaceae) (1.115)
S6		*Bupleurum spinosum* (Apiaceae) (37.18), *Populus* sp (Salicaceae) (20.575), *Salvia* sp (Lamiaceae) (17.42)	*Cytisus* sp (Fabaceae) (8.8), *Eragrostis pilosa* (Poaceae) (5.795), *Euphorbia* sp (Euphorbiaceae) (5.85)	*Artocarpus altilis* (Moraceae) (1.915), *Quercus* sp (Fagaceae) (2.475)
S7		*Bupleurum spinosum* (Apiaceae) (30.025), *Populus* sp (Salicaceae) (21.65)	*Eragrostis pilosa* (Poaceae) (3.015), *Eucalyptus* sp (Myrtaceae) (8.92), *Euphorbia* sp (Euphorbiaceae) (14.695), *Phytolacca americana* (Phytolaccaceae) (4.065), *Raphanus* sp (Brassicaceae) (6.585)	*Celtis* sp (0.875), *Citrus* sp (Rutaceae) (0.875), *Cytisus* sp (Fabaceae) (2.785), *Lactuca* sp (Asteraceae) (0.625), *Phoenix* sp (Arecaceae) (0.875), *Quercus* sp (Fagaceae) (2.295), *Salvia* sp (Lamiaceae) (2.715)
S8	*Bupleurum spinosum* (Apiaceae) (64.565)	*Cytisus* sp (Fabaceae) (22.95)	*Eucalyptus* sp (Myrtaceae) (7.675)	*Phytolacca americana* (Phytolaccaceae) (1.15), *Populus* sp (Salicaceae) (2.18), *Salvia* sp (Lamiaceae) (1.48)

S9	*Bupleurum spinosum* (Apiaceae) (55.02)	*Cytisus* sp (Fabaceae) (27.185)	*Eucalyptus* sp (Myrtaceae) (12.39)	*Artocarpus altilis* (Moraceae) (1.97), *Citrus* sp (Rutaceae) (2.08), *Populus* sp (Salicaceae) (1.355)
S10	*Bupleurum spinosum* (Apiaceae) (52.895)	*Cytisus* sp (Fabaceae) (36.04)	*Artocarpus altilis* (Moraceae) (5.6), *Populus* sp (Salicaceae) (5.465)	
S11	*Bupleurum spinosum* (Apiaceae) (51.05)	*Cytisus* sp (Fabaceae) (38.28)	*Populus* sp (Salicaceae) (7.775)	*Artocarpus altilis* (Moraceae) (0.995), *Eragrostis pilosa* (Poaceae) (1.9)
S12	*Bupleurum spinosum* (Apiaceae) (47.81)	*Cytisus* sp (Fabaceae) (29.82)	*Eragrostis pilosa* (Poaceae) (4.05), *Populus* sp (Salicaceae) (4.4)	*Artocarpus altilis* (Moraceae) (2.355), *Salvia* sp (Lamiaceae) (1.155)
S13	*Bupleurum spinosum* (Apiaceae) (45.945)	*Cytisus* sp (Fabaceae) (23.76)	*Eragrostis pilosa* (Poaceae) (11.915), *Populus* sp (Salicaceae) (12.26), *Salvia* sp (Lamiaceae) (6.105)	
S14	*Cytisus* sp (Fabaceae) (45.775)	*Bupleurum spinosum* (Apiaceae) (18.97), *Populus* sp (Salicaceae) (15.065)	*Eragrostis pilosa* (Poaceae) (3.33), *Eucalyptus* sp (Myrtaceae) (10.005), *Raphanus* sp (Brassicaceae) (4.125)	*Salvia* sp (Lamiaceae) (2.73)
S15	*Bupleurum spinosum* (Apiaceae) (56.995)	*Artocarpus altilis* (Moraceae) (18.1)	*Eragrostis pilosa* (Poaceae) (13.84), *Populus* sp (Salicaceae) (11.065)	
S16	*Bupleurum spinosum* (Apiaceae) (49.295)		*Artocarpus altilis* (Moraceae) (11.665), *Cytisus* sp (Fabaceae) (9.86), *Eragrostis pilosa* (Poaceae) (6.375), *Euphorbia* sp (Euphorbiaceae) (7.435), *Populus* sp (Salicaceae) (6.375), *Raphanus* sp (Brassicaceae) (4.125)	*Eucalyptus* sp (Myrtaceae) (2.52), *Phytolacca americana* (Phytolaccaceae) (2.35)
S17		*Artocarpus altilis* (Moraceae) (28.545), *Bupleurum spinosum* (Apiaceae) (22.02), *Eragrostis pilosa* (Poaceae) (26.335), *Populus* sp (Salicaceae) (19.995)		*Euphorbia* sp (Euphorbiaceae) (1.93), *Salvia* sp (Lamiaceae) (2.14)
S18	*Bupleurum spinosum* (Apiaceae) (68.6)		*Pinus* sp (Pinaceae) (3.55), *Populus* sp (Salicaceae) (7.4), *Prunus* sp (Rosaceae) (10.9), *Quercus* sp (Fagaceae) (4.9)	*Artocarpus altilis* (Moraceae) (2.45), *Erica* sp (Ericaceae) (1.1), *Eucalyptus* sp (Myrtaceae) (1.1)

An overview of the pollen analysis applied previously to the samples of the same botanical origin [17], revealed more contribution of *B. spinosum* pollen presenting an average of 62.50 ± 8.66% compared to the samples of the current work (51.02 ± 16.11%). Besides this quantitative difference, some pollen species were only identified in one of the two groups, making a qualitative difference between them. Namely, *Thymus* sp., *Opuntia* sp., *Phoenix* sp., *Olea* sp., *Epilobium* sp., *Cactus* sp. and *Acacia* sp. were identified only in the samples of the previous study, while the current work was specifically characterized by the presence of *Populus* sp., *Prunus* sp., *Quercus* sp., *Raphanus* sp., *Salvia* sp., *Euphorbia* sp., *Lactuca* sp., *Mimosa* sp., *Artocarpus altilis*, *Phytolacca* sp., *Pinus* sp., *Erica* sp., *Citrus* sp., *Celtis* sp. and *Cytisus* sp. The effect of the mentioned differences on the assessed parameters will be discussed with details in the multivariate analysis section.

The studied physicochemical parameters are summarized in Table 3. The pH values ranged from 3.74 to 4.39 with an average value of 4.03 ± 0.21 considering the 18 samples. The same average can be seen in the previously analysed samples (previous season). The lactonic acidity varied also amongst the analysed samples between 8.00 ± 1.49 mEq/kg and 16 ± 0.86 mEq/kg. The average values of the free and lactonic acidity (20.28 ± 6.36 mEq/kg - 12.11 ± 1.82 mEq/kg) were slightly lower than those observed in the previous work (25.70 ± 6.46 mEq/kg and 16.58 ± 1.40 mEq/kg).

The ash content and the electrical conductivity were evaluated. A correlation between the two parameters is reported in addition to their correlation with the mineral composition of honey samples [22]. The correlation can be seen by the fact that samples presenting low ash content had also low electrical conductivity, and *vice versa*. The lowest ash content percentage was 0.11 ± 0.02%, while the highest ash value was 0.32 ± 0.03%. For the electrical conductivity, the minimum and the maximum values, were 351.66 ± 0.57 µs/cm and 900.33 ± 3.05 µs/cm, respectively. Both assessed parameters can be used, not only to predict the richness on mineral elements, and so its nutritional value [23] but also for honey quality control purpose.

Table 3. Physicochemical characterization and sugar profile of the analysed honey samples.

Assessed parameter (Unit)	Means	SD	Min	Max
Ash content (%)	0.19	0.05	0.11	0.32
Water content (%)	19.59	0.81	18.40	21.13
Electrical conductivity µs/cm	507.63	144.95	351.66	900.33
pH	4.02	0.21	3.73	4.38
Free acidity (mEq/kg)	20.28	6.36	8.40	32.80

Lactonic Acidity (mEq/kg)	12.11	1.82	8.00	16.00
Total Acidity (mEq/kg)	32.39	7.37	18.40	43.30
Diastase (Shad number)	21.81	5.25	12.38	31.89
Melanoidins	0.94	0.30	0.40	1.45
Colour (mmPfund)	56.62	20.77	22.24	96.42
Fructose (g/100g)	37.43	2.42	33.52	41.83
Glucose (g/100g)	24.44	2.73	19.61	30.88
Fructose/Glucose Ratio	1.52	0.22	1.12	2.13
Melebiose (g/100g)	2.66	0.96	0.62	4.31
Turanose (g/100g)	1.71	0.28	1.08	2.05
Maltose (g/100g)	1.56	0.63	0.34	3.03
Arabinose (g/100g)	1.44	0.67	0.33	2.44
Trehalose (g/100g)	0.88	0.39	0.41	2.03
Xylose (g/100g)	0.31	0.04	0.25	0.42
Melezitose (g/100g)	0.51	0.01	0.50	0.51
Sucrose (g/100g)	-	-	-	-

Abbreviations: SD= standard deviation; Min=Minimal; Max=Maximal.

According to the international legislation the ash content and the electrical conductivity are not allowed to be more than 800 µS/cm and 0.6%, respectively in honey [24]. Considering this, all analysed Zantaz honey samples were within the established values, except for one sample that had a value of 900.33 ± 3.05 µs/cm for electrical conductivity. Furthermore, the comparison with the samples used in previous studies of our team showed no significant differences and the average value of the samples maintained almost the same: 0.20 ± 0.05% and 507.63 ± 144.95 µs/cm in comparison to the previous season 0.22 ± 0.06% and 529.4 ± 122.70 µs/cm.

The diastase activity of the samples analysed in the present work was very similar to that observed on the previous harvest, and the average values were 22.80 ± 5.55 Shade units/g and 22.21 ± 5.30 Shade units/g, successively. Although, an important variability could be seen amongst the analysed samples with a minimum activity of 12.38 ± 0.13 Shade units/g, and a maximum of 31.89 ± 0.73 Shade units/g, being all above the minimum value (8.0 Shade units/g) required for honey freshness confirmation by the European council [24].

The ripeness is another important step in the quality control of honey. Usually, the fast parameter used is the moisture content [8], which may be a factor favouring the growth and

development of undesirable germs. Therefore, a maximum value was fixed to be 20% [24]. In the present work eight of the analysed samples had moisture values between 20% and 21% (data not shown), and one sample presented a value of 21.13 ± 0.23% being the maximum observed. The remaining samples did not exceed the 20%, while the minimum value was 18.4 ± 0.20%.

Honey colour and melanoidins content are also important parameters in the characterization of honey samples. A well-established relationship is reported between the colour, bioactive compounds, and mineral content [3]. Both parameters ranged in the analysed samples between 22.24 ± 10.52 mm Pfund and 0.40 ± 0.02 AU as minimum values and maximum values of 96.42 ± 6.99 mm Pfund and 1.45 ± 0.03 for colour and melanoidins respectively.

The results of the sugars profiling are summarized in Table 3. In all samples the fructose prevailed, and the average of the ratio fructose/glucose was 1.56 ± 0.25 which is a usual value for honey [2]. The ratio variability ranged from a minimum of 1.13 to a maximum of 2.13. In terms of abundance, after fructose and glucose, the evaluated sugars were classified as follow: melebiose, turanose, maltose, arabinose, trehalose and xylose. While melezitose was detected in two samples, ramnose, erlose, and sucrose were below 0.2%, the detection threshold. In the samples of the previous study, the ratio fructose/glucose was 1.48 ± 0.17 (min = 1.25 and max = 1.74), lower than the current samples. Other differences involve, mainly, the big reduction in the melezitose content from 2.00 ± 0.30 g/100g honey, to be detected only in two samples. The relationship between this variability and the one of the pollen analyses will be probed in the multivariate analysis section.

When analysing the mineral content of Zantaz honeys, potassium prevailed in all samples, as reported in literature [3], followed by the calcium, and in a third position the sodium (Table 4). While the magnesium and iron occupied the fourth and the fifth places, cupper, manganese and zinc were present at very low levels with values equal or below 2 mg/kg, except one sample which presented an amount of 6.43 ± 0.073 mg/kg cupper. Sample S9 presented the highest amount of potassium, calcium and iron, and the second highest amount of magnesium with values of 1177.47 ± 18.29 mg/kg, 50.22 ± 1.006 mg/kg, 15.61 ± 0.08 mg/kg, and 50.22 ± 1.006 mg/kg, successively. The lowest amounts were observed in honey samples S5 (448.50 ± 2.03 mg/kg), S14 (94.18 ± 1.95 mg/kg), S8 (7.61 ± 0.01 mg/kg) and S6 (20.94 ± 0.57 mg/kg), successively for potassium, calcium, iron and magnesium. For the sodium, the minimum value was 38.39 ± 0.05 mg/kg observed in sample S4, while the maximum value was 163.38 ± 1.22 mg/kg in sample S14. In overall, the mineral content, based on the assessed elements, ranged between 0.067% (S10) and 0.148% (S9), being within the reported values (0.04%-0.2%) [3].

Table 4. Mineral content of honey samples.

	[K] mg/kg	[Ca] mg/kg	[Na] mg/kg	[Mg] mg/kg	[Fe] mg/kg	[Cu] mg/kg	[Mn] mg/kg	[Zn] mg/kg
S1	566.30±3.18^{f}	189.95±1.21^{b}	44.63±0.41^{k}	32.14±0.45^{def}	15.50±0.06^{b}	0.92±0.002^{j}	0.96±0.001^{d}	0.41±0.008^{k}
S2	679.34±3.40^{e}	170.73±7.62^{cde}	77.69±0.56^{c}	52.80±1.02^{a}	18.22±0.21^{a}	1.67±0.005^{d}	1.12±0.015^{b}	0.86±0.011^{f}
S3	702.39±6.87^{e}	221.12±17.72^{a}	39.82±0.30^{l}	34.69±0.62^{cd}	9.41±0.13^{g}	1.62±0.015^{de}	1.16±0.003^{a}	0.69±0.015^{i}
S4	492.35±2.04^{g}	124.15±1.41^{ij}	38.39±0.05^{m}	26.98±0.98^{gh}	11.93±0.19^{e}	0.89±0.022^{j}	0.75±0.014^{i}	0.36±0.003^{l}
S5	448.50±2.03^{gh}	138.78±3.22^{hi}	64.13±0.25^{e}	31.89±0.55^{def}	11.55±0.17^{ef}	1.02±0.007^{i}	0.69±0.007^{j}	0.26±0.008^{n}
S6	594.02±1.15^{f}	104.15±2.43^{kl}	64.97±0.12^{e}	20.94±0.57^{i}	6.78±0.054^{j}	1.06±0.002^{i}	0.46±0.005^{m}	0.44±0.004^{k}
S7	558.61±1.45^{f}	103.72±3.67^{kl}	58.04±0.04^{h}	27.78±0.61^{gh}	6.72±0.10^{j}	1.39±0.013^{f}	0.51±0.001^{l}	1.29±0.016^{c}
S8	755.55±0.95^{d}	124.41±2.52^{ij}	90.90±0.26^{b}	24.25±0.09^{hi}	7.61±0.01^{i}	1.07±0.008^{i}	0.67±0.007^{j}	1.72±0.002^{b}
S9	1177.47±18.29^{a}	173.79±4.71^{cd}	59.59±0.26^{g}	50.22±1.006^{a}	15.61±0.08^{b}	1.95±0.019^{b}	0.87±0.008^{f}	0.81±0.006^{g}
S10	429.15±2.31^{h}	153.43±1.88^{fgh}	51.23±0.48^{i}	24.35±1.89^{hi}	14.01±0.12^{d}	1.061±0.003^{i}	0.51±0.004^{l}	0.42±0.004^{k}
S11	584.34±0.94^{f}	169.82±0.98^{cde}	49.33±0.19^{j}	29.92±0.92^{efg}	18.23±0.15^{a}	1.80±0.023^{c}	0.88±0.012^{f}	0.88±0.014^{f}
S12	468.73±3.27^{gh}	180.08±2.14^{bc}	50.02±0.20^{j}	24.21±0.09^{hi}	15.59±0.15^{b}	1.57±0.009^{e}	0.84±0.005^{g}	0.32±0.014^{m}
S13	774.69±4.18^{d}	158.25±2.06^{efg}	77.20±0.22^{c}	44.71±2.41^{b}	14.52±0.10^{c}	0.89±0.010^{j}	0.79±0.003^{h}	1.02±0.010^{d}
S14	1013.45±5.64^{b}	94.18±1.95^{l}	163.38±1.22^{a}	36.77±1.44^{c}	7.84±0.05^{i}	6.43±0.073^{a}	0.74±0.002^{i}	2.02±0.010^{a}
S15	694.71±39.56^{e}	142.85±0.89^{gh}	44.01±0.18^{k}	30.31±1.14^{efg}	11.52±0.06^{f}	1.29±0.005g	0.93±0.006^{e}	0.35±0.006^{lm}
S16	704.48±5.23^{e}	94.65±1.66^{l}	62.31±0.19^{d}	32.80±0.95^{de}	8.75±0.12^{h}	1.69±0.014^{d}	0.62±0.002^{k}	0.94±0.005^{e}
S17	885.73±48.43^{c}	115.32±1.21^{jk}	74.28±0.15^{d}	37.29±1.71^{c}	11.90±0.09^{ef}	1.08±0.008^{i}	0.62±0.001^{k}	0.73±0.013^{h}
S18	554.21±2.18^{f}	159.95±0.43^{def}	57.10±0.07^{h}	28.78±2.38^{fg}	17.98±0.17^{a}	1.18±0.053^{h}	1.07±0.010^{c}	0.50±0.006^{j}

Values in the same column followed by the same letter are not significant different ($p < 0.05$) by the Tukey's multiple range test.

3.2. Multivariate analysis

For honey types with well-established monoflorality percentage, authors declared the importance of identified pollen species in shaping the final characteristics of honey samples [14]. An example is that of lavender honey [7,25], for which authors, after confirming that all analysed samples presented lavender pollen more than the monoflorality threshold (15%), found different clustering profiles using the pollen analysis or the physicochemical characteristics. Furthermore, when analysing the differences between the established clusters, the same authors found no significant contribution of lavender pollen, highlighting so the importance of other pollens in shaping the physicochemical features of a honey.

From the previous reference [7], a similar behaviour was expected to be seen in the case of Zantaz honey samples characterized by the prevalence of *Bupleurum spinosum* pollen. This expectation could be seen clearly considering the Figures 1 and 2, where specific pollen species correlate differently the assessed parameters. In other words, if we consider two honey samples presenting the same amount of *B. spinosum* pollen, but different secondary pollen species, both samples will share the characteristics provided by the main nectar, and other distinguished features matching other participating nectars. From this point of view, and as the pollen species varies greatly amongst the analysed samples, the determination of monoflorality threshold of Zantaz honey will not be a trivial task unless the maximum of parameters is considered, as well as the sensorial analysis.

Fig.1 illustrates the distribution of the 30 Zantaz honey samples belonging to both seasons based on their melissopalynological and physicochemical analyses. To retain a good representability of the given data three components were used for plotting. The three first components represented 23.97%, 20.78% and 15.87% respectively. Considering the two first components (Fig.1 (a)), a reasonable discrimination was seen between the 12 samples of the previous work (black circles) and the 18 honey samples of the current contribution (blue squares).

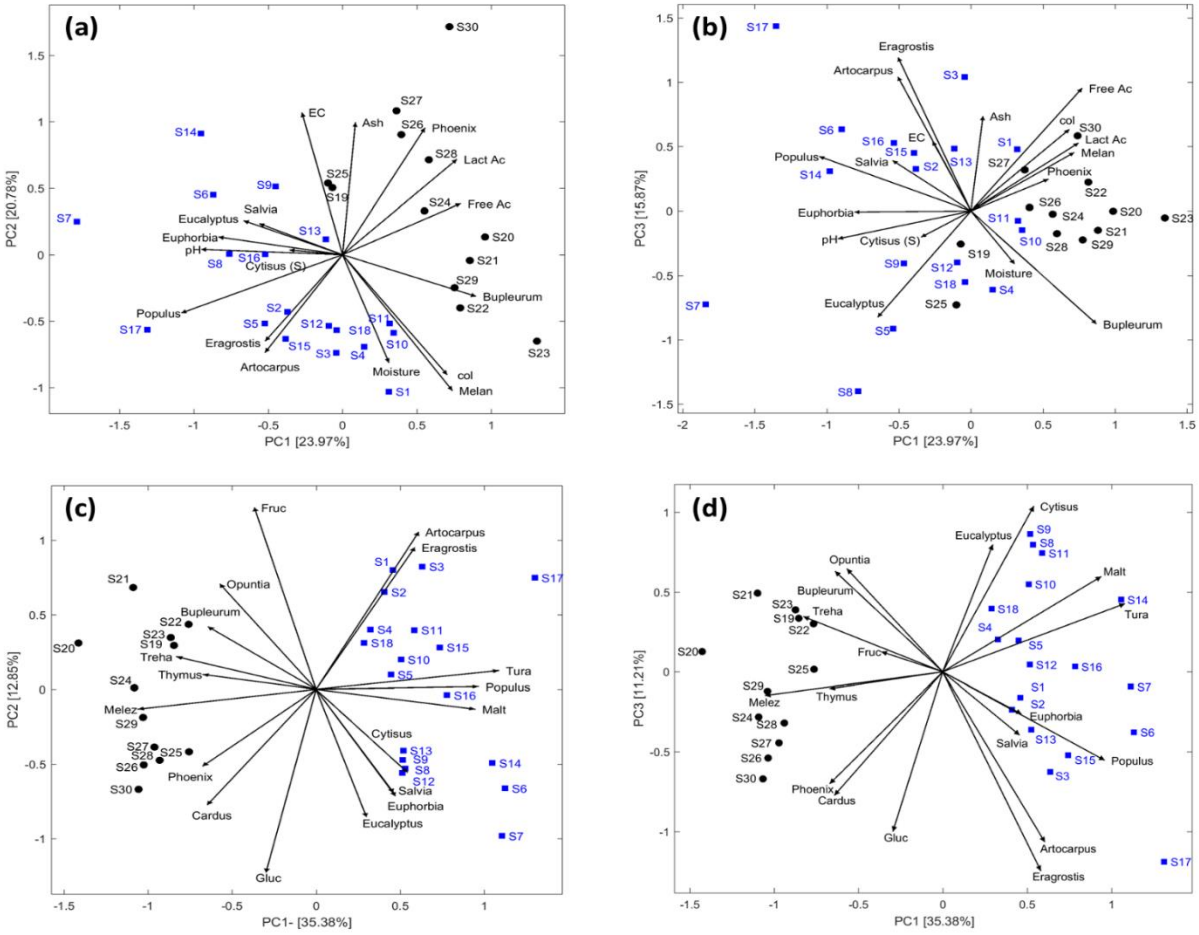

Figure 1. PCA of the pollen analysis and the physicochemical parameters assessed in the 30 Zantaz honey samples. (a) Plot using the first and the second component. (b) Plot using the first and the third components. (c) Plot using the first and the second component. (d) Plot using the first and the third components.

The samples of the previous season were characterised by the presence of more *B. spinosum* and *Phoenix.* ssp pollen, in comparison with the other samples, making them, based on the seen positive correlation, more acidic and darker. The current work samples presented, mainly, more *Eucalyptus* sp., *Euphorbia* sp., *Populus* sp., and so presented the opposite features: high pH values and light colour. A sub-group of those last samples, located in the bottom of the plot, was characterized by more *Eragostis* sp. and *Artocarpus* sp. Pollens and was characterized by low ash content and electrical conductivity.

Employing the first and the third PCs (Fig.1 (b)), the conserved information did not allow the same discrimination level as in Fig.1 (a), although it conserved the same correlation between the presence of pollen species and confirmed that there are a higher similarity between

samples with higher amounts of *Eragostis* and *Artocarpus* pollens, which were significantly different from those with higher amount of *B. spinosum* pollen.

To evaluate a possible effect of pollen variability on sugar content, another PCA was made using a data matrix fusing both parameters. Also, to improve the conservation of the given data, three components were employed. Considering the first and the second PCs (Fig.1 (c)), which explained 35.38% and 12.85% respectively, the samples of the two seasons were perfectly discriminated from each other by the PC1. The 12 samples of the previous works were characterized by the prevalence of *B. spinosum*, *Opuntia* sp., *Thymus* sp., *Phoenix* sp. and *Cardus* sp. pollens, and occupied the negative part of PC1. This pollen prevalence characterized the samples by higher levels in terms of trehalose and melezitose. In the 18 honey samples characterized in the present work, the *Artocarpus* sp., *Eragostis* sp., *Populus* sp., *Salvia* sp., *Euphorbia* sp. and *Eucalyptus* sp. pollens prevailed, and the samples presented higher amount of turanose and maltose. Considering the fructose and the glucose, they correlated oppositely, as observed by other authors [2] the PC2, presenting, so little participation to the mentioned discrimination

Besides being a good tool for the discrimination of the geographical origin, the mineral content can also suggest the botanical origin [26]. Therefore, a PCA was also employed to illustrate the relationship between the pollen species and the evaluated minerals. As the mineral content was only made for the 18 samples of the present work, their data was used for PCA, and the first three components conserved a sum of 67.61% of the given data. In overall no clear clustering could be seen amongst the analysed samples (Fig.2 (a)), except the discrimination of samples S14, presenting high percentage of *Cytisus* sp. pollen and high levels of Cu, Zn, Na and K. In addition, honey samples S6 and S7 were discriminated by their high amounts of *Salvia* sp., *Euphorbia* sp. and *Popolus* sp. pollens which were linked to the prevalence of Cu, Zn and Na. the presence of high amounts of *B. spinosum* pollen was linked to higher amounts of Ca, Fe and Mn.

When PC1 was plotted against PC3 (Fig.2 (b)), the same observation of Fig.2 (a) were made, as the first PC was the most representative in terms of data conservation. Furthermore, the presence of *Eragostis* sp. and *Artocapus* sp. was linked to the amount of Mg and the three variables were highly represented by the third PC, which explained 17.29% from the given data. Also, the relation between *Salvia* sp. and *Euphorbia* sp. and Cu, Na and Zn became clearer.

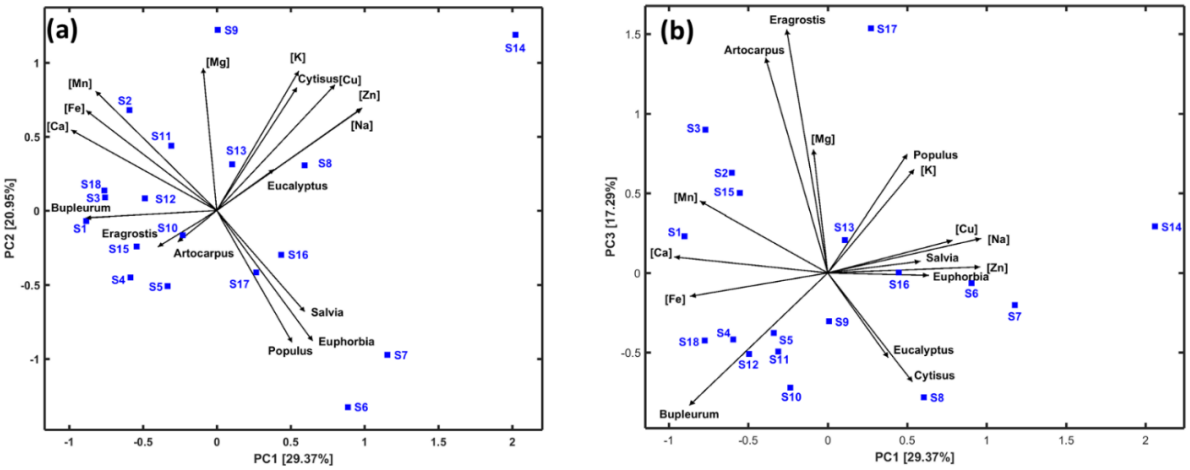

Figure 2. PCA of the pollen analysis and the mineral content of the 18 Zantaz honey samples. (a) Plot using the first and the second component. (b) Plot using the first and the third components.

To further understand how the presence of *B. spinosum* pollen, in addition to the other major represented pollen species, shape the final characteristics of Zantaz honey, a PCA integrating all the assessed physicochemical parameters was employed. Prior to running PCA, the data was subjected to the standardization. As the 18 samples of the present work were more characterized then those of the previous work, they were chosen to be used for the next section. The output is illustrated in Fig.3 (a).

The first two PCs explaining 40.02% and 21.74%, successively of the given data, were used for plotting. Considering PC1, two groups could be distinguished: a group labelled as black squares presenting an average value of 58±11.24% of *B. spinosum* pollen, and a second one, labelled in blue circles, presented an average of 40±15.98%. three samples of this group were above the average of *B. spinosum* pollen percentage and were misplaced in the left side of the plot. The samples were, namely, S8, S9 and S16, and shared with sample S7 the presence of *Eucalyptus* pollen. The presence of *Eucalyptus* sp. pollen is linked to the production of light-coloured honey, which may explain the divergence seen in the three mentioned samples compared to the remaining samples.

The observation of the variables plot showed on the onset of Fig.3 (a) confirmed the conclusion of PCA described previously (Fig.1 and Fig.2): The presence of *B. spinosum* pollen correlates positively the values of colour, melanoidins, moisture, iron, manganese and calcium. However, the data concerning the sugar must be confirmed in previous work compared samples with higher amount of *B. spinosum* pollen and low or quite null percentage of these pollens.

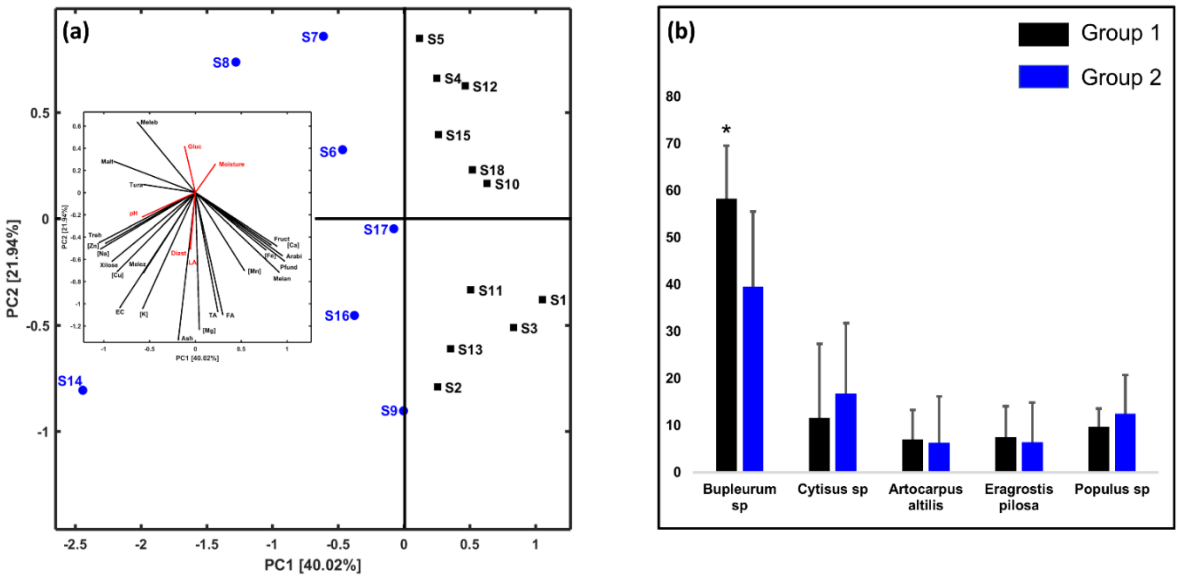

Figure 3. The discrimination amongst the 18 samples of the present work using a PCA integrating the information of all assessed parameters (a), and the possible effect of the most abundant characterized pollen species comparing the samples discriminated by the first PC using student t test (b). the onset of the figure (a) is the variable plot, and the red lines are plotted as facultative. (b) Comparison between the means of the groups discriminated in Fig. 3 (a) considering the main identified pollen species * $P < 0.05$.

To estimate the significance of the difference observed after the use of physicochemical parameters, the means of the major represented pollen species were compared between the two obtained groups, and the results were illustrated in Fig.3(b). *B. spinosum* pollen showed to play an important role in this discrimination, and the difference was significant at $p<0.05$. Another important feature is the prevalence of the *Cystus* sp. and *Populus* sp. pollens in the groups presenting low levels of *B. spinosum* pollen, although the difference was not significant.

Volatile composition of Zantaz honey and monoflorality threshold

Qualitatively speaking, ten of the identified compounds were detected in all 18 samples, 6 Straight-chain hydrocarbons, One straight-chain alcohol, one straight-chain aldehyde and two fatty acids (Table 5). From a quantitative point of view, straight-chain hydrocarbons was the most abundant family of compounds with an average of 36.68±11.50, followed by fatty acids with a level of 28.86±11.69 % considering all analysed samples. The remaining identified compounds constituted 18%.

Table 5. Minimum and maximum percentage range of main components (≥1%) identified in the volatiles isolated by hydrodistillation from different *Zantaz* honey samples.

	Components	RI	Occurrence	Mean	Sd	Min	Max
	heterocyclic aldehyde						
1	2-Furfural	825	15	1.09	0.44	0.50	2.10
2	5-Methyl furfural	938	2	0.60	0.40	0.20	1.00
	Straight-chain hydrocarbons						
3	*n*-Octane (C8)	800	12	0.75	0.68	0.20	2.80
4	*n*-Nonane (C9)	900	1	0.20	0.00	0.20	0.20
5	*n*-Pentadecane (C15)	1500	7	0.17	0.07	0.10	0.30
6	*n*-Heptadecane (C17)	1700	11	0.23	0.17	0.10	0.70
7	*n*-Nonadecane (C19)	1900	16	0.66	0.51	0.20	2.00
8	*n*-Heneicosane (C21)	2100	18	2.03	1.11	0.50	4.50
9	(Z)-9-Tricosene *	2287	18	1.46	0.62	0.50	3.00
10	*n*-Tricosane (C23)	2300	18	16.12	4.55	7.60	24.60
11	*n*-Tetracosane (C24)	2400	18	0.98	0.55	0.10	2.10
12	*n*-Pentacosane (C25)	2500	18	12.23	4.17	5.10	19.80
13	*n*-Heptacosane (C27)	2700	18	4.02	1.90	1.20	7.90
	straight-chain alcohol						
14	*n*-Hexanol	882	1	0.40	0.00	0.40	0.40
15	*n*-Octadecanol	2095	18	0.99	0.40	0.10	2.00
	straight-chain aldehyde						
16	*n*-Nonanal	1073	8	0.15	0.07	0.10	0.30
17	*n*-Octadecanal	2008	18	1.91	1.50	0.00	6.70
	Aromatic aldehyde						
18	Benzaldehyde	927	4	0.40	0.16	0.20	0.60
19	Benzene acetaldehyde	1002	14	0.65	1.42	0.10	5.70
20	Benzyl alcohol	1000	14	0.33	0.25	0.10	1.00
	Monoterpene hydrocarbons						
21	α-Pinene	930	3	0.17	0.05	0.10	0.20
22	Limonene	1009	7	0.30	0.25	0.10	0.90
23	**Oxygen-containing monoterpenes**						
24	1,8-Cineole	1005	8	0.39	0.25	0.20	0.90
25	Linalool	1074	7	0.14	0.07	0.10	0.30
26	α-Terpineol	1159	11	0.48	0.29	0.10	0.90
	Sesquiterpene hydrocarbons						
27	β-Caryophyllene	1414	3	0.37	0.24	0.20	0.70
	Oxygen-containing sesquiterpenes						
28	Viridiflorol	1569	2	0.20	0.00	0.20	0.20
29	α-Bisabolol	1656	1	0.30	0.00	0.30	0.30
	6 pelargonic acid						
30	Nonanoic acid	1263	2	0.45	0.25	0.20	0.70
	Fatty acids						
31	Oleic acid	2101	18	4.39	1.77	0.50	7.40
32	Linoleic acid	2108	18	24.42	11.64	4.30	51.50
	phenol						
33	3,4,5-Trimethyl phenol	1277	6	0.23	0.14	0.10	0.50
	UI						
34	UI A	1420	17	2.96	2.22	1.10	8.80

35	UI ≈A	1450	2	0.30	0.10	0.20	0.40
36	UI B	1484	17	1.15	0.52	0.60	2.50
37	UI C	1547	12	0.62	0.24	0.40	1.20
38	UI D	1553	7	0.50	0.25	0.20	1.00
39	UI G	2139	10	2.83	7.23	0.10	24.50
40	UI H	2142	16	2.34	3.19	0.50	14.20
	ester						
41	Methyl oleate	2096	16	1.57	0.81	0.30	3.20
42	Ethyl oleate	2137	16	10.86	15.15	0.40	48.50

Cluster analysis defined two poorly correlated clusters, Figure 4. Cluster A, with 17 out of the 18 samples analysed, included four subgroups, A1, A2, A3 and A4, dominated by straight-chain hydrocarbons and fatty acids in variable proportions. Cluster B included just one sample, with two yet unidentified components as main components. Mono- and sesquiterpene hydrocarbons, as well as oxygen-containing mono- and sesquiterpenes occurred always <1%.

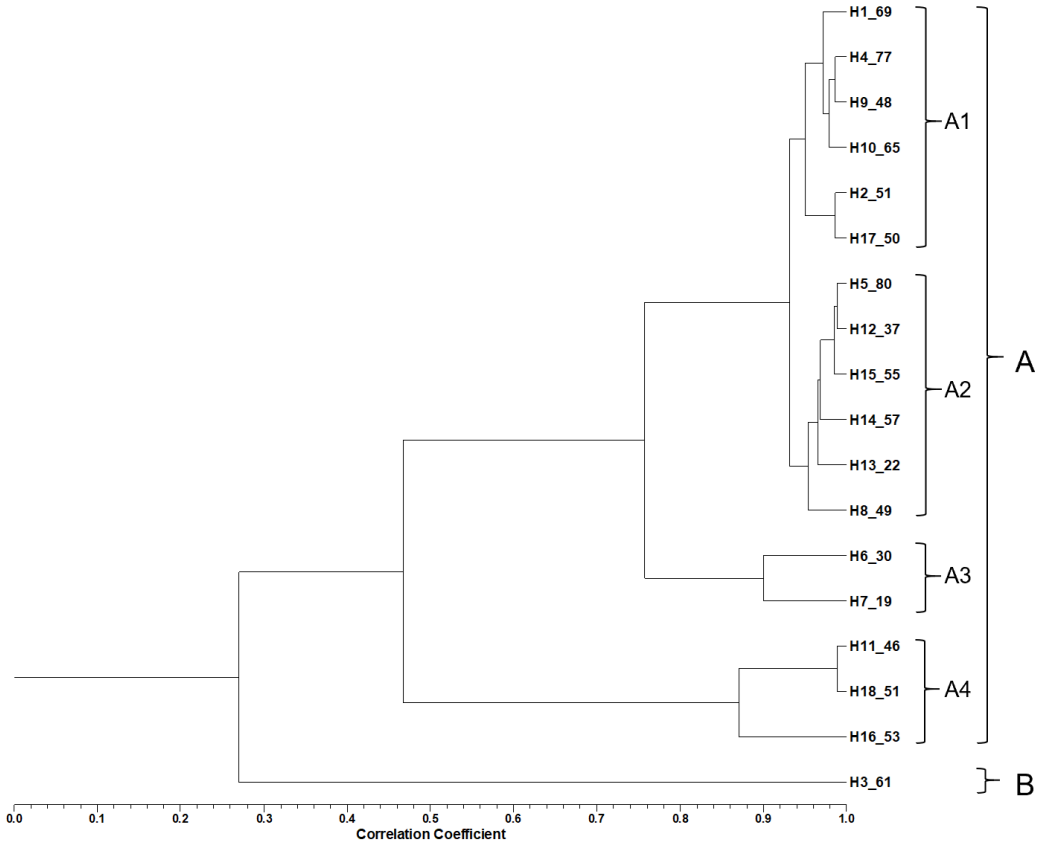

Figure 4. Dendrogram obtained by cluster analysis of the percentage composition of the volatiles isolated from the *Zantaz* honey, based on correlation and using unweighted pair-group method with arithmetic average (UPGMA).

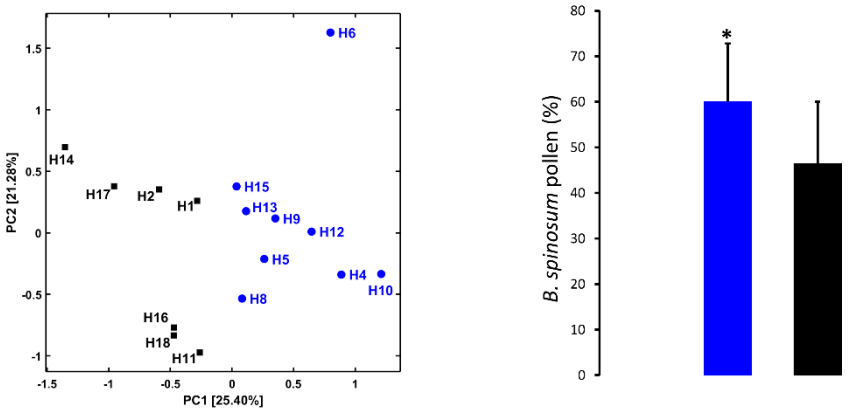

Figure 5. Score plot of the PCA using all identified volatile compounds. Bar plot comparing the means of *Bupleurum spinosum* pollen based on the clusters obtained in fig. 3. Student t test was used. *($p<0.05$)

Principal component analysis (PCA), Figure 2, of the volatile profile, showed two groups with significantly different means in terms of their *B. spinosum* pollen contents (60±13% against 46±14%) considering the second PC which explained 21% of the given data. This suggests the importance of *B. spinosum* plant in determining the final volatile profile of Zantaz honey, while no significant correlation was seen with specific compounds.

Further experiments should evaluate the volatiles from *B. spinosum* flowers and pollen, in order to evaluate how they contribute to the volatile profile of this type of honey.

4. Conclusion

In this second contribution to the characterization of the Moroccan Zantaz honey, known by the predominance of *Bupleurum spinosum* pollen, 83.33% of the analysed samples presented more than 45% of *B. spinosum* pollen, which is the general monofloral threshold for honey monoflorality declaration. Besides the pollen identification, the analysis concerned mainly the sugar and mineral profiling in addition to a panel of physicochemical parameters used for honey quality control. The results showed no important aberrant values in comparison to the previous study published by our team. While comparing the data of honey samples belonging to both seasons, an attempt to understand the relationship between the identified pollen species and the routinely assessed physicochemical parameters was performed using the clustering ability of PCA. The presence of *Bupleurum spinosum* pollen was correlated to the levels of acidity, colour intensity, trehalose, melezitose, Ca, Fe and Mn.

When integrating all assessed physicochemical parameters, a PCA conserving 61.96 % of the given data allowed discriminating the two groups of honey samples. The samples of the first group presented a mean of 58±11.24% in terms of *B.spinosum* pollen and was significantly higher ($p<0.05$) than the mean percentage obtained for the second group (40±15.98%). No

significant difference was seen in the case of other pollen species, suggesting the importance of *B. spinosum* nectar in shaping the acquired features of Zantaz honey samples. Considering the means of the two groups (58±11.24% and 40±15.98%), one can speculate that a Zantaz honey sample obey, at least, the general monoflorality threshold of 45%, though, a determinant conclusion can be made only after a detailed sensorial study.

5. References

[1] L. Bertacchini, M. Cocchi, M. Li Vigni, A. Marchetti, E. Salvatore, S. Sighinolfi, M. Silvestri, C. Durante, The Impact of Chemometrics on Food Traceability, 1st ed., Copyright © 2013 Elsevier B.V. All rights reserved., 2013. doi:10.1016/B978-0-444-59528-7.00010-7.

[2] O. Anjos, C. Iglesias, F. Peres, J. Martínez, Á. García, J. Taboada, Neural networks applied to discriminate botanical origin of honeys, Food Chem. 175 (2015) 128–136. doi:10.1016/j.foodchem.2014.11.121.

[3] P.M. Da Silva, C. Gauche, L.V. Gonzaga, A.C.O. Costa, R. Fett, Honey: Chemical composition, stability and authenticity, Food Chem. 196 (2016) 309–323. doi:10.1016/j.foodchem.2015.09.051.

[4] R. Briandet, E.K. Kemsley, R.H. Wilson, Discrimination of Arabica and Robusta in instant coffee by Fourier Transform Infrared Spectroscopy and Chemometrics, J. Agric. Food Chem. 44 (1996) 170–174. doi:10.1021/jf950305a.

[5] C. Sola-Larrañaga, I. Navarro-Blasco, Chemometric analysis of minerals and trace elements in raw cow milk from the community of Navarra, Spain, Food Chem. 112 (2009) 189–196. doi:10.1016/j.foodchem.2008.05.062.

[6] I.K. Karabagias, A.P. Louppis, S. Karabournioti, S. Kontakos, C. Papastephanou, M.G. Kontominas, Characterization and geographical discrimination of commercial Citrus spp. honeys produced in different Mediterranean countries based on minerals, volatile compounds and physicochemical parameters, using chemometrics, Elsevier Ltd, 2017. doi:10.1016/j.foodchem.2016.08.124.

[7] L.M. Estevinho, E.D. Chambó, A.P.R. Pereira, C.A.L. De Carvalho, V. De Alencar Arnaut De Toledo, Characterization of lavandula spp. honey using multivariate techniques, PLoS One. 11 (2016) 1–15. doi:10.1371/journal.pone.0162206.

[8] A. Terrab, M.J. Diéz, F.J. Heredia, Characterisation of Moroccan unifloral honeys by their physicochemical characteristics, Food Chem. 79 (2002) 373–379. doi:10.1016/S0308-8146(02)00189-9.

[9] E. Corbella, D. Cozzolino, Classification of the floral origin of Uruguayan honeys by chemical and physical characteristics combined with chemometrics, LWT - Food Sci. Technol. 39 (2006) 534–539. doi:10.1016/j.lwt.2005.03.011.

[10] J. Devillers, M. Morlot, M.H. Pham-Delègue, J.C. Doré, Classification of monofloral honeys based on their quality control data, Food Chem. 86 (2004) 305–312. doi:10.1016/j.foodchem.2003.09.029.

[11] J. Wang, X. Xue, X. Du, N. Cheng, L. Chen, J. Zhao, J. Zheng, W. Cao, Identification of Acacia honey adulteration with rape honey using liquid chromatography_electrochemical detection and chemometrics, Food Anal. Methods. 7 (2014) 2003–2012. doi:10.1007/s12161-014-9833-7.

[12] M. Bevilacqua, R. Bucci, A.D. Magrì, A.L. Magrì, R. Nescatelli, Chemometrics in food chemistry, Data Handl. Sci. Technol. 28 (2013) 171–233. doi:10.1016/B978-0-444-59528-7.00005-3.

[13] A. Pridal, L. Vorlova, Honey and its physical parameters, J. Anim. Sci. (2002) 439–444.

[14] M. Piana, L. Oddo, A. Bentabol, E. Bruneau, S. Bogdanov, C. Declerck, Sensory analysis applied to honey : state of the art, Apidologie. (2004) 26–37. doi:10.1051/apido:2004048.

[15] M. Pestorić, O. Šimurina, B. Filipčev, D. Jambrec, M. Belović, A. Mišan, N. Nedeljković, Relationship of physicochemical characteristics with sensory profile of cookies enriched with medicinal herbs, Int. J. Food Prop. 18 (2015) 2699–2712. doi:10.1080/10942912.2015.1004586.

[16] J. Louveaux, A. Maurizio, G. Vorwohl, Methods of melissopalynology, Bee World. 5 (1978) 139–153.

doi:10.1080/0005772X.1978.11097714.

[17] Y. Elamine, S. Aazza, B. Lyoussi, M. Dulce Antunes, L.M. Estevinho, O. Anjos, M. Resende, M.L. Faleiro, M.G. Miguel, Preliminary characterization of a Moroccan honey with a predominance of *Bupleurum spinosum* pollen, J. Apic. Res. 8839 (2017) 1–13. doi:10.1080/00218839.2016.1265759.

[18] S. Bogdanov, HARMONISED METHODS OF THE INTERNATIONAL IHC responsible for the methods :, (1997) 1–62.

[19] O.A. Naab, M.A. Tamame, M.A. Caccavari, Palynological and physicochemical characteristics of three unifloral honey types from central Argentina, Spanish J. Agric. Res. 6 (2008) 566–576.

[20] K. Brudzynski, D. Miotto, The recognition of high molecular weight melanoidins as the main components responsible for radical-scavenging capacity of unheated and heat-treated Canadian honeys, Food Chem. 125 (2011) 570–575. doi:10.1016/j.foodchem.2010.09.049.

[21] S. Bogdanov, P. Martin, C. Lüllmann, R. Borneck, C. Ch. Flamini, M. Morlot, J. Heretier, G. Vorwohl, H. Russmann, L. Persano-Oddo, A.. Sabatini, G.. Marcazzan, P. Marioleas, K. Tsigouri, J. Kerkvliet, A. Ortiz, T. Ivanov, Harmonised methods of the international honey commission, (2002). http://www.terezinka.cz/vcely/Med/IHCmethods_e.pdf.

[22] S. Aazza, B. Lyoussi, D. Antunes, M.G. Miguel, Physicochemical characterization and antioxidant activity of commercial Portuguese honeys, J. Food Sci. 78 (2013). doi:10.1111/1750-3841.12201.

[23] R. de O.R. Ribeiro, E.T. Mársico, E.F.O. de Jesus, C. da Silva Carneiro, C.A.C. Júnior, E. de Almeida, V.F. do N. Filho, Determination of trace elements in honey from different regions in Rio de Janeiro state (Brazil) by total reflection X-ray fluorescence, J. Food Sci. 79 (2014) 738–742. doi:10.1111/1750-3841.12363.

[24] European Union Directive (EU), Council Directive 2001/110 Relating to Honey, Off. J. Eur. Communities. 16 (2001) 1–12.

[25] O. Anjos, A.J.A. Santos, V. Paixão, L.M. Estevinho, Physicochemical characterization of Lavandula spp. honey with FT-Raman spectroscopy, Talanta. 178 (2018) 43–48. doi:10.1016/j.talanta.2017.08.099.

[26] B. Pasquini, M. Goodarzi, S. Orlandini, G. Beretta, S. Furlanetto, B. Dejaegher, Geographical characterisation of honeys according to their mineral content and antioxidant activity using a chemometric approach, Int. J. Food Sci. Technol. 49 (2014) 1351–1359. doi:10.1111/ijfs.12436.

Chapter III: Insight into the sensing mechanism of an impedance based electronic tongue for honey botanic origin discrimination

Chapter III. Insight into the sensing mechanism of an impedance based electronic tongue for honey botanic origin discrimination*

Abstract

An impedance based electronic tongue was developed and used to discriminate honey of different botanic origin. The e-tongue presented here is based on the small-signal frequency response of the electrical double-layer established between the honey solution and an array of four different sensing units composed by gold, carbon, indium-tin-oxide, and doped silicon. The ability of the e-tongue to discriminate honey of different floral origins was demonstrated by distinguishing honey from *Bupleurum* and *Lavandula* pollen prevalence. The honey fingerprint obtained with the e-tongue was validated by parallel melissopalenogical analysis and physico-chemical methods. It is demonstrated that the e-tongue is very sensitive to changes on the honey electrical conductivity. Small differences in electrical conductivity are introduced by the presence of ionisable organic acids and mineral salts. Moreover, we propose that the sensitivity of the tongue to changes in electrical conductivity can be explored to probe other complex liquid substances.

1. Introduction

Electronic tongue (e-tongue) technology is gaining attention from the scientific community as a method that can be applied in various fields, mainly to evaluate quality and authenticity of food products [1–5]. Over the last few years there have been extensive examples of sensitive, fast responding e-tongues for the classification and a certification of several types of beverages [3,6], olive oil [7], honey and coffee [8,9] and pollutants in water [3,4,10]. Detection of adulteration and counterfeiting of products is also being reported [4,11–14].

The e-tongue instrumentation is formed by an array of sensing units that can be composed by lipid membranes, ion-selective electrodes, conducting polymers or noble metals [3,15,16]. Because the identification of samples is most of the time a classification task, there has been a trend to use artificial intelligence and information visualization methods to enhance the performance of e-tongues[17]. Recent advances of e-tongues can be found in several reviews [4,15,16,18,19].

Several measuring methodologies can be used to address the sensing elements in e-tongue system. The most used are electrochemical measurements, namely, potentiometry,

* Y. Elamine, P.M.C. Inácio, B. Lyoussi, O. Anjos, L.M. Estevinho, M. da Graça Miguel, H.L. Gomes, Insight into the sensing mechanism of an impedance based electronic tongue for honey botanic origin discrimination, Sensors Actuators, B Chem. (2019). doi:10.1016/j.snb.2019.01.023.

amperometry and cyclic voltammetry. Advantages of the electrochemical methods include simple instrumentation and high sensitivity. On the other hand, there are some drawbacks as the use of intrinsically electroactive electrodes and the problem of integrating a stable, reliable miniaturized reference electrode. In 2002 Riul et al.[20] introduced the use of small-signal impedance measurements in electronic tongues. Low-signal impedance measurements offer the advantage of using materials composing the sensing units do not need to be electroactive and there is no need of a reference electrode. Impedance based e-tongues or impedimetric tongues are based on measuring the small-signal impedance of the electrical double-layer (EDL) or *Helmholtz layer* established at the electrode/electrolyte interface over a frequency range [21,22].

Honey is an attractive substance to be evaluated by e-tongues. The honey floral origin has a strong impact on the commercial value, thus the floral determination and certification play an important role in quality control. European legislation specifies that the commercialization of honey must made reference to the origin, whether blossom or plant (Codex Alimentarius Commission, 1970; Council Directive 74/409/EEC, 1974) [23,24]. Honey floral origin and honey adulteration are difficult to detect without performing laboratory analysis. Until now the most reliable method that can confirm honey botanic origin is known as mellissopalynogical analysis. This method basically counts the pollen species present in the honey. This is an expensive laboratorial analysis that requires trained people with scientific knowledge for visual identification of pollen species [25]. This method tends to be viewed as a reference tool in scientific research rather than the method of identifying the types of honey as merchandise. Other alternative methods for honey botanic origin characterization involve: the determination of the volatile fraction by gas chromatography–mass spectrometry [26,27], mineral content [28], physico-chemical parameters such as analysis of phenolic compounds [29,30], or phenolic profiling using liquid chromatography-tandem mass spectrometry [31–35]. These methods require rigorous sample preparation and expensive instrumentation. Because of this, the European Community is encouraging the development of harmonized analytical methods to verify the compliance with the quality specifications for different types of honeys. An e-tongue fulfils the requirements of a fast, inexpensive and a reliable tool to confirm that a particular sample belongs to a specific floral origin. It can be implemented in a portable hand instrument used in the field. Furthermore, an e-tongue will be an important asset to help the commercialization chain, to screen for counterfeiting and adulteration of honey.

Several studies reported the use of e-tongues to characterize honey. Wei et al.[36] used a commercial available potentiometric e-tongue (a-Astree from Alpha MOS company, France),

to classify honey of different floral and geographical origins. In other study the same group reported the use of a voltammetry electronic tongue to classify monofloral honeys of seven kinds of floral origins [37]. Dias et al. [11] reported on the use of an array with 20 all-solid-state potentiometric electrodes with polymeric membranes, to discriminate commercial honeys with different pollen profiles. A potentiometric e-tongue was also used as a tool to distinguish honey in three different states: raw, liquefied and pasteurize [38].

In addition to the use of e-tongues some authors use a combination of different measuring methods to probe honey. Chirantan et al. [14] used impedance spectroscopy complemented with optical methods as a tool for quantification of adulteration in different floral species of honey. Ulloa et al.[22] compared the performance of optical measurements with an electronic tongue and a study by Graziella et al. [21] demonstrated that impedance spectroscopy alone, also can be used to determine honey floral origin.

Despite the success of numerous studies on the use of e-tongues to probe honey, none of the above studies provides clear insight into the electrochemistry behind the ability of the tongues to discriminate the floral origin of honey samples.

It is also important to note, that although, the reported e-tongues use different measuring methods it is evident that all reported tongues share a common detecting mechanism. This mechanism relies on the establishment of an EDL or *Helmholtz* layer at the interface between a sensing electrode and the honey solution. The *Helmholtz* layer is the critical sensing layer in all the e-tongues reported for honey. Minute perturbations in this interfacial polarized layer can be probed by measuring the potential at the electrode (potentiometric measurements), by using voltage ramps (voltammetry) or by using small alternate signals (impedance spectroscopy).

This paper starts by presenting the individual characterization of the sensor units forming the electronic tongue. The stability and reproducibility of each sensing electrode is presented in detail. The ability of each electrode to discriminate different samples is then presented. While some materials such as gold, perform well, others such as doped *n*-type silicon have a low ability to discriminate the floral origin of honey samples. The e-tongue fingerprint was confirmed and validated by mellissopalenogical analysis in combination with standardized physico-chemical characterization involving the sugar content, the electrical conductivity, and the light absorbance. Finally, the discussion section highlights the major advantages of implementing a portable e-tongue instrument.

2. Material and methods

2.1. Honey samples

Eighteen samples of *Bupleurum* floral origin from Morocco were purchased in the summer of 2015 directly from beekeepers and from Taza and Fez beekeeper associations. This honey type was described for the first time by Elamine et al. [39] Thirty one honey samples with lavender floral origin were purchased from beekeepers from different regions in Portugal. The samples were stored in glass containers and kept under dark ambient conditions.

Electrical conductivity, moisture content, colour, glucose and fructose contents were determined according to the procedures described in literature [40]. The quantitative analysis of pollen spectrum in honey samples was performed according to the International Commission for Bee Botany (ICBB), as previously described [25]. For the e-tongue measurements, honey samples were diluted in Millipore water.

2.2. Experimental set-up and statistics

Fig.1 (a) shows a photograph of the e-tongue and Fig. 1 (b) shows a schematic diagram of the spatial arrangement of the sensing electrodes. The electrodes were placed inside of a 15 ml volume plastic vessel to hold 10 ml honey solution. Small-signal impedance measurements were carried out by an impedance analyser, a Fluke PM 6306. Both the capacitance (C_P) and resistance (R_P) were individually measured as function of frequency between each sensing electrode and a common counter electrode. The amplitude of the ac signal was 50 mV. Each honey sample is characterized by 8 impedance spectra. Each spectra measures 49 different frequencies from 60 Hz up to 1 MHz. A honey sample is then characterized by a total of 392 experimental data points. Principal Component Analysis (PCA) [41] was used to process the impedance data.

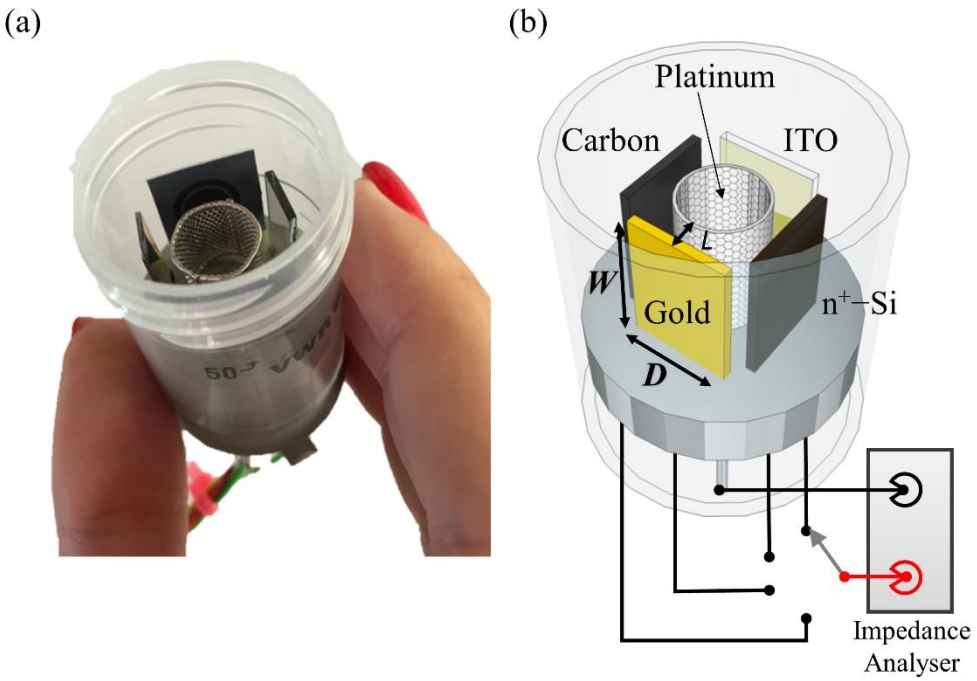

Figure. 1. (a) Photograph of an e-tongue, (b) schematic of the e-tongue showing the spatial distribution of the electrodes and electrical connections. Gold, ITO and n+- silicon electrodes have an individual active area of 1 cm2. The carbon electrode has an area of 0.39 cm2.

The e-tongue was formed by a set of four distinct sensing electrodes made of conducting materials. As conducting materials, we use (i) thermal evaporated thin film of gold on a glass slide, (ii) a carbon film, (iii) a glass slide coated with an Indium-Tin-Oxide (ITO) layer and (iv) a highly doped *n*-type silicon wafer (n^+-Si). A platinum grid was used as a common electrode. The carbon thin film electrodes are screen-printed on top of Valox and they are manufactured by Gwent Group Ltd (ref: BE2150327D2/001).

ANOVA statistical test was used for comparing the means of the used parameters and for the estimation of the significance level. The difference is considered significant when $p<0.05$.

The PCA analysis and dendrograms were carried out using MATLAB scripts.

2.3. Results and discussion

2.3.1. Characterization and equivalent circuit of the e-tongue

The sensing mechanism of an impedimetric tongue relies on measuring the impedance of the electrical double-layer (EDL) established at the electrode/honey solution interface. It is then important to demonstrate that our frequency observation window (60 Hz to 1 MHz) probes the EDL impedance and not only the honey bulk solution.

Fig. 2 (a) shows for the gold/honey solution/platinum electrode interface the frequency dependence of the capacitance (C_P) and the loss (G_P=1/(ωR_P) where ω is the angular frequency. Fig. 2 (b) shows the same impedance data but now represented as loss tangent (tan δ), which is given by tan δ=G_P/(C_P). The tan δ curve shows a Maxwell-Wagner relaxation at approximately 30 kHz. A Maxwell-Wagner relaxation is expected when the system being measured behaves as two different layers in series. From a physical point of view, the two layers in series are formed by the EDL in series with the bulk electrolyte layer. These two layers have very different impedances. The EDL has a significant higher capacitance. As the frequency increases, there is a transition from the high capacitive layer (interface) to the lower capacitive layer (bulk electrolyte). The relaxation is known as Maxwell-Wagner relaxation and gives rise to a peak in tan δ curve as function of the frequency as shown in Fig. 2 (b).

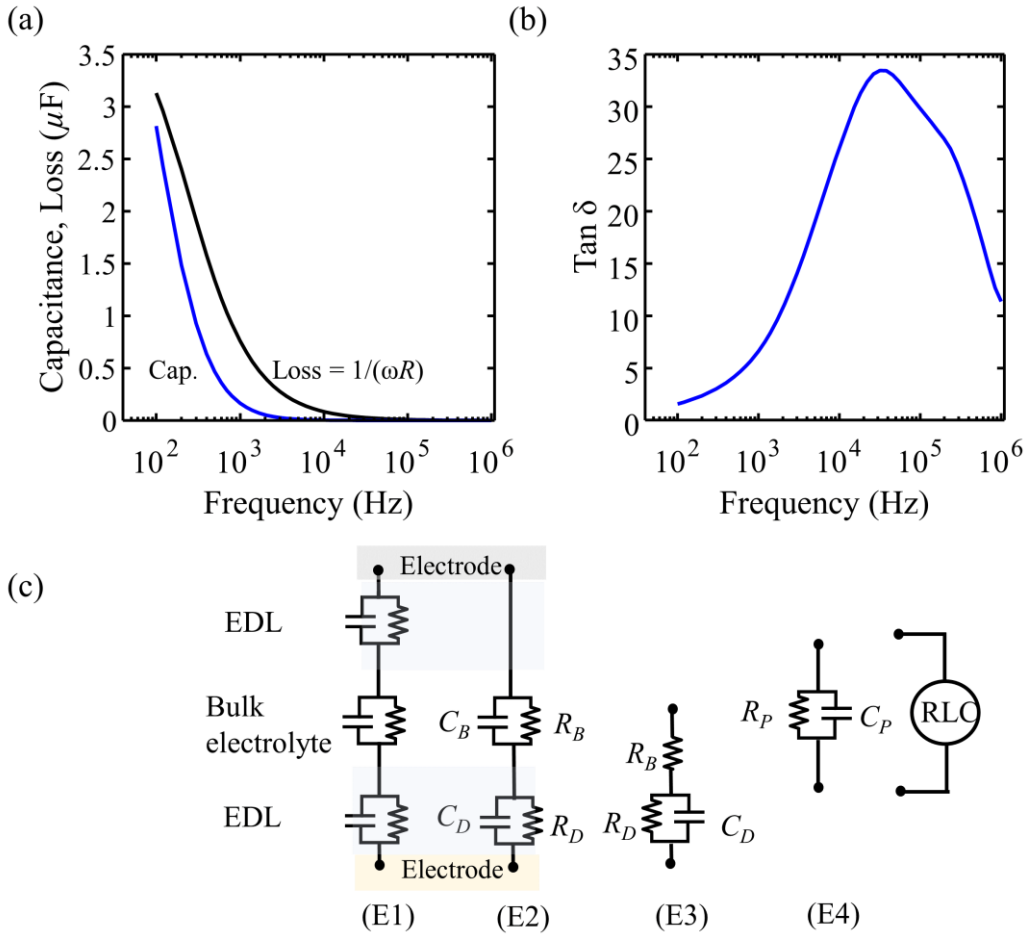

Figure. 2. Frequency dependence of the gold/honey/platinum impedance. (a) Frequency dependence of the capacitance (CP) and loss=1/(ωRP). (b) Frequency dependence of the tan δ (c) Equivalent circuit description of the electrodes immersed into the honey solution.

The above discussion can be proper understood with the help of equivalent circuits. From an electrical point of view, an EDL behaves as a capacitor. Therefore, the electrode in contact with the honey solution is modelled as a capacitor in parallel with a resistor. Since the

electrodes are made of different materials, two different EDLs layers are established, one at the sensing and other at the counter electrode (platinum) as shown in Fig. 2 (c) (circuit E1). Comparatively to the bulk electrolyte layer the two EDLs have higher impedance. Although, the two EDLs are different, for the sake of simplicity, the layers can be combined in a single equivalent circuit (circuit E2). The circuit E2 combines two EDLs layers by a C_D in parallel with R_D. The interfacial capacitance C_D models the extent to which the electrode is polarizable, and R_D takes into account the presence of Faradic currents. Faradic currents result from oxidation/reduction reactions occurring at the electrode/electrolyte interface. These reactions cause the transduction of charge carriers from electrons in the metal electrode to ions in the electrolyte. As it will be shown later the low frequency value for R_D is very high and the amplitude of the testing signals is only 50 mV. Therefore, Faradic currents are very small and can be neglected. The parallel network (R_D//C_D) of the double-layer appears in series with a honey solution resistance (R_B) and capacitance (C_B). The equivalent circuit described above and schematically represented in Fig. 2 (c) circuit (E2) allows us to understand the frequency response of the electrode/honey interface.

Because the distance (d) between sensing and counter electrode is relatively large (d=5 mm) C_B is very small and can be neglected. The overall system can them be described the equivalent circuit E3 on Fig. 2 (c). The circuit E3 is a reduction of the more complex model. In reality the interfacial capacitance is not a pure electrical capacitance. The capacitive element C_D is more accurately described as a Constant Phase Element (CPE) [42]. A complete equivalent model of the electrode/electrolyte interface should include the so-called "Warburg" diffusion element. A popular example is the Randles circuit [43]. The Randles circuit can simulate the low frequency dispersion on the impedance. However, the impact of the diffusion or constant phase elements on the data analysis carried out here is relatively small. For the sake of simplicity these elements are not included in the circuit model of Fig. 2. The circuit E3 represents the overall equivalent parallel resistance (R_P) and capacitance (C_P) measured externally using an impedance analyser or by a RCL meter.

The equivalent admittance Y_T of the series-parallel network is given by

$$Y_T = \frac{1}{R_P} + j\omega C_P \qquad (1)$$

Where R_P and C_P are the total parallel resistance and capacitance, respectively, given by

$$C_P(\omega) = \frac{R_D^2 C_D + R_B^2 C_B + \omega^2 R_D^2 R_B^2 C_D C_B (C_D + C_B)}{(R_D + R_B)^2 + \omega^2 R_D^2 R_B^2 \left(C_D + C_B\right)^2} \qquad (2)$$

$$R_P(\omega) = \frac{(R_D + R_B)^2 + \omega^2 R_D^2 R_B^2 (C_D + C_B)^2}{R_D + R_B + \omega^2 R_B R_D (R_D C_D^2 + R_B C_D^2)} \quad (3)$$

This circuit exhibits dispersion in capacitance centered around a relaxation frequency, f_r , given by

$$f_r = \frac{1/R_B + 1/R_D}{2\pi(C_B + C_D)} \quad (4)$$

Which for $R_D >> R_B$ reduces to

$$f_r = \frac{1}{2\pi R_B (C_B + C_D)} \quad (5)$$

The resistance R_B controls f_r. This frequency and depends on the electrolyte conductivity but also on the separation between electrodes and sensing area. According to Fig. 2 (b) the relaxation frequency is higher than 10 kHz. Therefore, it is demonstrated that the e-tongue is proper designed to record the Maxwell-Wagner relaxation and also to probe the impedance of the sensing electrode/honey interface, which dominates the impedance for frequencies lower than f_r.

2.3.2. Reproducibility and repeatability

Prior to use as an e-tongue instrument, the reproducibility and repeatability of the impedance of each electrode was evaluated. In order to inspect for impedance drifts as function of time, measurements were performed for periods as long as 6 hours. This time is significant longer that the time required to perform a full impedance characterization of a honey solution, which is less than 1 minute. Fig 3. (a) shows that for all electrodes the capacitance remains constant for a six hours long period. To inspect for reproducibility, 10 measurements were carried out using the same honey sample in a repeated fashion. Between measurements, the tongue was washed with ultrapure water and dried. The error bars in Fig. 3 (b) show the impedance variations for each electrode. The data is represented as tan δ. This impedance parameter is defined as $C_P/(\omega G_P)$. The ITO sensor showed the lowest reproducibility with a standard deviation of 50.43±11.83 (23.5%) in the peak of tan δ curve. The gold electrode is the sensor with the highest reproducible behaviour with a standard deviation of 33.01±0.37 (1.1%).

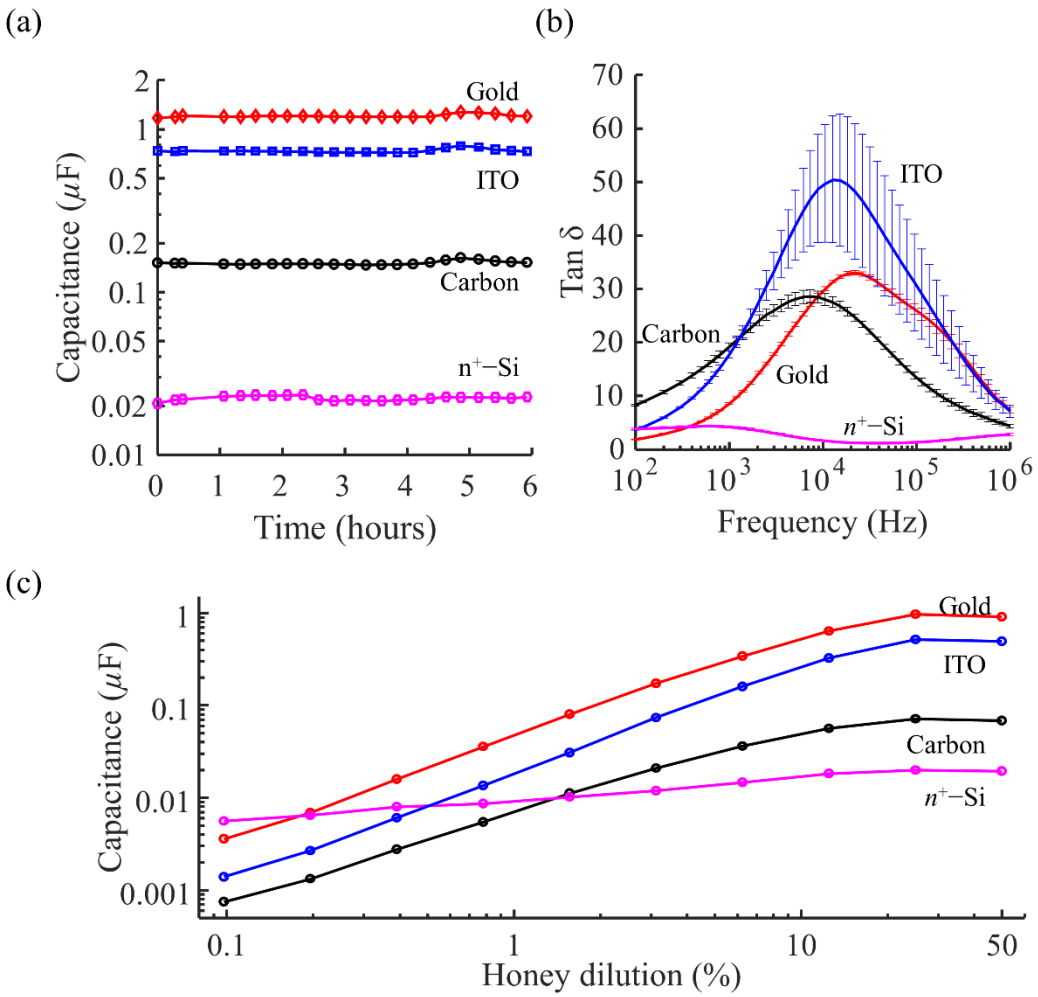

Figure. 3. E-tongue electrical stability and reproducibility. (a) Time dependence of the capacitance for all the sensing electrodes measured at 100 Hz. (b) the repeatability assay for all the sensors. Results are expressed as tan δ±standard deviation. (c) CP dependence on the honey dilution factor.

2.3.3. Study of individual sensing units forming the e-tongue.

In the following sections the e-tongue is used to characterize different samples of honey. The goal is to demonstrate the ability of the tongue to discriminate honey of different floral origin. The characterization was performed using the frequency dependence of the impedance. First the performance of each individual sensing electrode was evaluated. Fig. 4 shows the PCA using the tan δ data as function of frequency for each individual sensing electrode. In contrast to other e-tongue methodologies in which each sensor provides only one single value (potentiometry), impedance measurements provide an *m***n* matrix for each sensor. Here *m* corresponds to the tan δ values at different frequencies, and *n* to the number of honey samples. The number of frequency points (49) is equal to the number of honey samples.

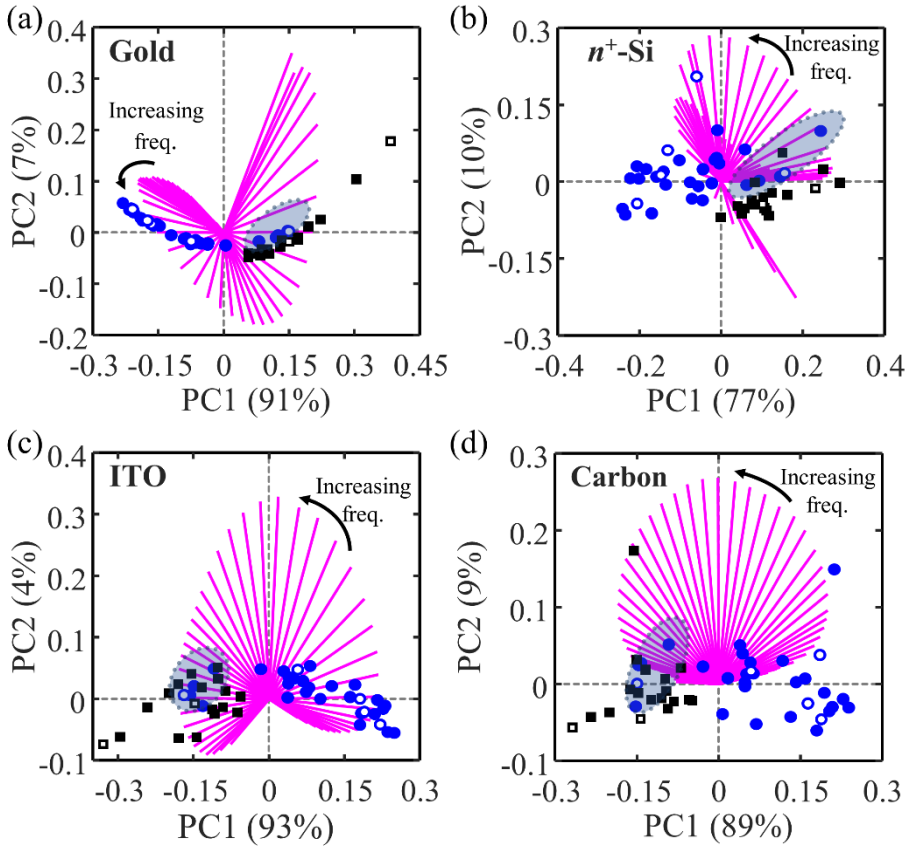

Figure. 4. PCA discrimination patterns for etongue. (a) Gold sensor. (b) n+-Si sensor. (c) ITO sensor. (d) Carbon sensor. Blue circles represent samples with lavender pollen prevalence; black squares samples with Bupleurum pollen prevalence. Empty black squares and empty blue circles represent odd samples purchased, as belonging to a specific floral origin but this origin was not confirmed using melissopalynology (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The black squares represent the Zandaz honey samples which have a *Bupleurum* pollen prevalence. The filled blue circles represent the samples of honey with lavender pollen prevalence. In addition, the plots in Fig. 4 also shown 4 odd samples that although have been purchased as lavender type (empty blue circles) or Zandaz honey type (empty black squares) their melissopalynological analysis did not show a prevalence of corresponding pollen species. In spite of that, physico-chemical methods confirm these odd samples indeed belong to their floral origin. These samples remain in the border of their corresponding cluster.

In addition to the experimental data extracted from the frequency response of tan δ, Fig. 4 (a) also shows a distribution of how tan δ values at specific frequencies correlate the obtained principal components. The pink lines in Fig. 4 show this correlation. The projection of a pink line end point on the XX axis is the relevance of tan δ value by the first principal component (PC1). In other words, the higher is a coordinate value on a given axis, more relevant is its relevance by the corresponding principal component, and more important is its role in discriminating honey samples. The signal frequency used to measure tan δ is an implicit parameter represented by the arrow in Fig. 4. The pattern created by the pink lines is different

for each electrode and this pattern reflects the ability of each electrode to discriminate different varieties of honey.

Two distinct clusters of data distribute along two well-separated curves, the blue circles follow a curve starting near the axis of the PC1 and bending upwards into the second quadrant (Fig. 4 (a)). These data points correspond to the lavender honey samples with normal electrical conductivity. Honey from Zandaz is represented by the black squares. The sample distribution also follows a curve starting near the origin but extending into the first quadrant. The two curves almost mix with each other over a small region, represented by the blue shadow area. Zandaz honey samples have a high tan δ value at high frequencies, which explain their location in the positive part of the PC1.

Fig. 4 (b) shows the PCA analysis of the tan δ data for the doped silicon (n^+-Si) sensor. This sensor has the lowest interfacial capacitance (0.02μF at 100 Hz). The first component (PC1) explained 77% and PC2 10% of the given data. In contrast, to the gold sensor, the n^+-Si sensor shows sparsely distributed data points in all the quadrants of the PCA plot. This is particular visible for the samples with lavender floral origin. The cluster from Zandaz honey samples aggregates into a narrow region located in the fourth quadrant. The shadow blue region includes the odd samples with lavender floral origin. Although, the n^+-Si sensor shows a broad distribution, particular for the honey with lavender origin, there is no mixing between the two clusters in the PCA plot. Samples from Zandaz cluster into a narrow region in the fourth quadrant of the PCA plot.

The performance of the indium tin oxide (ITO) sensing electrode is demonstrated in the plot (c) of Fig. 4. The two varieties of honey are now organized into two well-separated clusters in distinct quadrants. Honey with lavender floral origin is mostly located in the first quadrant while the honey with *Bupleurum* pollen prevalence is clustering in the second quadrant. The shadow blue regions show again the location of the 4 odd samples with lavender origin. The odd samples are now mixed into the Zandaz cluster.

Fig. 4 (d) shows the ability of the carbon electrode to discriminate the two types of honey. The honey samples are distributed into two clusters resemble to the distribution provided by the ITO electrode, however, in comparison with the ITO, the data points are more spread. The ability of the carbon electrode to discriminate honey with different origins is relatively poor. In a similar way as the ITO electrode the carbon electrode wrongly classifies the 4 odd lavender floral origin as samples with *Bupleurum* pollen prevalence.

In summary, the above analysis reveals that different electrodes have different ability to discriminate honey with different floral origin. The gold sensing electrode shows the highest ability to discriminate the two varieties of honey. The gold electrode organizes the data into two well-defined curves that diverge from the centre of the PCA plot into opposite directions. Comparatively to the gold electrode the data obtained by the ITO electrode is spread over the 4 quadrants but still organized into two well-separated clusters. The carbon and the n^+-Si electrodes have the lowest ability to discriminate honey samples.

2.3.4. Integration of the information provided by the four electrodes and validation of the e-tongue

Since, the e-tongue has four sensing electrodes, we may use the entire set of data from all the electrodes to construct PCA plots. Fig. 5 (a) shows the PCA plot constructed using the tan δ data from the 4 sensing electrodes. The discrimination of the two types of honey is now remarkably clear. The two varieties of honey distributed along two well-defined lines in distinct quadrants.

Fig. 5 (b) shows the PCA plot using the individual resistance (R_P) and the capacitance (C_P) impedance parameters. Comparatively to the tan δ data, the samples are still organized into two distinct clusters but spread over the PCA plot.

In order to evaluate how the electronic tongue performs in respect with other conventional physico-chemical methods. The honey samples were also characterized by, (i) light absorbance, (ii) water content, (iii) glucose and fructose contents and (iv) by electrical conductivity measurements. The higher is the honey conductivity the higher the mineral content [17] [26]. Light absorbance at several wavelengths was used to quantify melanoidins, phenolic and flavonoid contents [28]. It is important to state that the use of physico-chemical raw data does not provide a good discrimination between honey samples and the data must also be processed using PCA analysis. Fig. 5 (c) shows a PCA biplot of honey samples using normalized physico-chemical parameters mentioned above. The red lines show the relationship between the physico-chemical parameters with the principal components. The PC1 and the PC2 explained 60% and 17%, of the given data respectively, and correlated the electrical conductivity, the absorbance values in the first case and glucose, fructose and water contents in the second case. Honey samples with *Bupleurum* pollen prevalence (black squares) are discriminated from samples with lavender floral origin (blue circles) by the first component, correlating positively the electrical conductivity and the absorbance values.

Glucose, fructose and water contents showed little effect in discriminating the honey types (see Fig 5 (c), but enable to distinguish subgroups in each floral origin. Samples from both botanical origins with similarity in terms of glucose content have tendency to occupy the positive part of the second principal component, while those with fructose abundance occupied the negative part. Samples located in the red shadow region of the PCA plot have lavender floral origin. However, these samples are also characterized by an unusual high conductivity and color intensity compared to the remaining lavender samples. The PCA analysis classified them in a middle region between the two botanical honey types.

Fig. 5 (d) shows the PCA biplot of the data integrating both the information of the physicochemical characterization and the mellissopalenological analysis. The studied parameters/variables are shown in red lines. Only the important ones are labelled by their names. The first two components explained a sum of 98% of the given data, and correlated in the first component electrical conductivity as a major parameter. The second component correlated the major influencing pollen species, mainly *Lavandula* and *Bupleurum spinosum*, and allowed the discrimination between the samples of the different floral origins. The integration of the information provided by both characterization tools, makes the isolation of the odd lavender honey samples (samples in the red shadow region) clearer.

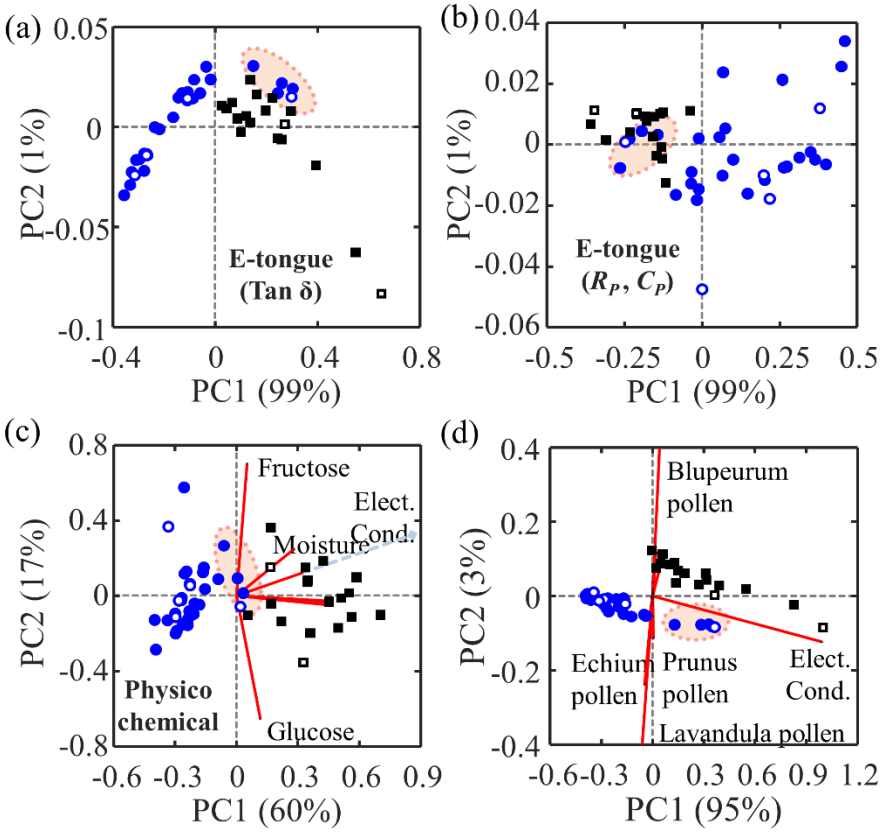

Figure. 5. The discrimination power of e-tongue in comparison with and the physicochemical parameters. (a) PCA biplot using the overall data (tan δ). (b) PCA biplot using the resistance (RP) and capacitance (CP) data. (c) PCA

biplot using normalized physicochemical characterizations (d) PCA biplot using both pollen data and physicochemical parameters. Black squares represent honey samples with Bupleurum pollen prevalence and blue circles represent lavender floral origin (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Honey from a specific floral origin does not have 100% of pollen from single specie but the overall composition has a percentage of other pollen species. It is known that depending of the percentage and composition of these secondary pollen species is possible to divide the honey into subgroups with different composition. These sub-groups with a specific dominant floral origin area not revealed by the PCA analysis. The identification of these subgroups is done by hierarchical clustering. A cluster tree known as dendrogram is used to represent data. The dendrogram looks for the correlation between samples and identifies sets of samples (subgroups) that are similar. Fig. 6 shows two dendrograms for the honey samples studied. The dendrogram have been made using the PCA analysis of the e-tongue data. The samples are arranged along the bottom of the dendrogram and referred to as honey sample numbers. The vertical axis is labelled distance (*D*) and measures the dissimilarity between honey samples. Highly similar subgroups are nearer the bottom of the dendrogram.

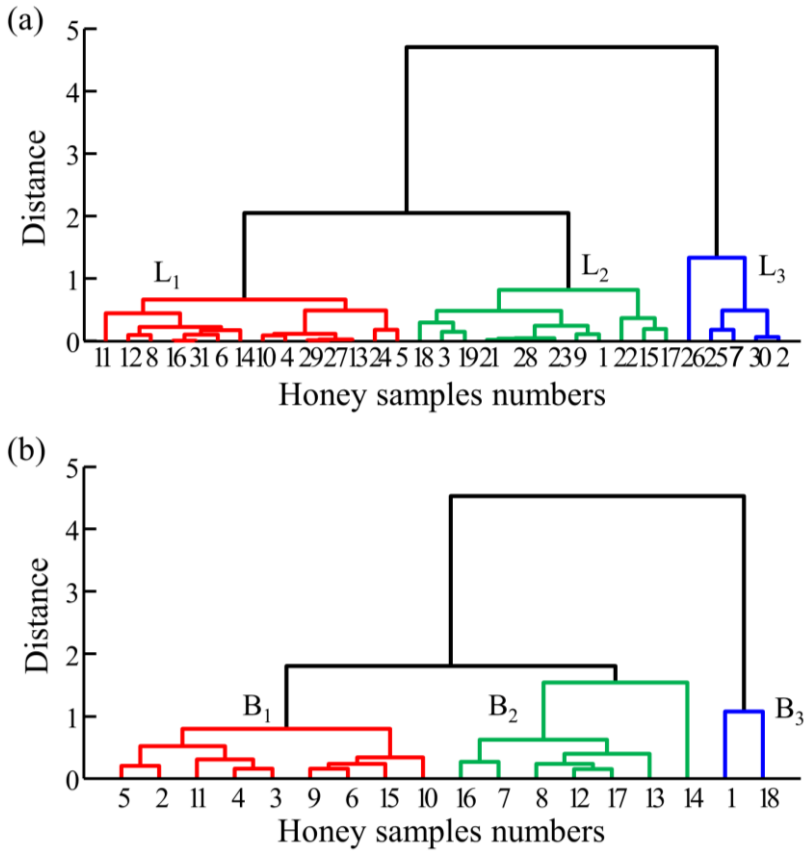

Figure. 6. Dendrogram of the scores resulted from the PCA in Fig. 4. (a) Dendrogram of Lavandula honey; The three clusters were labelled as L1, L2 and L3. (b): Dendrogram of Bupleurum honey; The three obtained clusters were labelled as B1, B2 and B3.

Fig. 6 (a) shows a dendrogram based on the data of the first two principal components of the e-tongue data of Fig. 5(a). The dendrogram reveals three main sub-groups within the

Lavandula honey group. These sub-groups are identified as L_1, L_2 and L_3. Similar analysis was carried out for *Bupleurum* based honey. Again, three different subgroups named B_1, B_2 and B_3 are distinguished. These subgroups are shown in Fig. 6 (b). The use of a dendrogram analysis of the e-tongue data shows that it is possible to identify subgroups within the main floral group.

It is now important to understand what the differences between subgroups are. This identification requires a detailed physico-chemical analysis of the samples belonging to each subgroup. Table I, presents the physico-chemical parameters of each subgroup. All subgroups have almost identical sugar and water contents. However, they have marked differences on the electrical conductivity and colour. Fig. 7 shows histograms for colour and electrical conductivity. The sub-group L_1 has the lowest electrical conductivity and the lighter colour, the subgroup L_3 has the highest electrical conductivity and colour intensity. L_2 has values of conductivity and colour in between the other two subgroups. Similar behaviour is showed for the *Bupleurum* based honey, the subgroups B_1, B_2 and B_3 are also characterized by differences on electrical conductivity and colour.

A comparison between the histogram for *Bupleurum* and *Lavandula* based honey also shows that while in lavender type of honey colour increases with honey conductivity, the opposite behaviour is observed for the *Bupleurum* based honey. In this case the higher the electrical conductivity more transparent becomes the *Bupleurum* based honey. Although colour and conductivity seems to be somehow linked, this relation depends on the type of secondary species contribution to the main honey composition. Further studies are required to elucidate this interesting relation between honey colour and electrical conductivity.

The physical chemical analysis reveals that the e-tongue is able to distinguish not only the floral origin of the honey, but furthermore, the tongue is also sensitive to changes in the electrical conductivity. Fig. 7 (a) shows that the e-tongue can distinguish differences in electrical conductivity of 100 μS/cm and differences in colour of 8 mm Pfund. The real discrimination sensitivity may even be lower. Further studies with samples with more similar values for conductivity and colour are required to evaluate the discrimination threshold of the impedimetric e-tongue.

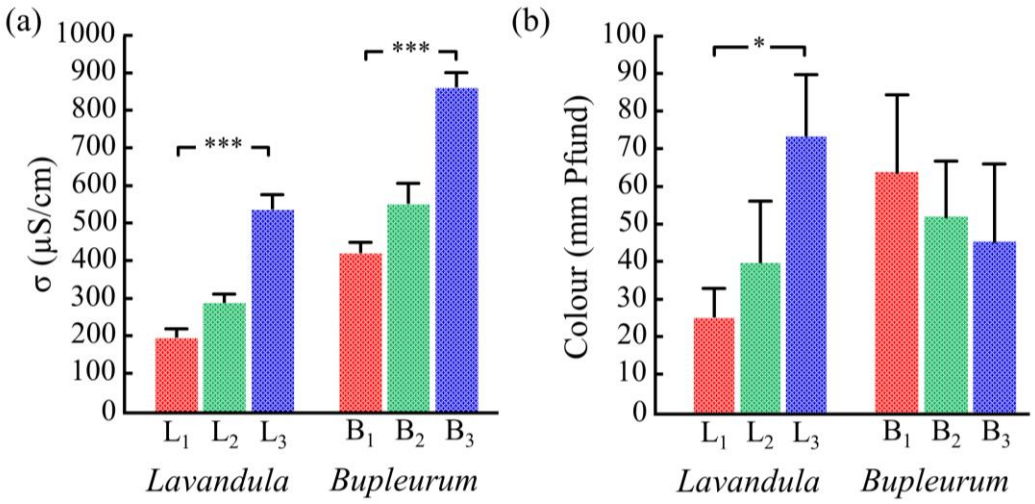

Figure. 7. Electrical conductivity and colour of the two honey subgroups L1, L2 and L3 (Fig. 6 (a)) for Lavandula honey and B1, B2 and B3 for Bupleurum honey (Fig. 6 (b)). (a) electrical conductivity and (b) colour for the two groups. The values are plotted as mean ± SD, difference was accepted to be significant with a α=0.05. *$p < 0.05$; ***$p < 0.001$.

Table 1 also shows that the sub-groups within each type of honey do not show differences in terms of moisture, glucose, and fructose contents. Therefore, we may conclude that the within a specific floral origin of honey, the sugar and water contents remains unchanged. The changes occur in the electrical conductivity which according to the literature is caused by the contribution of other botanical species [44,45]. This shed light why the e-tongue has a quite powerful discriminating power. The impedimetric tongue relies on the electrical properties of the electrical double-layer. The interfacial impedance of the EDL is strongly dependent on the electrical conductivity of the electrolyte solution. The interfacial capacitance and resistance vary dramatically upon changes on the electrolyte solution. In our study the changes in electrical conductivity amongst subgroups of the same honey are introduced by the presence of secondary botanical species. By detecting minute changes on the electrical conductivity, the e-tongue is able to distinguish variations matching the changes in the pollen composition.

Table 1. Melissopalenological, and physico-chemical characterization of the honey samples. Results are presented as Mean±SD.

	Honey from *Lavandula* floral origin			**Honey from *Bupleurum* floral origin**		
Subgroup	L_1	L_2	L_3	B_1	B_2	B_3
***Lavandula* pollen (%)**	37.76±8.39	42.41±18.79	31.71±11.55	-	-	-
***Bupleurum* pollen (%)**	-	-	-	58.18±14.63	46.64±12.67	34.13±15.16
Electrical conductivity (µS/cm)	186.76±23.35	278.22±33.15	522.48±45.53	405.11±33.65	540.71±56.70	853.16±47.16

Colour (mm Pfund)	25.28±7.12	39.91±15.97b	73.28±15.71	63.10±21.72	51.75±15.63	44.67±22.09
Moisture content (%)	18.36±0.67	18.88±0.49	18.76±0.50	19.73±0.60	19.58±1.03	19.05±0.35
Glucose (%)	24.03±4.53	22.80±1.82	24.64±2.49	24.04±1.87	25.28±3.65	23.24±0.07
Fructose (%)	36.14±2.86	35.57±1.95	34.99±2.99	38.00±2.17	37.47±2.46	34.72±1.20

The e-tongue ability to probe honey was evaluated with honey samples with identical sugar contents but with very different colour and pollen composition. Differences in electrical conductivity are somehow related with the colour and pollen composition as reported previously by other authors [44]. This relationship makes electrical conductivity a useful parameter for discriminating honeys of different floral origin [46]. It is also known that the acidity of any honey is directly related to its floral sources. Honey contains a number of different acids, including about 18 amino acids, many different organic acids, as well as aliphatic and aromatic acids. The aromatic acids greatly contribute to the flavour of honey [5]. Honey electrical conductivity is strongly related with the acidity and it has been reported that natural acidity of honeys may increase with the storage duration, especially when extracted from combs with propolis or when honey deteriorates due to fermentation [47]. Moreover, honey adulteration with sugar syrup lower its acidity while a honey sample adulterated with inverted sugar present a higher acidity [48]. Since the tongue developed and presented here is highly sensitive to ionisable species it should also be able to detect honey adulteration and ageing.

The e-tongue relies on the use of 4 different sensing electrodes. However, the performance comparison between the different electrodes shows that the use of gold electrode alone has the required discrimination ability and does not lower the overall e-tongue performance. The use of expensive platinum electrode can be discharged in future e-tongue implementations. All sensing electrodes were selected on terms of stability and reusability after proper washing with water.

3. Conclusions

An impedimetric electronic tongue was developed and successful applied to discriminate honeys with different floral origin. The tongue can distinguish *Bupleurum* and *Lavandula* based honeys with almost identical sugar and moisture contents.

The sensing unit is comprised of 4 different bare electrodes (not functionalized). A comparison between the different sensing electrodes tested, reveals that the best ability to

discriminate honey is achieved by the electrode with the highest interfacial capacitance per unit area, the gold electrode. However, the combined use of the four sensing electrodes provides the highest discriminating power. The e-tongue stability, reliability and reusability were also demonstrated.

The use of dendrograms complemented with physico-chemical analysis provides clear insight into the sensing mechanism. The e-tongue is essentially sensitive to changes in the honey electrical conductivity. Differences in electrical conductivity between samples with different floral origin are brought-in by the presence of ionisable organic acids and mineral salts.

Comparatively, to conventional conductivity measurements relying in bulk measurements, the e-tongue is extremely sensitive to minute changes on the electrical conductivity. The ultra-high sensitivity to ionisable substances occurs because the tongue probes the low-frequency impedance variations occurring in an ultra-thin interfacial polarizable electrode/electrolyte interface (*Helmholtz layer).*

The e-tongue configuration and the measuring methodology proposed here may be applied to a variety of other complex liquids where changes in ionisable species occur.

Acknowledgments

We gratefully acknowledge financial support from the Portuguese Foundation for Science and Technology (FCT), the Instituto de Telecomunicações (UID/Multi/04326/2013) and the Centro de Estudos Florestais (UID/AGR/00239/2013).

4. References

[1] H. Smyth, D. Cozzolino, Instrumental methods (Spectroscopy, Electronic Nose, and Tongue) as tools to predict taste and aroma in beverages: Advantages and limitations, Chem. Rev. 113 (2013) 1429–1440. doi:10.1021/cr300076c.

[2] P.D. Patel, (Bio)sensors for measurement of analytes implicated in food safety: A review, TrAC - Trends Anal. Chem. 21 (2002) 96–115. doi:10.1016/S0165-9936(01)00136-4.

[3] C.M. Daikuzono, C.A.R. Dantas, D. Volpati, C.J.L. Constantino, M.H.O. Piazzetta, A.L. Gobbi, D.M. Taylor, O.N. Oliveira, A. Riul, Microfluidic electronic tongue, Sensors Actuators B Chem. 207 (2015) 1129–1135. doi:10.1016/j.snb.2014.09.112.

[4] Y.. Vlasov, A.. Legin, A.. Rudnitskaya, A. D'Amico, C. Di Natale, «Electronic tongue» — new analytical tool for liquid analysis on the basis of non-specific sensors and methods of pattern recognition, Sensors Actuators B Chem. 65 (2000) 235–236. doi:10.1016/S0925-4005(99)00323-8.

[5] F. Winquist, P. Wide, I. Lundström, An electronic tongue based on voltammetry, Anal. Chim. Acta. 357 (1997) 21–31. doi:10.1016/S0003-2670(97)00498-4.

[6] A. Riul, H.C. De Sousa, R.R. Malmegrim, D.S. Dos Santos, A.C.P.L.F. Carvalho, F.J. Fonseca, O.N. Oliveira, L.H.C. Mattoso, Wine classification by taste sensors made from ultra-thin films and using neural networks, Sensors Actuators, B Chem. 98 (2004) 77–82. doi:10.1016/j.snb.2003.09.025.

[7] L.G. Dias, N. Rodrigues, A.C.A. Veloso, J.A. Pereira, Monovarietal extra - virgin olive oil classification : a fusion of human sensory attributes and an electronic tongue, Eur. Food Res. Technol. 242 (2016) 259–270. doi:10.1007/s00217-015-2537-4.

[8] A.M.S. Costa, M.M.C. Sobral, I. Delgadillo, A. Rudnitskaya, Electronic tongue as a rapid tool for the assessment of coffee flavour and chemical composition, in: IEEE SENSORS 2014 Proc., IEEE, 2014: pp. 2126–2129. doi:10.1109/ICSENS.2014.6985458.

[9] E.J. Ferreira, R.C.T. Pereira, A.C.B. Delbem, O.N.O. Jr, L.H.C. Mattoso, Random subspace method for analysing coffee with electronic tongue, Electron. Lett. 43 (2007) 5–7. doi:10.1049/el.

[10] F. Winquist, J. Olsson, M. Eriksson, Multicomponent analysis of drinking water by a voltammetric electronic tongue, Anal. Chim. Acta. 683 (2011) 192–197. doi:10.1016/j.aca.2010.10.027.

[11] L. a. Dias, A.M. Peres, M. Vilas-Boas, M. a. Rocha, L. Estevinho, A. a S.C. Machado, An electronic tongue for honey classification, Microchim. Acta. 163 (2008) 97–102. doi:10.1007/s00604-007-0923-8.

[12] K. Tiwari, B. Tudu, R. Bandyopadhyay, A. Chatterjee, Identification of monofloral honey using voltammetric electronic tongue, J. Food Eng. 117 (2013) 205–210. doi:10.1016/j.jfoodeng.2013.02.023.

[13] G. Pioggia, F. Di Francesco, A. Marchetti, M. Ferro, A. Ahluwalia, A composite sensor array impedentiometric electronic tongue. Part I. Characterization, Biosens. Bioelectron. 22 (2007) 2618–2623. doi:10.1016/j.bios.2006.10.031.

[14] C. Das, S. Chakraborty, K. Acharya, N. Kumar, FT-MIR supported Electrical impedance spectroscopy based study of sugar adulterated honeys from different floral origin, Talanta. 171 (2017) 327–334. doi:10.1016/j.talanta.2017.05.016.

[15] A. Riul, C.A.R. Dantas, C.M. Miyazaki, O.N. Oliveira, Recent advances in electronic tongues, Analyst. 135 (2010) 2481–2495. doi:10.1039/c0an00292e.

[16] L. Escuder-Gilabert, M. Peris, Review: Highlights in recent applications of electronic tongues in food analysis, Anal. Chim. Acta. 665 (2010) 15–25. doi:10.1016/j.aca.2010.03.017.

[17] C. Krantz-Rülcker, M. Stenberg, F. Winquist, I. Lundström, Electronic tongues for environmental monitoring based on sensor arrays and pattern recognition: A review, Anal. Chim. Acta. 426 (2001) 217–226. doi:10.1016/S0003-2670(00)00873-4.

[18] D. Ha, Q. Sun, K. Su, H. Wan, H. Li, N. Xu, F. Sun, L. Zhuang, N. Hu, P. Wang, Recent achievements in electronic tongue and bioelectronic tongue as taste sensors, Sensors Actuators B Chem. 207 (2015) 1136–1146. doi:10.1016/j.snb.2014.09.077.

[19] F. Winquist, Voltammetric electronic tongues – basic principles and applications, Microchim. Acta. 163 (2008) 3–10. doi:10.1007/s00604-007-0929-2.

[20] A. Riul, D.S. Dos Santos, K. Wohnrath, R. Di Tommazo, A.C.P.L.F. Carvalho, F.J. Fonseca, O.N. Oliveira, D.M. Taylor, L.H.C. Mattoso, Artificial taste sensor: Efficient combination of sensors made from Langmuir-Blodgett films of conducting polymers and a ruthenium complex and self-assembled films of an azobenzene-containing polymer, Langmuir. 18 (2002) 239–245. doi:10.1021/la011017d.

[21] G. Scandurra, G. Tripodi, A. Verzera, Impedance spectroscopy for rapid determination of honey floral origin, J. Food Eng. 119 (2013) 738–743. doi:10.1016/j.jfoodeng.2013.06.042.

[22] P.A. Ulloa, R. Guerra, A.M. Cavaco, A.M. Rosa da Costa, A.C. Figueira, A.F. Brigas, Determination of the botanical origin of honey by sensor fusion of impedance e-tongue and optical spectroscopy, Comput. Electron. Agric. 94 (2013) 1–11. doi:10.1016/j.compag.2013.03.001.

[23] Codex Alimentarius Commission, Codex Alimentarius Commission Standards, Codex Stan 12-1981. (2001) 1–8. doi:10.1007/978-3-540-88242-8.

[24] European Union Directive (EU), Council Directive 2001/110 Relating to Honey, Off. J. Eur. Communities. 16 (2001) 1–12.

[25] J. Louveaux, A. Maurizio, G. Vorwohl, Methods of melissopalynology, Bee World. 5 (1978) 139–153. doi:10.1080/0005772X.1978.11097714.

[26] V. Kaškonienė, P.R. Venskutonis, V. Čeksterytė, Composition of volatile compounds of honey of various floral origin and beebread collected in Lithuania, Food Chem. 111 (2008) 988–997. doi:10.1016/j.foodchem.2008.05.021.

[27] E. Alissandrakis, P. a. Tarantilis, P.C. Harizanis, M. Polissiou, Aroma investigation of unifloral Greek citrus honey using solid-phase microextraction coupled to gas chromatographic–mass spectrometric analysis, Food Chem. 100 (2007) 396–404. doi:10.1016/j.foodchem.2005.09.015.

[28] S. Bogdanov, M. Haldimann, W. Luginbühl, P. Gallmann, Minerals in honey: environmental, geographical and botanical aspects., J. Apic. Res. 46 (2007) 269–275. doi:10.3896/IBRA.1.46.4.11.

[29] I.K. Karabagias, A. V. Badeka, S. Kontakos, S. Karabournioti, M.G. Kontominas, Botanical discrimination of Greek unifloral honeys with physico-chemical and chemometric analyses, Food Chem. 165 (2014) 181–190. doi:10.1016/j.foodchem.2014.05.033.

[30] I.K. Karabagias, M. V. Vavoura, C. Nikolaou, A. V. Badeka, S. Kontakos, M.G. Kontominas, Floral authentication of Greek unifloral honeys based on the combination of phenolic compounds, physicochemical parameters and chemometrics, Food Res. Int. 62 (2014) 753–760. doi:10.1016/j.foodres.2014.04.015.

[31] I. Sergiel, P. Pohl, M. Biesaga, Characterisation of honeys according to their content of phenolic compounds using high performance liquid chromatography/tandem mass spectrometry, Food Chem. 145 (2014) 404–408. doi:10.1016/j.foodchem.2013.08.068.

[32] V. Kaškonienė, P.R. Venskutonis, Floral markers in honey of various botanical and geographic origins: a review, Compr. Rev. Food Sci. Food Saf. 9 (2010) 620–634. doi:10.1111/j.1541-4337.2010.00130.x.

[33] E. Anklam, A review of the analytical methods to determine the geographical and botanical origin of honey, Food Chem. 63 (1998) 549–562. doi:10.1016/S0308-8146(98)00057-0.

[34] S. Soares, J.S. Amaral, M.B.P.P. Oliveira, I. Mafra, A comprehensive review on the main honey uuthentication issues: production and origin, Compr. Rev. Food Sci. Food Saf. 16 (2017) 1072–1100. doi:10.1111/1541-4337.12278.

[35] L. PERSANO ODDO, R. PIRO, Main European unifloral honeys: descriptive sheets, Apidologie. 35 (2004) 38–81. doi:10.1051/apido:2004049.

[36] Z. Wei, J. Wang, W. Liao, Technique potential for classification of honey by electronic tongue, J. Food Eng. 94 (2009) 260–266. doi:10.1016/j.jfoodeng.2009.03.016.

[37] Z. Wei, J. Wang, Classification of monofloral honeys by voltammetric electronic tongue with chemometrics method, Electrochim. Acta. 56 (2011) 4907–4915. doi:10.1016/j.electacta.2011.02.065.

[38] I. Escriche, M. Kadar, E. Domenech, L. Gil-Sánchez, A potentiometric electronic tongue for the discrimination of honey according to the botanical origin. Comparison with traditional methodologies: Physicochemical parameters and volatile profile, J. Food Eng. 109 (2012) 449–456. doi:10.1016/j.jfoodeng.2011.10.036.

[39] Y. Elamine, S. Aazza, B. Lyoussi, M. Dulce Antunes, L.M. Estevinho, O. Anjos, M. Resende, M.L. Faleiro, M.G. Miguel, Preliminary characterization of a Moroccan honey with a predominance of Bupleurum spinosum pollen, J. Apic. Res. 57 (2018). doi:10.1080/00218839.2016.1265759.

[40] S. Bogdanov, B.P. Science, C. Criteria, HARMONISED METHODS OF THE INTERNATIONAL HONEY COMMISSION, (n.d.).

[41] L. Bertacchini, M. Cocchi, M. Li Vigni, A. Marchetti, E. Salvatore, S. Sighinolfi, M. Silvestri, C. Durante, The Impact of Chemometrics on Food Traceability, 1st ed., Copyright © 2013 Elsevier B.V. All rights reserved., 2013. doi:10.1016/B978-0-444-59528-7.00010-7.

[42] K.S. Cole, R.H. Cole, Dispersion and absorption in dielectrics I. Alternating current characteristics, J. Chem. Phys. 9 (1941) 341–351. doi:10.1063/1.1750906.

[43] J.E.B. Randles, Kinetics of rapid electrode reactions, Discuss. Faraday Soc. 1 (1947) 11. doi:10.1039/df9470100011.

[44] O. Anjos, C. Iglesias, F. Peres, J. Martínez, Á. García, J. Taboada, Neural networks applied to discriminate

botanical origin of honeys, Food Chem. 175 (2015) 128–136. doi:10.1016/j.foodchem.2014.11.121.

[45] A. Terrab, a. G. González, M.J. Díez, F.J. Heredia, Mineral content and electrical conductivity of the honeys produced in Northwest Morocco and their contribution to the characterisation of unifloral honeys, J. Sci. Food Agric. 83 (2003) 637–643. doi:10.1002/jsfa.1341.

[46] Sancho, M. T., Muniategui, S., Sánchez, M. P., Huidobro, J. F., Simal, J., Relationships between electrical conductivity and total and sulphated ash contents in Basque honeys, Apidologie. 22 (1991) 487–494. doi:10.1051/apido:19910501.

[47] J. Pourtallier, Y. Taliercio, Honey control analyses, Apiacta. (1972) 2–5.

[48] D. Yadata, Detection of the electrical conductivity and acidity of honey from different areas of Tepi, Food Sci. Technol. 2 (2014) 59–63. doi:10.13189/fst.2014.020501.

Part II. Pharmacological properties of *B. spinosum* honey

Chapter I. Effect of extreme heat processing on Zantaz' honey antioxidant activities.

Chapter I. Effect of extreme heat processing on Zantaz' honey antioxidant activities [*]

Abstract

The effect of an extreme heat processing on Zantaz honey samples was studied using a panel of physicochemical parameters, antioxidant activities and FTIR-ATR spectroscopy. Honey samples were heated at 121 °C for 30 minutes and the heat processing effect was confirmed indirectly through the assessment of Hydroxymethylfurfural content (HMF), for which the values increased significantly ($p<0.01$), and diastase activity, which was totally absent after the thermal processing. On the other hand, the effects of the heat on the antioxidant activities were diverse. Indeed, while the ability to scavenge 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid cation radicals ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was enhanced ($p < 0.05$ only for $ABTS^{\bullet+}$), after the heat processing, nitric oxide (NO) radicals scavenging activity was reduced drastically ($p < 0.01$). Regarding the chelating power, it was totally abolished following heating. Other activities showed no significant alteration. The initial values of antioxidant activities seem to be determinant in the changes occurring after the heat processing. Amongst the analysed parameters, following the heat processing, the honey colour was the variable where the influence of baseline values was the greatest. The spectral analyses confirmed that FTIR-ATR is a useful technique to discriminate the chemical differences occurring in honey after heat processing.

1. Introduction

The most important factors influencing honey marketing are those that are easily evaluated by customers, including rheology (the ability of honey to crystallize), color and shelf-lifetime. Having those characteristics into consideration, honey heating used to be a common practice to reach the desired rheological properties thereby avoiding the crystallization process as well as to increase the shelf-lifetime by elimination possible contaminant microorganisms [1,2].

In spite of the theoretical benefits of heating, it is important to mention that during thermal processing the composition of honey undergoes a series of changes that are linked to the interactions between its major (sugars) and minor (amino acids) compounds. Those reaction

[*] Y. Elamine, O. Anjos, L.M. Estevinho, B. Lyoussi, S. Aazza, M.G. Miguel, Effect of extreme heat processing on Zantaz' honey antioxidant activities, Journal of Food Science and Technology. (Accepted)

include the conversion of organic acids to alcohols or that of polyphenolic compounds into volatiles or even degradation of compounds like vitamins [3]. One of the reactions is the non-enzymatic browning, Maillard reaction, which in the ultimate stages, the degradation of Amadori products occur and generate Strecker aldehydes, pyrazines, pyrroles and furans, amongst others [4]. The condensation of the generated active molecules with amino-acids form high molecular weight brown polymers known as melanoidins [5]. Hydroxymethylfurfural (HMF) is considered to be the most important product and is therefore used for the confirmation of honey freshness when its values do not exceed 40 mg/kg [6]. In addition, in a recent review made by Shapla et al. (2018) about the influence of HMF levels present in honey on human health, the authors reported that it remains inconclusive because this compound has been described as exerting pernicious effects, such as mutagenic, genotoxic, organotoxic and enzyme inhibitory activities; but also beneficial effects including antioxidant, anti-allergic, anti-inflammatory, anti-hyperuricemic, and anti-hypoxic activities [7].

Several studies analysed the thermal effects on honey by evaluating the antioxidant activities and proved that the content of melanoidins correlated positively with honey antioxidant activity. The formation of melanoidins also explained the acquired activity after the heat processing [8,9]. The observed increase depends on heat processing degree, and showed different behaviours towards different temperatures and heating times [4]. Another influencing factor is honey botanic and geographical origin [10]. In their studies about the effect of melanoidins formation on honey antioxidant activity after heat processing, Brudzynski and Miotto (2011) observed that this phenomenon was more pronounced in light-coloured honey than in the dark-coloured samples [9].

While the increase in melanoidins' content is shown to increase the ability of honey to scavenge the free radicals used in the ORAC (Oxygen Radical Absorbance Capacity) assay [9], the degradation of other bioactive molecules may affect negatively the antioxidant ability toward other assays. [11] reported that the antioxidant activity of honey samples, determined using a linoleic acid-based lipid peroxidation assay, was drastically decreased following thermal treatment. The same authors linked the observed decrease to the decomposition of vitamins B1, B2, and C, and the destruction of catalase and peroxidase.

With the aim of better understanding heat effects on honey, we studied eight Zantaz honey samples produced in Morocco, mainly from Bupleurum spinosum. This honey was characterized in two previous contributions by the same group [12,13]. The collected samples

have been characterized regarding their physicochemical properties and antioxidant activities, before and after the heat processing at 121 °C during 30 min. To estimate the antioxidant activity it were used some of the most commonly employed assays like radical scavengers, metal-chelators and hydrogen donating agents [5]. The physicochemical characteristics were studied using spectroscopic methods among which Fourier-transform infrared-attenuated total reflection, which has the advantage of being a non-destructive technique that only needs very small amounts of sample [14].

2. Materials and Methods

2.1. Reagents and instruments

Sodium phosphate, sodium hydroxide, and sodium carbonate were purchased from Pronalab, Madalena, Portugal; hydrochloric acid was provided by Fisher Scientific UK Ltd, Loughborough, UK; Acetic acid, Ascorbic acid, Folin–Ciocalteu, fuchsin, potassium hydroxide, methanol, and 2-propanol were provided by Merck KGaA products, Darmstadt, Germany; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ferrozine, ferrous sulfate, gallic acid, nitroblue tetrazolium (NBT), ninhydrine, phenazine methosulfate (PMS), proline were purchased from Acros Organics, New Jersey, USA; aluminium chloride and acetic anhydride provided by Panreac Química, MontcadaReixac, Barcelona, Spain; Catechin was purchased from Fluka. Biochemika. Sigma-Aldrich. Steinheim, Germany; sulfuric acid, sodium acetate, starch, potassium iodide, I_2, sodium nitroprusside, available at Riedel-de-Haen, Germany; acetic acid was purchased from CHEM-Lab, Belgium; optic microscope (Leitz Messtechnik GmbH, Wetzlar, Germany); HPLC**:** Dionex ICS3000 equipment; microplate reader(Tecan Infinite M200, Tecan, Austria);; analytical balance (Shimadzu, Aux 220, Philippines); electric furnace (Cassel, Portugal);; Abbe Refractometer (HANNA, HI968601, Romania); HPLC (Hitachi, LaChrom Elite, Japan).

2.2. Sample preparation

In this study, eight monofloral honey samples (classified as monofloral according the methodology proposed by Elamine et al. (2019) [12] purchased from local Moroccan beekeepers were used. Each honey sample was divided into two aliquots: half was kept at room temperature and studied within one week (n = 8); and half were heat processed at 121 °C for 30 min (n=8). Both raw and heat-treated samples were prepared in distilled water (50%, w/v) and centrifuged at 5.000 *g*, 20 min. The temperature, the heating period as well as preparation of samples were selected based on a previous work claiming that the conditions were sufficient

to increase the degree of browning in both light- and medium-coloured honeys [9]. All measurements were performed in triplicate and the results were presented as mean ± SD (standard deviation).

2.3. Preliminary characterization

Moisture, diastase activity, HMF and proline content assessed following the Harmonized Methods of the International Honey Commission [15].

2.4. Colour, and melanoidins estimation

Colour and melanoidins estimations were determined by measuring the absorbance of aqueous solutions (10 g of honey in 20 mL of distilled water) at three wavelengths by using Shimadzu 160-UV spectrophotometer. The mm Pfund values of honey samples were obtained using the following algorithm: mm Pfund = -38.7 + 371.39 x A_{635} [16]. Melanoidins content was estimated based on the browning index (net absorbance =A_{450}-A_{720}) [9]. Spectrophotometric measurements were performed in a 1 cm quartz cell; results were expressed as absorbance units (AU).

2.5. Polyphenols

The total polyphenol content (TPC) was determined in honey solutions using the Folin–Ciocalteau method [17]. To eliminate a possible interference of sugars in the assay, sugar solutions were made considering the sugar profile of each 50% honey sample solution (w/v), used for the estimation of polyphenols content. The assay was made for both honey and sugar solutions, and the results were expressed as mean of gallic acid equivalent (GAE) mg ± SD (standard deviation) / 100 g honey, after the elimination of sugar interference. This interference was eliminated as follows: Abs = Abs (H) - Abs (S), where Abs is the absorbance considered for the calculation of the phenolic content; Abs (H) is the absorbance of the honey solution and the Abs (S) is the absorbance of the sugar solution.

2.6. Flavonoids

The total flavonoid content (TFC) of different honey samples was determined using the aluminium chloride assay according to Samatha et al. (2012) [18]. To study a possible interference of sugars in this assay the sugar solution made for polyphenol content estimation was also used. To avoid possible interaction of sample colour with the reaction's reagents, the blank was constituted by water (substituting the other components of the reaction) plus the sample as reported by Sancho et al. (2016) [19]. The resulting absorbance was subtracted from

the whole reaction absorbance before TFC estimation. The TFC was expressed in mg of catechin equivalents (CE) per 100 g of honey as the mean of three triplicate ± SD.

2.7. Total antioxidant activity by ammonium molybdate reduction method

Total antioxidant activity (TAA) was measured in different concentrations of samples according to the described by [20]. Honey solution was mixed with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After 90 minutes incubation at 95 ºC, the samples were cooled at room temperature and their absorbance values were measured at 695 nm in a Shimadzu 160-UV spectrophotometer. The antioxidant activity was expressed as the number of ascorbic acid equivalents (AAE)/100 g of honey.

2.8. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity

Fifty microliters of each sample at different concentrations was placed in a cuvette, and 2 mL of 60 μM methanolic solution of DPPH was added [21]. After 60 min incubation at room temperature, absorbance measurements were made at 517 nm using a Shimadzu 160-UV spectrophotometer (Tokyo, Japan). The percentage of inhibition of free radicals was calculated following the formula: $[(A_0 – A_1/ A_0) \times 100]$. A_0 is the absorbance of the negative control (blank sample containing the same amount of water and DPPH solution); A_1 is the absorbance of the sample. The IC_{50} was then determined from the resulting concentration-inhibition percentage plot, as honey concentration inhibiting 50% of the generated free radicals and was expressed as mg/mL.

2.9. $ABTS^{\bullet+}$ (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging activity

The determination of $ABTS^{\bullet+}$ radical scavenging capacity was carried out as reported previously [22]. The reaction of $ABTS^{\bullet+}$ aqueous solution (7 mM) with potassium persulfate ($K_2S_2O_8$) (2.45 mM) was done in the dark, for 16 h to generate $ABTS^{\bullet+}$ free radical. The absorbance of the resulted solution was adjusted to 0.7 at 734 nm. Twenty-five μL of honey dilutions were added to 275 μL $ABTS^{\bullet+}$ and the absorbance was read at 734 nm, after 6 min incubation. The IC_{50} values were calculated from three replications and the results were expressed as mean ± SD (mg/mL).

2.10. Reducing power

The reducing power of honey samples was evaluated as described previously [23]. In brief, honey solution in phosphate buffer (0.2 M, pH 6.6) was added to 0.5 mL of potassium ferricyanide (1%), and the mixture was incubated at 50 °C for 20 min. After the incubation

trichloroacetic acid solution (TCA) (10%) was added to the mixture. A centrifugation at 600 g was applied for 10 min, and the supernatant was mixed with H_2O and ferric chloride solution (0.1%) for 10 min. The absorbance was measured at 700 nm. Results were expressed as the absorbance of the reaction at the highest honey concentration.

2.11. Chelating metal ions

The chelating ability of honey samples against ferrous ions was evaluated as previously described in detail [22]. Briefly, honey solutions [different dilutions starting from 50% (w/v) solution] were incubated with 50 µL of iron(II) chloride, $FeCl_2$ (2 mM). The reaction was initiated by the addition of 200 µL ferrozine 5 mM, and after 10 min, the absorbance at 562 nm was measured. The IC_{50} values were calculated and expressed as mean ± SD (mg/mL) of three replicates.

2.12. Nitric oxide (NO) scavenging activity

The protocol was initiated by generating NO radicals from sodium nitroprusside (SNP) ($Na_2[Fe(CN)_5NO]$) and was measured by using the Griess reagent (N-(1-Naphthyl) ethylenediamine) [24]. Briefly, 50 µL of serially diluted honey solutions were added to 50 µL of 10 mM SNP in phosphate buffer saline (PBS) into a 96-well plate, and incubated at room temperature for 90 min. Finally, the remaining free fraction of NO radicals was revealed by adding 50 µL Griess reagent and absorbance was measured at 546 nm. The IC_{50} was calculated with slight different of what was explained in DPPH section: $[1-(A_{sample} - A_{sample\ blank})/ (A_{control} - A_{control\ blank})] \times 100$, where $(A_{sample} - A_{sample\ blank})$ is the difference in the absorbance of a sample, with or without 10 mM SNP, and $(A_{control} - A_{control\ blank})$ is the difference in the absorbance of the PBS control, with or without 10 mM SNP.

2.13. Superoxide anion radical (O2•-) scavenging activity (non-enzymatic method)

Superoxide anion radical $O_2^{\bullet-}$ ions were generated in a non-enzymatic phenazine methosulfatenicotinamide adenine dinucleotide (PMS-NADH) system based on the oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT) [25]. Honey solutions (25 µL) were mixed with 25 µL nitroblue tetrazolium (0.42 mg/mL) and 25 µL NADH (1.32 mg/ mL) and H2O (125 µL) followed by 25 µL phenazine methosulfate (PMS) (0.25 mg/mL). The percentage inhibition was calculated by the formula given bellow: $[(A_0 – A_1/ A_0) \times 100]$ in which A_1 and A_0 are absorbances of solvent with and without sample, respectively. The results were expressed as the mean ± SD (mg/mL) of three IC_{50} values (replicates).

2.14. FTIR-ATR (Fourier-transform infrared-attenuated total reflection) analysis

Honey samples were also analysed with FTIR-ATR spectroscopy. The spectral information was acquired in accordance with the procedure previously reported [26] with a Bruker spectrometer (Alpha) using a diamond single reflection attenuated total reflectance device. For spectra for different aliquot of the same samples were obtained with 32 scans per spectrum at a spectral resolution of 4 cm^{-1} in the wavenumber range from 4000 to 400 cm^{-1}. Prior analysis several pre-treated were tested namely: standard normal variate (SNV), multiplicative scatter correction (MSC), normalize, centred and scaled, first derived Savitzky–Golay (1st derived), second derived Savitzky–Golay (2nd derived), baseline and several combinations of 2 pre-treatments, to identify the better one to use in the further analysis.

2.15. Statistical analysis

To compare the means of assessed parameters before and after the heat processing, paired student t test was used. The difference was accepted to be statistically significant with $p<0.05$. The data processing was done on IBM SPSS Statistics 25 software.

Principal component analyses (PCA) with spectral information were performed using the Unscrambler® X, version: 10.5.46461.632 (CAMO Software AS, Oslo, Norway).

3. Results and discussion

3.1. Effect of heat processing on the physicochemical parameters

The application of thermal treatment on honey samples has been reported to affect highly its quality and it is proven that the kinetic of this process depends on the degree and heating time [6]. In this study, honey samples were heated at 121 °C for 30 minutes and the heat processing effect was confirmed indirectly through the assessment of a panel of physicochemical parameters including hydroxymethylfurfural (HMF) and diastase (Table 1). The heat processing, in all the analysed samples, significantly increased ($p < 0.01$) the HMF content and the mean values changed from 7.70±5.82 mg/kg to 20.11±11.91 mg/kg. Both values are below 40 mg/kg which is the maximum allowed by the Codex Alimentarius for the HMF content in honey [27]. In spite of that, the HMF content increased around 55% with the thermal processing what is a very significant quick increase. [6] reported the same pattern in floral honey submitted to a similar treatment (100 °C for 30 min) and suggested that the increase in the HMF content is more likely to be sensitive to primitive storage conditions rather than to overheating.

As expected, the use of decomposition heat processing [28] induced a drastic decrease in the diastase activity: the recorded value changed from 21.31 ± 5.94 shade unit/g in raw samples to values bellow the detection limits following processing. Theoretically, a process of proteins' decomposition induces an increase in the free fraction of amino acids, including the proline. In contrast, the presence of a digestion step (sulphuric acid and thermal treatment) in the process of quantifying the proline content suppresses the ability to see this increase. On the contrary, a decrease was seen in the proline content of the analysed samples after the heat processing, yet this result was not significant (Table 1).

In the present study, HMF, proline, diastase activity, honey colour, melanoidins, polyphenols and flavonoids contents were assessed. All these parameters changed after honeys' heating, although statistical significance was not always reached. The honey colour increased 38% in mm pfund while the melanoidins' content increased by 28%. Both parameters are directly linked to the core of this study, as they provide a tool to assess the formation of the brown pigments of the Millard reaction, known to occur in honey following a thermal treatment [29]. Both parameters showed a highly significant increase ($p < 0.001$), and their values increase from 60.94 ± 30.67 pfund to 83.92 ± 29.98 pfund for the colours and from 0.98 ± 0.38 to 1.25 ± 0.44 for melanoidins content.

Table 1. The physicochemical characterization and the antioxidant activities of honey sample before and after heat processing

Parameter	**Raw honey samples**		**After heat processing**	
	Mean±SD	**Range**	**Mean**	**Range**
Proline (mg/Kg)	817.10 ± 104.59	709.95 - 1007.49	773.65 ± 141.68	593.63 - 994.15
HMF (mg/Kg)	7.70 ± 5.82	1.07 - 15.97	20.10 ± 11.91	8.73 - 38.57
Diastase (Shade number)	21.31 ± 5.94	11.33 - 30.30	-	-
Colour (mm pfund)	60.94 ± 30.67	28.46 - 116.91	83.92 ± 29.98	54.75 - 131.58
Melanoidins	0.98 ± 0.38	0.56 - 1.55	1.25 ± 0.44	0.74 - 1.88
Water content (%)	18.97 ± 1.25	17.27 - 20.40	18.35 ± 0.77	17.27 - 19.13
Phenol (mg GAE/100 g)	96.05 ± 26.55	63.44 -130.04	96.78 ± 25.36	67.27 - 132.61
Flavonoids mg CE/100 g	23.70 ± 4.89	17.12 - 29.32	20.44 ± 3.41	14.52 - 25.59
TAA (mg EAA/ g Honey)	370.44 ± 51.97	278.53 - 442.68	377.25 ± 48.54	294.60 - 433.12
DPPH (IC_{50} mg/mL)	15.34 ± 7.55	7.94 - 27.13	12.70 ± 4.61	6.45 - 19.69
$ABTS^{\bullet+}$ IC_{50} (mg/mL)	7.00 ± 2.79	4.20 - 12.18	5.60± 2.10	3.53 - 9.26
Chelating Power IC_{50} (mg/mL)	46.11 ± 42.39	7.98 - 132.19	-	-
Reducing power (Max Abs)	1.77 ± 0.40	1.29 - 2.34	1.48 ± 0.17	1.21 - 1.74

Superoxide (IC_{50} mg/mL)	50.91 ± 28.30	15.02 - 88.43	50.88 ± 22.81	24.05 - 85.90
Nitric oxide (IC_{50} mg/mL)	125.89 ± 39.28	81.98 - 189.60	161.45 ± 43.37	110.81 - 222.83

-: Without activity

It has been reported that the polyphenols and flavonoids contents are involved in the antioxidant activities of honey [5]. Therefore, these compounds were assessed as quick predictive indicators of the changes occurring in antioxidant activities', either lost or acquired. It was observed, although, that the polyphenols' contents did not vary significantly with the heat treatment. Only the flavonoid content decreased significantly ($p < 0.01$) after the thermal treatment (around 14%). A significant decrease in the content of some flavonoids and phenolic acids had been reported ($p < 0.05$) following the industrial pasteurization process [30].

3.2. Effect of thermal treatment on the antioxidant activities

The effect of heat processing on the antioxidant activities is also presented in Table 1. Results are expressed as mean of all analysed samples for a given parameter, and the difference between the means was evaluated using a student t-test.

The antioxidant activity assessed through the DPPH methodology increased after the thermal treatment, with a decrease of the IC_{50} values from 15.34 ± 7.55 mg/mL to 12.70 ± 4.61 mg/mL (Table 1). Even though this increase was not significant in the present study, the same behaviour was reported previously in heather honey heated at 95ºC for 5 minutes [31]. The same authors reported that the increase was more pronounced in acacia honey samples ($p < 0.05$), suggesting that the change in the antioxidant activity following thermal treatment is influenced by the botanic origin of honey samples.

Similar results were obtained for the $ABTS^{\bullet+}$ antioxidant assay, where an increasing of 20% in the activity was observed after the heat processing. Still, in contrast to the registered in the DPPH assay, this difference was significant ($p < 0.05$). Both assays are used to evaluate the free radical scavenging capacity, but the $ABTS^{\bullet+}$ is more likely to be used for honey samples.

The heat processing applied in this study could abolish the ability of samples to chelate the iron ions. Indeed, following the chelating power experimental procedure the IC_{50} of the thermally treated samples could not be determined, in opposite to what occurred with raw fresh samples where IC_{50} was of 46.11 ± 42.39 mg/mL. Organic acid and flavonoids are known to possess metal chelating activity [32]**.** Therefore, the observed decrease in flavonoids content along with a possible deterioration of organic acids (not determined in the present work) may have an influence in the observed effect on iron chelating power after the thermal treatment.

The ability of honey samples to reduce the ferric ions was another activity that was negatively affected by the heat processing, reaching a value of 1.48 ± 0.17 (absorbance of the highest used concentration) compared to 1.77 ± 0.40 of the raw samples (Table 1); although, this difference did not achieve the level of significance (Table 1).

The heat processing negatively affected ($p < 0.01$), the ability of Zantaz honey samples to scavenge the NO free radicals. The corresponding IC_{50} values increased from 125.89 ± 39.28 mg/mL (in the raw unprocessed samples) to 161.45 ± 43.37 mg/mL (in honey samples submitted to heating). Such important decrease of the NO scavenging assay could be explained by the reduction of flavonoids content as a strong correlation between the two parameters was already reported in honey [33].

As for the superoxide activity, no significant changes were observed and the IC_{50} values were 50.91 ± 28.30 mg/mL before the heat processing and 50.88 ± 22.81 mg/mL after the heating process. The stability seen in this antioxidant assay suggests that the responsible compounds, which were not quantified in the present study, did not suffer the consequences of the heat treatment.

Table 2 illustrates the correlation matrix between the initial values obtained for the assessed parameters and the percentage of the changes within the same parameters following heat processing. The honey colour seems to be the most affected parameter, as a negative correlation could be seen between honey colour changes after heat processing, from one side; and the baseline values of colour ($p < 0.05$), melanoidins ($p < 0.01$), phenols ($p <0.01$), and HMF ($p < 0.05$), from the other side. In addition, the same parameter had also a positive correlation with the IC_{50} obtained with DPPH ($p < 0.01$), $ABTS^{\bullet+}$ ($p < 0.05$), superoxide ($p < 0.05$) and nitric oxide ($p < 0.05$) antioxidant activities.

Other feature that could be observed in the correlation matrix is the effect of the initial antioxidant activity and the resulting changes in such activity following heat processing. Negative correlations were obtained in the case of DPPH ($p < 0.05$), reducing power ($p < 0.01$) and superoxide ($p < 0.05$) antioxidant activities.

Higher percentages of colour changes were observed on honeys that were lighter prior heating. Similarly, higher colour changes occurred following heating in samples with baseline lower amounts of melanoidins and phenols, as well as lower capacity for scavenging DPPH, $ABTS^{\bullet+}$, superoxide and NO free radicals. Changes in the chemical and antioxidant attributes of honey after heating had already been reported [5,11]. The results of the present work suggest

that heating lighter honey, honey with lower amounts of phenols, or with lower antioxidant activity induces more accentuated changes than those observed in darker honeys or those already possessing higher amounts of phenols or higher antioxidant activity.

Table 2. Correlation matrix between baseline values and the percentage of change following heat processing.

	Proline %	HMF %	Colour %	Melanoidins %	Moisture %	Phenols %	Flavonoids %	TAA %	DPPH %	ABTS %
Proline	0.531	0.22	-0.291	-0.21	-0.543	-0.08	-0.146	-0.603	0.177	-0.495
HMF	-0.536	-0.7	0.736*	0.472	0.513	-0.013	0.498	0.688	-0.123	0.199
Colour	0.587	0.591	-0.797*	-0.618	-0.782*	-0.02	-0.25	-0.414	0.376	0.141
Melanoidins	0.548	0.579	-0.848**	-0.64	-0.708*	-0.215	-0.128	-0.313	0.465	0.147
Moisture	0.871**	0.398	-0.584	-0.244	-0.970**	0.286	-0.49	-0.482	0.452	-0.157
Phenols	0.459	0.417	-0.865**	-0.692	-0.625	-0.423	-0.076	-0.24	0.602	0.15
Flavonoids	0.818*	0.568	-0.684	-0.326	-0.867**	0.222	-0.591	-0.554	0.382	-0.113
DPPH	-0.599	-0.43	0.907**	0.478	0.669	0.324	0.072	-0.023	-0.754*	-0.195
ABTS	-0.667	-0.345	0.749*	0.353	0.791*	0.105	0.156	-0.082	-0.754*	-0.288
Chelating power	0.027	0.425	-0.242	-0.536	-0.286	0.073	0.138	-0.498	-0.318	0.045
Reducing	-0.315	0.379	0.29	-0.072	0.337	0.298	-0.22	-0.569	-0.848**	-0.104
Superoxide	0.047	-0.423	0.794*	0.591	0.008	0.61	-0.212	-0.337	-0.455	-0.555
Nitric Oxide	-0.376	-0.445	0.769*	0.481	0.38	0.531	-0.343	0.093	-0.438	0.12

* Correlation is significant at the $p< 0.05$ level. ** Correlation is significant at the $p < 0.01$ level.

3.3. Effect of heating evaluated by FTIR-ATR analysis

The spectra obtained for the honey samples, represented in Fig 1, are similar to those obtained by other authors [26,34].

The spectra were considered adequate for analysis when the difference between spectra for a particular sample was lower than 0.002 for 10 spectra. First derived Savitzky–Golay (1st derived) was used as pre-processed in the region between 1300 e 1800 cm^{-1} (Figure 1 (B) and (C). The validation method used was the cross-validation method. The criteria used to classify the samples was a ratio of calibrated to validated residual variance of 0.5; ratio of validated to calibrated residual variance of 0.75 and residual variance increase limit of 6%.

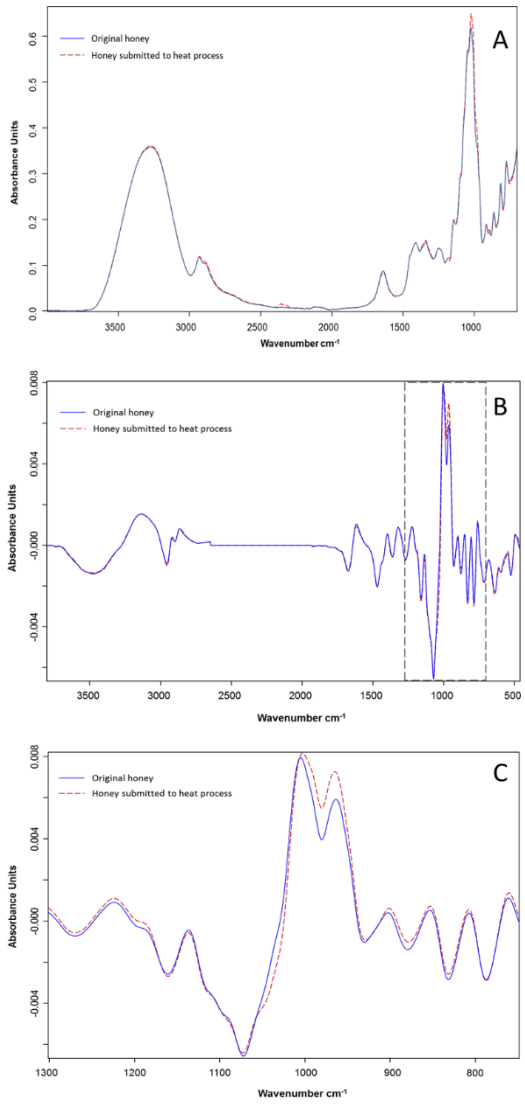

Figure 1. FTIR-ATR spectra for honey samples with and without heating. A) Average spectra obtained by FTIR-ATR, for honey with and without heating. B) Processed FTIR-ATR (All spectra). C) Processed FTIR-ATR (Region used in the analysis).

In a first screening, a PCA with all spectral regions (from 4000 cm^{-1} to 400 cm^{-1}) was performed. However, better results were obtained when the region from 800 to 1250 cm^{-1} was used as shown in the Figure 2. In this case, it was observed an important effect related to the heating processed that was explained by the PC-1 (83% of the total variation). Furthermore, in Figure 2, it is also observed a different behaviour for the different species, that could be explain by the effect of the botanical origin as explained before. The honey samples are all classified as Zantaz honey, but the secondary nectar plays also an important influence.

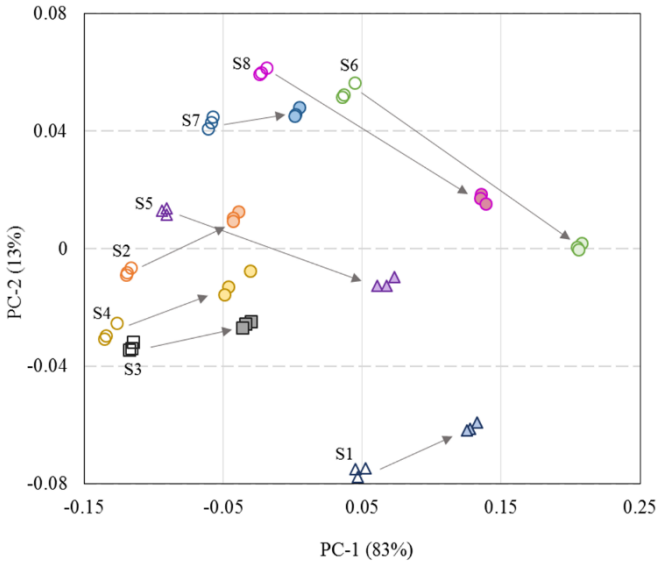

Figure 2. Score plot of two principal components from raw spectral data acquire with FTIR-ATR data of honey based on: a) marks without fill correspond to honey samples without heat processing, b) marks filled belong to samples after heat processing from 800 to 1250 cm^{-1}.

In order to better understand the influence of the botanical origin and the importance of the antioxidant activities in the differentiation of honey samples, a second PCA was performed with the data collected in this work and the secondary bee pollen observed in the honey samples (between 15% and 45%) (Figure 3).

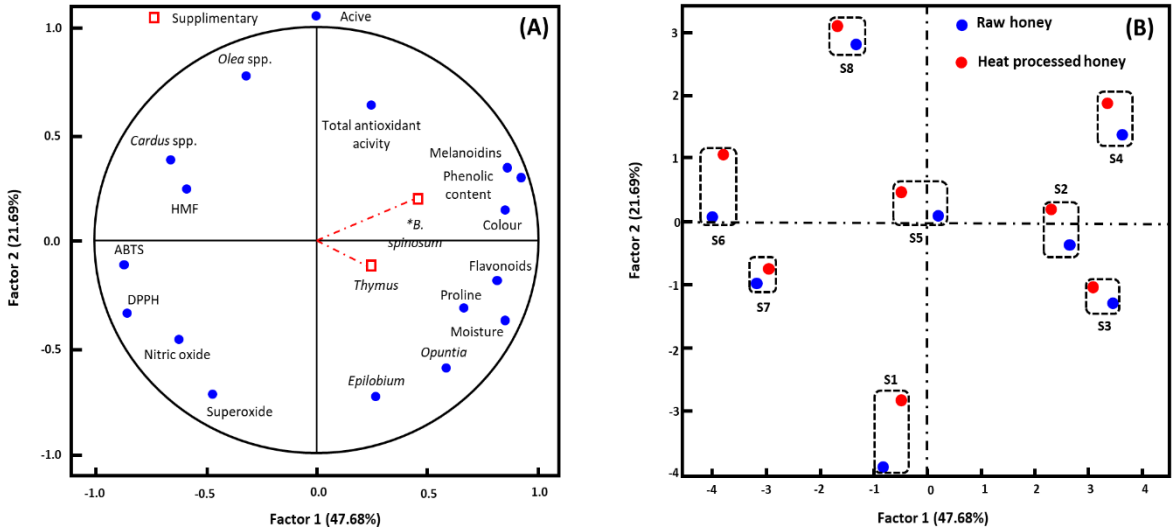

Figure 3. Plot of two principal components from all analysed parameter (A) and Scores (B) processing. Each rectangle refers to one honey sample before and after heat processing.

In the Figure 3 (A) it is possible to understand the effect of the secondary pollen namely *Cardus* spp, *Olea* spp and *Opuntia* spp in the antioxidant activity of the different honey samples. The quantity of pollen of *B. spinosum* does not influence the differentiation in the samples. The scores plot (Figure 3 (B)) show similar results to the observed in the Figure 3. There are differences between the samples after and before heating process; however, this variation was explained by the Factor 2 that explained only 21.7% of the total variance. Therefore, the spectral variance observed is influenced by the degradation of the phenol compounds but also related to the degradation of sugar by the Maillard reaction, also well explained by [5].

4. Conclusions

The existence of different tendencies amongst the assessed antioxidant activities following the thermal treatment suggests that each one is governed by distinct molecule or group of molecules. Additionally, radicals without homologies in biological systems, namely DPPH and $ABTS^{\bullet+}$, were increased by the heat processing. On the contrary, the capacity for scavenging NO radicals was reduced after heating honey samples; this may indicate that the ability of heated honey to scavenge this important mediator of the inflammation is reduced. Furthermore, the chelating ability of honey samples was totally removed by the heat processing. The antioxidant capacity of honey after heating is therefore also dependent on the method used for evaluating this property. Overall, it seems that heating Zantaz honey will not improve the antioxidant activity, at least when this biological property is assessed by the methodologies hereby reported. While a confirmation is needed for honeys with other floral origins, it may be important to avoid heat processing for samples destined for being applied in circumstances where the oxidative stress plays a role.

Author Contributions: OA, LME, BL, SA and MGM conceived and designed research; YE, OA, LME and MGM wrote the manuscript: YE, OA, LME and MGM conducted the experiments. MGM, OA and LME supervised the work. All authors read and approved the manuscript.

Funding: Thanks are due to Foundation for Science and Technology FCT, Portugal (FCT/MCTES) for the financial support to MeditBio (UID/BIA/04325/2013), CEF (Centro de Estudos Florestais (UID/AGR/00239/2019) and CIMO (UID/AGR/00690/2019)

Conflicts of Interest: The authors declare no conflict of interest.

5. References

[1] B. Abu-Jdayil, A. Al-Majeed Ghzawi, K.I.M. Al-Malah, S. Zaitoun, Heat effect on rheology of light- and dark-colored honey, J. Food Eng. 51 (2002) 33–38. doi:10.1016/S0260-8774(01)00034-6.

[2] E. Tosi, M. Ciappini, E. Ré, H. Lucero, Honey thermal treatment effects on hydroxymethylfurfural content, Food Chem. 77 (2002) 71–74. doi:10.1016/S0308-8146(01)00325-9.

[3] P.M. Da Silva, C. Gauche, L.V. Gonzaga, A.C.O. Costa, R. Fett, Honey: Chemical composition, stability and authenticity, Food Chem. 196 (2016) 309–323. doi:10.1016/j.foodchem.2015.09.051.

[4] N. Turkmen, F. Sari, E.S. Poyrazoglu, Y.S. Velioglu, Effects of prolonged heating on antioxidant activity and colour of honey, Food Chem. 95 (2006) 653–657. doi:10.1016/j.foodchem.2005.02.004.

[5] K. Brudzynski, D. Miotto, The relationship between the content of Maillard reaction-like products and bioactivity of Canadian honeys, Food Chem. 124 (2011) 869–874. doi:10.1016/j.foodchem.2010.07.009.

[6] I. Turhan, N. Tetik, M. Karhan, F. Gurel, H. Reyhan Tavukcuoglu, Quality of honeys influenced by thermal treatment, LWT - Food Sci. Technol. 41 (2008) 1396–1399. doi:10.1016/j.lwt.2007.09.008.

[7] U.M. Shapla, M. Solayman, N. Alam, M.I. Khalil, S.H. Gan, 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: effects on bees and human health, Chem. Cent. J. 12 (2018) 35. doi:10.1186/s13065-018-0408-3.

[8] K. Brudzynski, D. Miotto, Honey melanoidins: Analysis of the compositions of the high molecular weight melanoidins exhibiting radical-scavenging activity, Food Chem. 127 (2011) 1023–1030. doi:10.1016/j.foodchem.2011.01.075.

[9] K. Brudzynski, D. Miotto, The recognition of high molecular weight melanoidins as the main components responsible for radical-scavenging capacity of unheated and heat-treated Canadian honeys, Food Chem. 125 (2011) 570–575. doi:10.1016/j.foodchem.2010.09.049.

[10] O. Escuredo, M.S. Rodríguez-Flores, S. Rojo-Martínez, M.C. Seijo, Contribution to the chromatic characterization of unifloral honeys from Galicia (NW Spain), Foods. 8 (2019) 233. doi:10.3390/foods8070233.

[11] T. Nagai, M. Sakai, R. Inoue, H. Inoue, N. Suzuki, Antioxidative activities of some commercially honeys, royal jelly, and propolis, Food Chem. 75 (2001) 237–240. doi:10.1016/S0308-8146(01)00193-5.

[12] Y. Elamine, B. Lyoussi, O. Anjos, L.M. Estevinho, S. Aazza, J.D. Carlier, M.C. Costa, M.G. Miguel, Zantaz honey "monoflorality": Chemometric applied to the routinely assessed parameters, LWT - Food Sci. Technol. 106 (2019) 29–36. doi:10.1016/j.lwt.2019.02.039.

[13] Y. Elamine, S. Aazza, B. Lyoussi, M. Dulce Antunes, L.M. Estevinho, O. Anjos, M. Resende, M.L. Faleiro, M.G. Miguel, Preliminary characterization of a Moroccan honey with a predominance of Bupleurum spinosum pollen, J. Apic. Res. 57 (2018). doi:10.1080/00218839.2016.1265759.

[14] L. Salvador, M. Guijarro, D. Rubio, T. Guill, P.V. Jentzsch, V. Ciobot, L. Stolker, S. Ulic, V. Luis, P. Garrido, J. Bravo, L.R. Guerrero, Exploratory monitoring of the quality and authenticity of commercial honey in Ecuador, Foods. (2019) 1–13. doi:10.3390/foods8030105.

[15] S. Bogdanov, P. Martin, C. Lüllmann, R. Borneck, C. Ch. Flamini, M. Morlot, J. Heretier, G. Vorwohl, H. Russmann, L. Persano-Oddo, A.. Sabatini, G.. Marcazzan, P. Marioleas, K. Tsigouri, J. Kerkvliet, A. Ortiz, T. Ivanov, HARMONISED METHODS OF THE INTERNATIONAL HONEY COMMISSION, (2002).

[16] O.A. Naab, M.A. Tamame, M.A. Caccavari, Palynological and physicochemical characteristics of three unifloral honey types from central Argentina, Spanish J. Agric. Res. 6 (2008) 566–576.

[17] V.L. Singleton, J.A. Rossi Jr., Rossi J A Jr., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (1965) 144–158. doi:10.12691/ijebb-2-1-5.

[18] T. Samatha, R. Shyamsundarachary, P. Srinivas, N.R. Swamy, Quantification of total phenolic and total flavonoid contents in extracts of Oroxylum indicum L.Kurz, Asian J. Pharm. Clin. Res. 5 (2012) 177–179.

[19] M.T. Sancho, A. Pascual-Maté, E.G. Rodríguez-Morales, S.M. Osés, I. Escriche, Á. Periche, M.A. Fernández-Muiño, Critical assessment of antioxidant-related parameters of honey, Int. J. Food Sci. Technol. 51 (2016) 30–36. doi:10.1111/ijfs.12988.

[20] P. Prieto, M. Pineda, M. Aguilar, Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E, Anal. Biochem. 269 (1999) 337–341. doi:10.1006/abio.1999.4019.

[21] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, LWT - Food Sci. Technol. 28 (1995) 25–30. doi:10.1016/S0023-6438(95)80008-5.

[22] M.G. Miguel, S. Nunes, S.A. Dandlen, A.M. Cavaco, M.D. Antunes, Phenols and antioxidant activity of hydro-alcoholic extracts of propolis from Algarve, South of Portugal, Food Chem. Toxicol. 48 (2010) 3418–3423. doi:10.1016/j.fct.2010.09.014.

[23] Y. Chu, C. Chang, H. Hsu, Flavonoid content of several vegetables and their antioxidant activity, Food Sci. Technol. 566 (2000) 561–566. doi:https://doi.org/10.1002/(SICI)1097-0010(200004)80:5<561::AID-JSFA574>3.0.CO;2-%23.

[24] S.C. Ho, Y.L. Tang, S.M. Lin, Y.F. Liew, Evaluation of peroxynitrite-scavenging capacities of several commonly used fresh spices, Food Chem. 119 (2010) 1102–1107. doi:10.1016/j.foodchem.2009.08.020.

[25] S.M. Albano, M.G. Miguel, Biological activities of extracts of plants grown in Portugal, Ind. Crops Prod. 33 (2011) 338–343. doi:https://doi.org/10.1016/j.indcrop.2010.11.012.

[26] O. Anjos, M.G. Campos, P.C. Ruiz, P. Antunes, Application of FTIR-ATR spectroscopy to the quantification of sugar in honey, Food Chem. 169 (2015) 218–223. doi:10.1016/j.foodchem.2014.07.138.

[27] Codex Alimentarius Commission, Codex Alimentarius Commission Standards, Codex Stan 12-1981. (2001) 1–8. doi:10.1007/978-3-540-88242-8.

[28] E. Tosi, R. Martinet, M. Ortega, H. Lucero, E. Ré, Honey diastase activity modified by heating, Food Chem. 106 (2008) 883–887. doi:10.1016/j.foodchem.2007.04.025.

[29] H.Y. Wang, H. Qian, W.R. Yao, Melanoidins produced by the Maillard reaction: Structure and biological activity, Food Chem. 128 (2011) 573–584. doi:10.1016/j.foodchem.2011.03.075.

[30] I. Escriche, M. Kadar, M. Juan-Borrás, E. Domenech, Suitability of antioxidant capacity, flavonoids and phenolic acids for floral authentication of honey. Impact of industrial thermal treatment, Food Chem. 142 (2014) 135–143. doi:10.1016/j.foodchem.2013.07.033.

[31] S. Kowalski, Changes of antioxidant activity and formation of 5-hydroxymethylfurfural in honey during thermal and microwave processing, Food Chem. 141 (2013) 1378–1382. doi:10.1016/j.foodchem.2013.04.025.

[32] L.S. Chua, N.L.A. Rahaman, N.A. Adnan, T.T. Eddie Tan, Antioxidant activity of three honey samples in relation with their biochemical components, J. Anal. Methods Chem. 2013 (2013). doi:10.1155/2013/313798.

[33] G.O.L.M. Dor, M.F. Mahomoodally, Chemical profile and in vitro bioactivity of tropical honey from Mauritius, Asian Pacific J. Trop. Dis. 4 (2014) S1002–S1013. doi:10.1016/S2222-1808(14)60773-8.

[34] R. de O.R. Ribeiro, E.T. Mársico, E.F.O. de Jesus, C. da Silva Carneiro, C.A.C. Júnior, E. de Almeida, V.F. do N. Filho, Determination of trace elements in honey from different regions in Rio de Janeiro state (Brazil) by total reflection X-ray fluorescence, J. Food Sci. 79 (2014) 738–742. doi:10.1111/1750-3841.12363.

Chapter II. Antibacterial activity of Zantaz honey and influence of its physicochemical properties.

Chapter II. Antibacterial activity of Zantaz honey and the influence of its physicochemical properties

Abstract

Alternative antimicrobial strategies are urgently needed, which led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including honey. Honey antimicrobial activity depends on several factors, including hydrogen peroxide, plant derived metabolites, osmotic pressure caused by sugars in addition to its acidity. In addition, different bacterial strains may show different sensitivities toward same honey type. Zantaz honey samples, that is produced from *Bupleurum spinosum* plant, were characterized for their content on pollen, and were selected nine of them that presented *B. spinosum* pollen more than 45%. Sugars, minerals other physicochemical parameters used for quality control of honey were measured. Antimicrobial activity of the characterised samples was evaluated against *Escherichia coli* (466), *Pseudomonas aeruginosa* and *Staphylococcus aureusis.*

The preliminary analysis of honey samples showed that, according to the international quality standards, none of analysed samples showed alarming values, and all of them were within the established limits. The results showed that bacterial strains showed different sensitivities regarding honey samples. *S. aureus* was the most sensitive, with a MIC (minimum inhibitory concentration) CMI and CBM (minimum bactericidal concentration) of 62.5 mg/ml when using honey samples S2 S7 S8 S9, while S6 was the less active samples with both CMI and CBM of 250 mg/ml. *P. aeruginosa* showed an intermediate sensitivity, and *E. coli* was the most resistant with values of 250 mg/mL in four of honey samples. Analysing the correlation between the activity and the parameters, disaccharides showed a positive correlation in opposition to monosaccharaide for which the presence with linked to lower the antibacterial activities. From the mineral species only Na and Zn had positive correlation. The nectar provided by *B. spinosum* and *Cytisus sp* seems to be contributing to the antimicrobial activity of honey as a positive correlation was measured with their pollen frequencies.

Keywords.

Zantaz honey; antibacterial activity; physicochemical parameters.

1. Introduction

Honey is one of the oldest traditional medicines for the treatment of several human ailments including infectious diseases [1,2]. This activity was demonstrated for the first time

by Van Ketel in 1892 [3]. Since then, many studies have been published dealing with the antimicrobial activity of honey of different botanical origins, and the mechanisms underlined [4–6]. The emergence of multi-resistant bacteria has contributed to reintroduction of natural products, including honey, as antibacterial compounds [7].

The topic antibacterial activity is due to sugar content (high osmotic pressure), acidic behaviour (low pH), high viscosity, low water activity, low protein content, high contents on bacteriostatic and bactericidal factors like hydrogen peroxide (H_2O_2) and plant derived compounds [8]. Hydrogen peroxide that is produced by glucose-oxidase action on sugars was identified as main antibacterial compound in honey [9,10]. The mechanism of action is still not yet fully understood, but it is known that honey as well as other natural products may act at several levels. Authors reported that honeys act by DNA degrading activity through hydrogen peroxide and an unknown component(s) [11]. It was also shown that removing H_2O_2 eliminated bacteriostatic activities of both phenolics and H_2O_2 suggesting that the observed activity resulted from the combining chemistry between these compounds [11].

Nevertheless, the sensitivity of bacteria toward honey is also influenced by other phytochemical proprieties which may act also as antimicrobial agents [12]. Those parameters include colour, melanoidins, conductivity and mineral content [13,14]. Phytochemicals such as flavonoids, phenolic derivatives and aromatic acids originating from plants delivering the nectar, are also known to contribute to the antimicrobial activity of honey [15]. More recently, methylglyoxal and bee defensin-1 were discovered to determine the activity of some honeys [16].

Previous works highlighted the importance of honey physicochemical properties for its antibacterial activity and used them as a predictive tool for the selection process. It has been shown that antibacterial effect of honey increases with increasing water contents and reducing sugars in honey, while decreases with increasing EC (electrical conductivity) [17]. In addition, honeys of Fabaceae floral origin have a higher detrimental effect on bacteria compared to other spontaneous Saharan species. Hamada et al reported good correlation between honey conductivity and mineral content with its antibacterial activity when associated with essential oils [14].

In fact, honey antibacterial activity is not only dependent on honey properties but also on the targeted bacterial strain. Previous studies showed that bacteria have several sensitivities toward the same honey. Considering only the pH, the minimum values for growth inhibition

of some common pathogenic bacteria are pH 4.3 (Escherichia coli), pH 4.0 (Salmonella spp), pH 4.4 (Pseudomonas aeruginosa) and pH 4.5 (Streptococcus pyogenes) [5]. Studying the sensitivity of different bacterial strains toward Zantaz honey will be the first aim of the present manuscript. In addition, an attempt will be made to establish relationships between the assessed activities and physicochemical properties. This will be useful when filtering honey samples to select the best one for a given bacterial strain.

2. Material and methods

2.1. Sampling.

Honey samples were selected from a previous study of the same research group [18]. As the monoflorality threshold of Zantaz honey is not yet established with strong evidence, the general rule for monoflorality declaration was assumed and samples with *Bupleurum spinosum* pollen exceeding 45% were selected.

2.2. Physico-chemical characterization.

Physico-chemical characterization of Zantaz honey samples consisted on the determination of pH, Acidity (free, lactonic and total) ash content, conductivity, moisture, diastase activity, which were determined according the harmonized methods of the international honey commission [19]. In addition, colour intensity was estimated on the mmpfund scale by determination of the absorbance of honey solutions (50%) at 635 nm (A635) in a Shimadzu spectrophotometer [20]. The mm Pfund values and honey colour were obtained using the following algorithm, mm Pfund = −38.7 + 371.39 x A635. Additionally, honey colour was determined by spectrophotometry by calculating net absorbance (A560- A720). Melanoidins content was estimated based on the browning index (net absorbance=A450-A720) [21]. Spectrophotometric measurements were performed in a 1 cm quartz cell.

Total polyphenolic content was also assessed as an indicator of the bioactive fraction of honey. Polyphenols were determined using the Folin–Ciocalteu reagent [22] and gallic acid as a standard.

Sugars were analysed by High Performance Liquid Chromatography (HPLC) with refraction index (IR) detector according the methodology proposed by Bogdanov (1997) [23].

HPLC analysis was performed on analytical stainless-steel column, Purospher® STAR – NH2 with 4mm in diameter, 250mm length and with 5mm particle size, using a flow rate of 1.3 mL/min; mobile phase: acetonitrile: water (80:20, v/v); column and detector temperature: 30 °C; and sample volume injection of 10 mL. Different sugars (fructose, glucose, sucrose,

melezitose, turanose, maltose, xilose, rhamnose, arabinose, melibiose, trehalose and erlose) were identified and quantified by comparison of the retention times and the peak areas of the honey sugars with those of calibration curves of standard sugars. The values expressed in g/100 g of honey.

For mineral content determination, sample preparation is initiated by incineration of 5 g honey at 550 °C. After cooling, 5 mL of 0.1M nitric acid was added to the ash and the mixture was stirred on a heating plate to almost complete dryness. Then, 10 mL of the same nitric acid solution was added, and the mixture was brought up to a final volume of 25 mL with distilled water. Afterwards, Ca, Mg, Mn, Zn, Cu, and Fe were measured by flame atomic absorption, while Na, K by emission spectrometry (air-acetylene) using a novAA 350 (Analytik Jena, Germany). After the determination of mineral concentrations in the prepared solutions, their values were extrapolated to be expressed as mg/kg honey.

2.3. Antimicrobial activity.

Antimicrobial activity of honey samples was performed by determination of the Minimum Inhibitory Concentration (MIC) and the Minimal Bactericidal Concentration (MBC). *Escherichia coli* (466), *Pseudomonas aeruginosa* strains (Gram-negative bacilli bacteria) and *Staphylococcus aureus* (Gram-positive cocci bacteria) were isolated from Universitary Hospital Hassan II. Bacterial growth is carried out at 37 ° C in Mueller-Hinton Broth (MHB) liquid medium and on Mueller-Hinton Agar (MHA). Bacterial suspensions (inocula) were obtained by taking colonies from 24-hour cultures. These colonies were suspended in physiological saline (0.9% NaCl) and shacked for 15 seconds and the density was adjusted to a turbidity of 0.5 McFarland, which corresponds to an optical density of 0.08-0.13 measured at a wavelength of 625 nm. The final concentration will be approximately 108 CFU (colony forming unit)/ mL [24].

The MICs were determined by micro-dilution test in 96 well microplate according to NCCLS standards. Honey concentrations in wells ranged between 7.81 and 250 mg/ml, while this of inoculums was 5 × 105 CFU/ml, both in a final volume of 200 ul. After an incubation of 20 hours at 37 °C, 40 µl of triphenyltetrazolium chloride (TTC), was added to each well. This compound is reduced into formazan forming a red colour, indicating bacterial growth. After 2 hours of incubation, the MIC is the honey concentration that do not produce a red colour [24]. To determine MBC, a portion from each well corresponding or lower to the MIC was

sub-cultured on MHA and incubated for 24 hours at 37 °C. The MBC is defined as the lowest honey concentration that do not produce a bacterial growth [25].

2.4. Statistical analysis

The means were compared with ANOVA one-way test using IBM SPSS statistics 25. The test was then followed by a post hock tukey test and the differences were accepted to be different with $p<0.05$. Independent student t test was used when two by two comparison are needed. For correlation study, the r value of Pearson correlation was determined between honey bioactivities and physicochemical parameters. The obtained r values were grouped using the dendrogram function of MATLAB 2018a software and presented as heatmap of three colours: red (r = -1), white (r = 0) and blue (r = 1).

3. Results and discussion

Physicochemical properties of honey samples are summarized in Table 1. According to the international quality standards, none of analysed samples showed alarming values [26,27]. pH values ranging from 3.78 to 4.38, is one of the factors inhibiting the growth of microorganisms in honey [28]. Regarding moisture (water content), all honey samples showed values below the maximum allowed in honey (20%) [26,27]. Exceeding this limit is a favourable environment for the growth of undesirable microorganisms that detriment honey quality by fermentation process**.** Free acidity was always lower than 50 mEq/kg, whereas ash content and conductivity did not exceed 0.6% and 800 µS/cm respectively [29]. Diastase activity is useful for determining honey freshness, which at low values it may indicate submission to inadequate storage conditions or processing. A minimum of 0.8 shade unit is required in honey [26] and was respected in Zantaz honey.

Sugars are an important parameter to analyse in honey as they may represent more than 80% of its composition. All analysed samples showed a predominance of fructose with an average of 38.50±2.37 % followed by glucose with an average of 23.25±1.96 %. Sucrose, for which the presence indicate possible adulteration, was below 5% established by the international criteria as the maximum allowed in honey. Mineral content is an indicator of honey geographical origin and may reveal possible environmental pollution if toxic metals are detected [29,30]. Potassium was the most abundant element with values between 429.15 mg / kg and 1177.47 mg/kg followed by calcium with an average of 154.78 ± 20.90 mg/kg. The remaining minerals can be classified in descending order as follows Na > Mg > Fe > Cu > Mn

> Zn. It is worth mentioning that the physicochemical values highlighted in this section were similar to other Zantaz and Moroccan honeys [29–31].

Table 1. Physico-chemical features of the analysed honey samples.

		Means	Sd	Min	Max
General physicochemical properties	**Ash content (%)**	0.19	0.05	0.13	0.32
	Water content (%)	19.88	0.85	18.40	21.13
	Electrical conductivity µs/cm	454.80	85.50	351.66	652.33
	pH	4.05	0.21	3.78	4.38
	Free acidity (mEq/Kg)	18.41	5.72	8.40	26.40
	Lactonic acidity (mEq/Kg)	11.30	1.36	8.00	13.00
	Total acidity (mEq/Kg)	29.71	6.66	18.40	38.40
	Diastase (Shad number)	20.21	4.86	12.38	29.52
	Melanoidins	1.01	0.28	0.40	1.45
	Colour (mm Pfund)	62.71	18.98	25.98	96.42
Sugars (mg/kg)	**Fructose**	38.50	2.37	34.71	41.83
	Glucose	23.25	1.96	19.61	26.89
	Melibiose	2.80	0.92	1.44	4.31
	Turanose	1.79	0.20	1.29	2.04
	Maltose	1.53	0.47	0.95	2.45
	Arabinose	1.60	0.72	0.33	2.44
	Trehalose	0.82	0.29	0.50	1.50
	Xylose	0.30	0.03	0.25	0.36
Minerals (mg/Kg)	**K**	638.19	206.29	429.15	1177.47
	Ca	154.78	20.90	124.15	189.95
	Na	57.70	15.46	38.39	90.90
	Mg	33.16	9.55	24.25	52.80
	Fe	14.22	3.36	7.61	18.23
	Cu	1.29	0.36	0.89	1.95
	Mn	0.85	0.18	0.51	1.12
	Zn	0.66	0.42	0.26	1.72

Bacterial strains showed different sensitivity regarding honey samples (Table 2). *S. aureus* was the most sensitive, with both MIC and MBC of 62.5 mg/mL when using honey samples S2, S7, S8 and S9, while S6 was the less active samples with both MIC and MBC of 250 mg/mL. *P. aeruginosa* showed an intermediate sensitivity, and the threshold of 62.5 in

both MIC and MBC was reached only in the case of S7, and S9, but similarly S6 was the less active. *E. coli* showed to be the most resistant and the threshold of 62.5 mg/mL was not reached in any sample. This bacterial strain showed to be resistant in most samples with values MIC and MBC of 250 mg/mL with samples S4, S5, S6, and S10, similar to the results of MICs reported by other studies [14,32].

Honey composition determines it antibacterial activity, which makes it determined by phytogeographic origin [14,33]. Previous work reported that honey composition and pollen analysis might be useful to predict the antibacterial activity of honey harvested in the Algerian Sahara [17]. It has been shown that honey contributes to its antibacterial activity and may be used as predictive tools when selecting honey. The next section of the present contribution highlights possible influence of honey properties on its antibacterial activity.

Table 2. Antimicrobial activity of Zantaz honey samples against Gram-positive and Gram-negative bacteria.

	***Escherichia coli* (466)**		***Pseudomonas aeruginosa***		***Staphylococcus aureus***	
sample	**MIC (mg/mL)**	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**
S1	125	125	125	125	125	125
S2	125	125	62.5	125	62.5	62.5
S3	125	250	125	125	125	125
S4	250	250	125	125	125	125
S5	250	250	125	250	125	125
S6	250	250	250	250	250	250
S7	125	125	62.5	62.5	62.5	62.5
S8	125	125	125	125	62.5	62.5
S9	125	250	62.5	62.5	62.5	62.5
S10	250	250	125	125	125	125

The antibacterial activity differs between honeys and depends on the targeted bacteria [15]. Honey composition diversity makes it difficult to make choice to select the best honey against a given bacteria. Hence reviewing and re-evaluating in-depth the role of physiochemical properties of honey in determining its antibacterial activity.

To understand the effect of Zantaz honey physicochemical properties in determining its antibacterial activity, Pearson correlation was determined between each parameter and the assessed antibacterial activity. To simplify the output illustration, the r values of the correlation

were converted into colour scale, and similar correlations were clustered together using the clustering property of a dendrogram presentation heat map models was used.

Figure 1 illustrates the correlations between the physicochemical properties of Zantaz honeys and their respective antibacterial activity. The dendrogram presentation showed that, apart from water content, the parameters presented negative correlation with the measured MIC of at least two bacterial strains, making them contributors to the antibacterial activity of Zantaz honey. The observed contribution of melanoidins to honey antibacterial activity was previously reported employing correlations in other honeys [13,14,21]. Numerous authors have shown the antibacterial activity of honey can be attributed to the acidity [13,14,34]. In the case of *E. coli* 466 and *S. aureus*, a negative correlation was obtained between their MIC values and Ash content suggesting ash content contribution, but not in the case of *P. aeruginosa.* Still through figure 1, the results of MIC against the strains used were positively influenced by the phenolic content, but a significant negative correlation was observed only in the case of *E. coli* (ATB: 57). Many studies have shown that antibacterial activity of honey can be attributed to the presence of flavonoids and phenols [9,35]. The positive correlation between the moisture of honey and its antibacterial activity, may be explained by the diluting effect. Increasing honey moisture will consequently dilute the contents of other components.

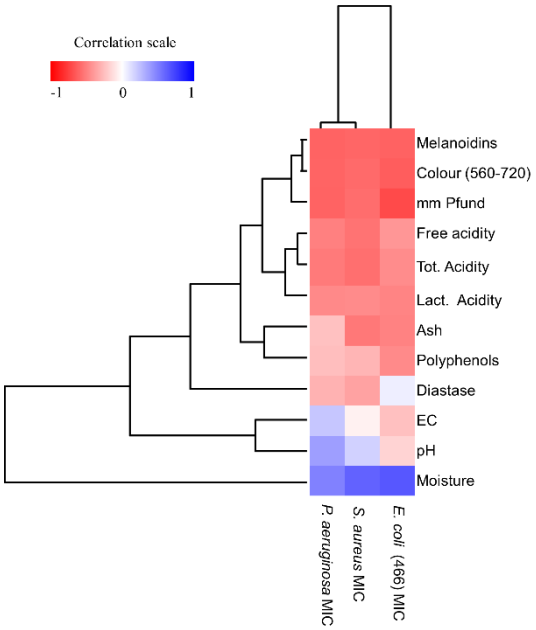

Figure 1: Heat map illustrating the correlation between the physiochemical properties and the assessed antimicrobial activities.

Mineral elements showed also a contribution to the antibacterial activity of honey except for Na and Zn (fig 2 (a)). Regarding potassium, the most abundant elements, it showed the same influence as does the conductivity, for which the use may be enough to replace the K evaluation. The bacteria showed similar sensitivities toward both measurements. *E. coli* was the most sensitive.

Major sugars (glucose and fructose) present in Zantaz honey correlated negatively the bacterial growth (negative correlation with the MICs). Though, *E. coli* was more sensitive to glucose contents than do the other bacteria. While this group of sugars was formed by two hexoses and one aldopentose, the group that seems to favourite bacterial growth was formed by four disaccharides and one aldopentose (fig 2 (b)). As a result of high sugar in honey, it acts in an osmotic manner which causes hypertonicity that induce osmotic dehydration of microorganisms [16,34].

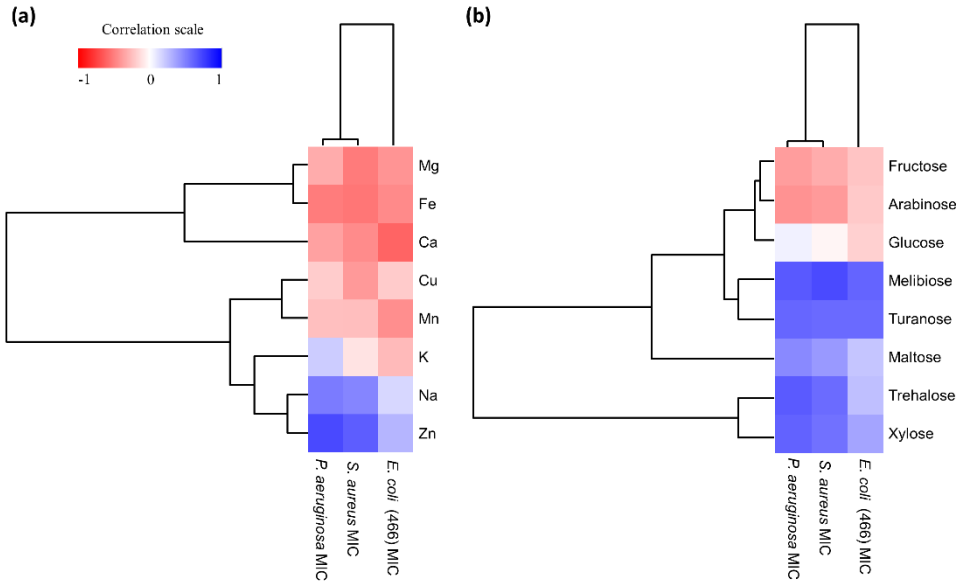

Figure 2: (a) sugars heatmap; (b) minerals heatmap.

4. Conclusion

Analysing Zantaz honey samples with *B. spinosum* pollen predominance from Morocco showed to be in accordance with the international legislation for both freshness and adulteration absence.

Zantaz honey has antibacterial activity depending on its chemical composition and physical properties. Melanoidins, acidity, phenolic compounds, hexoses and divalent minerals showed to be the best parameters to select the best Zantaz honey sample for antibacterial

activity. The sensitivity of bacterial strains varied also. *S. aureus* was the most sensitive to Zantaz honey followed *P. aeruginosa*, while *E. coli* was the most resistive.

5. References

[1] M.D. Mandal, S. Mandal, Honey: Its medicinal property and antibacterial activity, Asian Pac. J. Trop. Biomed. 1 (2011) 154–160. doi:10.1016/S2221-1691(11)60016-6.

[2] T. Eteraf-Oskouei, M. Najafi, Traditional and modern uses of natural honey in human diseases: A review, Iran. J. Basic Med. Sci. 16 (2013) 731–742.

[3] J.H. Dustmann, Antibacterial Effect of Honey*, Apiacta. (1979) 7–11. doi:10.1038/ncomms3838.5.

[4] D. Cianciosi, T. Forbes-Hernández, S. Afrin, M. Gasparrini, P. Reboredo-Rodriguez, P. Manna, J. Zhang, L. Bravo Lamas, S. Martínez Flórez, P. Agudo Toyos, J. Quiles, F. Giampieri, M. Battino, Phenolic compounds in honey and their associated health benefits: a review, Molecules. 23 (2018) 2322. doi:10.3390/molecules23092322.

[5] M. Aurongzeb, K. Azim, Antimicrobial properties of natural honey : a review of literature Antimicrobial properties of natural honey : a review of literature, Pak. J. Biochem. Mol. Biol. (2016).

[6] J. Alvarez-Suarez, M. Gasparrini, T. Forbes-Hernández, L. Mazzoni, F. Giampieri, The composition and biological activity of honey: a focus on manuka honey, Foods. 3 (2014) 420–432. doi:10.3390/foods3030420.

[7] P. Combarros-Fuertes, L.M. Estevinho, L.G. Dias, J.M. Castro, F.A. Tomás-Barberán, M.E. Tornadijo, J.M. Fresno-Baro, Bioactive components and antioxidant and antibacterial activities of different varieties of honey: a screening prior to clinical application, J. Agric. Food Chem. 67 (2019) 688–698. doi:10.1021/acs.jafc.8b05436.

[8] C. Libonatti, S. Varela, M. Basualdo, Antibacterial activity of honey: A review of honey around the world, J. Microbiol. Antimicrob. 6 (2014) 51–56. doi:10.5897/jma2014.0308.

[9] N.A. Albaridi, Antibacterial potency of honey, Int. J. Microbiol. 2019 (2019). doi:10.1155/2019/2464507.

[10] F.C. Bizerra, P.I. Da Silva, M.A.F. Hayashi, Exploring the antibacterial properties of honey and its potential, Front. Microbiol. 3 (2012) 2–3. doi:10.3389/fmicb.2012.00398.

[11] K. Brudzynski, K. Abubaker, D. Miotto, Unraveling a mechanism of honey antibacterial action: Polyphenol/H 2O 2-induced oxidative effect on bacterial cell growth and on DNA degradation, Food Chem. 133 (2012) 329–336. doi:10.1016/j.foodchem.2012.01.035.

[12] K. Brudzynski, K. Abubaker, L. St-Martin, A. Castle, Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey, Front. Microbiol. 2 (2011) 1–9. doi:10.3389/fmicb.2011.00213.

[13] A. Moussa, A. Saad, The influence of botanical origin and physico-chemical parameters on the antifungal activity of Algerian honey, J. Plant Pathol. Microbiol. 03 (2012). doi:10.4172/2157-7471.1000132.

[14] H. Imtara, Y. Elamine, B. Lyoussi, Honey antibacterial effect boosting using origanum vulgare L. essential oil, Evidence-Based Complement. Altern. Med. 2018 (2018). doi:10.1155/2018/7842583.

[15] P. Molan, The antibacterial activity of honey 1. The nature of the antibacterial activity, Bee World. 73 (1992) 5–28.

[16] P.H.S. Kwakman, S.A.J. Zaat, Antibacterial components of honey, IUBMB Life. 64 (2012) 48–55. doi:10.1002/iub.578.

[17] H. Laallam, L. Boughediri, S. Bissati, T. Menasria, M.S. Mouzaoui, S. Hadjadj, R. Hammoudi, H. Chenchouni, Modeling the synergistic antibacterial effects of honey characteristics of different botanical origins from the Sahara Desert of Algeria, Front. Microbiol. 6 (2015) 1–12. doi:10.3389/fmicb.2015.01239.

[18] Y. Elamine, B. Lyoussi, O. Anjos, L.M. Estevinho, S. Aazza, J.D. Carlier, M.C. Costa, M.G. Miguel,

Zantaz honey “monoflorality”: Chemometric applied to the routinely assessed parameters, LWT - Food Sci. Technol. 106 (2019) 29–36. doi:10.1016/j.lwt.2019.02.039.

[19] S. Bogdanov, P. Martin, C. Lüllmann, R. Borneck, C. Ch. Flamini, M. Morlot, J. Heretier, G. Vorwohl, H. Russmann, L. Persano-Oddo, A.. Sabatini, G.. Marcazzan, P. Marioleas, K. Tsigouri, J. Kerkvliet, A. Ortiz, T. Ivanov, HARMONISED METHODS OF THE INTERNATIONAL HONEY COMMISSION, (2002).

[20] O.A. Naab, M.A. Tamame, M.A. Caccavari, Palynological and physicochemical characteristics of three unifloral honey types from central Argentina, Spanish J. Agric. Res. 6 (2008) 566–576.

[21] K. Brudzynski, D. Miotto, The relationship between the content of Maillard reaction-like products and bioactivity of Canadian honeys, Food Chem. 124 (2011) 869–874. doi:10.1016/j.foodchem.2010.07.009.

[22] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, in: Lipids, 1999: pp. 152–178. doi:10.1016/S0076-6879(99)99017-1.

[23] S. Bogdanov, HARMONISED METHODS OF THE INTERNATIONAL IHC responsible for the methods :, (1997) 1–62.

[24] D. Dimitrijević, Antioxidant and antimicrobial activity of different extracts from leaves and roots of Jovibarba heuffelii (Schott.) A. Löve and D. Löve, J. Med. Plants Res. 6 (2012) 4804–4810. doi:10.5897/jmpr12.239.

[25] R.R. Hafidh, Inhibition of Growth of Highly Resistant Bacterial and Fungal Pathogens by a Natural Product, Open Microbiol. J. 5 (2011) 96–106. doi:10.2174/1874285801105010096.

[26] COUNCIL DIRECTIVE 2001/110/EC of 20 December 2001 relating to honey, Off. J. Eur. Communities. (2002) 47–52.

[27] Codex Alimentarius Commission, Codex Alimentarius Commission Standards, Codex Stan 12-1981. (2001) 1–8. doi:10.1007/978-3-540-88242-8.

[28] A. Terrab, M.J. Diéz, F.J. Heredia, Characterisation of Moroccan unifloral honeys by their physicochemical characteristics, Food Chem. 79 (2002) 373–379. doi:10.1016/S0308-8146(02)00189-9.

[29] H. Imtara, Y. Elamine, B. Lyoussi, Physicochemical characterization and antioxidant activity of Palestinian honey samples, Food Sci. Nutr. (2018). doi:10.1002/fsn3.754.

[30] Y. Elamine, S. Aazza, B. Lyoussi, M. Dulce Antunes, L.M. Estevinho, O. Anjos, M. Resende, M.L. Faleiro, M.G. Miguel, Preliminary characterization of a Moroccan honey with a predominance of Bupleurum spinosum pollen, J. Apic. Res. 57 (2018). doi:10.1080/00218839.2016.1265759.

[31] S. Aazza, B. Lyoussi, D. Antunes, M.G. Miguel, Physicochemical characterization and antioxidant activity of 17 commercial Moroccan honeys, Int. J. Food Sci. Nutr. 65 (2014) 449–457. doi:10.3109/09637486.2013.873888.

[32] L. Boukraa, Additive activity of royal jelly and honey against Pseudomonas aeruginosa, Altern. Med. Rev. 13 (2008) 330–333.

[33] E.K. Nishio, J.M. Ribeiro, A.G. Oliveira, C.G.T.J. Andrade, E.A. Proni, R.K.T. Kobayashi, G. Nakazato, Antibacterial synergic effect of honey from two stingless bees: Scaptotrigona bipunctata Lepeletier, 1836, and S. postica Latreille, 1807, Sci. Rep. 6 (2016) 1–8. doi:10.1038/srep21641.

[34] K.S. Kumar, D. Bhowmik, Medicinal uses and health benefits of Honey: An overview, J Chem Pharm Res. 2 (2010) 385–395.

[35] M. Johnston, M. McBride, D. Dahiya, R. Owusu-Apenten, P. Singh Nigam, Antibacterial activity of Manuka honey and its components: An overview, AIMS Microbiol. 4 (2018) 655–664. doi:10.3934/microbiol.2018.4.655.

Chapter III. Physicochemical and functional characteristics of a Moroccan Zantaz honey rich in methyl syringate

Chapter III. Physicochemical and functional characteristics of a Moroccan Zantaz honey rich in methyl syringate*

Abstract

Zantaz honey is produced from the melliferous plant *Bupleurum spinosum (Apiaceae)*, that grows at the summit of high mountains in Morocco and other Mediterranean countries. Previous works of our group revealed that this honey scavenge free radicals *in vitro*. Methyl syringate was the predominant phenolic compound reaching more than 50% of all identified compounds. In the second place comes the epicatechin. This combination may be a characteristic of this honey.

Antiproliferative activity of Zantaz honey was evaluated in THP-1 cells from acute monocytic leukaemia. First, as honey interferes with the MTT reagent inducing an underestimation of its antiproliferative activity, a base line absorbance was made of reference solutions of honey with MTT. The interference was dose dependent and increased the cell viability estimation 1.42 times at a honey concentration of 0.02g/mL and 11.24 times at 0.1 g/mL after an incubation of 24h. While sugars interference, evaluated through identical artificial sugar solutions, was between 1.31 to 2.18 times lower than the entire honey, positive influence was seen in the case of free acidity ($p<0.05$), Colour intensity, fructose and phenolic compounds mainly the methyl syringate content.

Key words

Zantaz honey, antiproliferative activity, cell-based antioxidant activity, MTT assay, interference

1. Introduction

Nowadays, in food chemistry, research for new functional foods is one of the leading tendencies. This trend is encouraged by scientific evidences that prove that food is an important factor in slowing or inhibiting diseases, such as cancer and degenerative ones [1]. Honey is an example of potential functional food because of its richness in nutritional and bioactive compounds [2].

* This chapter will be submitted to Food Chemistry journal after few modifications and the agreement of all co-authors.

Honey bioactivity is conditioned by its botanical origin since it determines its main bioactive fractions [3,4]. The different botanical origins makes it a complex mixture of compounds, being sugars the major part (75% monosaccharides, 10–15% disaccharides and small amounts of other sugars), mixed in water (from 15g to 21g /100 g) [5]. Minor compounds are vitamins, enzymes, amino acids, minerals, organic acids and aroma compounds [6]. Secondary metabolites of plants delivering the nectar such as phenolic acids and flavonoids are the most important bioactive fraction of honey [7]. Many works have reported antibacterial [2], antifungal [8], and wound healing properties [9], amongst others [7], of different honeys. The presence of specific compounds such as methyl syringate in Manuka honey determines its bioactivates and its botanical origin.

Honey from *Bupleurum spinosum* (*Apiaceae*) melliferous plant is a dark coloured honey, with *in vitro* antioxidant activity [10]. This activity, due to phenolics from *B. spinosum* and possibly other plants, suggest this honey as a possible functional food useful in the treatment of diseases involving oxidative stress. Nevertheless, no previous information has been published about the phenolic composition of Zantaz honey. Such information may provide further characterising biological properties of Zantaz honey and help its botanical discrimination.

In the last years, cell-based assays have been extended to determine the bioactivity of functional compounds, since they provide more formation than in-tube assays, such as for example the bioavailability of the functional compounds [11]. Antiproliferative or immunomodulatory activity of different honeys have been reported against THP-1 and other cancer cells [12]. Honey can be used in its original form or after extraction of bioactive fractions [13–15].

The aim of the present contribution it to test Zantaz honey antioxidant activity on Caco-2 cells and its antiproliferative activity on THP-1 cells. Possible effects of identified compounds will be assessed throughout the appropriate statistical tools. In addition, a focus will be provided on Zantaz honey phenolic composition and it is possible use as finger print for the botanical origin declaration.

2. Material and methods

2.1. Honey samples and physicochemical characterisation.

Six honey samples were purchased from Moroccan beekeepers from Fez and Taza regions. pH, free acidity, lactonic acidity, ash content, electrical conductivity, moisture content and diastase activity were determined according to the International Honey Commission (IHC) [16]. Colour and melanoidins contents were determined as previously described [17].

2.2. Determination of sugar composition

High Performance Liquid Chromatography (HPLC) with refraction index (IR) detector was used for the identification of sugars (fructose, glucose, sucrose, melezitose, turanose, maltose, xylose, ramnose, arabinose, melibiose, trehalose and erlose) according to [16]. Sugars identification and quantification was made by comparison of retention times and peak areas with those of standards.

2.3. Determination of phenolic composition.

Analysis of phenolics was carried out by RP-HPLC and detection at 254 nm, using an Ultrasphere ODS column (4.6 mm x 25 mm and 5 µm particle size) (Beckman-Coulter, Orange County, CA, USA). Elution of polyphenols was carried out at 1 mL/min using the following gradient of methanol in water adjusted to pH 3.0 with phosphoric acid: 0 to 70 min, gradient from 0 to 70% methanol; 70 to 75 min, 70% methanol; 75 to 80 min gradient to 100% methanol; 80 to 85 min, 100% methanol.

The identification of the major compound which did not correspond to any of available standards was carried out by LC/HRMS using an Agilent 1200 Rapid Resolution HPLC interfaced to a Bruker maXis mass spectrometer as described by Pérez-Victoria, Martín, & Reyes (2016). The obtained molecular formula/exact mass was searched against the Chapman and Hall Dictionary of Natural Products database.

2.4. Cell culture.

THP-1 and Caco-2 cells were obtained from the European Cell Culture Collection and cultured under standard cell cultured conditions (5% CO_2 at 37 °C) in DMEM medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 100 U/ml penicillin, and 100 mg/mL streptomycin. THP-1 cells were subcultured every three days, while Caco-2 cells once a week.

2.5. Determination of the antiproliferative activity of Zantaz honey.

THP-1 cell line, derived from human monocytic leukaemia**,** normally, grows in suspension, unless they differentiated to macrophages upon stimulation [19]. The antiproliferative assay relying on 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) reagent reduction is widely used [20]. THP-1 cells were seeded in 96-well plates (10^4/well) and incubated with different honey concentrations from 0.02 to 0.10 g/mL, for 24 h and 72 h. Cells were exposed to MTT by addition of fresh medium containing the reagent so that the final concentration was 0.5 mg/mL and were incubated for 1 h in the CO2 incubator. Reduced MTT was solubilized by addition of the same volume of 0.1 N HCl in isopropanol. Absorbance at 570 nm with a background reference wavelength of 630 nm was measured using a plate reader (Abs_1).

To avoid the interference of honey in MTT assay, the absorbance at 570 nm of honey reaction with MTT was measured in the absence of cells (Abs_2). This absorbance was used to correct the cell viability overestimation following the formula: $Abs = Abs_1 – Abs_2$. Negative control (no honey) was considered 100% cell viability and was used to estimate cell viability for honey dilutions. The obtained curve (Cell viability vs honey concentration) was used to calculation the IC_{50}, the honey concentration inducing 50% of the antiproliferative activity. The expressed results are the average of 5 replicates.

2.6. Caco-2 based antioxidant activity.

Caco-2 cell line derived from a colon tumour were used to evaluate antioxidant activity of honey samples by measuring the 2,2′-Azo-bis-aminopropane (ABAP)-induced production of free radicals using the dichlorofluorescein (DCF) assay. Cells were seeded in 96 well microplates (100 µl, 10^4 cells/well) and incubated with honey samples until reaching confluency. When they are allowed to grow to confluence, Caco-2 cells lose their cancerous phenotype and differentiate into enterocyte-like cells [21]. Growth medium was removed, and wells were washed with HBSS before addition of DCFH-DA (50 µl, 25 µM in HBSS). Plates were preincubated at 37 °C for 1 h and washed with HBSS (100 µl) followed by addition of 285 µM ABAP in HBSS (100 µl). Plates were then incubated in a Fluoroskan Ascent plate-reader (Thermo Scientific, MA, USA) at 37 °C and fluorescence (excitation at 485 nm, emission at 555 nm) was measured every 10 min for 180 min. Each plate included a positive control (cells treated with DCFH-DA and the free radical generator ABAP) and negative control (cells treated only with DCFH-DA). The negative control was included in order to prevent overestimation of free radicals due to photo oxidation of DCFH. Lower fluorescence

emission in the presence of honey indicates a lower free radical concentration due to the antioxidant activity of the honey. Antioxidant activity was calculated as CAA of each honey samples as shown in the following equation: $CAA = (AUC_c - AUC_s)/AUC_c$. Furthermore, the calibration curve of Quercetin (concentration vs CAA) was used to calculate the quercetin equivalency for honey samples.

2.7. Statistical analysis.

The means were compared with ANOVA one-way test using IBM SPSS statistics 25. The test was then followed by a post hock tukey test and the differences were accepted to be different with $p<0.05$. Independent student t test was used when two by two comparison are needed. For correlation study, the r value of Pearson correlation was determined between honey bioactivities and physicochemical parameters. The obtained r values were grouped using the dendrogram function of MATLAB 2018a software and presented as heatmap of three colours: red (r = -1), white (r = 0) and blue (r = 1). $1/IC_{50}$ was calculated for antiproliferative activity to avoid negative correlation with possible antiproliferation agents and simplify though results interpretation.

3. Results and discussion

3.1. Preliminary characterization: sugars, phenolic compounds, minerals and other physicochemical properties.

Table 1 shows the average physicochemical properties of Zantaz honey samples. From a quality control point of view, and according to the international standards [22], both minimum and maximum measured values did not show undesirable characteristics. Average ash content and conductivity were below the maximum allowed of 800 μS/cm and 0.6%, respectively. All analysed samples had a diastase activity above 8.0 Shade units/g required for honey authenticity. Being lower than this threshold may indicate heat processing or inadequate storage conditions. For moisture content, some samples presented values slightly exceeding the maximum of 20% allowed in honey, but without being significant. Acidic behaviour of honey was also measured, and the pH values ranged between 3.78 and 4.22. In addition, total acidity was withing the established limits varying between 12.85 and 19.10 mEq/Kg.

Sugars contents determine the nutritional value of honey represent the main fraction. Fructose and glucose were the predominant sugars with average values of 38.79±2.65 g/100g and 22.15±1.27 g/100g, respectively, being in accordance with previous studies. Sucrose contents are useful for the determination of honey adulteration. Zantaz honey samples have

sucrose below 5%, the maximum generally allowed in honey [22]. Melezitose presence may indicate the presence of a honeydew source among the participating botanic origins [23].

The contamination of nectar by the apiaries surrounding sands is the main source of its mineral content. Hence, it is useful for the determination of its geographical origins [24]. In terms of abundance, the analysed minerals in zantaz honey samples can be ordered as follow K> Ca> Na> Mg> Fe> Cu> Mn> Zn. This order has already been reported previously in Zantaz honey [17] and other Moroccan honeys at least considering the first four elements [25].

In addition to their use for quality control of the analysed samples, the above-mentioned parameters will be useful to assess their contribution to the interference of honey in the MTT assay or its bioactivities. Details will be provided in correlation and multivariate analysis section.

Table 1. Physicochemical characterisation of Zantaz honey. results are the average ± sd of six different samples.

	Parameter	Mean ± SD	Min	Max
Physicochemical properties	**Conductivity (µs/cm)**	429.16 ± 55.78	351.66	527
	Ash content (g/100g)	0.18 ± 0.03	0.13	0.22
	pH	3.99 ± 0.18	3.78	4.22
	Free acidity (mEq/Kg)	19.21 ± 4.14	14.7	26.2
	Lactonic Acidity (mEq/Kg)	11.92 ± 0.73	11	13
	Total Acidity (mEq/Kg)	15.56 ± 2.2	12.85	19.1
	Diastase activity (Shad number)	22.12 ± 4.32	15.82	29.52
	Melanoidins	1.09 ± 0.13	0.86	1.25
	Colour (mm Pfund)	67.65 ± 7.56	51.93	74.25
	Water content (%)	19.86 ± 0.92	18.4	21.13
Minerals contents (mg/Kg)	**K**	572.35 ± 94.69	429.15	694.71
	Ca	153.49 ± 16.23	124.15	170.73
	Na	52.96 ± 12.5	38.39	77.69
	Mg	32.19 ± 9.43	24.35	52.8
	Fe	15.32 ± 2.93	11.52	18.23
	Cu	1.32 ± 0.32	0.89	1.8
	Mn	0.88 ± 0.2	0.51	1.12
	Zn	0.56 ± 0.22	0.35	0.88
Sugars (g/100 g)	**Fructose**	38.7 ± 2.65	33.52	41.83

Glucose	22.15 ± 1.27	19.61	23.42
Melibiose	2.84 ± 1.00	1.44	4.28
Turanose	1.9 ± 0.11	1.77	2.05
Arabinose	1.69 ± 0.78	t	2.44
Maltose	1.63 ± 0.67	0.95	3.03
Trehalose	1.03 ± 0.46	0.68	2.03
Melezitose	0.19 ± 0.22	t	0.51
Sucrose	t	t	t

Polyphenols, resulting from plants secondary metabolism, are heterogeneous class of chemical compounds, that can be divided into flavonoids and non-flavonoids (mainly phenolic acids) [7]. Both groups are found in honey and are useful for determining the botanical origin of honey especially in the case of monofloral types. They are also responsible of the honey bioactivity [6,7]. RP-HPLC analysis of Zantaz honey showed the presence of 19 compounds (table 2). Methyl syringate was the most abundant phenolic compound. Its contents ranged from 277.93 µg/g in sample H5 to 443.89 µg/g in H6, representing 40.44% and 57.88% of all polyphenols respectively. Such high amounts are not usual in Moroccan honeys. In contrast, similar high concentrations (above 122.6 mg/kg) are a characteristic of Manuka honey [26]). Epicatechin (flavan-3-ol) was the second abundant phenolic compounds with an average value of 77.05 ± 47.87 µg/g. Their derivatives syringic acid and catechin were respectively the following most abundant compounds.

In addition to the above-mentioned use of individual phenolics for floral origin determination, the integration of all identified phenolic compounds at their specific proportions is a chemical fingerprint that can distinguish a botanical origin from another [27].

Table 2. Phenolic composition of studied *Zantaz* honey samples.

Phenolic compounds (µg/g)	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**Means ± SD**	**Min**	**Max**
Methyl syringate	327.20	336.62	285.92	370.81	277.93	443.89	340.39 ± 55.82	277.93	443.89
Epicatechin	54.90	62.58	56.08	78.81	179.29	30.67	77.05 ± 47.87	30.67	179.29
Syringic acid	31.49	39.53	25.16	32.33	53.07	105.86	47.91 ± 27.34	25.16	105.86
Catechin	66.13	54.52	24.35	14.56	37.64	26.68	37.31 ± 17.92	14.56	66.13
4- Coumaric acid	18.83	11.90	17.43	19.11	49.43	5.13	20.31 ± 13.91	5.13	49.43
Gallic acid	22.76	14.66	18.04	15.32	0.00	34.40	17.53 ± 10.27	0.00	34.40
Quercetin	12.26	13.23	7.53	10.67	10.39	42.77	16.14 ± 12.04	7.53	42.77
Apigenin	13.85	15.50	19.61	33.38	1.25	7.00	15.10 ± 10.11	1.25	33.38
Luteolin	0.00	11.60	22.08	14.04	21.22	16.08	14.17 ± 7.34	0.00	22.08
Kaempferol	19.11	5.24	7.51	10.51	15.96	19.64	12.99 ± 5.58	5.24	19.64
Naringenin	0.00	42.41	0.00	19.82	0.77	0.00	10.50 ± 15.97	0.00	42.41
Formononetin	3.26	0.87	4.92	10.01	9.04	17.88	7.66 ± 5.55	0.87	17.88
Genistein	6.61	0.00	0.00	24.00	5.40	3.36	6.56 ± 8.18	0.00	24.00
3- Coumaric acid	7.03	5.85	2.40	8.38	8.66	2.72	5.84 ± 2.50	2.40	8.66
Daidzein + Pelargonidin	1.84	3.29	6.63	3.52	6.09	8.16	4.92 ± 2.19	1.84	8.16
2- Coumaric acid	0.45	0.89	1.75	1.12	8.17	1.66	2.34 ± 2.64	0.45	8.17
Biochanin A	1.71	0.86	0.96	4.10	2.83	1.03	1.92 ± 1.19	0.86	4.10
Cyanidin	0.39	0.31	1.13	2.30	0.19	0.00	0.72 ± 0.79	0.00	2.30
Total phenolic content	**587.82**	**619.86**	**501.49**	**672.78**	**687.31**	**766.91**	**639.36 ± 83.39**	**501.49**	**766.91**
Methyl syringate (%)	**55.66**	**54.31**	**57.01**	**55.12**	**40.44**	**57.88**	**53.40 ± 5.92**	**40.44**	**57.88**

3.2. Honey interferences in the MTT assay.

Previous work on prostate cancer cells PC3 and DU145 reported an interference of honey with the MTT assay inducing an increase of absorbance and suggested possible influence of reducing sugars and phenolic compounds [28]. The increase in absorbance overestimates cell count which causes an underestimation of honey antiproliferative activity. This behaviour may also be linked to the fact that honey absorbs light at similar wavelengths to that used for the MTT assay. In attached cells adding a PBS (phosphate buffered saline) washing step before the addition of MTT removes the fraction of honey that is not absorbed by cells and solves the issue [28]. These authors applied a PBS washing step before the addition of the MTT reagent.

Preliminary analysis of zantaz honey antiproliferative activity with the MTT assay, showed an increase in THP-1 cells viability, in a dose dependent manner, with the increase in honey concentration (blue line in fig.1 (a)). At highest honey concentration assayed (0.1g/mL) cell viability reached 140% of the control. However, microscopy observation of these cells showed an opposite behaviour and the number of cells in fact decreases with the increase of honey concentration. In contrast, after 72h of incubation the antiproliferative activity of honey can be seen in MTT assay (red line of figure 1 (b)) as well as in visual inspection photos (bottom of figure 1 (b)). Still the activity seems to be more pronounced when using the visual evaluation.

Application of the suggested washing step cannot be made in the MTT protocol when cells in suspension, such as THP-1, are used. Instead of the PBS washing step, we determined and subtracted the interference produced by the reaction of honey with the MTT to determine the real absorbance of MTT. As a result of this interference elimination the antiproliferative activity of honey samples was observed even after the first 24h of incubation (red line of fig.1 (a)). In comparison to positive control (absence of honey), the number of cells was below 20% of this control at the highest honey concentration assayed. These results are in accordance the visual observation of cells. After 72h incubation, the antiproliferative activity of honey samples were more pronounced when the absorbance baseline was used to correct the initial values. The cell viability was below 20% at lower honey concentration (0.03g/mL) in comparison to that of 24h incubation (0.07 g/mL). This result is presented by the red curve of figure 1 (b).

The difference between corrected and raw data were significant ($p<0.001$) in all concentrations after 24 hours of incubation. It worth mentioning that the interference was attenuated after 72h. After 24h the caused overestimation was 1.42 times higher than the

corrected estimation at a honey concentration of 0.02g/mL and 11.24 times at 0.1 g/mL (fig. 1 (a)), showing the dose dependent behaviour of the interference. After 72h, the ratios at same concentrations were only 1.17 and 4.59 while maintaining a dose dependency manner.

Based on the obtained results, no determinant conclusion can be made about this decrease in the interference, although it can be suggested that it may be due to the slight evaporation known to occur in the medium after hours of incubation [29]. Another suggestion is that the cells absorb honey compounds that are responsible for the interference also in a time dependent manner.

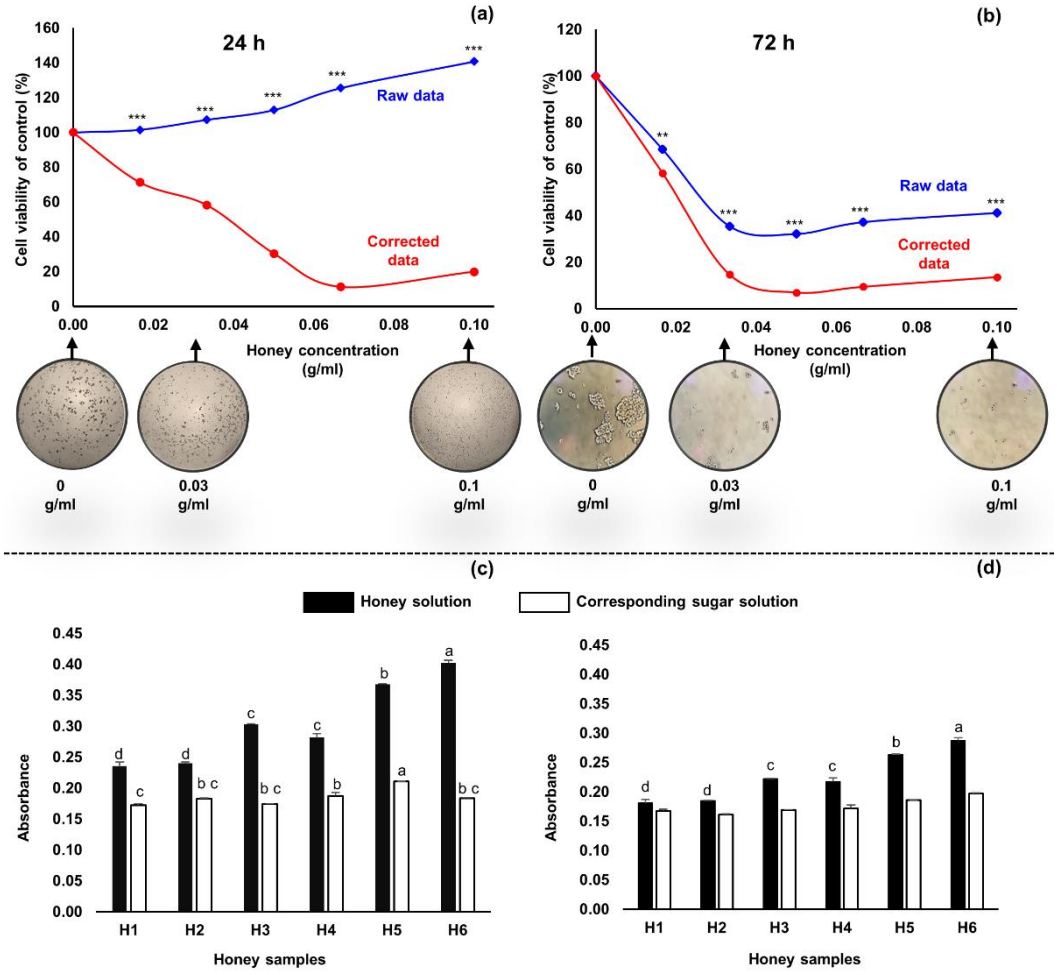

Figure 1. interference of honey with the MTT assay. Comparison between raw and corrected data with corresponding visual inspections of THP-1 cells after 24h of incubation (a) and after 72 hours (b). ** $p<0.01$; ***$p<0.001$. Sugars contribution to the interference in comparison to the whole honey after 24 hours (c) and 72 hours (d). Samples or sugars solutions flagged with the same alphabet are not significantly different ($p<0.05$).

A previous study suggested that the interference of honey in the MTT assay was due to the reduction of MTT by sugars in honey and highlighted it by employing an artificial honey [28]. Their results showed that the used sugar solution increased the MTT absorbance by 57.0%. In the present contribution, a corresponding sugars solution was made for each honey sample by mixing its major sugars at correct ratios in DMEM. Figure 1 (c) shows that in all analysed samples the interference in the MTT Assay was not due entirely to sugars but varied greatly amongst honey samples. Honey absorbance was 131±0.1% that of the sugar solution in H2, while it was 218 ± 3.6% in H6. After an incubation of 72h the values decreased to be 108±1% and 146 ± 3.7%. The decrease of this ratio was due to a decrease in honey interference with th MTT. The absorbance of sugar solution remained almost constant comparing the two incubation times (24h and 72h). It worth mentioning that lowering honey concentration decreased its interference in the MTT assay, which may be an option in the case of honey samples with high antiproliferative activity as high dilution will be required.

3.3. Antiproliferative and antioxidant activities of Zantaz honey.

Antiproliferative activity was studied on THP-1 cells. After 24 h of incubation with Zantaz honey, the viability of THP-1 cells decreased significantly in a dose dependent manner (fig. 2 (a)). The concentration that inhibits the growth of THP-1 cells by 50 % (IC_{50}) was determined (Fig. 2 (b)). The highest activity was observed in sample H1 with and IC_{50} of 30 ± 0.97mg/ml while sample H5 showed the lowest activity, with an IC_{50} of 43 ± 4.79 mg/mL. The observed difference was statistically different ($p < 0.05$).

With respect to the antioxidant activity, Zantaz honey samples showed clear differences among them. Three statistically distinguishable population groups could be observed. A group with highly antioxidant samples formed by H2 and H6. An intermediate sample is H1, and the last group formed by samples H3, H4, and H5. The difference between the three groups was statistically significant ($p < 0.05$). Wan et al. (2015) compared the antioxidant activity of pure phytochemicals at both *in vitro* and *in vivo* and observed positive correlation between Caco-2 antioxidant assay and rats' plasma ORAC (oxygen radical absorbance capacity) [11].

In both cell-based assays, the activity is mostly be due to the presence of phenolic compounds, such as methyl syringate. Similarly, in *Asphodelus* honeys, antioxidant activities *in vitro* and *in vivo* were related to the high phenolic content including methyl syringate [30,31]. The anti-proliferative activity of manuka honey on murine melanoma (B16.F1), colorectal carcinoma (CT26) and human breast cancer (MCF-7) cells acted through activation of a

caspase 9-dependent apoptosis, induction of caspase 3 which encoded for reduced Bcl-2 expression, and DNA fragmentation and finally apoptosis [26]. *In vivo*, manuka decreased tumour volume by increasing the apoptosis in a mouse melanoma model and reduced colonic inflammation in inflammatory bowel disease in rats [32].

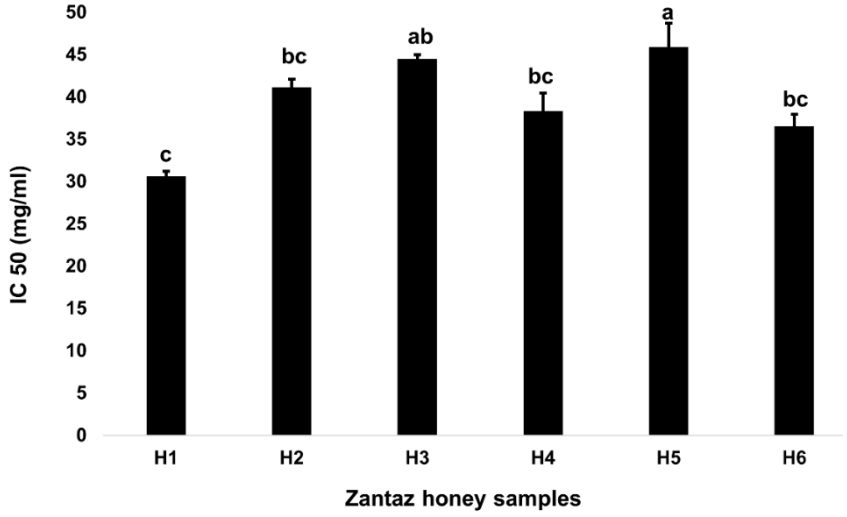

Figure 2. Antiproliferative activity of Zantaz honey samples after 24 hours incubation with THP-1 cells.

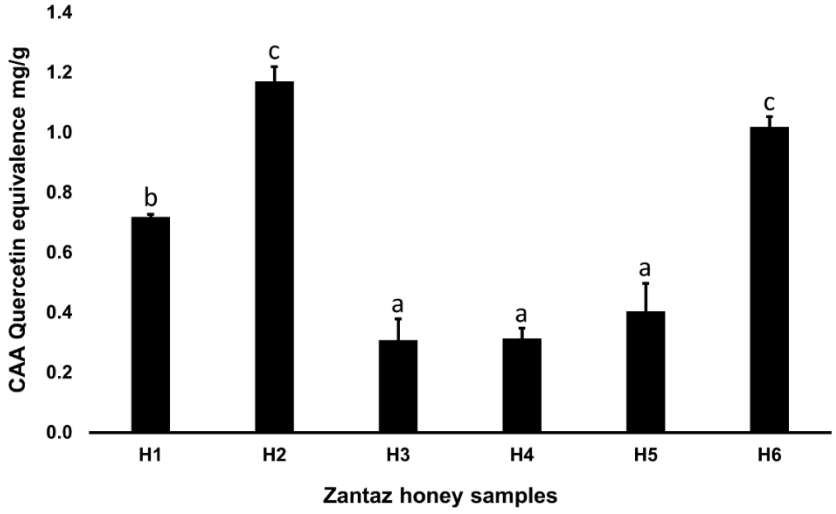

Figure 3. Antioxidant activity of Zantaz honey samples in Caco-2 cells expressed as cellular antioxidant activity (CAA) quercetin equivalence mg/g of honey.

3.4. Correlations and multivariate analysis.

Honey is a complex mixture of many compounds of different nature. To identify which compounds in Zantaz honey were more responsible of the antiproliferative and antioxidant activity, a correlation and multivariate analysis of these activities with the physicochemical characteristics of honey was carried out.

To study possible effects of honey physico-chemical properties, on its interference with MTT, antiproliferative and cell-based antioxidant activities, r value of correlation test was determined for each parameter and activity. Only compounds identified in all samples at significant amounts were selected. For phenolic compounds and mineral, a threshold of 10 mg/kg was established for the selection process. The clustered correlation matrix is presented as a heat map in figure 4.

Four clusters are recognized in the obtained dendrogram. The first group of parameters was formed by catechin, pH, minerals (K, Mg, Na, Fe and Ca) and hence their representative parameters ash content and conductivity. Those parameters showed positive correlation with the measured bioactivities, while they correlated negatively the interference. It was expected that catechin will have also a positive correlation with the interference. An explanation is that catechin bioavailability was fast which may be due to the presence of sugars or other compounds. This suggestion is in accordance with previous study that showed catechin bioavailability enhancement in formulation with sucrose and ascorbic acid [33].

The elements of the second group were free acidity, two reducing sugars (fructose and arabinose) and five phenolic compounds (syringic acid, quercetin, methyl syringate, gallic acid and kaempferol), which are the main contributors to honey colour, and explain though the presence of colour intensity and melanoidins in the same group. The elements of this cluster possess positive correlation with both bioactivities and honey interference with the MTT assay. Abel & Baird (2018) reported that the interference with the MTT assay is mainly due to the presence of reducing sugars and phenolic compounds [28]. In addition, the phenolic compounds are known to possess both antioxidant and antiproliferative activities *in vitro*, in cells and *in vivo* models [6,7,14,15,30,34,35]

Melanoidins are the final products of the Maillard reactions (MR), which takes place in foods containing reducing sugars and compounds possessing free amino groups, are also known to possess antiproliferative against rhabdomyosarcoma-medulloblastoma, glioma,

breast, colon, and lung carcinoma. Melanoidins decrease cancer cell motility, inducing remarkable morphological changes and apoptotic cell death [36].

The elements of the third group showed the opposite behaviour to that of group 1. The positive correlation of honey interference with lactonic acidity was expected because the same behaviour was seen also in the case of free acidity. Also, epicatechin, 4-coumaric acid and luteolin showed a positive correlation with the interference but showed to have negative correlation with bioactivities. A suggested explanation is that the matrix, and the existence of other phenolics might reduce the bioavailability of some phenolic compounds [37]. This explains the interaction of the phenolic compounds at the extracellular level by their positive correlation with the interference in the MTT assay, and the absence of their intracellular bioactivities.

The last group was formed by elements presenting negative correlation with the cell-based antioxidant activity and honey interference with the MTT while they seemed to induce its antiproliferative activity (positive correlation with $1/IC_{50}$). This group is formed by reducing sugars (glucose, maltose, melibiose and turanose) and none reducing sugars (trehalose). Unexpectedly, two reducing sugars showed negative correlation with the interference glucose and melibiose. In the first case, taking into account that in honey if a nectar is rich in fructose, then it is less in glucose, and because in our case fructose as it is major, it was translated by negative correlation with glucose and interference. The same can be said about melibiose in addition to its being a minor sugar.

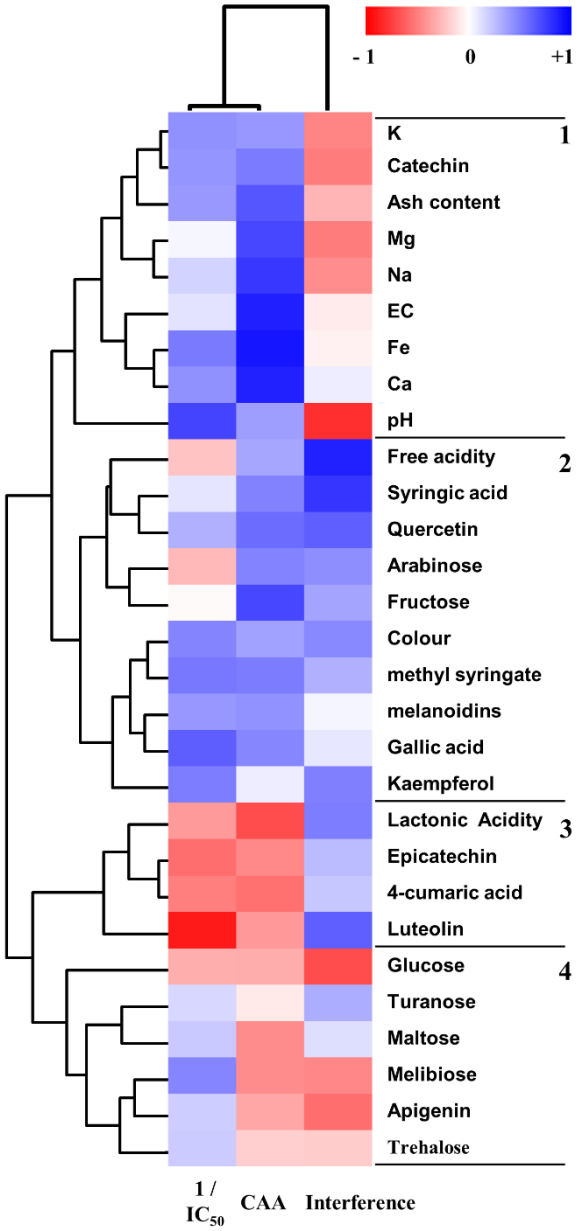

Figure 4. Heat map of the evaluated correlation r value supported with the clustering ability of dendrogram presentation. Significant correlation at $p<0.05$ is flagged by a star "*".

4. Conclusion

Zantaz honey samples presented high amounts of methyl syringate, which may be considered as floral origin determination when the geographical region is known. The observed amounts were even higher than those found in Manuka honey. regarding its bioactivity, Zantaz honey possess both antioxidant and antiproliferative activity, which seems to be due to its content on phenolic compounds.

Employing a reference solution to correct the increase seen in the absorbance of MTT with honey seems to be promising. The overestimation of cell viability was reduced, and the final output was similar to visual inspection observations.

Acknowledgments

5. References

[1] A. Bach-Faig, E.M. Berry, D. Lairon, J. Reguant, A. Trichopoulou, S. Dernini, F.X. Medina, M. Battino, R. Belahsen, G. Miranda, L. Serra-Majem, J. Aranceta, T. Atinmo, J.M. Barros, S. Benjelloun, I. Bertomeu-Galindo, B. Burlingame, M. Caballero-Bartolí, C. Clapés-Badrinas, S. Couto, I. Elmadfa, R. Estruch, A. Faig, F. Fidanza, S. Franceschi, J. Hautvast, E. Helsing, D. Julià-Llobet, C. La Vecchia, A. Lemtouni, A. Mariné, M.A. Martínez-González, R. Mokni, F. Mombiela, I. Noain, B. Obrador, G. Pekcan, S. Piscopo, B. Raidó-Quintana, E. Ros, S. Sáez-Almendros, J. Salas-Salvadó, F. Sensat, D. Trichopoulos, J.A. Tur, M.D. Vaz Da Almeida, W.C. Willett, M.J. Amiot-Carlin, A. Bellio, C. Cannella, R. Capone, D. Cassi, L.M. Donini, C. Lacirignola, G. Maiani, M. Mancini, N. Merendino, M. Padilla, S. Padulosi, Mediterranean diet pyramid today. Science and cultural updates, Public Health Nutr. 14 (2011) 2274–2284. doi:10.1017/S1368980011002515.

[2] P. Szweda, Antimicrobial activity of honey, in: Honey Anal., InTech, 2017. doi:10.5772/67117.

[3] A.A.M. De-melo, A.A.M. De-melo, L.B. De Almeida-muradian, M.T. Sancho, A. Pascual-maté, A. Alexandre, L.B. De Almeida-muradian, Composition and properties of Apis mellifera honey : A review Composition and properties of Apis mellifera honey : A review, J. Apic. Res. (2017) 1–33. doi:10.1080/00218839.2017.1338444.

[4] C. Chen, L. Wang, R. Wang, X. Luo, Y. Li, J. Li, Y. Li, Z. Chen, Phenolic contents , cellular antioxidant activity and antiproliferative capacity of different varieties of oats, Food Chem. 239 (2018) 260–267. doi:10.1016/j.foodchem.2017.06.104.

[5] P. Missio, C. Gauche, L.V. Gonzaga, A. Carolina, O. Costa, Honey : Chemical composition , stability and authenticity Honey : Chemical composition , stability and authenticity, FOOD Chem. 196 (2016) 309–323. doi:10.1016/j.foodchem.2015.09.051.

[6] S. Bogdanov, T. Jurendic, R. Sieber, P. Gallmann, Honey for nutrition and health: A review, J. Am. Coll. Nutr. 27 (2008) 677–689. doi:10.1080/07315724.2008.10719745.

[7] D. Cianciosi, T. Forbes-Hernández, S. Afrin, M. Gasparrini, P. Reboredo-Rodriguez, P. Manna, J. Zhang, L. Bravo Lamas, S. Martínez Flórez, P. Agudo Toyos, J. Quiles, F. Giampieri, M. Battino, Phenolic compounds in honey and their associated health benefits: a review, Molecules. 23 (2018) 2322. doi:10.3390/molecules23092322.

[8] J. Irish, D.A. Carter, T. Shokohi, S.E. Blair, Honey has an antifungal effect against Candida species, Med. Mycol. 44 (2006) 289–291. doi:10.1080/13693780500417037.

[9] B. Minden-Birkenmaier, G. Bowlin, Honey-based templates in wound healing and tissue engineering, Bioengineering. 5 (2018) 46. doi:10.3390/bioengineering5020046.

[10] Y. Elamine, S. Aazza, B. Lyoussi, M. Dulce Antunes, L.M. Estevinho, O. Anjos, M. Resende, M.L. Faleiro, M.G. Miguel, Preliminary characterization of a Moroccan honey with a predominance of Bupleurum spinosum pollen, J. Apic. Res. 57 (2018). doi:10.1080/00218839.2016.1265759.

[11] H. Wan, D. Liu, X. Yu, H. Sun, Y. Li, A Caco-2 cell-based quantitative antioxidant activity assay for antioxidants, Food Chem. 175 (2015) 601–608. doi:10.1016/j.foodchem.2014.11.128.

[12] L. Porcza, C. Simms, M. Chopra, Honey and cancer: current status and future directions, Diseases. 4 (2016) 30. doi:10.3390/diseases4040030.

[13] S. Jaganathan, A. Balaji, M. Vellayappan, M. Asokan, A. Subramanian, A. John, E. Supriyanto, S. Razak, M. Marvibaigi, A review on antiproliferative and apoptotic activities of natural honey, Anticancer. Agents Med. Chem. 15 (2014) 48–56. doi:10.2174/18715206146661407220847 47.

[14] S.K. Jaganathan, M. Mandal, Antiproliferative effects of honey and of its polyphenols: a review, J. Biomed. Biotechnol. 2009 (2009) 1–13. doi:10.1155/2009/830616.

[15] S. Ahmed, N.H. Othman, Honey as a potential natural anticancer agent: a review of its mechanisms, Evidence-Based Complement. Altern. Med. 2013 (2013) 1–7. doi:10.1155/2013/829070.

[16] S. Bogdanov, P. Martin, C. Lüllmann, R. Borneck, C. Ch. Flamini, M. Morlot, J. Heretier, G. Vorwohl, H. Russmann, L. Persano-Oddo, A.. Sabatini, G.. Marcazzan, P. Marioleas, K. Tsigouri, J. Kerkvliet, A. Ortiz, T. Ivanov, HARMONISED METHODS OF THE INTERNATIONAL IHC responsible for the methods :, (1997) 1–59.

[17] Y. Elamine, B. Lyoussi, O. Anjos, L.M. Estevinho, S. Aazza, J.D. Carlier, M.C. Costa, M.G. Miguel, Zantaz honey "monoflorality": Chemometric applied to the routinely assessed parameters, LWT - Food Sci. Technol. 106 (2019) 29–36. doi:10.1016/j.lwt.2019.02.039.

[18] I. Pérez-Victoria, J. Martín, F. Reyes, Combined LC/UV/MS and NMR Strategies for the Dereplication of Marine Natural Products, Planta Med. 82 (2016) 857–871. doi:10.1055/s-0042-101763.

[19] S. Tsuchiya, Y. Yamaguchi, Y. Kobayashi, Establishment and characterization of a human acute monocytic leukemia cell line (THP-1), Int. J. Cancer. 176 (1980) 171–176.

[20] J. Weyermann, D. Lochmann, A. Zimmer, A practical note on the use of cytotoxicity assays, Int. J. Pharm. 288 (2005) 369–376. doi:10.1016/j.ijpharm.2004.09.018.

[21] J. Girón-Calle, J. Vioque, J. Pedroche, M. Alaiz, M.M. Yust, C. Megías, F. Millán, Chickpea protein hydrolysate as a substitute for serum in cell culture, Cytotechnology. 57 (2008) 263–272. doi:10.1007/s10616-008-9170-z.

[22] COUNCIL DIRECTIVE 2001/110/EC of 20 December 2001 relating to honey, Off. J. Eur. Communities. (2002) 47–52.

[23] S. BOGDANOV, K. RUOFF, L.P. ODDO, Physico-chemical methods for the characterisation of unifloral honeys : a review, Apidologie. 35 (2004) 4–17. doi:10.1051/apido.

[24] B. Pasquini, M. Goodarzi, S. Orlandini, G. Beretta, S. Furlanetto, B. Dejaegher, Geographical characterisation of honeys according to their mineral content and antioxidant activity using a chemometric approach, Int. J. Food Sci. Technol. 49 (2014) 1351–1359. doi:10.1111/ijfs.12436.

[25] S. Aazza, B. Lyoussi, D. Antunes, M.G. Miguel, Physicochemical characterization and antioxidant activity of 17 commercial Moroccan honeys, Int. J. Food Sci. Nutr. 65 (2014) 449–457. doi:10.3109/09637486.2013.873888.

[26] S. Patel, S. Cichello, Manuka honey: an emerging natural food with medicinal use, Nat. Products Bioprospect. 3 (2013) 121–128. doi:10.1007/s13659-013-0018-7.

[27] J. Bertoncelj, T. Polak, U. Kropf, M. Korošec, T. Golob, LC-DAD-ESI/MS analysis of flavonoids and abscisic acid with chemometric approach for the classification of Slovenian honey, Food Chem. 127 (2011) 296–302. doi:10.1016/j.foodchem.2011.01.003.

[28] S.D.A. Abel, S.K. Baird, Honey is cytotoxic towards prostate cancer cells but interacts with the MTT reagent : Considerations for the choice of cell viability assay, Food Chem. 241 (2018) 70–78. doi:10.1016/j.foodchem.2017.08.083.

[29] C.R. Warren, Rapid measurement of chlorophylls with a microplate reader, J. Plant Nutr. 31 (2008) 1321–1332. doi:10.1080/01904160802135092.

[30] Z. Jubri, N.B. Abdul Rahim, G.J. Aan, Manuka honey protects middle-aged rats from oxidative damage, Clinics. 68 (2013) 1446–1454. doi:10.6061/clinics/2013(11)11.

[31] J.M. Stephens, R.C. Schlothauer, B.D. Morris, D. Yang, L. Fearnley, D.R. Greenwood, K.M. Loomes, Phenolic compounds and methylglyoxal in some New Zealand manuka and kanuka honeys, Food Chem. 120 (2010) 78–86. doi:10.1016/j.foodchem.2009.09.074.

[32] J. Alvarez-Suarez, M. Gasparrini, T. Forbes-Hernández, L. Mazzoni, F. Giampieri, The composition and biological activity of honey: a focus on manuka honey, Foods. 3 (2014) 420–432. doi:10.3390/foods3030420.

[33] C.M. Peters, R.J. Green, E.M. Janle, M.G. Ferruzzi, Formulation with ascorbic acid and sucrose modulates catechin bioavailability from green tea, Food Res. Int. 43 (2010) 95–102. doi:10.1016/j.foodres.2009.08.016.

[34] V. Baltrušaityte, P.R. Venskutonis, V. Čeksteryte, Radical scavenging activity of different floral origin honey and beebread phenolic extracts, Food Chem. 101 (2007) 502–514. doi:10.1016/j.foodchem.2006.02.007.

[35] J.M. Alvarez-Suarez, S. Tulipani, S. Romandini, E. Bertoli, M. Battino, Contribution of honey in nutrition and human health: A review, Med. J. Nutrition Metab. 3 (2010) 15–23. doi:10.1007/s12349-009-0051-6.

[36] E. Langner, W. Rzeski, Biological properties of melanoidins: a review, Int. J. Food Prop. 17 (2014) 344–353. doi:10.1080/10942912.2011.631253.

[37] M. D'Archivio, C. Filesi, R. Varì, B. Scazzocchio, R. Masella, Bioavailability of the polyphenols: Status and controversies, Int. J. Mol. Sci. 11 (2010) 1321–1342. doi:10.3390/ijms11041321.

Part III. Phytochemical and functional characterization of propolis samples

Chapter I. Phytochemical characterization and antiproliferative activity of propolis samples obtained from hives in the surrounding area of *Bupleurum spinosum* melliferous plant

Chapter I. Phytochemical characterization and antiproliferative activity of propolis samples obtained from hives in the surrounding area of *Bupleurum spinosum* melliferous plant [*]

Abstract

Propolis samples were collected form hives during the flowering season of *Bupleurum spinosum,* which is known by the production of the Moroccan Zantaz honey. World wild propolis is known to be rich in bioactive compounds and to possess several biological activities *in vitro* and *in vivo*. The surrounding areas of collected sample is mainly occupied by *Bupleurum*, *Quercus, Juniperus, Cedrus,* which contribute specifically to the quality of this propolis.

High Performance Liquid Chromatography-Dyode Array Detector (HPLC-DAD) analysis showed the presence of hydrophobic phenolic compounds. The antiproliferative activity was tested in THP-1 cells, a phenotype of leukaemia. The IC_{50} was as low as 0.60±0.07 µg/mL. 1.23±0.02 ug/mL was the highest measured value, while one sample had an activity similar to that of the DMSO (vehicle). Based on their phenolic profiles assessed as area under curve, samples with the lowest area had the lowest antiproliferative activity and vice versa. although the correlation was not significant.

Key words

Propolis, THP-1 cells, antiproliferative activity, HPLC, *B. spinosum*

1. Introduction

Honeybee is known to produce several products with nutritional and health benefits [1]. While honey is the first one considering the nutritional value with its high levels of sugars, minerals and amino acids [2,3], propolis might be the first one in terms of bioactive compounds [1,4]. Propolis, from the greek ‘pro’=‘in front’ or ‘in defence’ and ‘polis’=‘the city’, meaning ‘defence of the hive’, is a strongly adhesive natural product manufactured by honey bees from the buds or exudates of plants near their hives [4]. Propolis is used by bees to seal holes in hives, smooth out the internal walls, making the hive defensible, and as a barrier against external invaders, diseases, thermal insulation, humidity and wind [5]

[*] The work done in this chapter will be improved by the addition of detailed composition before considering for publication

Propolis is composed of resin (~50%), wax (~30%), essential oils (~10%) and pollen (~5%)[4]. The geobotanical and climatic components are the most influencing factors on determining propolis chemical composition which determine its functional properties [6]. Pollen is introduced in propolis unintentionally by the bee-workers during nectar foraging or by wind. Hence, pollen analysis cannot be used as strong characterization tool, as the plant delivering the resinous material are not necessarily providing the pollen. Although, pollen analysis in propolis can be used as guiding tool for the determination of melliferous plants in the surrounding areas and help determining the plants delivering the resin. D'Albore (1979) analysed 56 samples of propolis from different countries and continents, and showed that in most cases, it was possible to determine their phytogeographical origin by pollen analysis. Later on, Barth (1998), focused on analysing Brazilian propolis and emphasised the importance of pollen grains from anemophilous plants in characterising the geographical origin of this propolis [7].

Poplar, birch, green, red, 'Clusia', 'Pacific' and Mediterranean are the seven main types of propolis distinguishable based on their major compounds and geobotanical source [4]. The Moroccan propolis belong to the Mediterranean type. The total phenolic content in propolis can reach up to 238.9 mg GAE/g (gallic acid equivalent)/g EEP (ethanolic extract propolis) for samples collected in South Korea [8]. High amounts of phenolic compounds were also reported for Moroccan propolis with a content of 265.37mg ferulic acid equivalent in a sample purchased in Outat Elhaj region [9]. This region is known by the commercialization of Zantaz honey collected from surrounding mountains suggesting that the propolis belongs to the same type studied in the present paper.

The high phenolic content of propolis determines its bioactivity against several health issues, especially those based on oxidative stress [1,10]. This property is known as chemoprotective effect, and can be proved by the ability of propolis to attenuate the side effects of different circumstances, such as cytopenia, radiation or chemotherapy in animal models [4].

Antiproliferative activity of propolis is widely reported against several cancer lines *in vitro* and *in vivo* employing propolis of different geobotanical origins. Propolis from Italy had apoptosis-like in androgen-resistant prostate cancer cell line DU145 [11]. Croatian propolis and some of its polyphenolic derivative compounds had cytotoxic activity on tumour cells of transplantable mammary carcinoma mouse model and prolong survival of mice [12]. Furthermore, propolis and its phenolic compounds can protect from the lethal effect of irradiation in animal models suggesting that propolis could be an adjunct to anticancer drugs

to boost their antitumor activity and decrease chemo/radiotherapeutic side effect [13,14]. Propolis from Turkey had anticarcinogenic and antimitotic effect against human breast carcinoma, hepatocellular carcinoma, prostate adenocarcinoma, colon adenocarcinoma, HL-60 myeloid leukemia, lung carcinoma and mammary adenocarcinoma cells line [4].

In Morocco, several studies aimed the characterisation and some biological activities of propolis [9,15–17], however no work has been focused on propolis samples originating from hives in surrounding areas of *B. spinosum* populations. Antioxidant, anti-inflammatory, antibacterial, and antidiabetic activities were reported for Moroccan propolis samples from different regions and botanical sources. Regarding antitumoral activity, propolis samples form Morocco showed antiproliferative activity against human tumour cell lines (MCF7- breast adenocarcinoma, NCI-H460- non-small cell lung carcinoma, HeLa- cervical carcinoma, HepG2- hepatocellular carcinoma, and MM127- malignant melanoma), as well as non-tumour cells (a porcine liver primary culture-PLP2) [18]. The resulted cytotoxicity was as low as 9 ug/mL in the case HeLa cell lines with samples that showed less phenolic compounds in comparison to the other samples. It was suggested that this observation may be due to low content on specific phenolic compound responsible of the desired activity.

The complication of in vivo studies due to financial and ethical constraints, implicated the development of *in vitro* experiments [19]. However, using *in vitro* antioxidant activity using free radicals may be misleading as it does not implicate the bioavailability principles [20]. Hence cell lines derived from mouse or humans, originating from cancerous cells, are important intermediate tool between the two ways. Cell based assays are tools to study cellular functions, mechanisms and responses, as well as signalling pathways and nutrient and drug transport [19].

THP-1 cell line is derived from human leukemia monocytic cell line, which has been used to study monocyte/macrophage functions, mechanisms, signalling pathways, and nutrient and drug transport. In addition, this type of cells grows in suspension and is easy to manipulate due to its fast doubling time (35 to 50 h) compared to that of PBMC-derived monocytes [19].

The selection of adequate sample, that presents specific attributes is then important before its application, and it can be made by a screening of its active fraction. The objective of the present study is to identify the pollen grains present in propolis samples collected in the surrounding areas of *Bupleurum spinosum* melliferous plant, known by the production of the so called Zantaz honey in Morocco. The present work aimed also the characterisation of phenolic composition of the collected samples and their antiproliferative activity.

2. Material and methods

2.1. Samples preparation.

Figure 1 illustrate the sample preparation. Hydroalcoholic extracts were prepared for 6 Moroccan propolis samples from hives in the surrounding areas of *Bupleurum spinosum*. Three grams of each sample was subjected to maceration in 30 ml of 70% hydroalcoholic solution for 72h. The obtained solution was centrifuged to eliminate the wax and debris, before their dry under N_2. The dry maters were solved in DMSO (dimethyl sulfoxide) at a concentration of 5 mg/mL which were used to prepare the propolis solution by applicating a 1/1000 (v/v) dilution in DMEM medium to final concentration of 5 µg/mL.

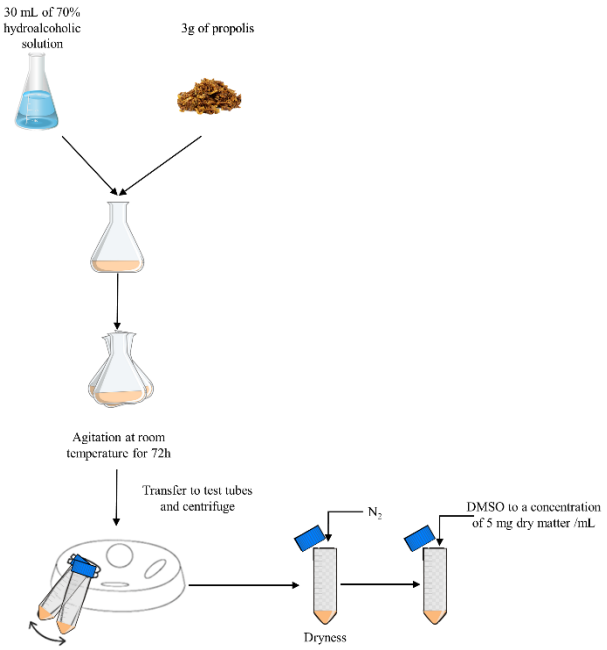

Figure 1. Preparation procedure of propolis samples.

2.2. Phenolic composition

Analysis of phenolics was carried out by RP-HPLC and detection at 254 nm, using an Ultrasphere ODS column (4.6 mm x 25 mm and 5 µm particle size) (Beckman-Coulter, Orange County, CA, USA). Elution of polyphenols was carried out at 1 mL/min using the following gradient of methanol in water adjusted to pH 3.0 with phosphoric acid: 0 to 70 min, gradient from 0 to 70% methanol; 70 to 75 min, 70% methanol; 75 to 80 min gradient to 100% methanol; 80 to 85 min, 100% methanol. Twenty two compounds were used as standards.

2.3. Cell culture and antiproliferative activity of propolis.

THP-1 cells were obtained from the European Cell Culture Collection and cultured under standard cell cultured conditions (5% CO_2 at 37 °C) in DMEM medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. THP-1 cells were subcultured every three days.

THP-1 cell line, derived from human monocytic leukaemia**,** normally, grows in suspension, unless they differentiated to macrophages upon stimulation [21]. The antiproliferative assay relying on 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) reagent reduction is widely used [22]. THP-1 cells were seeded in 96-well plates (10^4/well) and incubated with different propolis concentrations from 0.25 to 1.5 µg/mL, for 24 h and 72 h. Cells were exposed to MTT by addition of fresh medium containing the reagent so that the final concentration was 0.5 mg/ml and were incubated for 1 h in the CO2 incubator. Reduced MTT was solubilised by addition of the same volume of 0.1 N HCl in isopropanol. Absorbance at 570 nm with a background reference wavelength of 630 nm was measured using a plate reader. Negative control (no propolis) was considered 100% cell viability and was used to estimate cell viability for propolis dilutions. The obtained curve of cell viability *vs*. propolis concentrations was used to deduce the IC_{50}, the porpolis concentration inducing 50% of the antiproliferative activity. The expressed results are the average of 5 replicates.

2.4. Statistical analysis.

The means were compared with ANOVA one-way test using IBM SPSS statistics 25. The test was then followed by a post hock tukey test and the differences were accepted to be different with $p<0.05$. Independent student t test was used when two by two comparison are needed. For correlation study, the r value of Pearson correlation was determined between honey bioactivities and obtained areas of phenolic compounds.

3. Results and discussion

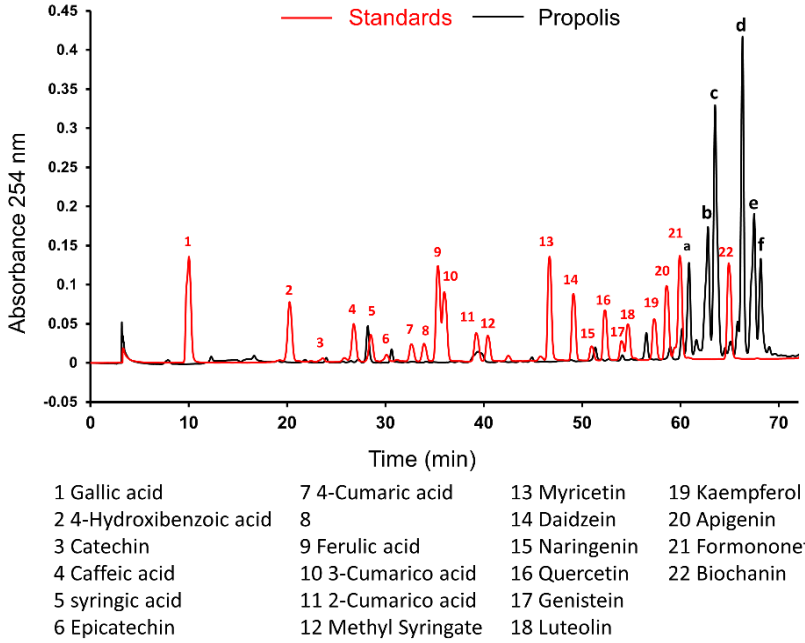

Figure 2. HPLC-DAD profile of one propolis sample with the available standards.

Figure 2 is a HPLC-DAD profile of both propolis samples (black line) and the employed standards (red line). None of the twenty-two standards was present in propolis at detectable levels. The majority of propolis compounds appears after 50 min of running time. This region is known by the presence of hydrophobic phenolic compounds.

For seeking of simplicity, the detected compounds were designated by alphabet letters from "a" to "f", and their respective areas will be used to perform correlation study with propolis anti-proliferative activity. Table 1 shows the compounds designation, retention time and respective area. Considering the sum of the areas, P6 and P1 had the lowest values with areas of 23932380 and 32844765 respectively. The remaining samples showed roughly similar areas ranging from 38430308 and 45907629.

Table 1. Detected compounds in propolis samples and their respective areas obtained by HPLC-DAD.

	RT (min)	P1	P2	P3	P4	P5	P6
a	**60.1**	2754582	2760643	1940918	2448782	2522157	1331765
b	**62.85**	4853618	5590439	6076182	6905899	7994612	3420211
c	**63.48**	8618301	9695273	10333598	8697385	11947359	4978374
d	**66.36**	8255115	11102520	10067961	8863675	11413657	7743751
e	**67.58**	5314275	6210161	6568377	5994464	8277513	4362013

f	**68.23**	3048874	3763621	3443272	3209575	3752331	2096266
sum	**-**	**32844765**	**39122657**	**38430308**	**36119780**	**45907629**	**23932380**

Propolis samples collected from hives in the surrounding areas of *B. spinosum* showed distinguishable effect regarding their antiproliferative activity against THP-1 cells. after 24h of incubation, cell viability decreased in a dose dependent manner as can be observed in figure 3 (a). The difference between samples has also the same dose dependency behaviour and reaches its maximum at a honey concentration of 1.5 µg/mL. Cell viability at 1.5 µg/mL is illustrated in figure 3 (b) and shows significant differences between propolis samples and the used vehicle (DMSO) except for sample P6. The measured values ranged between 60±8% in P1 and 91±2% in P6, while DMSO at the same volumes used to solve propolis samples did not cause cell viability reduction.

After 72h of incubation the cell viability decrease becomes clearer with the increase of propolis concentration in the medium as can be observed in figure 3 (c). at the highest concentration of propolis, five of the analysed samples decreased THP-1 cells viability by more than 50% allowing the determination of IC_{50} for conclusion elaboration. For sample S6 and the DMSO, there were no significant decrease, and THP-1 cells viability remained almost 100%. Figure 3 (d) illustrate the concentration that inhibited the growth of 50% of THP-cells (IC_{50}).

Samples P3 exhibited the highest antiproliferative activity by an IC_{50} value of 0.60 ± 0.07 µg/mL followed by P4 and P5 with IC_{50} values of 0.83 ± 0.02 µg/mL and 0.99 ± 0.01 µg/mL respectively. P1 and P2 exhibited slightly higher IC_{50} in comparison to previous samples with values of 1.23 ± 0.02 µg/mL and 1.15 ± 0.09 µg/mL respectively but remain lower than those reported for other Moroccan propolis samples on other cancer cell lines [18].

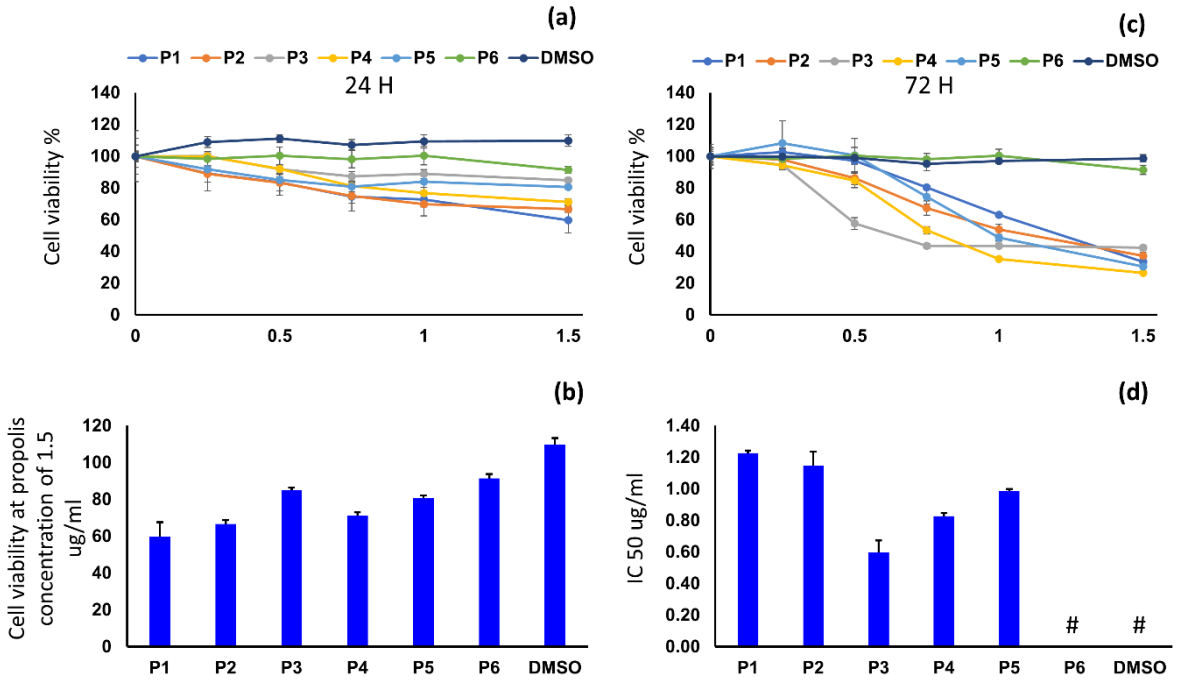

Figure 3. Antiproliferative activity of propolis samples against THP-1 cells. (a) Cell viability in function of propolis concentration after 24h of incubation. (b) Cell viability at 1.5 µg/mL of propolis after 24h of incubation. (c) Cell viability in function of propolis concentration after 72h of incubation. (d) Cell viability at 1.5 µg/ml of propolis after 72h of incubation. # IC_{50} determination was not possible because of the low activity.

Table 2. Pearson's correlation between the areas of the detected phenolic compounds and antiproliferative activity of propolis samples.

		a	b	c	d	e	f	sum
IC50	Pearson's Correlation	.964**	-0.389	-0.235	-0.069	-0.434	-0.019	-0.284
	Significance	0.008	0.517	0.703	0.912	0.465	0.976	0.643
	N	5	5	5	5	5	5	5

**. Correlation is significant at the 0.01 level (2-tailed).

Pearson's correlation between the antiproliferative activity and the areas of detected phenolics is summarized in table 2. Overall, no significant correlation between propolis antiproliferative activity and the compounds individually or considering the sum. Nevertheless, samples P1 and P6 that showed the lowest antiproliferative activity, had also less phenolic content considering the sum of the areas. It worth mentioning that previous work on antiproliferative activity of Moroccan propolis reported a nonsignificant correlation between the measured activity on NCI-H460, HepG2 and MCF-7 cells lines and total phenolics, and a significance was seen only with specific compounds [18].

The compounds designated as “a” had a significant positive correlation with the antiproliferative activity. Two suggested explanation may be given. The first one is that the compound “a” induce the proliferation of THP-cells. The second is that this compound is present in propolis samples at levels negatively related to other compounds, and the observed positive correlation is an artefact and not real. This suggestion seems to be stronger as it can be strengthened by the negative correlation existing between the compound “a” and the remaining compounds (data not shown). In other words, as propolis has several botanical sources, if one source is predominant its compounds will also be predominant and will govern the bioactivity.

4. Conclusion

Propolis samples collected from hives in the surrounding areas of B. spinosum exhibited very interesting antiproliferative activity. The measured IC_{50} on THP-1 cells was as low as 0.60±0.07 µg/mL. Although no significant correlation was observed with the phenolic profile information, propolis sample with very low phenolic content presented a very low antiproliferative activity.

Acknowledgment

5. References

[1] V.R. Pasupuleti, L. Sammugam, N. Ramesh, S.H. Gan, Honey, propolis, and royal jelly: a comprehensive review of their biological actions and health benefits, Oxid. Med. Cell. Longev. 2017 (2017). doi:10.1155/2017/1259510.

[2] S. Patel, S. Cichello, Manuka honey: an emerging natural food with medicinal use, Nat. Products Bioprospect. 3 (2013) 121–128. doi:10.1007/s13659-013-0018-7.

[3] J.M. Alvarez-Suarez, S. Tulipani, S. Romandini, E. Bertoli, M. Battino, Contribution of honey in nutrition and human health: A review, Med. J. Nutrition Metab. 3 (2010) 15–23. doi:10.1007/s12349-009-0051-6.

[4] S. El-Guendouz, B. Lyoussi, M.G. Miguel, Insight on propolis from mediterranean countries: chemical composition, biological activities and application fields, Chem. Biodivers. (2019). doi:10.1002/cbdv.201900094.

[5] A.F.N. Ramos, J.L. Miranda, Propolis: a review of its anti-inflammatory and healing actions, J. Venom. Anim. Toxins Incl. Trop. Dis. 13 (2007) 697–710.

[6] G. RICCIARDELLI d’ALBORE, L’ORIGINE GÉOGRAPHIQUE DE LA PROPOLIS, Apidologie. 10 (1979) 241–267. doi:10.1051/apido:19790303.

[7] O.M. Barth, Pollen analysis of Brazilian propolis, Grana. 37 (1998) 97–101.

doi:10.1080/00173139809362650.

[8] X. Wang, K. Sankarapandian, Y. Cheng, S.O. Woo, H.W. Kwon, Relationship between total phenolic contents and biological properties of propolis from 20 different regions in South Korea, BMC Complement. Altern. Med. (2016) 1–12. doi:10.1186/s12906-016-1043-y.

[9] S. El-Guendouz, S. Aazza, B. Lyoussi, V. Bankova, M. Popova, L. Neto, M.L. Faleiro, M. Da Graça Miguel, Moroccan propolis: a natural antioxidant, antibacterial, and antibiofilm against staphylococcus aureus with no induction of resistance after continuous exposure, Evidence-Based Complement. Altern. Med. 2018 (2018). doi:10.1155/2018/9759240.

[10] J.M. Sforcin, Biological properties and therapeutic applications of propolis, Phytother Res. 905 (2016) 894–905. doi:10.1002/ptr.5605.

[11] C. Scifo, V. Cardile, A. Russo, R. Consoli, C. Vancheri, F. Capasso, A. Vanella, M. Renis, Resveratrol and propolis as necrosis or apoptosis inducers in human prostate carcinoma cells, Oncol Res. 14 (2004) 415–426. doi:10.3727/0965040041791437.

[12] N. Oršolić, I. Bašić, Immunomodulation by water-soluble derivative of propolis: a factor of antitumor reactivity, J. Ethnopharmacol. 84 (2003) 265–273. doi:10.1016/S0378-8741(02)00329-X.

[13] N. Oršolić, V. Benković, A. Horvat-Knežević, N. Kopjar, I. Kosalec, M. Bakmaz, Ž. Mihaljević, K. Bendelja, I. Bašić, Assessment by survival analysis of the radioprotective properties of propolis and its polyphenolic compounds, Biol. Pharm. Bull. 30 (2007) 946–951. doi:10.1248/bpb.30.946.

[14] N. Oršolic, I. Bašic, Antitumor , hematostimulative and radioprotective action of water-soluble derivative of propolis (WSDP), 59 (2005) 561–570. doi:10.1016/j.biopha.2005.03.013.

[15] N. El Menyiy, N. Al Waili, M. Bakour, H. Al-Waili, B. Lyoussi, Protective effect of propolis in proteinuria, crystaluria, nephrotoxicity and hepatotoxicity induced by ethylene glycol ingestion, Arch. Med. Res. 47 (2016) 526–534. doi:10.1016/j.arcmed.2016.12.010.

[16] S. El-guendouz, L. Badiaa, Potential therapeutic effect of Moroccan propolis in hyperglycemia, dyslipidemia, and hepatorenal dysfunction in diabetic rats, Iran. J. Basic Med. Sci. (2019). doi:10.22038/ijbms.2019.33549.8004.

[17] S. El-Guendouz, S. Aazza, B. Lyoussi, M.D. Antunes, M.L. Faleiro, M.G. Miguel, Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis, Int. J. Food Sci. Technol. 51 (2016) 1762–1773. doi:10.1111/ijfs.13133.

[18] S.I. Falcão, R.C. Calhelha, S. Touzani, B. Lyoussi, I.C.F.R. Ferreira, M. Vilas-Boas, In Vitro interactions of Moroccan propolis phytochemical's on human tumor cell lines and anti-inflammatory properties, Biomolecules. 9 (2019) 315. doi:10.3390/biom9080315.

[19] W. Chanput, J.J. Mes, H.J. Wichers, THP-1 cell line: An in vitro cell model for immune modulation approach, Int. Immunopharmacol. (2014) 1–9. doi:10.1016/j.intimp.2014.08.002.

[20] F. Shahidi, H. Peng, Bioaccessibility and bioavailability of phenolic compounds, J. Food Bioact. 4 (2018). doi:10.31665/jfb.2018.4162.

[21] S. Tsuchiya, Y. Yamaguchi, Y. Kobayashi, Establishment and characterization of a human acute monocytic leukemia cell line (THP-1), Int. J. Cancer. 176 (1980) 171–176.

[22] J. Weyermann, D. Lochmann, A. Zimmer, A practical note on the use of cytotoxicity assays, Int. J. Pharm. 288 (2005) 369–376. doi:10.1016/j.ijpharm.2004.09.018.

General conclusion: Discussion and perspectives

1. Introduction

In Morocco, Beekeeping and its related sciences are attracting more attention from both beekeepers and scientific community. The valorisation of bee products starts from determining their characterizing properties which discriminate them for labelling, bioactivity evaluation or just to determine their suitability for human uses. Such characterization can be made by individual analysis of established parameters, or by employment of sophisticated mathematical tools to elaborate more determinant conclusion.

Nowadays the characterization is passing from traditional techniques, requiring costly tools and trained professionals to low cost and reliable technique that may lack the specificity but provide enough information to solve classification, discrimination problems for examples. The e-tongue technology is one of the promising techniques in this context.

Other valorization step is the determination of biological activities, and pharmacological properties of bee-products. Their nutritional values and their richness in bioactive compounds make them potential for the discovery of functional properties.

During the elaboration of the present thesis, a focus was made about the characterization of two products, namely honey and propolis. Both products are collected in the surrounding areas of *B. spinosum*. This last chapter summarizes the most relevant results, discussion and perspectives.

2. Zantaz honey: Quality control and monoflorality declaration.

2.1. Preliminary characterization

First screening of mellissopalynological, and physicochemical characterization was made for a Moroccan honey type with *B. spinosum* as main pollen source. This honey is called *zandaz*, *zentaz*, *aguerbaz* or *airbaz* honey, referring to the berbere name of *B. spinosum* Gouan (Syn. *Bupleurum fruticescens* subsp. *spinosum* (Gouan) O. Bolos & Vigo). This species can be found in the siliceous high peaks of eastern Anti-Atlas, generally above 2000 m a.s.l. [1]. Some authors also reported the presence of this species at high altitudes of the High Atlas (above approximately 2900 m a.s.l.) and in some mountains of the middle Atlas [2] where the samples of this study were mainly collected. The flowering period occurs during August–September [3]

In the present thesis, *Zantaz* honey samples belonging to two different years were analysed. This characterization aimed the comparison of Zantaz honey to the international quality control standards, the determination of its characterizing properties, and the

determination of the influence of *B. spinosum* pollen presence on the acquired physical and chemical properties.

The comparison of pollen profiles revealed more contribution of *B. spinosum* pollen presenting an average of 62.50 ± 8.66% in the samples collected between 2011 and 2013 (season 1) and those collected during 2015 (51.02 ± 16.11%) (season 2). Besides this quantitative difference, some pollen species were only identified in one of the two groups, making a qualitative difference between them. Namely, *Thymus* sp., *Opuntia* sp., *Phoenix* sp., *Olea* sp., *Epilobium* sp., *Cactus* sp. and *Acacia* sp. were identified only in the samples of the first sampling, while the second group was specifically characterized by the presence of *Populus* sp., *Prunus* sp., *Quercus* sp., *Raphanus* sp., *Salvia* sp., *Euphorbia* sp., *Lactuca* sp., *Mimosa* sp., *Artocarpus altilis, Phytolacca* sp., *Pinus* sp., *Erica* sp., *Citrus* sp., *Celtis* sp. and *Cytisus* sp.

From a quality point of view, the variability of botanical sources did not alter Zantaz honey parameters, and almost all analysed samples were within the established limits [4]. However, the mentioned qualitative and quantitative differences of pollen profiles were translated into differences in honey parameters. No difference was observed in the case of pH, diastase activity between the two seasons. In contrast, lactonic acidity, ash content and the electrical conductivity were slightly lower in samples of the season 2 in comparison to the first one.

Regarding sugar analysis, in the samples of the season 1 was lower than that of season 2 (1.48 ± 0.17 vs 1.52 ± 0.22). While melezitose has been detected in all samples of season 1, it has been detected in only two samples of season 2. This none reducing trisaccharide is characteristic of honeydew honey [5]. Its presence in Zantaz honey suggest the participation of a honeydew component in the composition of this honey type. This suggestion can be strengthened by the observed increase in the electrical conductivity in the samples that present high melezitose level. Electrical conductivity is another parameter used to discriminate blossom honey from honeydew types being higher than 800mS/cm^2 in honeydew.

When analysing the mineral content of Zantaz honeys, potassium prevailed in all samples, as reported in literature [6], followed by the calcium, and in a third position the sodium. While the magnesium and iron occupied the fourth and the fifth places, cupper, manganese and zinc were present at very low levels with values equal or below 2 mg/kg, except one sample which presented an amount of 6.43 ± 0.073 mg/kg cupper. The comparison was

not possible in this case as no mineral analysis have been performed for samples of season 1. In overall, the mineral content, based on the assessed elements, ranged between 0.067% (S10) and 0.148% (S9), being within the reported values (0.04%-0.2%) [6].

2.2. Chemometrics for monoflorality determination

The difference between samples of the two seasons become clearer when the analysed parameters are integrated using chemometric tools. The samples of season 1 were discriminated from those of season 1 by the presence of more *B. spinosum* and *Phoenix.* ssp pollen, making them, based on the seen positive correlation, more acidic and darker. Samples of season 2 presented, mainly, more *Eucalyptus* sp., *Euphorbia* sp., *Populus* sp., and so presented the opposite features: high pH values and light colour.

The prevalence of *B. spinosum, Opuntia* sp., *Thymus* sp., *Phoenix* sp. and *Cardus* sp. Pollens in samples of season 1 characterized them by higher contents of trehalose and melezitose. In the 18 honey samples of season *Artocarpus* sp., *Eragostis* sp., *Populus* sp., *Salvia* sp., *Euphorbia* sp. and *Eucalyptus* sp. pollens prevailed, and the samples presented higher amount of turanose and maltose. Little participation to the mentioned discrimination was observed regarding fructose and glucose contents.

The integration of all analysed parameters except the pollen data, of samples of season 2 allowed the discrimination of two groups of samples. A first group presenting an average value of 58±11.24% of *B. spinosum* pollen, and a second one, presented an average of 40±15.98%. To estimate the significance of the difference observed after the use of physicochemical parameters, the means of the major represented pollen species were compared between the two obtained groups. *B. spinosum* pollen showed to play an important role in this discrimination, and the difference was significant at $p<0.05$. Another important feature is the prevalence of the *Cystus* sp. and *Populus* sp. pollens in the groups presenting low levels of *B. spinosum* pollen, although the difference was not significant. This suggest the importance of *B. spinosum* nectar in shaping the acquired features of Zantaz honey samples. Considering the means of the two groups (58±11.24% and 40±15.98%), one can speculate that a Zantaz honey sample obey, at least, the general monoflorality threshold of 45%, though, a determinant conclusion can be made only after a detailed sensorial study.

2.3. Electronic tongue technology

Honey floral origin and honey adulteration are difficult to detect without performing the above-mentioned laboratory analysis. Until now the most reliable method that can confirm

honey botanic origin is known as mellissopalynogical analysis especially when combined to other parameters such as the determination of the volatile fraction [7,8], mineral content [9], physico-chemical parameters [10,11], phenolic profiling [12–16]. These methods require rigorous sample preparation and expensive instrumentation. Because of this, the European Community is encouraging the development of harmonized analytical methods to verify the compliance with the quality specifications for different types of honeys.

An e-tongue fulfils the requirements of a fast, inexpensive and a reliable tool to confirm that a particular sample belongs to a specific floral origin. It can be implemented in a portable hand instrument used in the field. Furthermore, an e-tongue will be an important asset to help the commercialization chain, to screen for counterfeiting and adulteration of honey.

In present thesis a contribution has been made to develop an impedance based electronic tongue for honey botanical origin discrimination. The e-tongue was built of four working electrodes, namely thermal evaporated thin film of gold on a glass slide, a carbon film, a glass slide coated with an Indium-Tin-Oxide (ITO) layer and a highly doped *n*-type silicon wafer (n^+-Si). A platinum grid was used as a common electrode. The e-tongue fingerprint was confirmed and validated by mellissopalenogical analysis in combination with standardized physico-chemical characterization involving the sugar content, the electrical conductivity, and the light absorbance.

The individual characterization of the sensors' stability showed that for all electrodes the capacitance remains constant for a six hours long period. To inspect for reproducibility, 10 measurements were carried out using the same honey sample in a repeated fashion. The ITO sensor showed the lowest reproducibility with a standard deviation of 50.43±11.83 (23.5%) in the peak of tan δ curve. The gold electrode is the sensor with the highest reproducible behaviour with a standard deviation of 33.01±0.37 (1.1%). In summary, and regarding the botanical discrimination of honey samples, it has been revealed that different electrodes have different ability to discriminate honey with different floral origin. The gold sensing electrode shows the highest ability to discriminate the two varieties of honey. The carbon and the n^+-Si electrodes have the lowest ability to discriminate honey samples.

The integration of information provided by the four sensing units showed that the e-tongue could even distinguish subgroups in used honey types with significant differences in terms of main pollen frequency. The physical chemical analysis reveals that the e-tongue is sensitive to changes in the electrical conductivity and detect differences of 100 μS/cm and differences in colour of 8 mm Pfund.

3. Honey biological activities.

3.1. Effect of heat processing.

honey heating used to be a common practice to reach the desired rheological properties thereby avoiding the crystallization process as well as to increase the shelf-lifetime by elimination possible contaminant microorganisms [17,18]. In spite of benefits of heating, the composition of honey undergoes a series of changes that are linked to the interactions between its major and minor compounds leading to their degradation or formation of new molecules [5,18,19]. Those changes may have different effects on honey antioxidant activities dependent on the used radicals.

In the present thesis Zantaz honey colour increased 38% in mm pfund while the melanoidins' content increased by 28%. Both parameters are directly linked to the core of this study, as they provide a tool to assess the formation of the brown pigments of the Millard reaction, known to occur in honey following a thermal treatment [21]. Both parameters showed a highly significant increase ($p < 0.001$). It was observed that the polyphenols' contents did not vary significantly with the heat treatment, while the content of flavonoids decreased significantly ($p < 0.01$) after the thermal treatment (around 14%).

The above-mentioned parameters reflect the bioactive fraction of honey, and their changes following heat processing affected in different ways the antioxidant activities. DPPH has been increased after the thermal treatment, with a decrease of the IC_{50} values from 15.34 ± 7.55 mg/mL to 12.70 ± 4.61 mg/mL. Similarly, ABTS antioxidant activity has been increased by 20% in a significant way ($p < 0.05$). Both assays are used to evaluate the free radical scavenging capacity, but the ABTS is more likely to be used for honey samples.

In contrast, heat processing application could abolish totally the ability of samples to chelate the iron ions at the same concentration used for raw samples. This may be explained by the reduction in flavonoids as they are known to be responsible of honey metal chelating activity [22]**.** The ability of honey samples to reduce the ferric ions was another activity that was negatively affected by the heat processing, although, this difference was not significance. The heat processing negatively affected ($p < 0.01$), the ability of Zantaz honey samples to scavenge the NO free radicals, while superoxide activity, remained unchanged.

The data of this section was assisted with FTIR-ATR analysis. In a first screening, a PCA with all spectral regions (from 4000 cm^{-1} to 400 cm^{-1}) was performed. However, better results were obtained when the region from 800 to 1250 cm^{-1}, suggesting that this spectrum region is

most sensitive to heat processing. Furthermore, when FTIR-ATR was compared to pollen analysis it has been observed a different behaviour for different pollen species, that could be explain by the effect of the botanical origin. The honey samples are all classified as Zantaz honey, but the secondary nectar plays also an important influence.

3.2. Antimicrobial activity.

The topic antibacterial activity is due to sugar content (high osmotic pressure), acidic behaviour (low pH), high viscosity, low water activity, low protein content, high contents on bacteriostatic and bactericidal factors like hydrogen peroxide (H_2O_2) and plant derived compounds [23]. Nevertheless, the sensitivity of bacteria toward honey is also influenced by other phytochemical proprieties which may act also as antimicrobial agents [24]. Those parameters include colour, melanoidins, conductivity and mineral content [25,26]. Phytochemicals such as flavonoids, phenolic derivatives and aromatic acids originating from plants delivering the nectar, are also known to contribute to the antimicrobial activity of honey [27].

The preliminary analysis of honey samples showed that, according to the international quality standards, none of analysed samples showed alarming values, and all of them were within the established limits. The results showed that bacterial strains showed different sensitivities regarding honey samples. *S. Aureus* was the most sensitive, with a CMI and CBM of 62.5 mg/ml when using honey samples s2 s7 s8 s9, while S6 was the less active samples with both CMI and CBM of 250 mg/ml. *P. aeruginosa* showed an intermediate sensitivity, and *E. coli* was the most resistant and with values of 250 mg/ml with four of honey samples. Analysing the correlation with the analysed parameters, disaccharides showed a positive correlation in opposition to monosaccharaide for which the presence with linked to lower the antibacterial activities. From the mineral species only, Na and Zn had positive correlation with the measured activity.

3.3. Antiproliferative and antioxidant activities.

In the last years, cell-based assays have been extended to determine the bioactivity of functional compounds, since they provide more formation than in-tube assays, such as for example the bioavailability of the functional compounds [11]. Antiproliferative or immunomodulatory activity of different honeys have been reported against THP-1 and other cancer cells [12]. Honey can be used in its original form or after extraction of bioactive fractions [13–15].

Antiproliferative activity of Zantaz honey was evaluated in THP-1 cells from acute monocytic leukemia, while antioxidant activity was evaluated using Caco-2 cells. First, as honey interferes with the MTT reagent inducing an underestimation of its antiproliferative activity, a base line absorbance was made of reference solutions of honey with MTT. The interference was dose dependent and increased the cell viability estimation 1.42 times at a honey concentration of 0.02g/mL and 11.24 times at 0.1 g/mL after an incubation of 24h. After 24 h of incubation with Zantaz honey, the viability of THP-1 cells decreased significantly in a dose dependent manner. IC_{50} was determined and the Highest activity was in the order of 30 ± 0.97mg/ml. With respect to the antioxidant activity, Zantaz honey samples showed clear differences between them. Three statistically distinguishable population groups could be observed with statistical significance ($p<0.05$). Wan et al. (2015) compared the antioxidant activity of pure phytochemicals at both *in vitro* and *in vivo* and observed positive correlation between caco-2 antioxidant assay and rats' plasma ORAC.

RT-HPLC analysis of Zantaz honey showed the presence of 19 compounds. Methyl syringate was the most abundant phenolic compound. Its contents ranged from 277.93 µg/g to 443.89 µg/g, representing up to 57.88% of all polyphenols. Such high amounts are not usual in Moroccan honeys. In contrast, similar high concentrations (above 122.6 mg/kg) are a characteristic of Manuka honey [29]. Epicatechin (flavan-3-ol) was the second abundant phenolic compounds with an average value of 77.05 ± 47.87 µg/g. Their derivatives syringic acid and catechin were respectively the following most abundant compounds.

Regarding the correlation between the analysed properties and the evaluated activities, four groups of groups are recognized. The first group of parameters was formed by catechin, pH, minerals (K, Mg, Na, Fe and Ca) and hence their representative parameters ash content and conductivity. Those parameters showed positive correlation with the measured bioactivities, while they correlated negatively the interference. The elements of the second group were free acidity, two reducing sugars (Fructose and Arabinose) and five phenolic compounds (Syringic acid, Quercetin, methyl syringate, Gallic acid and Kaempferol), which are the main contributors to honey colour, and possess positive correlation with both bioactivities and honey interference with the MTT assay. Abel & Baird (2018) reported that the interference with the MTT Assay is mainly due to the presence of reducing sugars and phenolic compounds. In addition, the phenolic compounds are known to possess both antioxidant and antiproliferative activities *in vitro*, in cells and *in vivo* models [31–37]

Lactonic acidity, Epicatechin, 4-cumaric acid and Luteolin showed a positive correlation with the interference but showed to have negative correlation with bioactivities. A suggested explanation is that the matrix, and the existence of other phenolics might reduce the bioavailability of some phenolic compounds [38]. This explains the interaction of the phenolic compounds at the extracellular level by their positive correlation with the interference in the MTT assay, and the absence of their intracellular bioactivities. The last group was formed by elements presenting negative correlation with the cell-based antioxidant activity and honey interference with the MTT while they seemed to induce its antiproliferative activity (positive correlation with $1/IC_{50}$). This group is formed by reducing sugars (glucose, maltose, melibiose and turanose) and none reducing sugars (trehalose).

4. Propolis phytochemical characterization and pharmacological activities.

Propolis samples were collected form hives during the flowering season of *Bupleurum spinosum*, which is known by the production of the Moroccan Zantaz honey. world wild propolis is known to be rich in bioactive compounds and to possess several biological activities *in vitro* and *in vivo.* Such interest dependent on propolis botanical source. The surrounding areas of collected sample is mainly occupied by *Bupleurum, Quercus, Juniperus, cedrus,* which contribute specifically to the quality of this propolis.

After 24h of incubation with THP-1 cells, cell viability decreased in a dose dependent manner and reaches its maximum at a honey concentration of 1.5 µg/mL. After 72h of incubation the cell viability decrease becomes clearer. The highest antiproliferative activity evaluated by IC_{50} was as low as 0.60 ± 0.07 µg/mL. This was lower than those reported for other Moroccan propolis samples on other cancer cell lines [39].

Pearson's correlation between the antiproliferative activity and the areas of detected phenolics showed positive but non-significant correlations. Nevertheless, samples with low antiproliferative activity, had also less phenolic content considering the sum of the areas. It worth mentioning that previous work on antiproliferative activity of Moroccan propolis reported a nonsignificant correlation between the measured activity on NCI-H460, HepG2 and MCF-7 cells lines and total phenolics, and a significance was seen only with specific compounds [39].

5. Perspectives

The monofloral characteristic of Zantaz honey will only be confirmed after performing the sensorial analysis to complete the work that have been done in the present thesis. The

sensorial analysis assisted by pollen, physical and chemical analysis will provide more evidence about the monofloralirty threshold. Such a step will highlight the properties that characterize better Zantaz honey to help future characterization works.

The contribution about the e-tongue technology revealed different sensitivity of the used sensing units. This encourages screening for other material to build more performant e-tongue. In addition, the e-tongue shoed high sensitivity toward electrical conductivity of honey samples, suggesting the importance of mineral component in determining obtained output. This observation suggests the use of the e-tongue can be extended to the discrimination of honey geographical origins. A validation will be needed using mineral analysis.

Regarding biological activities of Zantaz honey, the important achievement is the identification of methyl syringate as major phenolic compound. The positive correlation between this compound and antioxidant and antiproliferative activity will be the guiding idea for future pharmacological studies.

The propolis collected form hives in the surrounding areas of *B. spinosum* showed a very interesting antiproliferative activity, with an IC_{50} below 1 µg/mL. Such properties require further investigation to determine the responsible compound (s) and isolate them if possible.

6. References

[1] J. Peltier, Ecologie de quelques especes climaciques dans le Sous (Maroc Occidental), Doc. Cartogr. Ecol. XXVI (1983) 61–82.

[2] F. Nassif., B. El Amiri., Goat production systems in a mountainous community of the Middle Atlas, Morocco, Options Méditerranéennes. Séries A. Mediterr. Semin. 100 (2011) 199–203.

[3] A. Khabbach, A. Libiad, E. Mohamed, Melliferous flora and apiculture in the pre-Rif of the province of Taza (north of Morocco), Luna Azul. (2013) 240–256. doi:10.17151/luaz.2015.40.14.

[4] European Union Directive (EU), Council Directive 2001/110 Relating to Honey, Off. J. Eur. Communities. 16 (2001) 1–12.

[5] S. Bogdanov, K. Ruoff, L. Persano Oddo, Physico-chemical methods for the characterisation of unifloral honeys: a review, Apidologie. 35 (2004) S4–S17. doi:10.1051/apido:2004047.

[6] P.M. Da Silva, C. Gauche, L.V. Gonzaga, A.C.O. Costa, R. Fett, Honey: Chemical composition, stability and authenticity, Food Chem. 196 (2016) 309–323. doi:10.1016/j.foodchem.2015.09.051.

[7] V. Kaškonienė, P.R. Venskutonis, V. Čeksterytė, Composition of volatile compounds of honey of various floral origin and beebread collected in Lithuania, Food Chem. 111 (2008) 988–997. doi:10.1016/j.foodchem.2008.05.021.

[8] E. Alissandrakis, P. a. Tarantilis, P.C. Harizanis, M. Polissiou, Aroma investigation of unifloral Greek citrus honey using solid-phase microextraction coupled to gas chromatographic–mass spectrometric analysis, Food Chem. 100 (2007) 396–404. doi:10.1016/j.foodchem.2005.09.015.

[9] S. Bogdanov, M. Haldimann, W. Luginbühl, P. Gallmann, Minerals in honey: environmental, geographical and botanical aspects., J. Apic. Res. 46 (2007) 269–275. doi:10.3896/IBRA.1.46.4.11.

[10] I.K. Karabagias, A. V. Badeka, S. Kontakos, S. Karabournioti, M.G. Kontominas, Botanical discrimination of Greek unifloral honeys with physico-chemical and chemometric analyses, Food Chem. 165 (2014) 181–190. doi:10.1016/j.foodchem.2014.05.033.

[11] I.K. Karabagias, M. V. Vavoura, C. Nikolaou, A. V. Badeka, S. Kontakos, M.G. Kontominas, Floral authentication of Greek unifloral honeys based on the combination of phenolic compounds, physicochemical parameters and chemometrics, Food Res. Int. 62 (2014) 753–760. doi:10.1016/j.foodres.2014.04.015.

[12] I. Sergiel, P. Pohl, M. Biesaga, Characterisation of honeys according to their content of phenolic compounds using high performance liquid chromatography/tandem mass spectrometry, Food Chem. 145 (2014) 404–408. doi:10.1016/j.foodchem.2013.08.068.

[13] V. Kaškonienė, P.R. Venskutonis, Floral markers in honey of various botanical and geographic origins: a review, Compr. Rev. Food Sci. Food Saf. 9 (2010) 620–634. doi:10.1111/j.1541-4337.2010.00130.x.

[14] E. Anklam, A review of the analytical methods to determine the geographical and botanical origin of honey, Food Chem. 63 (1998) 549–562. doi:10.1016/S0308-8146(98)00057-0.

[15] S. Soares, J.S. Amaral, M.B.P.P. Oliveira, I. Mafra, A comprehensive review on the main honey uuthentication issues: production and origin, Compr. Rev. Food Sci. Food Saf. 16 (2017) 1072–1100. doi:10.1111/1541-4337.12278.

[16] L. PERSANO ODDO, R. PIRO, Main European unifloral honeys: descriptive sheets, Apidologie. 35 (2004) 38–81. doi:10.1051/apido:2004049.

[17] B. Abu-Jdayil, A. Al-Majeed Ghzawi, K.I.M. Al-Malah, S. Zaitoun, Heat effect on rheology of light- and dark-colored honey, J. Food Eng. 51 (2002) 33–38. doi:10.1016/S0260-8774(01)00034-6.

[18] E. Tosi, M. Ciappini, E. Ré, H. Lucero, Honey thermal treatment effects on hydroxymethylfurfural content, Food Chem. 77 (2002) 71–74. doi:10.1016/S0308-8146(01)00325-9.

[19] N. Turkmen, F. Sari, E.S. Poyrazoglu, Y.S. Velioglu, Effects of prolonged heating on antioxidant activity and colour of honey, Food Chem. 95 (2006) 653–657. doi:10.1016/j.foodchem.2005.02.004.

[20] I. Turhan, N. Tetik, M. Karhan, F. Gurel, H. Reyhan Tavukcuoglu, Quality of honeys influenced by thermal treatment, LWT - Food Sci. Technol. 41 (2008) 1396–1399. doi:10.1016/j.lwt.2007.09.008.

[21] H.Y. Wang, H. Qian, W.R. Yao, Melanoidins produced by the Maillard reaction: Structure and biological activity, Food Chem. 128 (2011) 573–584. doi:10.1016/j.foodchem.2011.03.075.

[22] L.S. Chua, N.L.A. Rahaman, N.A. Adnan, T.T. Eddie Tan, Antioxidant activity of three honey samples in relation with their biochemical components, J. Anal. Methods Chem. 2013 (2013). doi:10.1155/2013/313798.

[23] C. Libonatti, S. Varela, M. Basualdo, Antibacterial activity of honey: A review of honey around the world, J. Microbiol. Antimicrob. 6 (2014) 51–56. doi:10.5897/jma2014.0308.

[24] K. Brudzynski, K. Abubaker, L. St-Martin, A. Castle, Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey, Front. Microbiol. 2 (2011) 1–9. doi:10.3389/fmicb.2011.00213.

[25] A. Moussa, A. Saad, The influence of botanical origin and physico-chemical parameters on the antifungal activity of Algerian honey, J. Plant Pathol. Microbiol. 03 (2012). doi:10.4172/2157-7471.1000132.

[26] H. Imtara, Y. Elamine, B. Lyoussi, Honey antibacterial effect boosting using origanum vulgare L. essential oil, Evidence-Based Complement. Altern. Med. 2018 (2018). doi:10.1155/2018/7842583.

[27] P. Molan, The antibacterial activity of honey 1. The nature of the antibacterial activity, Bee World. 73

(1992) 5–28.

[28] H. Wan, D. Liu, X. Yu, H. Sun, Y. Li, A Caco-2 cell-based quantitative antioxidant activity assay for antioxidants, Food Chem. 175 (2015) 601–608. doi:10.1016/j.foodchem.2014.11.128.

[29] S. Patel, S. Cichello, Manuka honey: an emerging natural food with medicinal use, Nat. Products Bioprospect. 3 (2013) 121–128. doi:10.1007/s13659-013-0018-7.

[30] S.D.A. Abel, S.K. Baird, Honey is cytotoxic towards prostate cancer cells but interacts with the MTT reagent : Considerations for the choice of cell viability assay, Food Chem. 241 (2018) 70–78. doi:10.1016/j.foodchem.2017.08.083.

[31] V. Baltrušaityte, P.R. Venskutonis, V. Čeksteryte, Radical scavenging activity of different floral origin honey and beebread phenolic extracts, Food Chem. 101 (2007) 502–514. doi:10.1016/j.foodchem.2006.02.007.

[32] S. Bogdanov, T. Jurendic, R. Sieber, P. Gallmann, Honey for nutrition and health: A review, J. Am. Coll. Nutr. 27 (2008) 677–689. doi:10.1080/07315724.2008.10719745.

[33] S.K. Jaganathan, M. Mandal, Antiproliferative effects of honey and of its polyphenols: a review, J. Biomed. Biotechnol. 2009 (2009) 1–13. doi:10.1155/2009/830616.

[34] J.M. Alvarez-Suarez, S. Tulipani, S. Romandini, E. Bertoli, M. Battino, Contribution of honey in nutrition and human health: A review, Med. J. Nutrition Metab. 3 (2010) 15–23. doi:10.1007/s12349-009-0051-6.

[35] S. Ahmed, N.H. Othman, Honey as a potential natural anticancer agent: a review of its mechanisms, Evidence-Based Complement. Altern. Med. 2013 (2013) 1–7. doi:10.1155/2013/829070.

[36] Z. Jubri, N.B. Abdul Rahim, G.J. Aan, Manuka honey protects middle-aged rats from oxidative damage, Clinics. 68 (2013) 1446–1454. doi:10.6061/clinics/2013(11)11.

[37] D. Cianciosi, T. Forbes-Hernández, S. Afrin, M. Gasparrini, P. Reboredo-Rodriguez, P. Manna, J. Zhang, L. Bravo Lamas, S. Martínez Flórez, P. Agudo Toyos, J. Quiles, F. Giampieri, M. Battino, Phenolic compounds in honey and their associated health benefits: a review, Molecules. 23 (2018) 2322. doi:10.3390/molecules23092322.

[38] M. D'Archivio, C. Filesi, R. Varì, B. Scazzocchio, R. Masella, Bioavailability of the polyphenols: Status and controversies, Int. J. Mol. Sci. 11 (2010) 1321–1342. doi:10.3390/ijms11041321.

[39] S.I. Falcão, R.C. Calhelha, S. Touzani, B. Lyoussi, I.C.F.R. Ferreira, M. Vilas-Boas, In Vitro interactions of Moroccan propolis phytochemical's on human tumor cell lines and anti-inflammatory properties, Biomolecules. 9 (2019) 315. doi:10.3390/biom9080315.

Annexes

1- Articles of the present thesis

Journal of Apicultural Research, 2017
http://dx.doi.org/10.1080/00218839.2016.1265759

IBRA INTERNATIONAL BEE RESEARCH ASSOCIATION

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH ARTICLE

Preliminary characterization of a Moroccan honey with a predominance of *Bupleurum spinosum* pollen

Youssef Elamine[a], Smail Aazza[a], Badiâa Lyoussi[a], Maria Dulce Antunes[b], Letícia M Estevinho[c,d], Ofélia Anjos[e,f,g], Mafalda Resende[h], Maria Leonor Faleiro[i] and Maria Graça Miguel[b]*

[a]*Laboratory of Physiology-Pharmacology-Environmental Health, Faculty of Sciences Dhar El Mehraz, University Sidi Mohamed Ben Abdallah, Fez, Morocco;* [b]*FCT, Universidade do Algarve, Faro, Portugal;* [c]*Escola Superior Agrária, Instituto Politécnico de Bragança, Apartado Bragança, Portugal;* [d]*Centre of Molecular and Environmental Biology, University of Minho, Braga, Portugal;* [e]*Instituto Politécnico de Castelo Branco, Castelo Branco, Portugal;* [f]*CEF, Universidade de Lisboa, ISA, Lisboa, Portugal;* [g]*Centro de Biotecnologia de Plantas da Beira Interior, Castelo Branco, Portugal;* [h]*CATAA – Centro de Apoio Tecnológico Agro-Alimentar de Castelo Branco, Zona Industrial de Castelo Branco – Rua A, Castelo Branco, Portugal;* [i]*FCT, Center for Biomedical Research, Universidade do Algarve, Faro, Portugal*

(*Received 29 April 2016; accepted 18 November 2016*)

Honey with *Bupleurum spinosum* (zandaz) as a main pollen source has not been the subject of previous detailed study. Therefore, twelve Moroccan samples of this honey were subjected to melissopalynological, physicochemical and microbiological quality characterization, as well as antioxidant activity assessment. From a quality point of view, almost all samples were within the limits established by Codex Alimentarius, and/or the European legislation. All samples presented predominance of *B. spinosum* pollen (more than 48%). Relatively high levels of trehalose (1.3–4.0 g/100 g) and melezitose (1.5–2.8 g/100 g) were detected. Those sugars, not common in monofloral honeys, could be used as an important factor to discriminate zandaz honey. Flavonoid content correlated positively with the honey color, melanoidin and polyphenol content, and negatively with the IC_{50} values of scavenging ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) free radicals, while proline amount correlated negatively with IC_{50} values of nitric oxide scavenging activity and chelating power. This correlation supports the use of anti-oxidant activities as important variables for PCA (principal component analysis). Both components explained 70% from the given data, and showed certain homogeneity upon analyzed samples independent of the region, suggesting the importance of *B. spinosum* nectar in the resulting honey characteristics.

Caracterización preliminar de una miel marroquí con predominio del polen de *Bupleurum spinosum*

La miel con *Bupleurum spinosum* (pendejo) como principal fuente de polen no ha sido objeto de un estudio detallado previo. Por lo tanto, doce muestras marroquíes de esta miel fueron sometidas a una caracterización melisopalinológica, físico-química y microbiológica, así como a la evaluación de su actividad antioxidante. Desde el punto de vista de la calidad, casi todas las muestras estaban dentro de los límites establecidos por el Codex Alimentarius y/o la legislación europea. Todas las muestras presentaron predominio de polen de *B. spinosum* (más del 48%). Se detectaron niveles relativamente altos de trehalosa (1.3-4.0 g/100 g) y melecitosa (1.5-2.8 g/100 g). Esos azúcares, no comunes en mieles monoflorales, podrían ser utilizados como un factor importante para discriminar la miel de pendejo. El contenido de flavonoides se correlacionó positivamente con el color de la miel, el contenido de melanoidina y polifenol y negativamente con los valores de IC_{50} de la actividad de barrido de los radicales libres de ABTS (2,2'-azino-bis (3-etilbenzotiazolina-6-sulfónico), mientras que la cantidad de prolina se correlacionó negativamente con los valores de IC_{50} de la actividad de barrido de ON (óxido nítrico) y el poder quelante. Esta correlación apoya el uso de las actividades antioxidantes como variables importantes para el APC (análisis de componentes principales). Ambos componentes explican el 70% de los datos obtenidos y muestran cierta homogeneidad en las muestras analizadas independientemente de la región, lo que sugiere la importancia del néctar de *B. spinosum* en las características de la miel resultante.

Keywords: *Bupleurum spinosum*; Zandaz honey; melissopalynology; physicochemical analysis; antioxidant activity; botanic origin

Introduction

The floral origin determination of honey is very important in the field of apicultural research, due to its direct link with customer demand. For the purpose of consumer protection, the honey floral origin labeling has been required by the European Community legislation, i.e., Directive EEC/74/409 amended by the Proposal COM/95/0722 (1996).

Melissopalynolgical, sensory and physico-chemical analyzes together are needed for establishing the botanical denomination of a honey, because when considered individually, each one has its own limitations. Regarding melissopalynolgical analysis, whereas some honey types need high percentage of pollen to be considered as monofloral (90% for chestnut honey), for others only 15% is sufficient to declare their botanical origin

*Corresponding author. Email: mgmiguel@ualg.pt

© 2017 International Bee Research Association

LWT - Food Science and Technology 106 (2019) 29–36

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Zantaz honey "monoflorality": Chemometric applied to the routinely assessed parameters

Youssef Elamine[a,b], Badiâa Lyoussi[a], Ofélia Anjos[c,d,*], Leticia M. Estevinho[e], Smail Aazza[a,f], Jorge D. Carlier[g], Maria C. Costa[g], Maria G. Miguel[b]

[a] Laboratory of Physiology-Pharmacology-Environmental Health, Faculty of Sciences Dhar El Mehraz, BP 1796 Atlas, University Sidi Mohamed Ben Abdallah, Fez 30 000, Morocco
[b] Universidade do Algarve, FCT, MeditBio, Edf 8, Campus de Gambelas, 8005-139, Faro, Portugal
[c] Instituto Politécnico de Castelo Branco, Castelo Branco, 6001-909, Portugal
[d] Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, Lisboa, 1349-017, Portugal
[e] CIMO, Mountain Research Center, Polytechnic Institute of Bragança, Campus Santa Apolónia, 5301-855, Bragança, Portugal
[f] Laboratory of Phytochemistry, National Agency of Medicinal and Aromatic Plants, BP 159, Taounate, Morocco
[g] Centro de Ciências do Mar, Universidade do Algarve, FCT, Campus de Gambelas, Faro, 8005-139, Portugal

ARTICLE INFO

Keywords:
Bupleurum spinosum
Monoflorality threshold
Melissopalynology
Principal component analysis

ABSTRACT

The comparison of Zantaz honey samples harvested in Morocco in two different years was performed through chemometric analysis of routinely assessed parameters. The objective was to study how the pollen profile of this newly reported honey shapes its physicochemical characteristics as well as to determine its monoflorality threshold, which has not yet been defined. The predominance of *B. spinosum* pollen was confirmed in the majority of samples reaching 45%, generally requested for monoflorality declaration. The principal component analysis (PCA) was used for clustering and variables correlations. Pollen qualitative and quantitative differences could discriminate the samples belonging to both seasons when combined with the sugar analysis (59.44%) better than the combination with physicochemical parameters (pH, acidity, ash content, electrical conductivity and color) (60.62%). Positive correlation between the presence of *B. spinosum* pollen and melanoidins, color, fructose, moisture, trehalose, melezitose, iron, manganese and calcium could be seen. Integrating all the parameters except the pollen data allowed distinguishing two groups with significant differences ($P < 0.05$) in *B. spinosum* representability (58 ± 11.24% against 40 ± 15.98%). This may suggest that 40% is the monoflorality threshold of the Moroccan Zantaz honey, although a confirmation with sensorial analysis is required.

1. Introduction

Chemometrics application to food chemistry parameters is attracting more attention from the scientific community due to its power on the elaboration of conclusions about the traceability (Bertacchini et al., 2013) and the classification/clustering problems (Anjos et al., 2015a; Silva, Gauche, Gonzaga, Costa, & Fett, 2016). These techniques were previously applied on the analysis of several food products, among which coffee (Briandet, Kemsley, & Wilson, 1996) and amino acids content in honey (Zhao et al., 2018). Honey does not make an exception; indeed the chemometric tools were not only used as a discriminatory tool between honeys with different geographical (Estevinho, Chambó, Pereira, Carvalho, Alencar, 2016; Karabagias et al., 2017a, 2017b) or botanical origins (Corbella & Cozzolino, 2006; Devillers, Morlot, Pham-Delègue, & Doré, 2004; Terrab, Díez, & Heridia, 2002) but also as an effective way to detect honeys' adulteration (Wang et al., 2014). Several tools can be employed depending on the study aim, implicating the filtration of the parameters. This parameters' filtration helps choosing the adequate parameters/variables carrying the adequate information as well as the elimination of the "noising" parameters (Bevilacqua, Bucci, Magrì, Magrì, & Nescatelli, 2013).

Honey monoflorality determination can be done using the combination of pollen spectrum analysis, physicochemical parameters and sensorial qualities (Bogdanov, Ruoff, & Oddo, 2004). This last step is highly expensive, requiring panels of assessors which go through controlled experimental protocols. The results need to be statistically reproducible to be considered (Piana et al., 2004). In fact, a predictive relationship has been established between physicochemical characteristics and sensory properties (Pestorić et al., 2015), although this should

* Corresponding author.Instituto Politécnico de Castelo Branco, Castelo Branco, 6001-909, Portugal
E-mail address: ofelia@ipcb.pt (O. Anjos).

https://doi.org/10.1016/j.lwt.2019.02.039
Received 11 August 2018; Received in revised form 23 January 2019; Accepted 12 February 2019
Available online 12 February 2019
0023-6438/ © 2019 Elsevier Ltd. All rights reserved.

Sensors & Actuators: B. Chemical 285 (2019) 24–33

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Insight into the sensing mechanism of an impedance based electronic tongue for honey botanic origin discrimination

Youssef Elamine[a,b], Pedro M.C. Inácio[b,c], Badiâa Lyoussi[a], Ofélia Anjos[d,e,f], Leticia M. Estevinho[g], Maria da Graça Miguel[b], Henrique L. Gomes[b,c,*]

[a] Laboratory of Physiology-Pharmacology-Environmental Health, Faculty of Sciences Dhar El Mehraz, BP 1796 Atlas, University Sidi Mohamed Ben Abdallah, Fez 30 000, Morocco
[b] Universidade do Algarve, Faculdade de Ciências e Tecnologia, 8005-139 Faro, Portugal
[c] Instituto de Telecomunicações, Avenida Rovisco, Pais 1, 1049-001 Lisboa, Portugal
[d] Instituto Politécnico de Castelo Branco, Castelo Branco 6001-909, Portugal
[e] Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, Lisboa 1349-017, Portugal
[f] Centro de Biotecnologia de Plantas da Beira Interior, Castelo Branco 6001-909, Portugal
[g] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, 5300-252 Bragança, Portugal

ARTICLE INFO

Keywords:
Electronic tongue
Small signal impedance
Electrical-double-layer
Honey

ABSTRACT

An impedance based electronic tongue was developed and used to discriminate honey of different botanic origin. The e-tongue presented here is based on the small-signal frequency response of the electrical double-layer established between the honey solution and an array of four different sensing units composed by gold, carbon, indium-tin-oxide, and doped silicon. The ability of the e-tongue to discriminate honey of different floral origins was demonstrated by distinguishing honey from *Bupleurum* and *Lavandula* pollen prevalence. The honey fingerprint obtained with the e-tongue was validated by parallel melissopalenogical analysis and physico-chemical methods. It is demonstrated that the e-tongue is very sensitive to changes on the honey electrical conductivity. Small differences in electrical conductivity are introduced by the presence of ionisable organic acids and mineral salts. Moreover, we propose that the sensitivity of the tongue to changes in electrical conductivity can be explored to probe other complex liquid substances.

1. Introduction

Electronic tongue (e-tongue) technology is gaining attention from the scientific community as a method that can be applied in various fields, mainly to evaluate quality and authenticity of food products [1–5]. Over the last few years there have been extensive examples of sensitive, fast responding e-tongues for the classification and a certification of several types of beverages [3,6], olive oil [7], honey and coffee [8,9] and pollutants in water [3,4,10]. Detection of adulteration and counterfeiting of products is also being reported [4,11–15].

The e-tongue instrumentation is formed by an array of sensing units that can be composed by lipid membranes, ion-selective electrodes, conducting polymers or noble metals [3,16,17]. Because the identification of samples is most of the time a classification task, there has been a trend to use artificial intelligence and information visualization methods to enhance the performance of e-tongues [18]. Recent advances of e-tongues can be found in several reviews [4,16,17,19,20].

Several measuring methodologies can be used to address the sensing elements in e-tongue system. The most-used employed are electrochemical measurements, namely, potentiometry, amperometry and cyclic voltammetry. In 2002 Riul et al. [21] introduced the use of small-signal impedance measurements in electronic tongues. Low-signal impedance measurements offer the advantage of using materials composing the sensing units do not need to be electroactive and there is no need of a reference electrode. Impedance based e-tongues or impedimetric tongues are based on measuring the small-signal impedance of the electrical double-layer (EDL) or Helmholtz layer established at the electrode/electrolyte interface over a frequency range [22,23].

Honey is an attractive substance to be evaluated by e-tongues. The honey floral origin has a strong impact on the commercial value, thus the floral determination and certification play an important role in quality control. European legislation specifies that the commercialization of honey must made reference to the origin, whether blossom or plant (Codex Alimentarius Commission, 1970; Council Directive 74/

* Corresponding author at: Universidade do Algarve, Faculdade de Ciências e Tecnologia, 8005-139 Faro, Portugal.
E-mail address: hgomes@ualg.pt (H.L. Gomes).

https://doi.org/10.1016/j.snb.2019.01.023
Received 11 September 2018; Received in revised form 30 December 2018; Accepted 6 January 2019
Available online 07 January 2019
0925-4005/ © 2019 Published by Elsevier B.V.