UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

"Studies on honey from the Algarve in view of its valorization"

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the degree of PhD in Chemistry

By

Pablo A. Ulloa Fuentes

Under the supervision of Amadeu F. Brigas, Ph.D.

Under the co-supervision of Ana C. Figueira, Ph.D.

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If the bee disappeared off the surface

of the globe the men would

only have four years of live left

Albert Einstein

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Abstract

Several studies were conducted with different Portuguese honey samples from South area (Algarve) in view of exploiting its properties and increasing the commercial value of some types of honey. Initially, the effects of honey storage for long periods were evaluated in terms of physicochemical parameters and bioactivity (Chapter 3) and it was concluded that after three years most of the properties of honey remained unchanged except for the freshness indicators, which were, as expected, reasonably far from the regulated values.

Several commercial samples of honey with different floral origins were subjected to the conventional studies, including melissopalynology pollen analysis, physiochemical and biological (antioxidant properties) analysis. Honey from strawberry tree (*Arbutus unedo* L.), a typical plant in Algarve, was the focus of this study. This kind of honey has low acceptability by consumers probably because of unawareness of its existence and/or of its beneficial biological properties. Also, it may not be an obvious preference, due to its exquisite bitter taste, thus being locally called "bitter honey" (Chapter 4). Artisanal bitter honey was compared with other commercial honeys that are appreciated by Portuguese consumers (Chapter 5). This research consisted in the study of the physicochemical parameters, bioactive compounds and sensorial evaluation; done in comparison with sunflower (*Helianthus annuus*) honey, french lavender (*Lavandula stoechas*) honey, orange blossom (*Citrus* spp.) honey and commercial strawberry tree honey.

Finally, in the last stage of the work (Chapter 6), new methods for determination of botanical origin of honey were explored as potential alternatives to the traditional analysis method (melissopalynological). These non-invasive techniques namely electronic tongue, UV-Vis spectroscopy and Vis-NIR spectroscopy, were used with the help of multivariate analysis (principal component analysis, PCA), for the interpretation of data's obtained with above techniques. It is hoped that this research will represent an enrichment of knowledge on honey from the Algarve and, especially for *Arbutus unedo* honey, it has been demonstrated that it has the characteristics of similar gourmet honey from Italy existing, therefore, the potential of increasing its commercial value, which would also be advantageous for the interior region of the Algarve where, since the Arab occupation, the strawberry tree is abundant.

Keywords: honey, strawberry tree (*Arbutus unedo* L.) honey, storage, pollen analysis, physicochemical parameters, bioactive compounds, antioxidant activity, non-invasive techniques, PCA.

Resumo

O termo mel refere-se normalmente a um produto natural, de tonalidade âmbar, viscoso e doce, produzido por abelhas *Apis mellifera L.* a partir do néctar da flor de plantas, designando-se por isso néctar de mel. Existe também o mel de melaço, que é obtido a partir de secreções de plantas ou mesmo de excreções de insectos sugadores de partes vivas das plantas. As abelhas, são atraídas pelo cheiro das flores na altura da polinização, sugam o néctar da flor para o papo, adicionam-lhe secreções e processam-no enzimaticamente e depositam-no nos favos. O mel, no seu perfeito estado de maturação é constituído maioritariamente por uma mistura de açúcares (85-95%) e água (16-18%) e outros produtos minoritários, como proteínas, aminoácidos livres, ácidos orgânicos, compostos fenólicos, vitaminas e minerais.

Na produção de mel, as abelhas *Apis melífera* L. coletoras viajam cerca de 25 km, mais de uma dúzia de vezes por dia, recolhendo até 40 mg néctar e transportam-no para as suas colónias. É importante referir que em cada viajem uma abelha visita entre 50 e mil flores, sendo fiel à espécie botânica que escolheu. O néctar é transferido para as abelhas obreiras, que lhe adicionam mais enzimas. As enzimas diástase e invertase, transformam oligossacárideos como a sacrose em monossacárideos como frutose e glucose. A glucose oxidase, oxida a glucose ao ácido correspondente com formação de peróxido de hidrogénio que é usado para combater a degradação bacteriana. A transformação de néctar em mel começa de imediato, na vesícula melífera das abelhas, prosseguindo na colmeia até à fase em que é depositado nos alvéolos, depois de ter perdido grande parte da água existente no néctar. A evaporação ocorre pela ventilação provocada pelo bater de asas das abelhas a 35 °C, que é a temperatura interna da colmeia.

O mel faz parte da história da humanidade e até da mitologia. Existem registos da sua utilização em 2100 A. C. na Babilónia, Índia e Egito, sendo também referido na bíblia “terra do leite e do mel”. No seculo XI era usado na Europa como forma de pagamento de tributos e na América Latina a apicultura já existia antes da invasão dos europeus. A sua importância histórica e social, que não se limita a produtos alimentares, pois é usado desde a antiguidade como conservante alimentar, produto medicinal, protetor de madeira, etc., propagou-se até aos nossos

dias em que o consumo de mel está generalizado por todo o mundo. Tem havido um incremento na sua produção, em parte atribuído ao reconhecimento científico das suas propriedades medicinais, maioritariamente resultantes do seu conteúdo polifenólico. Estes compostos têm confirmada a sua eficiência na diminuição do risco de doenças degenerativas reduzindo o stress oxidativo e inibindo a oxidação macromolecular. A tendência geral parece ser, que os consumidores esclarecidos preferem, tal como em relação a outros produtos alimentares, produtos típicos com autenticidade regional e com garantia de qualidade.

Assim, a qualidade do mel e a sua classificação são factores primordiais na sua valorização. Para a atribuição da origem floral, botânica, é necessária uma análise melissopalinológica e análises físico-químicas complementares. A análise melissopalinológica é uma técnica morosa que requer um analista qualificado e muito experiente pois necessita de uma coleção tão completa quanto possível de pólens, e um conhecimento profundo da morfologia e abundância do pólen da suposta região. Normalmente, o mel é considerado monofloral quando o pólen da mesma origem está presente em mais de 45% (predominante). Uma descrição completa é também informativa em relação aos outros tipos de pólen presentes: secundário, 16-45%; pólen minoritário mais relevante, 3-15%; outros pólens minoritários, < 3%. Também se verifica que, para algumas flores, a quantidade de pólen (frequência) de pólen está sobrerepresentada, i.e. a percentagem de pólen nos sedimentos é superior à correspondente percentagem no néctar. E o inverso também se verifica.

Uma parte do trabalho descrito nesta tese debruçou-se especificamente sobre este aspecto, tendo sido possível obter, por métodos não clássicos e expeditos, uma boa indicação da origem floral. Resumidamente, é possível por métodos não-invasivos, diferenciar completamente o mel de medronho do de outros méis estudados.

Outro aspecto investigado, em rigor o primeiro, foi o da estabilidade ou frescura do mel. Como alimento, o mel é considerado um produto bastante estável durante cerca de um ano, apenas com pequenas alterações no sabor, algum escurecimento e alguma eventual cristalização, não sendo esta sinal de degradação. Recorrendo a instrumentação analítica, detecta-se também alguma variação na constituição de alguns componentes, como a percentagem de

hidroximetilfurfural (HMF), e atividade diástase (DA). Estes parâmetros são por isso usados como indicadores de frescura do mel. A magnitude das alterações ocorridas no mel com a passagem do tempo depende, obviamente, das condições de armazenamento e do tipo e qualidade do mel. Neste trabalho foram estudadas várias amostras de mel oriundo do Sotavento Algarvio, produzido diretamente pelos apicultores locais, e estudado duas semanas depois da colheita de 2006, portanto, antes de o trabalho desta tese ser iniciado. Foi de particular relevância averiguar se o mel, na sua constituição, ainda respeitava o estabelecido na extensa Legislação Nacional e Europeia. Em 2009, ano em que este trabalho foi iniciado (capítulo 3), as amostras anteriormente recolhidas e guardadas durante três anos à temperatura ambiente (20 ± 3 ºC) e ao abrigo da luz, foram estudadas quanto aos seguintes parâmetros físico-químicos: teor de cinzas, condutividade elétrica, pH, acidez total, cor, teor de prolina, humidade, sólidos solúveis, atividade da agua (a_w), DA e teor de HMF. Foi também estudada a atividade biológica dessas amostras. Este estudo de 2009, revelou que as amostras de mel, divididas em oito categorias: *Arbutus unedo* (medronho), *Erica* sp. (mel de queiró), *Cistus ladanifer* (esteva), *Rosmarinus* (alecrim), *Palmae* (palmeira), *Carduus* (cardo), *Ceratonia* (alfarroba), e *Citrus* spp. (laranjeira), estavam em razoável estado de conservação, dentro dos limites legais para uma boa parte dos parâmetros fisico-quimicos estudados: pH, acidez total, sólidos solúveis, condutividade elétrica, humidade e atividade da agua. No que diz respeito ao aspecto, verificou-se que tinha havido um escurecimento (diminuição do valor de L^*) o que naturalmente terá resultado do aumento do teor de melanoidinas (reação de Maillard). No entanto, como esperado, os valores de HMF e DA, que são usados como indicadores do grau de frescura, estavam foram do limites legais (máx 40 mg/kg e não menos do 8 Gothe, respectivamente). Apesar de não apresentarem sinais de deterioração, é evidente que um período de três anos de armazenamento é excessivo para que o mel possa ser consumido como produto alimentar. No entanto, os teores fenólico e flavonoico, estavam bem preservados ao fim deste período, sendo comparáveis a teores obtido em amostras de mel frescas de outras regiões.

No capítulo 4 é descrita a caracterização de mel de medronho (*Arbutus unedo* L.). Esta planta, abundante no Algarve e bastante valorizada desde a ocupação árabe da península, são atribuídos

muitos benefícios a nível medicinal. Na região Algarvia, como no resto do país, a produção de medronho destina-se quase exclusivamente à produção de aguardente de medronho e seus derivados. O mel de medronho, paradoxalmente, é muito pouco apreciado a nível local e quase desconhecido a nível nacional, provavelmente graças ao seu carácter *exquisite* e sabor ligeiramente amargo. Esta situação é completamente diferente da encontrada na ilha de Sardenha (Italia), onde o mel amargo é muito valorizado, e comercializado a preço 4-8 vezes superiore a outros tipos de mel. É tratado como uma especialidade graças às suas características organolépticas e às propriedades biológicas que lhe são atribuídas. Em Portugal, contrariamente, a outros produtos do medronho, este mel tem sido praticamente ignorado tanto comercialmente como em estudos científicos. Neste capítulo é descrita a caracterização físico-química do mel de medronho, os resultados da análise melissopalinológica e, ainda, os resultados de atividade biológica. Estes estudos foram realizados tendo em vista a valorização deste tipo de mel graças a presumível presença acentuada de compostos antioxidantes.

Os apicultores locais forneceram amostras de mel que classificaram como mel de medronho, tendo a análise melissopalinológica confirmado que este era de facto o pólen predominante (31,96%). O estudo físico-químico, incidindo sobre os parâmetros acima referidos, indicou que se tratava de néctar de mel, em todas as amostras. Revelou também que este tipo de mel obedece aos requisitos legais em vigor e que não difere significativamente do seu congénere italiano. A atividade antioxidante do mel de medronho foi confirmada. Na realidade ela é várias vezes superior à de outros tipos de mel, muito mais populares. Estes resultados podem ser usados para demostrar os aspectos atrativos do mel de medronho a um público mais esclarecido. Estes aspectos foram discutidos com dirigentes de uma associação de apicultores que se mostraram interessados nos resultados.

No seguimento deste trabalho com mel de medronho (capítulo 5), foi feito um estudo comparativo com quatro amostras de mel comercial: flor de laranjeira (*Citrus* spp.), girassol (*Helaiantus annuus*), rosmaninho (*Lavandula stoechas*) e medronho (*Arbutus unedo* L.). Além dos estudos físico-químicos e biológicos, foi também feito um estudo sensorial com um painel de avaliadores com uma boa experiência na evaluação de productos alimentares. De acordo com

os resultados da análise melissopalinológica todas as amostras comerciais foram confirmadas seus origen florais. A amostra de mel etiquetado como mel de laranjeira continha 30,2% de pólen de *Citrus* spp.; a amostra de girassol indicava conter 24,6% (*Helliantus annuus*), o mel etiquetado como mel de rosmaninho, tinha o conteudo de polen de 54,2% de *Lavandula stoechas*. Finalmente, a amostra etiquetada comercialmente como mel amargo monofloral provinha de 20,4% de pólen de *Arbutos unedo* L. Todas as amostras indicam conter maioritariamente mel da proveniência indicada no rótulo.

O teor de HMF, um dos indicadores de frescura, estava acima do estipulado (40 mg/kg) em duas das amostras comerciais: 61,3 mg/kg para o mel de medronho e 68,1 mg/kg para o mel de girassol. Este facto pode resultar de armazenamento inapropriado ou prolongado e, também, de processamento a temperaturas demasiado elevadas. As outras duas amostras apresentavam teores de HMF bastante mais baixos: 16,7 e 24,4 mg/kg para as amostras de mel de laranjeira e de rosmaninho, respectivamente. O outro indicador de frescura, DA, também não respeitava a regulamentação em todas as amostras (menor ou igual a 8 Gothe). As amostras de mel de girassol e de rosmaninho tinham praticamente os mesmos valores (7,6 Gothe). A legislação diferencia alguns tipos de mel que podem ter combinações diferentes de HMF e DA. Assim, quando o conteúdo enzimático do mel é baixo por natureza, o valor mínimo de DA é 3 Gothe. Nestes casos, DA entre 3 e 8 Gothe, o teor do outro indicador de frescura, HMF, não pode ser superior a 15 mg/kg. Sendo assim, só o mel de rosmaninho estaria próximo da conformidade. É evidente que, não obstante a legislação existir e ser clara, o mel adquirido mesmo em grande exposição (*e.g.* hipermercado) é de qualidade muito inferior ao estipulado por lei. Apenas um tipo de mel, laranjeira, apresentava indicadores de frescura aceitáveis.

Este estudo revelou que o mel de medronho comercial apresentava um elevado teor em fenóis e flavonóides: 91,83 mg acido galico equivalente (GAE)/100g e 4,49 mg quercitina equivalente (QE)/100 g respectivamente, valores bastante superiores aos observados para os outros tipos de mel. Estes estudos revelam que o mel amargo, tal como a planta donde provem o pólen, medronho, tem na sua constituição uma variedades de substâncias de potencial medicinal muito elevado. Esta possibilidade esteve na base deste estudo, que nos apraz ter comprovado, e seria

muito desejável que os resultados aqui apresentados contribuíssem para a valorização deste tipo mel à semelhança do que acontece na zona de Sardenha, em que o mel amargo é uma raridade. No entanto, em Portugal, parece que o consumidor não tem conhecimento nem consciência do valor medicinal do mel amargo. Nesta tese também se apresenta um estudo sensorial em que se compara mel amargo artesanal com vários tipos de mel comercial. De acordo com um painel de provadores, a preferência pelos diferentes tipos de mel foi: rosmaninho > girassol > medronho (comercial) > laranjeira > medronho (artesanal). A ordem não é surpreendente pois o medronho comercial parece ser uma mistura de medronho com outros tipos de mel. É possível que o tratamento exagerado do ponto de vista térmico no mel de medronho comercial, conforme descrito anteriormente, tenha produzido compostos (caramelizados) que mascaram algum do sabor amargo original.

Finalmente, esta tese debruça-se sobre métodos alternativos de análise (capítulo 6), aplicáveis de forma genérica na diferenciação de amostras e que, apesar de não se basearem em normas estabelecidas e oficialmente reconhecidas para o mel, podem responder de forma mais eficiente e apropriada em questões de verificação de autenticidade e/ou de adulteração. Pelo menos, podem ser usadas como primeiros indicadores de frescura e origem floral, como já foram demostrados em outros estudos. A sua principal vantagem é que não necessitam de equipamento dispendioso nem análise morosa. Fundamentam-se no tratamento estatístico de um número geralmente elevado de variáveis obtidas através de medidas eléctricas e/ou ópticas. Não é essencial que os "sinais" observados se relacionem com propriedades físico-químicas ou biológicas no sentido tradicional mas é importante que haja um agrupamento de resultados consistentemente próximos e isolados para cada amostra. Este tratamento estatístico é designado por "análise de componentes principais". No âmbito desta abordagem, entre outros, foi feito um estudo exploratório com várias técnicas não-invasivas para determinação da origem floral, um dos mais relevantes aspectos na caraterização do mel e, simultaneamente, o mais difícil de assegurar de forma expedita. Neste trabalho, depois de um estudo com uma "língua electrónica" constituída por elétrodos de ouro, platina, alumínio e óxido de estanho e índio, foi feito um estudo espectroscópico nas gamas do visível-infravermelho próximo (NIR) e visível-

ultravioleta. O tratamento estatístico do complexo e extenso número de resultados foi feito com software especializado. Resumidamente, foi possível diferenciar sem qualquer ambiguidade mel de medronho e mel de girassol. Infelizmente, os resultados não foram tão satisfatórios para o mel de laranjeira e de rosmaninho. Genericamente as técnicas usadas deram resultados semelhantes, sendo a língua electrónica a que parece demonstrar melhor desempenho. Isto sugere que há um grande grau de sobreposição entre a informação fornecida pelos três métodos e portanto pouca complementaridade entre eles. De qualquer forma, o principal objectivo do estudo era o mel de medronho e este diferencia-se perfeitamente de todos os outros. Este trabalho exploratório deverá ser continuado com mais amostras, mais variáveis e promovido como um método certificado com valor legal na caraterização e avaliação da origem e qualidade do mel.

Este trabalho abordou a caracterização de vários tipos de mel. Verificou-se que o mel disponível no mercado português não faz justiça à tradicional qualidade do mel Algarvio o que, a médio/longo prazo, contribuirá para a sua desvalorização. Este facto pode decorrer da incapacidade das instituições legais em verificar os principais parâmetros de qualidade e de origem floral. No que respeita ao mel de medronho, considerou-se importante estudá-lo pela importância histórica, económica e social da planta *Arbutus unedo* L. no Sul de Portugal e na região mediterrânica e, também, pelo potencial que o mel amargo tem intrinsecamente pela sua bioactividade e pelo seu sabor. Amargo é uma característica que pode ser muito valorizada tanto no mel em si, enquanto alimento, como na preservação de alimentos ou para fins medicinais.

Palavras chave: mel, caracterização físico-química, análise de pólen, medronho, compostos bioativos, tempo de armazenamento, capacidade antioxidante.

List of Symbols

Symbols

$(\alpha)_D^{20}$	optical rotation unit
$\bar{x}$	average
$\dot{\gamma}$	shear rate (1/s)
µg/mL	microgram/milliliter
µm	micrometer
µS	microSiemens
a^*	colour space co-ordinate (degree of greenness/redness)
Abs	absorbance
Ag	silver
Ag_2CO_3	silver carbonate
Ag_2O	silver oxide
AgCl	silver chloride
Al	aluminium
$AlCl_3$	aluminium trichloride
Au	gold
a_w	water activity
b^*	colour space co-ordinate (degree of blueness/yellowness)
$C_4H_6O_3$	acetic anhydride
$C_6H_6O_3$	hydroxymethylfurfural
cm	centimeter
Cu	cupper
Cu_2O	cuprous oxide
E_a	activity energy (kJ/mol)
g	gram
g/L	gram/liter
H_2SO_4	sulfuric acid
H_3PO_4	phosphoric acid
K	Kelvin
kg	kilogram
L^*	colour space co-ordinate (degree of lightness)
meq/kg	millequivalent/kilogram
mg	milligram
mg/kg	milligram/kilogram
min	minute
mL	milliliter
mL/min	milliliter/minute
mm	millimeter

mS/cm	milliSiemens/centimeter
Na_2CO_3	sodium carbonate
NaOH	sodium hydroxide
nF	nanoFaradio
nm	nanometer (referred absorbance)
p <0.05	significant level (5% error)
Pd	paladium
pK_a	acid dissociation constant
Pt	platinum
R^2	coefficient of determination
rpm	revolutions per minute
Ti	titanium
W	tungsten
w/v	weight/volume
w/w	weight/weight
A_{620}	absorbance at 620 nm
A_{635}	absorbance at 635 nm
Δ E	differences of color
η	viscosity (Pa s)
τ	yield stress (Pa)

List of Abbreviations

Abbreviations

AEAC	Ascorbic acid Equivalent Antioxidant Capacity
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
CA	Cluster Analysis
CCA	Canonical Correlation Analysis
CEs	Catequin Equivalents (referred flavonoid content)
CQVR	Centro de Química de Vila Real
CS	Coefficient of Supersaturation (referred to crystallization)
D	Predominant pollen > 45% of the pollen grains
DA	Diastase Activity (Gothe)
DAs	Discriminant Analysis
DFA	Discriminant Function Analysis
DN	Diastase Number (Gothe)
DNSA	3,5-dinitrosalicyllic acid (reagent)
DPPH	2,2-diphenyl-2-picryl-hydrazyl (reagent)
EC	Electrical Conductivity (mS/cm)
e-nose	Electronic nose
ERH	Equilibrium Relative Humidity (%)
e-tongue	Electronic tongue
EU	European Union
FCM	Folin-Ciocalteu Method
FTIR	Fourier Transform Infrared Spectrometry
GAE	Gallic Acid Equivalent
GC-FID	Gas Chromatography with Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectrometry
HGA	Homogentisic Acid (2,5-dihydroxypenylacetic acid)
HMF	Hydroxymethylfurfural
HPAEC	High Performance Anion-Exchange Chromatography
HPLC	High Performance Liquid Chromatographic
IHC	International Honey Commission
IN	Invertase Number
ITO	Indium Thin Oxide
KF	Karl Fischer titration
LDA	Linear Discriminant Analysis
M	Important minor pollen (3–15% pollen grains)
MC	Moisture content (%)

MPCA	Multi-way Principal Component Analysis
NIR	Near-Infrared
NMR	Nuclear Magnetic Resonance
OR	Optical Rotation
PCA	Principal Components Analysis
PDO	Protected Denomination of Origin
PGI	Protected Geographical Indication
PLS	Partial Least Square
QE	Quercitin Equivalent
QEAC	Quercitin Equivalent Antioxidant Capacity
RI	Refractive Index
S	Secondary pollen (16–45% pollen grains)
SD	Standard Deviation
SPME	Solid Phase Micro-extraction
T	Minor pollen (less than 3% pollen grains)
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TSG	Traditional Specialty Guaranteed
TSS	Total Soluble Solids (ºBrix)
UTAD	Universidade de Trás-os-Montes e Alto Douro
UV	Ultraviolet Visible
WLF	Williams Landel Ferry

Table of content

CHAPTER 1

INTRODUCTION AND REVIEW

"Spider caves" art ruprecht

Chapter One: Introduction and Review

1.1. Honey

Honey is a natural food produced by *Apis mellifera* L. bees from the nectar of blossom (nectar honey) or from the secretions of living plants or excretions of plant sucking insects of the living part of plant (honeydew honey) that transform and combine this with specific substances of their own, and leave it in the honey comb to ripen and mature. Honey is composed primarily by a mixture of sugars (85–95%) and water (16–18%) approximately, and minority compounds (proteins, free amino acids, organics acids, phenolic compounds, vitamins and minerals) (Sanz, et al., 2005; Pires et al., 2009; Kahraman et al., 2010; Castro-Vázquez et al., 2010).

Apis mellifera L. bee foragers collect nectar and honeydew from plants and carry it by means of their honey sac and bring it to their colony. When a worker bee drinks the nectar from flowers, a maximum of about 25 mg of nectar is stored at the bottom of the esophagus in a widened region called the honey stomach or honey sac. On their way they already add enzymes from their two glands, salivary and the hypopharengeal, and transfer the nectar to colony bees. These nurse bees pass it over to each other and finally fill the honey into the combs. During this process the bees fan with their wings, thus lowering honey´s humidity, and when the water contents reaches 30–40% the honey is filled into the combs. During this process the bees add additional enzymes to the honey. These enzymes include diastase and invertase, both of which break down larger saccharides, especially invertase, into monosacharides (fructose and glucose). While glucose oxidase oxidates the glucose to gluconic acid and hydrogen peroxide; the latter acting as an agent against bacterial spoilage. Thus, chemical transformation of the nectar into honey begins almost immediately. The warm colony temperature (35 ºC), and further fanning lowers further the honey humidity. Bees also suck out the honey and deposit it back into the combs, and by this process further lower the water content of the honey. This transformation process takes place in 1 to 3 days. Generally, when the ripe honey has a humidity lower than 20% the bees cap the combs, thus preventing an absortion of moisture by the ripe honey. Only rarely, under

very humid or tropical conditions can honey with more than 20% be capped by bees. The aim of the beekeeper is to harvest honey with less than 18% humidity (Ball, 2007).

1.2. Types of honey

Honeys are classified in to two kinds: nectar honey and honeydew. Nectar is a sugar solution of varying concentrations: from 5–80%. About 95% of the dry substances are sugars, the rest are amino acids (0.05%), minerals (0.02–0.45%), small amounts of organic acids, vitamins and aroma compounds. The sugar value ranges widely, from 0.0005 to 8 mg. The sugars composition is also typical for each plant species, the principal sugar being sucrose, glucose and fructose. Most plants have nectars consisting predominantly of fructose and glucose (60–85%); but in some plants the nectar is mainly sucrose (*e.g.* acacia clover, lavender) (Lazaridou et al., 2004; De la Fuente et al., 2011). The sugar concentration depends on different climatic factors such as temperature, soil humidity and season. When humidity is higher the nectar quantity is greater, but the sugar concentration is smaller. Temperature plays also a very important role. Optimum temperatures are 10 to 30 ºC. Strong winds reduce nectar secretion. The nectar secretion depends also on the day time. Therefore it is not possible to foresee nectar production. Maximum secretion is noon and early afternoon. Bees prefer nectar with higher sugar contents, *e.g.* around 50% and will not forage if it is below 5%. Bees gather nectar for their energy needs. The higher the sugar value of a plant, the more it is visited by bees for foraging (Weryszko-Chmielewska and Chwil, 2007; Bentabol et al., 2011).

Honeydew is the secretion product of plant-sucking insects (*Hemiptera*, mostly aphids). These insects pierce the foliage or other covering parts of the plant and feed on the sap. The ingested sap is passed through the insect´s gut, and the surplus is excreted as droplets of honeydew, which are gathered by the bees. There are different sorts of honeydew-producing insects. Most plants are trees; the coniferous trees yield worldwide the highest amounts of honeydew. However, other plants, *e.g.* cotton, lucerne and sunflower can also provide honeydew (Sanz et

al., 2005; Bentabol et al., 2011). Honeydew is a solution with varying sugar concentration (5–60%), containing mainly sucrose, besides higher sugars (oligosaccharides). There are also smaller amounts of amino acids, proteins, minerals, acids and vitamins. In addition, honeydew contains cells of algae and fungi, however there are not specific for the honeydew origin. Some insects produce high amounts of the trisaccharide melezitose, which is only very slightly soluble in water, thus yielding honey, which can crystallize in the combs (Bentabol et al., 2011). Honeydew production is even less predictable than the nectar flow, as it depends on the build-up of plant sucking insects. By evaluating the populations of the plant-sucking insects before the honeydew flow, the potential for a possible honey flow can be estimated. However, the honeydew flow depends also on favorable weather conditions during the honey flow period (Bentabol et al., 2011; De la Fuente et al., 2011). Table 1.1 shows differences in composition between nectar and honeydew honey for some physicochemical parameters these kinds of honeys.

Table 1.1 Differences in composition and some physicochemical parameters of nectar honey and honeydew honey; values (g/100 g) (from Bogdanov, 2009; Bentanol et al., 2011).

Component	**Nectar Honey**	**Honeydew Honey**
Water	15–20	15–20
Carbohydrates	72–85	73–83
Fructose	30–45	28–40
Glucose	20–40	22.9–40.7
Sucrose	0.1–4.8	0.2–7.6
Melezitose	<0.1	0.3–22.0
Erlose	0.56	0.16–1
Threhalose	1.2–2.4	1.2–2.9
Turanose	1.0–2.1	0.7–2.2
Other disaccharides (maltose)	28	16
Other oligosaccharides	0.5–1.0	0.1–6.0
Total sugars	79.7	80.5
Acids	0.2–0.8	0.8–1.5
Water activity	0.56–0.61	0.57–0.61
Minerals	0.1–0.5	0.6–2
Amino acids, proteins	0.2–0.4	0.4–0.7
pH	3.5–4.5	4.5–6.5
Optical rotation ($\propto^{D}_{20^\circ}$)	Levorotatory (-)	Dextrorotatory (+)
Diastase activity (º Gothe)	8.9–35.9	4.7–25.8

1.3. Classification of honey

Nowadays the current tendency is to differentiate honeys from different floral sources, in order to obtain a standard of quality and authenticity for this product that allows it to be distinctive in the market. In Europe this has led to the introduction of several official regulations for agricultural products, such as protected denomination of origin (PDO), protected geographical indication (PGI), and traditional specialty guaranteed (TSG) certifications, which allows certain products to be labeled with the names of their geographical areas of production (European Regulations EC/510, 2006; Castro-Vázquez et al., 2010).

The classification of honey is of great importance for quality and, in addition, to determination of control the geographical and botanical origin; therefore of pollen analysis (melissopalynology) is necessary. The melissopalynology and other physicochemical parameters are also used (Devillers et al., 2004; Serrano et al., 2004). The nectar honey always includes numerous pollen grains (mainly from the plants species foraged by honey bees) and honeydew elements (like wax tubes, algae and fungal spores) that altogether provide a food fingerprint of the environment where the honey comes from (Lieux, 1980; Von der Ohe et al, 2004). Besides, pollen analysis provides some important information about honey extraction and filtration, fermentation, some kinds of adulteration and hygienic aspects such as contamination with mineral dust, soot, or starch grains (Louveaux et al., 1978, Von der Ohe et al., 2004). However, this method is time-consuming, requires specialized knowledge and expertise, and involves a laborious counting procedure, with interpretation of results and an identification of botanical origin being rather difficult. Particular difficulties in this method are associated with need of good experience and knowledge of pollen morphology and availability of a comprehensive collection of pollen grains (Cotte et al., 2003; Nozal et al., 2005; Kaškonienė and Venskutonis, 2010a).

The determination of the geographical origin is established by the presence of characteristic pollens that are limited to a certain region. More often, the region in which the honey was

produced can be determined from the presence of certain pollen combinations (honey types). The pollen spectrum present in a honey depends on the floral agricultural and forest conditions where it was produced (Louveaux et al., 1978; Ruoff et al., 2007).

Melissopalynological determination of botanical origin of honey is based on the relative frequency of the pollen from nectar–secreting plants and honeydew elements in it by microscopic examination (figure 1.1). However, there are some problems related to this method: different plant species produce different proportions of pollen, the amount of pollen can vary from season to season, the nectar yield can be different in male and female flowers, pollen filtered out in the bee´s honey sac, bees can take pollen without collecting nectar, most of the pollen can be collected from plants that cannot be the sources of honey, filtering of honey for packaging for sale, and the straining of honey (Anklam, 1998). The very different levels of abundance of a given pollen type in the nectar of the plant that produces it, in addition to others sources of variability, such as secondary, tertiary and quaternary enrichment, requires particular caution in the interpretation of melissopalynological results. Secondary enrichment is defined as the inclusion of pollen inside the hive, while tertiary enrichment can occur during the extraction process of the honey and quaternary enrichment can come from aerial contamination (Louveaux et al., 1978; Von der Ohe et al., 2004, Kaškonienė and Venskutonis, 2010a).

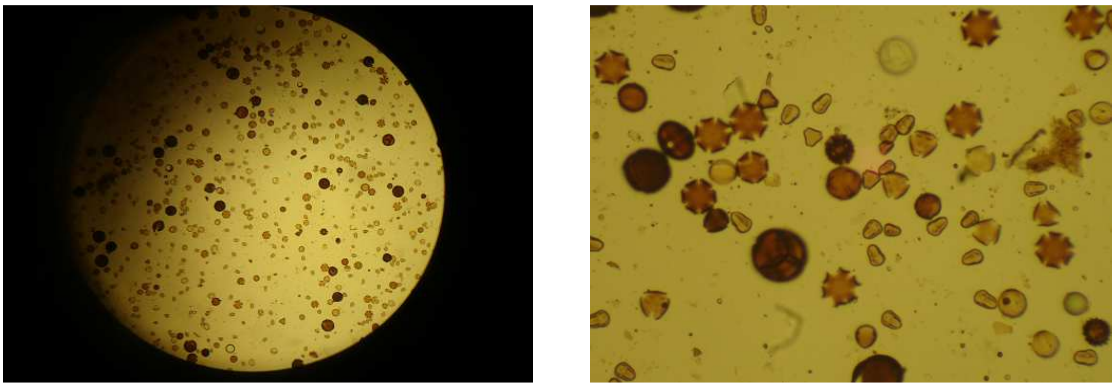


Figure 1.1 Photography showing the variety of pollen that has a type of honey.

In general, a honey is considered as coming predominantly from a given botanical origin (unifloral honey) if the relative frequency of the pollen of that *taxon* exceeds 45%. The honey can be classified as unifloral or multifloral, but this depend on pollen grains frequencies (Louveaux et al., 1978; Persano Oddo et al., 1995; Valle et al., 2007). Classes are designated such as:

- Predominant pollen (D): more than 45% of the pollen grains counted.
- Secondary pollen (S): 16–45%.
- Important minor pollen (M): 3–15%.
- Minor pollen (T): less than 3%.

Some flowers have special cases for the amount of pollen (frequency pollen), because are over-represented, *i.e.* the percentage of pollen in the sediments is higher than the percentage of the corresponding nectar in the honey; with some other pollens the situations is reversed they are under-represented (Louveaux et al., 1978; Von der Ohe et al., 2004).

The table 1.2 describes examples for some flowers that showed under, normal and over-represented pollen.

Table 1.2 Relative level of abundance and relative frequency of the main pollen types in various unifloral honeys (from Von der Ohe et al., 2004; with modifications).

Botanical origin	**Under-represented pollen (%)**	**Pollen which can be under-represented in some cases (%)**	**Normally represented pollen (%)**	**Over-represented pollen (%)**
Arbutus	8–20			
Brassica napus				>60
Calluna		10–70		
Carduus	5–25			
Castanea				>86
Citrus	2–42			
Erica			>45	
Eryobotrya			>45	
Eucalyptus				>83
Hedysarum			>50	
Helianthus		12–92		
Lavandula latifolia	15–42			
Lavandula x *intermedia*	1–20			
Medicago	1–10			
Phacelia				>60
Rhodedendron		15–77		
Robinia		7–60		
Rosmarinus		10–57		
Taraxacum	5–40			
Thymus		13–68		
Tilia		1–56		

1.4. Production system

Honey is a semi-solid that is extracted from combs and apiaries; contains pollens, beeswax, and other undesirable materials, besides yeast, that are to be removed to obtain better product quality and shelf life. Hence, honey is processed before packaging in bottle or other containers. The type of equipment used and steps followed in processing, however, depend on the scale of operation (Subramanian et al., 2007; Babarinde et al., 2011).

The honey can be obtained from combs by three types of different processes of production and according of these productions the honey is designated (Bogdanov, 2009):

- *Extracted honey*: is obtained by centrifuging decapped broodless combs. This is most of the honey which is marketed in most countries of the World.
- *Pressed honey*: is obtained by pressing broodless combs.
- *Drained honey*: is obtained by draining decapped broodless combs.

The methods of harvesting honey have different effects on the final quality and acceptability and may create an impression of adulteration when on shelves. In the research made by Babarinde et al., (2011) investigated of the effect of two harvesting methods on physicochemical and microbial quality of Nigerian honeys. A comparison of the traditional (honey in wood logs) and modern methods (extraction of hives) of harvesting was performed. The results revealed that the honeys harvested using a modern method had a better quality, which related to physicochemical and microbiological attributes.

After harvesting, there are two important stages of honey processing: filtration and heating. The separation of pollens, beeswax, and other materials are normally done through straining and pressure filtration. Heat or thermal processing of honey eliminates the microorganisms responsible for spoilage and reduces the moisture content to a level that retards the initiation of

fermentation process. The flow diagram of the general process for conventional honey processing in provided in figure 1.2. The physical separation of undesirable, suspended matter is done before thermally processing of honey (Subramanian et al., 2007).

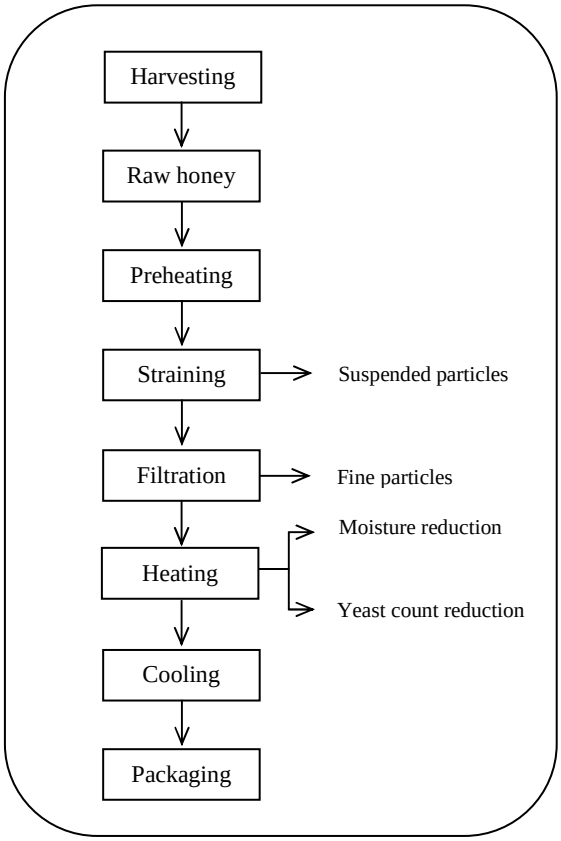


Figure 1.2 Conventional method of processing honey (from Subramanian et al., 2007; with modifications).

1.4.1. Straining

The straining operation to remove suspended solids (including large wax particles) is carried out either manually or by mechanical means. The method and the equipment used for straining depends on the size of the operation. In small-scale operations, straining is done using cloth or nylon bags, which are frequently cleaned to remove the suspended particles. In large-scale operations, the straining operation is combined with the preheating (up to 40 ºC) operation in jacketed fitted with a stirrer (Subramanian et al., 2007).

1.4.2. Filtration

The strained honey is further processed using pressure filters. Typically, a polypropylene micro filter of 80 µm is used as a filter medium. The honey temperature is maintained between 50–55 ºC, which prevents the melting of the beeswax. Large-scale processors subject honey to coarse filtration, centrifugal clarification, fine filtration and blending, prior to filling (Subramanian et al., 2007).

1.4.3. Thermal processing (heating)

Honey generally contains osmophilic (sugar-tolerant) yeast in higher or less amounts and ferment, if the moisture content is high enough and storage temperature is favorable. The unprocessed honey tends to ferment within a few days of storage at ambient temperature because of its high moisture content and yeast count. To prevent fermentation and facilitate handling, honey is heat processed before storage. Heat processing of honey eliminates the microorganisms responsible for spoilage and reduces the moisture content to a level that retards the fermentation process, reduces viscosity and breaks down crystals (Anklam, 1998; Fallico et al., 2004; Subramanian et al., 2007). The thermal process is performed with a preheating to 40–50 ºC in an air-ventilated chamber or immersion of honey drums in hot water, straining, filtering/clarification, and indirect heating of the filtered honey at 60–65 ºC for 25–30 minutes in a tubular heat exchanger followed by rapid cooling in order to protect its thermo-label parameters (enzymes, biological substances) as well natural colour and flavor (Fallico et al., 2004).

Researches has shown that the effect of processing with moderate temperatures (68–80 ºC) for a few minutes affects the honey´s hydroxymethylfurfural (HMF) content and diastase activity. The effects of heat are cumulative; thus, the effects of processing and storing honey are often considered together (Wang et al., 2004).

Singh and Bath (1998), analyzed the relationship between heating and HMF formation in different kinds of honey. This research showed that HMF formation increased with the increment of temperatures and this behaviour was described using a model of second order polynomials. Tosi et al., (2002) reported that during thermal processing, the time-temperature combination is crucial for maintaining the HMF content below the maximum permissible by legislations (< 40 mg/kg) (EU, 2001; Codex Alimentarius Commission, 2001).

Thermal processing increases the HMF content in honey, also affects the physicochemical characteristics and sensory quality. Gupta et al., (1992) studied the effect of processing and storage temperatures in these two parameters. The results obtained have shown that colour was significantly affected by the storage temperatures and period, with a maximum of deterioration at a storage temperature of 40 ºC. However, granulation was completely eliminated in honey storage over storage conditions. Evaluation of honey samples stored for six months showed a comparatively higher overall sensory score for unheated honey stored at 5 ºC.

Crystallization is an undesirable property during handling, processing, and marketing, except for certain purposes such as in the production of creamed honey. Glucose is the principal component that crystallizes in honey as it exists in a supersaturated state. There are diverse methods to stop crystallization of honey: storage at freezing temperature (-40 ºC), heat treatment to dissolve crystals and crystal nuclei, removal of air bubbles, dust and pollen particles by filtration, filling at higher temperatures (> 45 ºC) to avoid air bubbles incorporation during filling, addition of inhibitors such as isobutiric and ascorbic acid, and adjusting the glucose to fructose ratios or water content (Bhandari et al., 1999). Ultrasound processing has also been reported for preventing crystallization in honey, and the various attempts made are discussed under a separate heading. Presence of air bubbles in the packaging containers can cause nucleation and crystallization of honey, whilst filling at higher temperatures eliminates air bubbles and avoids air incorporation during packing, due to low viscosity (Subramanian et al., 2007).

Although, honey is thermally processed to avoid fermentation by yeast, it could result in a decrease of the original quality; with temperatures abuse honey is deteriorated due to an increment of the hydroxymethylfurfural content and a lowering of the enzyme activity. An excessive amount of HMF has been considered an evidence of over-heating, implying a darkening of colour and a loss of freshness of honey. The α-amilase enzymes of honey are also used as an indicator of quality, because of their sensitivity to heat. The enzyme content in honey is measured as the diastase activity and is expressed as a diastase number (DN). The European legislation for honey specifies a minimum DN of 8 in processed honey (EU, 2001; Codex Alimentarius Commission, 2001; Subramanian et al., 2007).

Other studies have also analyzed the effects of different processing methods (microwave, infrared heat, ultrasound, membrane) in the physicochemical, microbiological, crystallization and sensory parameters on honey. These types of techniques preserve the honey´s quality and save energy consumption, some, even improve the appearance of honey, for example clear and more transparent (Thrasyvoulou et al., 1994; Bath and Singh, 2001; Barhate et al., 2003; Hebbar et al., 2003; Subramanian et al., 2007).

1.5. Chemical composition of honey

1.5.1. Moisture

Water content in honey is important first of all because it affects its storage potential. While nectar or honeydew is processed by bees into honey its water content drops from the initial 80–85% of total weight of the product to 16–20%. A number of factors influence the final value of that parameter in honey produced by honeybee colonies, such as low air humidity, medium abundance of nectar flow, good colony strength and ventilation of the beehive (Semkiw et al., 2008a; Gallina et al., 2010).

A water content below 17% prevents fermentation and guaranties good honey storage, regardless of the count of osmophilic yeast (*Saccharomyces* spp.) present in the product. These act on fructose and glucose, producing gaseous carbon dioxide, with foam formation, and ethanol, which, in the presence of oxygen, produces acetic acid (Tosi et al., 2002; Chirife et al., 2006; Zamora et al., 2006). The danger of honey fermentation is greatest when moisture content exceeds 20%. The acceptable water content of honey is specified by honey quality standard, both in the World standard (Codex Alimentarius Commission, 2001) and in the European Communities (EU, 2001; Semkiw et al., 2008a). This process is essentially dependent on the initial load of microorganisms and the storage time–temperature (Jiménez et al., 1994).

Moisture influences the rate of fermentation, granulation, and honey flavor. A reduction of the moisture content to levels below 17% is considered to be a safe level for retarding yeast activity. The moisture content of honey depends on the environmental conditions and the manipulation at the period of harvest. A high moisture content could accelerate crystallisation in certain types of honey (Yanniotis et al., 2006; Subramanian et al., 2007). Some unifloral honeys, like sunflower, heather and strawberry tree tend to have higher water contents than others (Chirife et al., 2006). The beekeeper can estimate the moisture content by a simple test: a honey comb with open brood is punched by fist – if the honey does not splash, then the honey is ready for harvest. There are two official methods for moisture determination: a gravimetric determination after oven drying and the refractive index determination with an evaluation of moisture percentage by using an empirical formula or a relative conversion table (Bogdanov, 2002; Gallina et al., 2010). Isengard et al., (2001), and Isengard and Schultheiβ (2003) have used an alternative method to determine the moisture content, which was the Karl-Fischer (KF) titration, for honeys that cannot be heated or treated prior to analysis. The results obtained showed a good correlation, when compared with the official methods. This technique (KF) might be an alternative for the measure the of moisture content in “creamy honeys”.

1.5.2. Carbohydrates

In general, honey is a supersaturated sugar solution; sugars are the main constituents of honey, accounting for about 90–95 g/100 dry matter; it is produced by bees from nectar, which is transformed through the action of several enzymes (Mora and Marioli, 2001). Conversions catalysed by the enzyme pool result in a complex mixture of monosaccharides, disaccharides, trisaccharides and other oligosaccharides (table 1.3). Fructose (38%) and glucose (31%) are the major constituents of honeys (figure 1.3a), the former being the dominant component in almost all types. These carbohydrates range from 65% to 80% of the total soluble solids; except for some types of honeys such as dandelion (*Taraxacum officinale*), blue curl (*Trichostema lanceolatum*), and rape (*Brassica napus*), where glucose is present in higher amounts (Kaškonienė and Venskutonis, 2010a; Kaškonienė et al., 2010b). Honey disaccharides ranging from 10% to 15% are mainly constituted by regioisomers of α-glucosyl glucose and α-glucosyl fructose; disaccharides with β-glycosideic linkages are present in minor amounts while fructosyl-fructoses are very scarce: as well other disaccharides present in honey, albeit in very small quantities (cellobiose, gentiobiose, isomaltose, maltose, kojibiose, palatinose, neotrehalose, nigerose, turanose, laminaribiose, maltulose, melibiose, thehalose, sucrose) (Weston and Brocklebank, 1999; Kaškonienė and Venskutonis, 2010a; Kaškonienė et al., 2010b; Ouchemoukh et al., 2010). The more abundant trisaccharides (erlose, isopanose, panose) are derivatives of sucrose such raffinose and melezitose (figure 1.4), which have been used to distinguish between nectar and honeydew honey. In addition, some tetrasaccharides have also been identified in some samples of honey (Mateo and Bosch-Reig, 1997; Sanz et al., 2004; Cotte et al., 2004a; 2004b; Ruiz-Matute et al., 2010; Ouchemoukh et al., 2010; De la Fuente et al., 2011; Kamal and Klein, 2011).

Table 1.3 Some carbohydrates found in floral honeys and honeydew honeys (from Kamal and Klein, 2011; De la Fuente et al., 2011).

Types of Carbohydrates			
Monosaccharides	**Disaccharides**	**Trisaccharides**	**Tetrasaccharides**
fructose	cellobiose	centose	fructosyl-isomelezitose
glucose	gentiobiose	erlose	isomaltotetraose
	isomaltose	4-α-gentiobiosylglucose	isopanose
	isomaltulose	3-α-isomaltosylglucose	maltotetraose
	isomelezitose	isomaltotriose	nystose
	kojibiose	isopanose	stachyose
	laminaribiose	isomelezitose	α-4´-glucosyl-erlose
	leucrose	kestoses	α-6´-glucosyl-erlose
	maltose	laminaritriose	
	maltulose	maltotriose	
	melibiose	melezitose	
	neotrehalose	panose	
	nigerose	raffinose	
	palatinose	theanderose	
	sacarose		
	thehalose		
	turanose		

The highest content of sucrose (figure 1.3b) and lowest content of invert sugars (glucose and fructose) characterize the badly mature of honey or else about bees were fed with sucrose (Diminš et al., 2008).

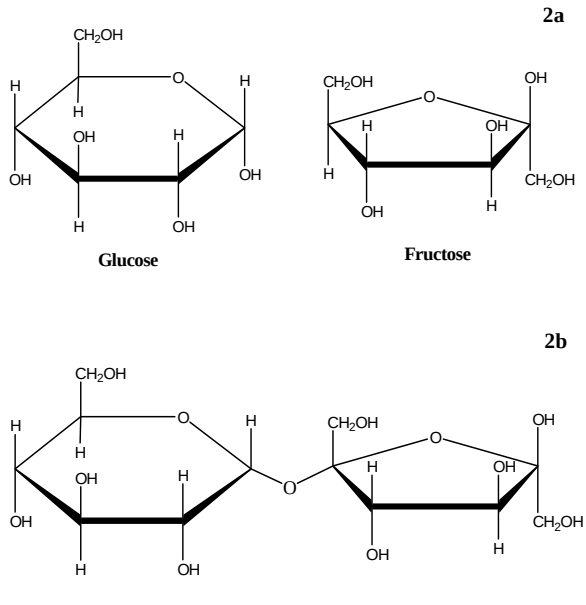
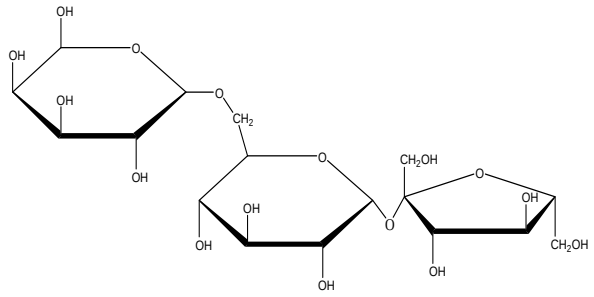


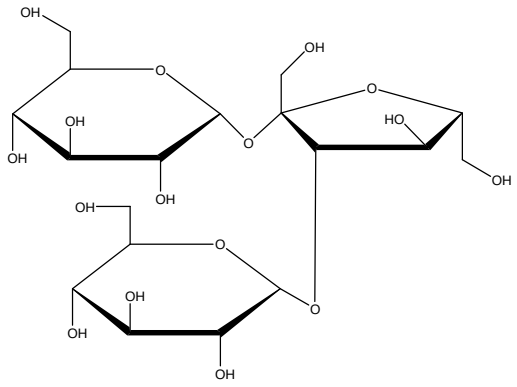
Figure 1.3 Structures of monosaccharides (glucose–fructose, 2a) and disaccharide (sucrose, 2b) found in honey.

Sugars in honey are responsible for properties such as viscosity, hygroscopy, specific rotation, energy value and crystallization phenomena. The ratio of glucose to fructose partially determines the crystallizations speed of honey (Conforti et al., 2006).

Each carbohydrate on honey are chiral compounds which have optical activity that can be used to identify the type of honey (nectar or honeydew honey) depending on relationships and contents of the different carbohydrates in honey. This property is detailed in the section of physicochemical properties (please refer to section 1.6.7. Optical rotation). Normally, nectar honey shows a levorotatory behaviour in comparison with honeydew, which has dextrorotatory optical rotation (Dinkov et al., 2004). This is a consequence of a normally higher percentage of fructose (~38%), which shows a negative specific rotation over glucose; the overall value for the optical rotation is a result of the values of the different honey sugars. The honeydew honey is characterized by higher concentration of oligosaccharides, mainly trisaccharides (Dinkov, 2003; Nanda et al., 2003; Nozal et al., 2005).



Raffinose



Melezitose

Figure 1.4 Structures of trissacharides (raffinose–melezitose) found in honeydew honey.

Characterization of honey sugars may be a useful approach for detecting honey adulteration by the use of commercially available carbohydrate materials. Honey can be adulterated by adding its invert syrup, corn syrup or high fructose corn syrup. It has been reported that honey produced with corn syrup or high fructose corn is distinguished by its high amount of oligosaccharides (Anklam, 1998; Ryback-Chmielewska, 2007a; 2007b; Kaškoniene et al., 2010b). Several investigations have suggested the use of the sugar composition to establish honey authenticity, geographical and botanical origin (Swallow and Low, 1990; Sanz et al., 2004; Nozal et al., 2005; De la Fuente et al., 2007; De la Fuente et al., 2011). Sugar composition can be determined by different methods, *e.g.* High Performance Anion-Exchange Chromatography (HPAEC) (Weston and Brocklebank, 1999; Cotte et al., 2004b), High Performance Liquid Chromatographic (HPLC) with Refractometric detection (Rybak-Chmielewska, 2007b; Kamal and Klein, 2011); HPLC with Pulsed Amperometric detection (Goodal et al., 1995; Ouchemoukh et al., 2010), Gas Chromatography with Flame Ionization Detector (GC-FID) (Cotte et al., 2003; 2004a; Sanz et al., 2004; Kaškonienė et al., 2010b), GC with Mass Spectrometry (GC-MS) (De la Fuente et al.,2007; 2011), Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FTIR), Near-Infrared (NIR) and dispersive Raman Spectroscopy (Batsoulis et al.,2005), etc. Each method possesses advantages and disadvantages, but the gas chromatographic method has advantages when compared with the rest, since it is quite simple and does not require any preliminary isolation of sugars. It does however involve derivatisation reaction to turn sugars into more volatile compounds (Kaškonienė et al., 2010b).

Some carbohydrates have been used as floral markers for certain types of honeys; for example the carbohydrate alcohol perseitol (D-glycero-D-galacto-heptitol) was identified in avocado honey by De la Fuente et al., (2007). Researchers have determined carbohydrates predominantly in honey´s. Cotte et al., (2004a) found that maltose and turanose were predominant in acacia; maltulose and turanose in chestnut and linden; turanose and trehalose in fir; and sucrose and maltose in lavender origin honey. Nozal et al., (2005) found the presence of erlose and nigerose

in heather honey; forest honey showed higher amounts of threhalose and, elezitose, spike lavender honey was specific by the presence of isomaltose; while French lavender and thyme honeys were characterized by the presence of panose.

The EU (2001) and Codex Alimentarius Commission (2001) legislations establish some requirements with respect to the amount of reducing sugars: the sugars content (expressed as total fructose and glucose) should not be lower than 60% in nectar honeys and 45% in honeydew honeys; sucrose content should not be higher than 5% for nectar and honeydew honeys; but there are exceptions for honeys in which the sucrose content may reach as much as 10% for example: false acacia (*Robinia pseudiaccacia*), alfalfa (*Medicago sativa*), red gum (*Eucalyptus camandulensis*), leatherwood (*Eucrypis lucida*, *Eucryphia milliganii*), Citrus spp., and even 15% lavender (*Lavendula* spp.), borage (*Borago officinalis*) (Rybak-Chmielewska and Szczęsna, 2003).

1.5.3. Aroma compounds

Aroma is one of the distinguishable characteristics of honeys collected from different flowers. Therefore, many studies have been carried out in order to specify the volatile compounds that are most closely associated with a particularly type of honey and consequently would be helpful for a fast and reliable identification of its botanical source (Castro-Vásquez et al., 2007; Kaškonienė and Venskutonis, 2010a). In general, the aroma of honey is formed by volatile compounds that may come from the nectar or honeydew collected by bees; consequently, it may largely depend on the plant of honey origin and on the state of honey maturity. In addition, flavor constituents may be formed by the honeybee, as well as during thermal processing and/or storage of honey (Pontes, 2007; Soria et al., 2008). Although volatile compounds do not comprise a specific category of compounds that are related to their chemical structure, the overwhelming majority of studies on honey, volatiles deal with all constituents, which are

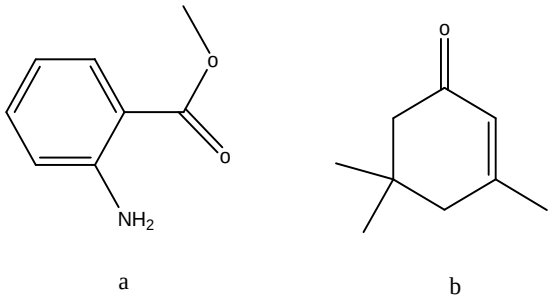
present in the honey headspace or may be distilled from the product (Cuevas-Glory et la., 2007; Bogdanov, 2009).

Although the identification of such compounds would be highly advantageous, there is not always an agreement on the compounds suggested as markers, since there may be differences, even within a single type of monofloral honey, due to the plant variety, the geographical origin or local beekeeping practices (Castro-Vázquez et al., 2009; Kropf et al., 2010; Escriche et al., 2011a).

Usually, unifloral honeys possess highly individual aroma profiles as compared with multifloral honey, due to the presence of characteristic volatile organic components deriving from the original nectar sources. Volatile honey compounds belong to seven major groups; aldehydes, ketones, acids, alcohols, esters, hydrocarbons, and cyclic compounds (Alissandrakis et al., 2003; Alissandrakis et al., 2005a; Pontes, 2007; Čajka et al., 2007). Although the presence of some aldehydes and alcohols will reflect on the product´s quality and may also be a consequence of microbiological activity, pyran and furan derivates arise from Maillard reactions or dehydration of sugar in an acid medium, heat exposure, and honey aging, some other compounds, such as linear aldehydes are considered as being characteristic compounds associated with a certain floral origin (Castro-Vázquez et al., 2009). Typical volatile components can be identified for honeys from a definite floral source; such compounds are specified as floral markers of the corresponding honey (Cuevas-Glory et al., 2007).

Several researchers have identified some single volatile compounds or groups of compounds as being characteristic of a particular floral origin and thus useful as “floral markers”, indicative of the honey floral type, *e.g.* hydroxyketones (acetoin, 5-hydroxy-2,7-dimethyl-4-octanone), sulfur compounds, *p*-cymene derivates are characteristic of eucalyptus honey (Castro-Vázquez et al., 2007; 2009; Alissandraki et al., 2011); acetophenone, 1-phenylethanol, 2-acetophenone and 3-aminoacetophenone are characteristic of chestnut honey (Jerković et al., 2011; Castro-Vázquez

et al., 2007, Alissandraki et al., 2011); 3,9-epoxy-1-*p*-menthadiene, *t*-8-*p*-menthen-1.2-diol and *cis*-rose oxide an characteristic of lime tree honey; hexanal, nerolidol oxide, coumarin and heptanal being the major flavour compounds in lavender honeys (Castro-Vázquez et al., 2007, 2009); and linalool derivates, limonyl alcohol, methyl anthranilate (figure 1.5a) having been reported as markers of citrus honeys (Ferreres et al., 1994; Bonvehí and Coll , 1995; Castro-Vázquez et al., 2009). Norisoprenoid, benzene, dehydrovomifoliol, phenylacetic acid, benzoic acid and 4-methoxybenzaldehyde have been found to be important markers for heather honeys (Castro-Vázquez et al., 2007; 2009), while isophorone (figure 1.5b) is characteristic of strawberry tree honey (De la Fuente et al., 2007); acetone, furfural and benzaldehyde characteristics of acacia honey and rosemary honey may be identified by the presence of acetone, 2-pentanone and 4-oxoisophorone (Radovic et al., 2001).



a b

Figure 1.5 Structure of volatile compounds: methyl anthranilate (a) and isophorone (b) found in citrus honey and strawberry tree honey, respectively.

The most widely-used method for determining the volatile compounds composition of honeys is based on GC-MS, although a prior fractionation step is required to separate the volatile compounds from the sugar matrix and water content (Castro-Vázquez et al., 2007, Cuevas-Glory et al., 2007). For the previous extraction of the volatile fraction different techniques may be used, with the most popular being the solid phase micro-extraction (SPME) to reviewed studies (Čajka et al., 2007; Ceballos et al., 2010; Kaškonienė and Venskutonis, 2010a), the simultaneous steam distillation-dichloromethane extraction, also called Likens-Nickerson

(Castro-Vázquez et al., 2009), the purge and trap technique (Radovic et al., 2001), or the ultrasonic solvent extractions (Alissandrakis et al., 2011, Jerković et al., 2011).

1.5.4. Minerals

The mineral content in honey ranges from 0.1% to 1.0%. In comparison with nectar honeys, honeydew honeys are higher in minerals, resulting in higher electrical conductivities. No discussion of honey is complete without including the mineral content. Here again, if we can judge from publications, about three-quarters of the work on the subject has been done in Europe. The principal component in the minerals is potassium, with an average of about one third of the total; followed by calcium, magnesium, sodium, sulfur and phosphorus. Trace elements include iron, copper, zinc and manganese (Terrab et al., 2004b; Lachman et al., 2007; Bogdanov, 2009). Table 1.4 shows the average mineral contents for two honeys classified according to colour (light and dark honey). Usually, light-colored honeys have lower mineral content, while dark-colored honeys generally have high mineral contents (Conti, 2000; Al et al., 2009; Gomes et al., 2010a).

Table 1.4 Mineral content of light honey and dark honey (from White, 1956; Ball, 2007).

Mineral	Light honey (mg/kg)	Dark honey (mg/kg)
Potassium	205	1676
Chlorine	52	113
Sulfur	58	100
Calcium	49	51
Sodium	18	76
Phosphorus	35	47
Magnesium	19	35
Silica	22	36
Iron	2.4	9.4
Manganese	0.3	4.09
Copper	0.3	0.56

Several investigations have shown that the trace element content of honey depends mainly on the botanical origin, geographical conditions, soil composition, environmental pollution and extraction techniques (Nanda et al., 2003; Conti, 2000).

1.5.5. Enzymes

The enzymes in honey are constituents of great interest and importance. Present only in the merest traces, they have profound effects on the nature and characteristics of honey. They may arise from the nectar, or be added by the bee from salivary and hypopharyngeal glands of foraging bees, in addition, they may be added by the bees, when the nectar is passed from bee to bee before being stored in the cells hive. Some enzyme activity might also be traced back to the pollen content, and some might come from microorganisms present in the nectar or honey (White, 1956; Persano Oddo et al., 1999; Babacan et al., 2002; Bogdanov et al., 2004). Enzymes in honey are an interesting factor, because they are a possible mean to distinguish between natural or artificial honeys; besides, is one parameter to determine the freshness of honey (Babacan and Rand, 2005). The predominant enzymes that have been reported in honey include invertase (α-glucosidase), diastase (α-amylase), and glucose oxidase. Other enzymes such as catalase, lactase and acid phosphatase, are generally present in lesser amounts and have not received the same analytical attention as the above mentioned enzymes (Persano Oddo et al., 1999; Babacan and Rand, 2005; Özcan et al., 2006).

Invertase (α-glucosidase, a glycoprotein) is the enzyme which is added to the nectar by the bee from its hypopharyngeal glands. It catalyzes the hydrolysis of sucrose to its monosaccharides (glucose and fructose), a vital step in the ripening of nectar to honey, and is more heat-sensitive than diastase which is also present in honey. The amount of excreted invertase depends on many aspects such as the age, physiological stage, nectar collection period, condition of a bee colony, pollen consumption, temperature and intensity or type of honey flow. Invertase has been considered responsible for most of the chemical changes that take place during the conversion

of nectar to honey (Persano Oddo, et al., 1999; Bonvehí et al., 2000; Vorlová and Přidal, 2002; Babacan and Rand, 2005).

Glucose oxidase is another enzyme added to honey by bees. This enzyme is considered mainly as responsible for the antimicrobial effect of honey (together with catalase), through the production of hydrogen peroxide and gluconic acid, by hydrolysis of the dextrose (glucose). The hydrogen peroxide has been identified as the inhibitor of bacterial growing. As for gluconic acid, in equilibrium with gluconolactone, it is the main acid and the reason behind the low pH values of about 4 for honey (Babacan and Rand, 2005; Bogdanov, 2009).

The α-amylase probably has the greatest attention, because of the importance related to the diastase activity of honey in Europe. It is a freshness indicator because the enzyme activity is decreased by heat or aging (Nagai et al., 2009). The origin of this enzyme has been attributed to the salivary secretions of bees or to its presence in pollen, or nectar; but the most widely accepted theory attributes its origin in honey to salivary secretions of bees (Persano Oddo et al., 1990; Babacan and Rand, 2005).

White et al., (1964) and White (1994) demonstrated that invertase was destroyed more quickly than amylase when honey was heated, so invertase activity could be a better indicator of honey quality than diastase activity. The activity of these enzymes varies greatly in honeys from different floral origins. It is known that some nectars is naturally much thicker than others and therefore require less manipulation by the bees in the hive to attain that thick consistency (Bonvehí, et al., 2000).

The enzyme sensitivity towards temperature is very high but the speed of enzyme destruction at temperatures below 15 ºC is very low. At 20 ºC the invertase decrease is 1.5 to 1.7% per month. At higher temperatures the loss of invertase activity in dependence on temperature may be even

higher. Seven to eight invertase isoenzymes are known (for example in acacia honeys) (Vorlová and Přidal, 2002).

Babacan et al., (2002) reported the effects of heat treatments and pH on honey amylase. The amylase activity was evaluated at 2 temperatures (63 ºC and 85 ºC). After treatment it was confirmed that the honey amylase was heat resistant (85 ºC for about 5 min) and the optimum pH for honey amylase was from pH 5.3 to 5.6.

In some European countries the invertase activity is a parameter that is not normally considered for these ends, in spite of being more sensitive to heating than diastase (Persano Oddo et al., 1999). Sometimes, invertase activities are given in sucrose hydrolysis units (sucrose number) or invertase number (IN). IN is expressed in grams of sucrose hydrolyzed per 100 g of honey in 1 h at 40 ºC, and has to higher or equal to 4 (White et al., 1964; Bogdanov, 2002; Sánchez et al., 2001; Orantes-Bermejo et al., 2009).

Studies (Krauze and Krauze, 1991) have shown diastase activity to be the best honey aging indicator, as this is doubly stable and considering that the activity of invertase depends on the management by beekeepers of honey treatment (Laude et al., 1991).

The International Honey Commission, (IHC; Bogdanov, 2002) suggests the diastase activity determination as a perspective criterion of the quality and freshness of honey. The limit established by IHC, Codex Alimentarius Commission (2001) and European Union (2001) was for it to be higher than or equal to 8, expressed as diastase number (DN). DN is Schade scale, which corresponds to the Gothe scale number, and is defined as g starch hydrolysed in 1 h at 40 ºC per 100 g of honey; and has to be at least 3 for honeys with a low natural enzyme content (Tosi et al, 2008). Study made by Persano Oddo et al., (1990) showed that some kinds of honey (*e.g. Robina, Citrus, Erica, Taraxacum* and *Arbutus*) had very low enzyme contents. Various explanations for the low enzymatic activities for these types of honey have been suggested, such

as the poor processing of nectar by the bees during abundant nectar flow or the seasonal activity of the pharyngeal glands (White, 1994; Babacan et al., 2002).

Consequently, the variability in amount of added enzymes depends on various factors such as the nectar collection period (and consequently the physiological stage of the colony); the abundance of nectar flow and its sugar content (a high flow of concentrated nectar leads to a lower enzyme content); the age of the bees (when the honey bee becomes a forager its glands produce more digestive enzymes), the diet, the strength of the colony, the temperature, etc. (Persano Oddo et al., 1999; Vorlavá and Přidal, 2002; Bogdanov et al., 2004).

1.5.6. Organic acids

A variety of organic compounds are known to be present in honey. The acid content of honey is relatively low but it is important for the honey flavor, texture and stability against microbial spoilage. Besides, the organic acids have the capacity to chelate metals and hence can synergistically enhance the action of other antioxidants, such as phenolic compounds (Ghendolf et al., 2002).

Formic acid and citric acid had for many years been considered to be principal acids in honey (White, 1956; Ball, 2007). However, it is now understood that gluconic acid (2,3,4,5,6-pentahydroxyhexanoic acid) is the predominant acid in honey. It is produced by glucose oxidase from glucose oxidation.

Researchers have reported the presence of malic acid, the volatile acids butyric, valeric, citric, acetic, formic, caproic and capric, and also lactic, malic, tartaric, oxalic and succinic acids (Anklan, 1998, Ghendolf et al., 2002).

Anklan (1998) described the organic acids´ concentration for Italian unifloral and multifloaral honeys. The mean concentrations of organic acids were: gluconic acid (2–12 g/kg), pyruvic acid (9–78 mg/kg), malic acid (69–145 mg/kg), citric acid (64–160 mg/kg), succinic acid (12–48 mg/kg) and fumaric acid (0.5–2.6 mg/kg).

1.5.7. Vitamins

Early determinations of the vitamin contents by feeding tests were generally rather negative. Later, examinations using chemical and microbiological methods gave small but definite values for the various vitamins in honey. There is no doubt that honey contains variable amounts of water-soluble vitamins (table 1.5): thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin (vitamin B_3), pantothenic acid (vitamin B_5), pyridoxine (vitamin B_6), ascorbic acid (vitamin C), folic acid (vitamin B_9) and biotin (vitamin H). In the other hand the fat-soluble vitamins: phylloquinone (vitamin K_1) and menaquinone (vitamin K_2) (Pontes, 2007; Ciulu et al., 2011). There is also no doubt that the amounts of these factors commonly found in honey are nutritionally insignificant to humans. In addition, honey processing, for example filtration, reduces its vitamin content due to the almost complete removal of pollen, besides the temperatures of processing. Another factor that causes vitamine loss is the oxidation of ascorbic acid by hydrogen peroxide produced by glucose oxidase and catalase (White, 1956; Ball, 2007; Ciulu et al., 2011).

Table 1.5 Vitamin content of honey (from White 1956; Ball, 2007).

Vitamin	Concentration (mg/kg)
Thiamin (B_1)	0.06
Riboflavin (B_2)	0.63
Niacin (B_3)	3.2
Pantothenic acid (B_5)	0.96
Pyridoxine (B_6)	3.2
Ascorbic acid (C)	22

1.5.8. Proteins and Amino acid

The honey´s proteins and amino acid contents are attributable both to animal and vegetal sources, the major of these being from pollen. Honey has a low percentage of protein (0.1–0.2%), but honeys with richer protein contents are from *Calluna vulgaris*. The amount of nitrogen in honey is low; on the average 0.04% (White, 1956, Anklam, 1998, Ball, 2007). The amino acid honey composition is described by several authors as a suitable method to determine the botanical differentiation of honeys (Pérez et al., 2007). Amino acids account for 1% (w/w), and proline is the major contributor with 50–85% of the total amino acids (Pérez et al., 2007; Bogdanov, 2009). Besides proline, there are other 26 amino acids such as glutamic acid, alanine, arginine, phenylalanine, cistine, glicine, tyrosine, leucine and isoleucine. Their relative proportions depend on the honey origin (nectar or honeydew). Since pollen is the main source of honey amino acids, the amino acid profile of a honey could be characteristic of its botanical origin (White, 1956; Hermosín et al., 2003). Proline is a unique amino acid that comes mainly from the honeybee during the conversion of nectar into honey. The amount of proline in honey has been suggested as an indicator of honey ripeness, together with other determinations also related to the honeybee, such as invertase and glucose oxidase activities. This component can be important for judging the honey quality; normally in honeys the proline content should be higher than 200 mg/kg, whilst values below 180 mg/kg mean that the honey is probably adulterated (Azeredo et al., 2003; Bogdanov, 2009).

Conte et al., (1998) studied 92 samples of honeys from 17 botanical sources; their amino acids were isolated and, after derivatization, analyzed by gas chromatography. All the samples showed a majority of proline as the main amino acid, and important amounts of phenylalanine, aspartic acid and asparagines, glutamic acid and glutamine. For the thyme honey, higher levels of serine, tyrosine and lysine were found; as for rosemary honey, tyrosine was the main amino acid, with also high amounts of proline and phenylalanine being observed (Hermosín et al., 2003).

Amino acids are known to react slowly (or more rapidly by heating) with sugar to produce yellow or brown compounds (melanoidins, the final products of the Maillard reaction). Part of the darkening of honey with age or heating may be due to this (White, 1956).

Some authors suggested that amino acids can be used for the characterization and differentiation of honeys sources. Cotte et al., (2004b) suggested that high amounts of threonine and phenylalanine are characteristic of sunflower and lavender honeys, respectively, while Hermosín et al., (2003) found that lavender honey contained a high concentration of tyrosine. The differences in the content and the composition of amino acids in honey can be explained by this dependency on its geographic origin (Kaškonienė and Venskutonis, 2010a).

Iglesias et al., (2004) used amino acids for the discrimination of honeydew and nectar honeys. This study showed that the content in glutamic acid and tryptophan enables the differentiation between nectar and honeydew honeys. Pérez et al., (2007) found that concentration of proline and phenylalanine were higher in nectar honeys than in honeydew honeys, in addition honey samples with high amino acid contents showed a higher antioxidant capacities.

The variation of this parameter in different unifloral honeys is quite high and it is not possible to classify honey only on the basis of their proline content. However, this parameter has been suggested as a complementary method to the melissopalynological analysis (Bogdanov et al., 2004).

1.6. Physicochemical properties

1.6.1. Colour

The colour of honeys, varies widely from water white, through amber tones, to almost black, with possible typical hues in some honey types, such as bright yellow, greenish or reddish (White, 1984). This is a physical property perceived most immediately by the consumer, and is a major factor that determines its price in the World market (Gonzales et al., 1999). The price of honey depends to a great extent on honey colour, light honeys like acacia and citrus generally achieving the highest prices (White, 1984; Gonzales et al., 1999). Bogdanov et al., (2004) reported that in Germany, Austria and Switzerland, dark honeydew honeys are especially appreciated and Murphy et al., (2000) concluded that the Irish consumer appreciated dark honeys.

Bogdanov et al., (2004) and Terrab et al., (2004c) reported that the colour of honey is one of the parameters of higher variability and is mainly determined by the botanical origin, but also depends on other factors for example ash content, conditions of processing (temperature) and conditions of storage (temperature and time).

Honey is classified by the U.S. Department of Agriculture into seven colour categories (table 1.6) according to the Pfund scale (White, 1984).

Table 1.6 Colour classifications of honey, according to the Pfund scale (from White 1984).

Categories colour	Pfund scale (mm)
Water white	< 8
Extra white	9 – 17
White	18 – 34
Extra light amber	35 – 50
Light amber	51 – 85
Amber	86 – 114
Dark amber	> 114

It has been previously reported by Al et al. (2009) and Gomes et al. (2010a) that light-colored honeys usually have low ash contents, while dark-colored honeys generally have higher ash contents (Abu-Jdayil et al., 2002). In addition, light colored honey types usually contain about 0.2% colloidal matter, on the other hand dark colored types may contain nearly 1%; in addition, dark honeys have a higher enzyme content that light honeys (White, 1984; Abu-Jdayil et al., 2002).

The colour intensity in honey is related to pigments (carotenoids, flavonoids, etc.), and as a method of fact, the increase of the colour intensity seems to be related to an increase of the concentration of flavonoids (Alvarez-Suarez et al., 2010).

1.6.2. Refractive index

The moisture content and the total soluble solids (TSS) in honey, are determined by measuring its refractive index (RI) using a refractometer at 20 ºC (normally about value of 1.49) and the corresponding moisture content (%) is calculated using the relationship between the refractive index and the water content (AOAC, 1990). This relation found in International Honey Commission (Bogdanov, 2002). In general, the RI increases with the increase in the solid content (Idris et al., 2011).

The TSS in honey is typically about 83 Brix degrees (º Brix), that indicates the amount in grams of sucrose, dissolved in 100 grams of solution (Ball, 2007).

1.6.3. Electrical conductivity

The electrical conductivity (EC) of honey is related to the mineral content, organic acids, proteins and polyol contents. This parameter has been useful to identify the different floral origins of honey (nectar or honeydew honeys), and can indicate whether bees have been artificially fed with sugar (Sancho et al., 1991b; Sanz-Cervera and Sanz-Cervera, 1994; Bogdanov et al., 2004). Lower EC values have been suggested for nectar honeys and the highest for honeydew honeys (Bogdanov, 2002). However, exceptions should be applied to some nectar honeys, *e.g.*, strawberry tree (*Arbutus unedo*), bell heather (*Erica*), eucalyptus (*Eucalyptus*), lime (*Tilia sp*.), ling heather (*Calluna vulgaris*) or manuka (*Leptospermum scoparium*) (Bogdanov, 2009; Kaškonienė et al., 2010b; Codex Alimentarius Commission, 2001).

According to the legislation (EU, 2001; Codex Alimentarius Commission, 2001) the electrical conductivity values for the nectar honey should be less than 0.80 mS/cm (with few exceptions) and for honeydew should be higher than 0.80 mS/cm.

Several authors found a linear correlation between electrical conductivity and the ash content (Sancho et al., 1991a, 1991b, 1992a; Accorti et al., 1987) and established that the determination of the ash content can be indirectly performed by measuring the EC and this has been recently included in the international standards, replacing the determination of the ash content (Codex Alimentarius Commission, 2001). The relationship between these two factors has established that higher ash contents resulted in higher electrical conductivities in honey.

1.6.4. Hydroxymethylfurfural

Hydroxymethylfurfural (HMF) is a cyclic aldehyde that is produced by dehydration of sugars under acidic conditions (caramelisation, figure 1.6) during thermal treatment (Risner et al., 2006; Turhan, 2009; Khalil et al., 2010a; Capuano and Fogliano, 2011). HMF is usually absent in fresh and untreated foods, but its concentration is also reported to increase as a result of the heating process or due to long-term storage (White, 1979, Khalil et al., 2010a). For this reason, the presence of HMF is recognized as a parameter related to the freshness and quality of several products such as: bakery products, coffee, fruit juices, caramel products, dried fruit, vinegar, cereals, milk, cookies and honey (Wunderlin et al., 1998, Sanz et al., 2003; Risner et al., 2007; Perez-Locas and Yaylayan, 2008; Capuano and Fogliano, 2011).

Generally, HMF is not present or is in very low concentration in fresh honey, with its content increasing during conditioning and storage. Honey processing requires heating both to reduce viscosity to facilitate handling and pouring, and to prevent crystallization or fermentation (Tosi et al., 2002; Yao et al., 2003; Ajlouni and Sujirapinyokul, 2010).

Several factors influence the formation of HMF in honey: temperature and time heating, storage conditions, use of metallic containers and chemical properties of honey, which are related to the floral source from which the honey has been extracted. These indicate pH, total acidity and mineral content (Anam and Dart, 1995; Bath and Singh, 1999; Tosi et al., 2002; Fallico et al., 2004; Turhan, 2009; Khalil et al., 2010a).

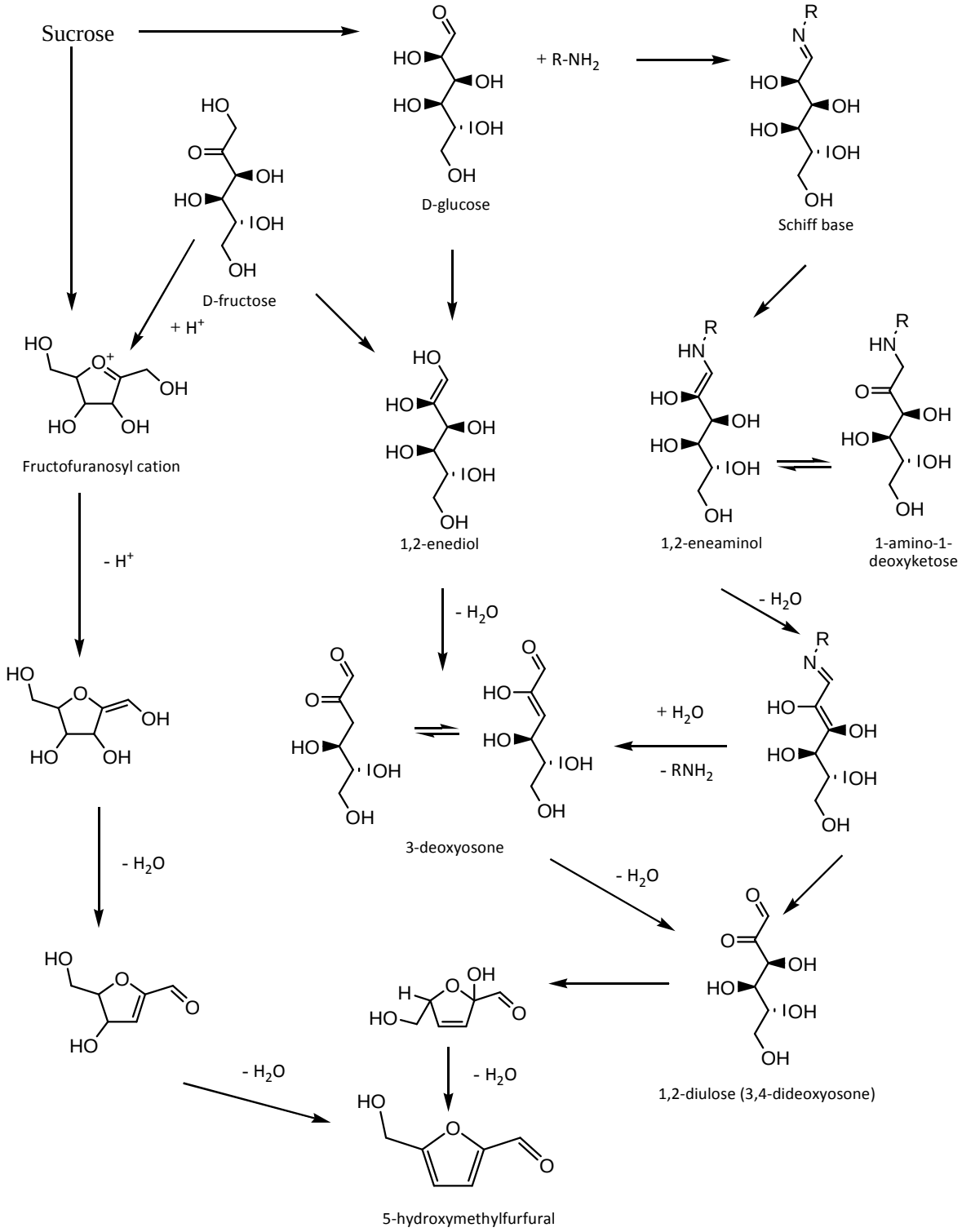


Figure 1.6 Scheme for the formation of hydroxymethylfurfural (from Capuano and Fogliano, 2011).

Fallico et al., (2004) determined the HMF level during the heating process at three temperatures (50, 70 and 100 ºC) in four different unifloral Sicilian honeys; in addition, the kinetics of HMF formation was also investigated. The results indicate a non-equivalence among different honey types and composition with regard to the heating treatment. Another study (Turham, 2009) analyzed of effects of storage (one year at 20 ± 5 ºC) on HMF formation and diastase activity in honey. The kinetics of the HMF formation after heating process was studied at six different temperatures (90, 105, 120, 135, 150 and 165 ºC). The results have shown that after storage the HMF level was significantly incremented but have not exceeded the limit imposed by legislation (< 40 mg/kg). The HMF formation increased with the temperature; therefore the conditions of processing (temperature/time) must be as low and short as possible. The same conclusions were also obtained by Tosi et al., (2002) for Argentine honeys under different thermal treatments.

Ajlouni and Sujirapinyokul (2010) concluded that HMF formation in honey is not only influenced by heating, but also honey composition, pH value and floral source can contribute.

Khalil et al., (2010a) analyzed the HMF content for Malaysian honeys, as well as the correlation of HMF formation with some physicochemical parameters (pH, free acids, lactone and total acidity). Results showed that honey samples stored at room temperature (25–30 ºC) from 12 to 24 months, exceeded the limits established by international regulations (Codex Alimentarius Commission, 2001; EU, 2001). It is recommended for honey to be consumed within six months to one year after extraction, regardless of the honey type. In addition, it was also found their physicochemical parameters (free acids and total acidity) were strongly correlated with HMF content (Bulut and Kilic, 2009).

Codex Alimentarius Commission (2001) establishes that the HMF content of honey after processing and/or blending must not be higher than 80 mg/kg. The European Union (2001) fixes a limit of 40 mg/kg for HMF in honey, with the following exceptions: 80 mg/kg for honey

coming from countries or regions with tropical temperatures, and 15 mg/kg for honey with low enzymatic level (3–8 Gothe units) (Zappalà et al., 2005). The International Honey Commission (Bogdanov, 2002) recommends three methods for the determination of HMF. These include two spectrophotometric methods which are widely used in routine analysis, but require a somewhat extensive sample preparation and the use of hazardous chemicals; the other method recommended is HPLC (Zappalà et al., 2005; Risner et al., 2006).

1.6.5. pH and Acidity

Honey is deceptively acidic, as the high sugar content tends to mask the acidity in the taste. The average pH of honey is 3.9 (with a typical range of 3.4 to 6.1). The pH of nectar honeys varies between from 3.3 to 4.6; an exception is the chestnut honey with a relatively high pH value of 5 to 6. Honeydew honeys, due to their higher mineral content, have a higher pH value, varying from 4.5 to 6.5. Honey is a buffer, which means that its pH does not change by the addition of small quantities of acids and bases. The buffer capacity is due to the content of phosphates, carbonates and other mineral salts (Bogdanov et al., 2004, Bogdanov, 2009).

Gluconic acid is the predominating acid in honey. However, it is present as its internal ester, a lactone, and does not contribute to honey´s active acidity. Gluconic acid has a pK_a of 3.7 (compared to the pK_a of acetic acid of 4.75) and is also a good chelating agent. Other acids that have been indentified in honey include acetic, butyric, citric, formic, lactic, maleic, pyruvic, malic, oxalic and succinic acids (White, 1956; Ball, 2007).

The acidity is due to the presence of these organic acids in equilibrium with their lactones and some inorganic ions such as phosphates, chlorides and sulfates, with their corresponding acids constituents of honey (Pérez-Arquillúe et al., 1994). There are three component of acidities: free acidity, lactone acidity and total acidity. The last is a result of the sum of the free acidity with

the lactone acidity. The ratio between lactone acidity/free acidity is an indicator of the botanical origin of honey (nectar or honeydew honey).

According to the legislations (Codex Alimentarius Commission, 2001; EU, 2001) a limit for the total acidity in honey of 50 meq/kg has been established.

1.6.6. Water activity (a_w)

The water activity value is a unit proportional to the free water content in food. Since in honey a part of the water presents is bound to sugars *via* hydrogen bonds and is unavailable for microorganisms, the a_w values of honey vary between 0.55 and 0.65; honeys with an a_w value < 0.60 are microbiologically stable. The higher water content of the honey the more likely is its fermentation and spoilage (Bhandari et al., 1999; Lazaridou et al., 2004; Zamora et al., 2006). Nowadays (a_w), it is the better quality criteria for honey than the water content, because it will indicate its free water content, which is microbiologically available to eventually cause fermentation. However, the simple and fast measurement of the water content has proven sufficient for assaying the fermentation risk of honey. The water activity needed for the development of microorganism is below 0.98 and depends on the class of microorganisms (around 0.70 for mould; 0.80 for yeast and 0.90 for bacteria). Osmophilic yeasts are specialists which need high sugar concentrations and are able to grow to in a minimal water activity up to 0.60. Such osmophilic yeasts cause honey fermentation (Gleiter et al., 2006; Chirife et al., 2006).

The water activity value is also related to quality problems (*e.g.* stability, viscosity and crystallization of honey) (Gleiter et al., 2006; Zamora et al., 2006; Abramović et al., 2008; Bogdanov, 2009).

1.6.7. Optical rotation

Honey is optically active. This property is due to the individual sugars present. The specific rotation of the carbohydrate fructose is -92.4º (levorotatory, negative value), the glucose +52.7º (dextrorotatory, positive value), the sucrose +66.5º, the maltose +130.4º, the melezitose +88.2º and the erlose +121.8º (García-Alvarez et al., 2002; Dinkov, 2003; Diminš et al., 2008; Bogdanov, 2009). The overall optical rotation depends on the concentration of the various sugars in honey. Measurements of specific rotation are currently used in Greece, Italy and UK to distinguish the botanical origin (nectar honey or honeydew) and to detect the adulteration of honey (Dinkov, 2003; Nanda et al., 2003). Normally nectar honeys show a levorotatory behaviour and honeydew honeys a dextrorotatory behaviour (Ryback-Chmielewska, 2007a; Kaškoniene et al., 2010b).

1.6.8. Viscosity

The rheological behaviour of honey has been investigated for in shelf-life, proper handling, packing and processing issues (Lazaridu et al., 2004; Ahmed et al., 2007). The honey viscosity depends on the water content, floral source, amount and size of crystals and, finally, the temperature (Zaitoun et al., 2001; Yanniotis et al., 2006; Wei et al., 2010). Honeys with higher water contents flow faster than those with lower ones (Goméz-Díaz et al., 2009).

The composition of honey generally has some effects on honey viscosity. Some studies, have reported honey as are Newtonian liquid (Junzheng and Changying, 1998; Bhandari et al., 1999; Abu-Jdayil et al., 2002; Lazaridou et al., 2004). However, there are a few honeys which show different characteristics regarding viscosity: heather (*Calluna vulgaris*), buckwheat (*Fagopyrum esculentum*), white clover (*Trifolium repens*) and manuka (*Leptospermum scoparium*) honeys are described as thyrotrophic which means they are gel–like (extremely viscous) when standing still and turn liquid when agitated or stirred; while dilatancy has been detected in Nigerian

honey and several eucalyptus honeys (*e.g. Eucalyptus fisifolia*) (Mossel et al., 2000; Yanniotis et al., 2006; Bogdanov, 2009).

The viscosity of honey decreases rapidly as its temperature rises. The Arrhenius model and the Williams Landel Ferry (WLF) model are typically used to describe the temperature–dependence, where the viscosity decreased with temperature increase due to reduced molecular friction and hydrodynamic forces (Bhandari et al., 1999; Mossel et al., 2000; Sopade et al., 2002). A 1% change in moisture content has been shown to have the same effect on viscosity as a 3.5 ºC change in temperature (Zaitoun et al., 2001; Recondo et al., 2006; Juszczak and Fortuna, 2006; Cohen and Weihs, 2010). Mossel et al., (2003) concluded that the carbohydrate concentration is the major factor contributing to unifloral Australian honey´s viscosity.

1.7. Bioactive compounds

Honey is considered as a natural product which is part of the traditional medicine since ancient times and also used as a natural food preservative, therapeutic properties and sweetening agent (Schramm et al., 2003; Bogdanov, 2009; Ferreira et al., 2009; Feás et al., 2010). The beneficial role of honey is partially attributed to its bioactive compound´s activities. Researchers have demonstrated the potential for honey to reduce enzymatic browning in fruits and vegetables (Chen et al., 2000; Rasmussen et al., 2008; De la Rosa et al., 2011), prevent lipid oxidation in meats (Antony et al., 2002; McKibben and Engeseth, 2002; Nagai et al., 2006), antibacterial activity (Molan, 1992; Bogdanov, 1997; Irish et al., 2011), anti-inflammatory effects (Bogdanov, 2009), treatment of skin wounds (Cooper et al., 2002; Blasa et al., 2006), reduction of the risk of cancer, cardiovascular diseases, and cataracts (Khalil et al., 2010b; Lachman et al., 2010). Bioactive compounds present in honey include enzymes and other different substances. The amount and type of these compounds depends largely upon the floral source/variety of the

honey, seasonal and environmental factors, as well as conditions of processing and storage (Gheldof et al., 2002; Khalil et al., 2010; Lachman et al., 2010; Silici et al., 2010).

1.7.1. Antioxidant properties

Antioxidants play an important role in human health by fighting damage caused by oxidizing agents and by food, where they specifically retard deterioration, rancidity or discoloration due to oxidation caused by light, heat and some metals (Johnston et al., 2005; Idris et al., 2011; Wang et al., 2011). The development of oxidative stress, which has been implicated in over 100 disorders, can be removed by enzymatic and non-enzymatic antioxidative mechanisms. Honey has been found to show a significant antioxidant activity, including enzymes (*e.g.* glucose oxidase and catalase) and non-enzymic substances (*e.g.* ascorbic acids, carotenoid derivates, α-tocopherol, organic acids, Maillard reaction products, amino acids, proteins, and polyphenols) (Nagai et al., 2001; 2006; Idris et al., 2011).

Polyphenols are products of the secondary metabolism of plants and are divided into several classes, *i.e.* phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavonols, flavones, flavanols, flavanones, isoflavones, proanthocyanidins) these being the more important classes; stilbenes, and lignans (El Gharras, 2009; Pyrzynska and Biesaga, 2009). Chemically, phenols can be defined as substances that possess and aromatic ring bound with one or more hydroxyl-substituent, including their functional derivates (Viuda-Martos et al., 2008; Idris et al., 2011).

Phenolic substances act as antioxidants by preventing the oxidation, as metal chelators, anti-mutagens and anti-carcinogens, antimicrobial agents, important oxygen reservoirs and substrates for browning reactions and clarifying agents. Specifically, the flavonoids behave as antioxidants in a variety of ways, including the direct trapping of reactive oxygen species, inhibition of enzymes responsible for producing superoxide anions, chelation of transition

metals involved in the process of forming radicals and prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals (Vela et al., 2007; Pyrzynska and Biesaga, 2009). Additionally, phenolics are responsible for colour, taste, astringency and bitterness (Viuda-Martos et al., 2008; El Gharras, 2009; Isla et al., 2010).

Some of the phenolic acids that can be present in honeys are: caffeic, coumaric, ferrulic, ellagic, chlorogenic; and flavonoids are (figure 1.7): chrysin, pinocembrim, pinobanksin, quercitin, kaempferol, lutein, galangin, apigenin, hesperetin, myricetin (Bertoncelj et al., 2007; Estevinho et al., 2008; Kenjerić et al., 2008).

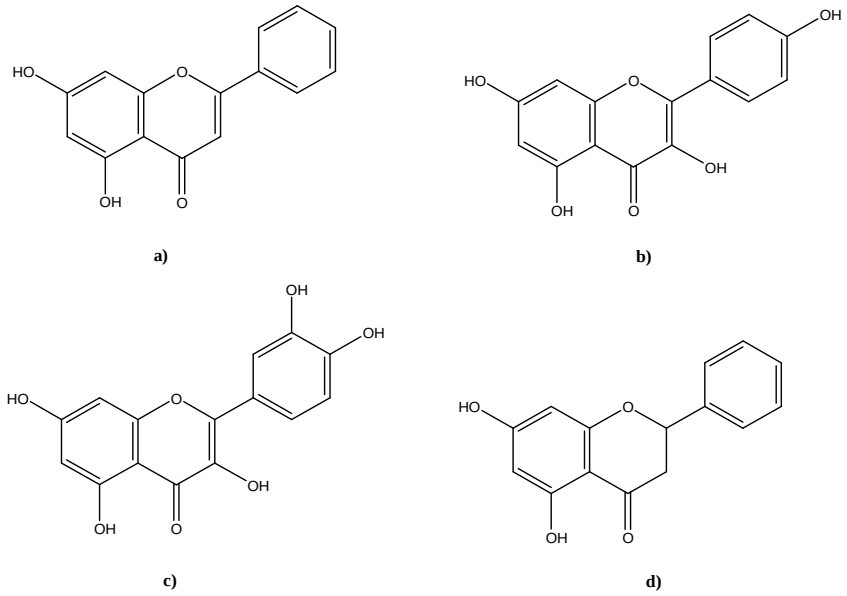


Figure 1.7 Chemical structures of some flavonoids: **a)** apigenin, **b)** kaempferol, **c)** quercitin, **d)**pinocembrin.

Several researchers reported that the antioxidant capacity of honey is due mainly to the phenolic compounds and flavonoids they contain (Aljadi and Kamaruddin, 2004; Bertoncelj et al., 2007; Lachman et al., 2010; Silici et al., 2010), and there is a high degree of correlation between these substances and the antioxidant capacity of honey, although a synergic action between several

compounds cannot be ruled out: ascorbic acid, α-tocopherol and Maillard reaction products (melanoidins) (Küçük et al., 2007; Brudzynski and Miotto, 2011b; Wang et al., 2011).

Researchers have demonstrated a positive and highly significant correlation between the colour of honey with darker honeys having higher total phenolic content and consequently higher antioxidant capacities (Beretta et al., 2005; Isla et al., 2011, Brudzynski and Miotto, 2011c). The colour of honey is the result of the combination of the phenol content, non-enzymatic browning or the product from the Maillard reaction (Viuda-Martos et al., 2008; Brudzynski and Miotto, 2011c).

Other compounds that have been reported to have antioxidant capacity are the melanoidinis. They are defined as carbohydrate-based, nitrogen-containing high molecular weight polymers formed in the final stage of the Maillard reaction that involves amino groups of free amino acids/proteins and the carbonyl groups of reducing sugars (Antony et al., 2002). These products are generated during the thermal processing of food and contribute to some of its physical properties, such as colour, aroma and texture, which make food more palatable to consumers (Brudzynski and Miotto, 2011b; 2011c; Wang et al., 2011). The mechanisms suggested for the antioxidant activity of melanoidins include the reduction of hydroperoxides to products unable to form free radicals, inactivation of free radicals formed during oxidative degradation of unsaturated fatty acids, scavenging of active oxygen and chelating of heavy metals by the reaction products (Antony et al., 2002; Wang et al., 2011).

Antony et al., (2002) and Turkmen et al., (2006) showed that the prolonged heating of honey led to the formation of melanoidin compounds and a concomitant increase in the antioxidant capacity.

1.7.2. Antibacterial properties

The antimicrobial activity (bacteriostatic and bactericidal) of honey on microorganisms and fungi is attributed to acidity, high osmolarity and chemical composition (such as hydrogen peroxide, organic acids, volatiles, phenolic compounds, beewax, nectar, pollen and propolis) (White, 1956; Wahdan, 1998; Weston, 2000; Lee et al., 2008; Viuda-Martos et al., 2008; Irish et al., 2011).

Variations in the type and level of antimicrobial activity in honey are associated with their floral origin, geographical location of the floral source, colony age and health (White, 1956; Bogdanov, 1997). The antibacterial activity of nectar honey is more influenced by heat, light and storage than the honeydew honey (Bogdanov, 2009).

The main component to which of the antimicrobial activity has been attributed is hydrogen peroxide (Estevinho et al., 2008; Lee et al., 2011). This component is determined by the relative levels of the enzymes glucose oxidase and catalase. The glucose oxidase enzyme is activated by unripe and a dilution of honey generates hydrogen peroxide, and is inactivated by heating and by exposure to light (Molan, 1992; Bogdanov, 1997). The higher the glucose oxidase level, the higher the peroxide content, whilst the higher the catalase content, the lower the peroxide level, since this enzyme destroys hydrogen peroxide (Weston, 2000; Irish et al., 2008). This phenomenon was checked by Torres et al., (2004) in Colombian honeys, finding that after being treated with catalase sample have lost their antimicrobial activity against bacteria and fungi.

Non-peroxide factors may also contribute to the antimicrobial activity of honey. Components such as lyzozyme, phenolic acids, and flavonoids are also present in honey (Escuredo et al., 2012). Flavonoids are derived from propolis, a resinous material collected by bees from gum exudates of trees, and used as an antibacterial agent in hives (Taormina et al., 2001). Phenolic acids are known to inhibit growth of a wide range of gram-negative (Gram –) and gram-positive

(Gram +) bacteria; for example caffeic acid against *Staphylococcus aureus*, *Proteus vulgaris*, *Mycobacterium tuberculosis* (Wahdan et al., 1998). The phenolic compounds have an action mechanism on microorganisms that involves degrading the cytoplasm membrane of the bacteria, which leads to the loss of potassium ions with the damage caused provoking cell autolysis (Viuda-Martos, 2008; Escuredo et al., 2012). In general, the Gram + bacteria are more sensitive to the honey antimicrobial action than the Gram – bacteria (Cooper et al., 2002; Mundo et al., 2004; Estevinho 2008; Alvarez-Suarez et al., 2010; Isla et al., 2011).

Physicochemical properties of the honey also have a responsibility on the antimicrobial activity, with the acidity: the pH of honey being low enough to slow down or prevent the growth of many species of bacteria, and osmoloraty, with the high sugar contents making the water unavailable for microorganisms (Molan, 1992; Wahdan, 1998).

The antibacterial activities of honey were reviewed by several researchers (table 1.7). Taormina et al., (2001) investigated honey from six floral sources for their inhibitory activity against *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Shigella sonnei*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*. The results revealed that the development of zones of inhibition of growth depend on the type and concentration of honey, as well as on the test pathogen. Alvarez-Suarez et al., (2010) analyzed the activity of five monofloral Cuban honeys against four bacterial strains: two Gram + (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram – (*Pseudomonas aeruginosa* and *Escherichia coli*). The results showed that dark honeys presented a higher antimicrobial activity than clear honeys; this can be attributed to the differences in the phenolic compound´s profile. Similar results were obtained by Isla et al., (2011).

Table 1.7 Factors that might contribute to the antibacterial activity of honey (from Snowdon and Cliver, 1996).

Factors
High osmotic pressure, low water activity (a_w)
Low pH-acidic environment
Glucose oxidase system – forms hydrogen peroxide
Low protein content
High carbon to nitrogen ratio
Low redox potential, due to high content of reducing sugars
Viscosity opposes convection currents and limits dissolved oxygen
Chemical agents
Pinocembrim, galangin, caffeic acid phenethyl ester, quercitin, ferulic acid (phenolic compounds)
Terpenes
Benzyl alcohol
Volatile substances (perhaps phytochemicals influenced by bee enzymes)

1.8. Changes in honey during storage

Honey is considered to be a relatively stable foodstuff, with only minor changes in flavor, colour visual aspect (crystallization) and composition taking place during several years of storage (White et al., 1961). The rate with which these changes occur depends on several different factors (*e.g.* temperature, light, oxygen, and composition of honey) (White et al., 1964).

There are two parameters that can be used to establish the freshness of honey or can be used to judge processing and storage conditions; those are the diastase activity and the hydroxymethylfurfural content (Ajlouni et al, 2010; Sancho et al, 1992b). The formation of hydroxymethylfurfural with time results from the sugar degradation of hexoses or nonenzymatic browning (Maillard reaction), under some factors; it is absent in fresh honey but its concentration tends to increase as the honey ages or during processing. Several conditions have

an influence in the increment of HMF concentration during the honey shelf-life, these include the following: the use of metallic containers and some of its physicochemical properties, which are related to the floral source from which the honey has been extracted (at pH 5 or lower, total acidity, mineral content, UV light), presence of organic acids and low water activity (Anklam et al, 1998; Wunderlin et al, 1998; Fallico et al., 2004; Spano et al, 2006; Turhan et al., 2008; Turhan, 2009; Ajlouni et al, 2010).

Anam and Dart (1995), reported that different metal ions had different effects in the production of HMF; for example, manganese had the highest effect, as opposed zinc, magnesium and iron. Also, this effect is greater at higher temperatures. Therefore, the use of metallic containers made from these metals can accelerate the HMF production during storage, which might lead to an excess of the recommended maximum limit. In the acid–catalysed formation of HMF from simple sugars in honey, a proton assists in the reaction by drawing electrons to itself, thus weakening the bond to be broken. The metal ions in the honey can therefore coordinate to several donor atoms whereas a proton usually coordinates to only one. Both acid and metal ion catalysis may take place in honey solutions to which metal ions have been added.

During long shipping or during long storage, darkening of honey may occur, and parallel changes in its organoleptic properties have detrimental effects on its quality, masking its original aroma, which promotes the loss of competitiveness in the World market. The rate of darkening has been related to the composition of honey and to the storage temperature. Of the compositional factors, the ratio of glucose to fructose, the nitrogen content, free amino acids, and moisture content have been cited as possible factors determining the rate of darkening (Gonzales et al., 1999). The darkening that occurs in honey could be due to: (a) an increment of melanoidins (Maillard reaction), (b) a combination of tannates and other oxydized polyphenols with ferrum salts; (c) the instability of fructose (caramelization reaction) (Lynn et al., 1936).

The activity of diastase is closely related to its structure and can be modified by denaturation, brought about by heating. Denaturation may be considered as a discontinuous phenomenon with various intermediate or transition states between the natural or native state and the completely denatured state (Cheftel et al., 1989; Tosi et al., 2008). White et al., (1963) evaluated the diastase and invertase activities in honeys subjected to different temperatures. The results led them to conclude that invertase is more heat-sensitive than diastase and that the storage time has the same effect as the heating on both enzymes activity. Also, the best conditions for storing raw honey would seem to be at below 10 ºC.

Sancho et al., (2001) analyzed the invertase activities of Spanish honeys over two years. Samples were stored in darkness at room temperature for up 24 months. Results showed that invertase activity had decreased with time, according to exponential (56% of samples), linear (25%), logarithmic (11%), inverse (5%) and quadratic models (3%).

One other change that can ocurr is spontaneous fermentation induced by osmophilic yeasts. This process depends on the initial load of microorganisms, the conditions of storage (temperature/time), and moisture content of the honey (Jímenez et al., 1994; Diminš, et al, 2006). Fermentation processes are indeed virtually annulled when the moisture content is < 17.1%, while for higher values they are dependent on the number of osmotic yeasts (> 1000/g of honey for 17.1% < moisture < 18% and > 10/g honey for 18.1% moisture < 19% (Conti, 2000).

Changes in carbohydrate composition were reported by White et al., (1961; 1964) for various unifloral honeys stored under different conditions (2 years at 23 ºC to 29 ºC) The results showed that 9% of the monosaccharides were converted to oligosaccharides; it was also observed that glucose declined faster than fructose. Rybak-Chmielewska (2007a) studied changes in carbohydrate after a half-year storage at 4 ºC and 20 ºC. The results indicated that honey stored under refrigeration (4 ºC) was more stable when compared to honeys at room temperature (the sucrose content dropped by as much as 79% compared to its initial value).

Castro-Vázquez et al., (2008) evaluated the changes produced in physicochemical parameters, volatile composition, sensory properties, and carbohydrates in a citrus honey stored for 12 months at 10, 20 and 40 ºC. The results showed that the storage conditions (40 ºC for one year) produced the higher changes in volatile compounds, with the disappearance of terpenes and derivates present in fresh citrus honey; changes in the disaccharides content (specifically a maltose increment) and sensory properties (the appeareance of a "toasted caramel" aroma appear and increment of acidity) detected by tasters.

Qamer et al., (2009) evaluated the effect of shelf-life on physicochemical parameters of sidder/ber honeys from Pakistan. The results obtained showed that after one year at room temperature the pH, total acidity, diastase activity, HMF, proline content, electrical conductivity, invertase activity values fell within the limits prescribed for good quality, but there were significant changes.

Moreira et al., (2010) performed a study of the effect of storage under tropical conditions (temperatures ranging 35 to 40 ºC for 3 and 6 months) on in the volatile compounds of Brazilian honeys. During storage time a number of changes were evidenced, namely reduction of the carbohydrate concentration, an increment of alcohol concentration (*e.g.* octadecanol and benzenemethanol) due to the degradation of lipid oxidative products or by aldehyde reduction processes catalyzed by enzymes; an increment and formation of furan derivates from Maillard reaction was also reported.

As previously commented, honey has several bioactive compounds with antioxidant and antibacterial properties. These compounds also exhibit changes with storage time. For example the study by Brudzynski and Kim (2011a) where the antibacterial activity was monitored (3–6 months) in Canadian honeys of different floral sources, which were screened for *Escherichia coli* and *Bacillus subtilis* after storage at 24 ºC in the dark for 1–3 years. After 2 years the results showed a drastic reduction in the antibacterial capacities against *Escherichia coli* and *Bacillus*

subtilis resistance to honey action. This reduction related to the diminished/concentrations of active phytochemicals due their sensitivities to storage conditions.

The antioxidant property changes have been analyzed after of storage. Wang et al., (2004) evaluated the antioxidant capacities of clover and buckwheat honey samples after being storage for 6 months at 4 ºC and at -20 ºC. The results obtained showed that the antioxidant capacity of honeys was reduced after that storage period, with no impact of storage temperature or container type detected at the end point of the storage period. However, this does not include the impact that might have occurred during early time periods of storage.

One of the most important changes that may occur during storage is crystallization. Crystallization is a complex phenomenon, being a matter of interest of beekeepers, honey handlers, and processors (Kabbani et al., 2011). When crystallization occurs during storage in a undesirable and uncontrolled fashion, it causes the product to be cloudy and, therefore, less appealing to consumers, but it is possible to obtain a desirable product through controlled crystallization, as “creamed honey”, in which there are a large number of crystals of very small size, so that they will not be perceived by the palate (Yao et al., 2003; Conforti et al., 2006). This product is obtained by seeding a liquid honey with 10% of crystallized honey at low temperature, so that crystals act as nuclei for growth, imparting a smoothess and resulting very pleasant to taste (Cavia et al., 2007).

Fructose determines the hygroscopic features of honey, whilst glucose influences the speed of honey crystallization. In honey that suffered a partial crystallization, the top liquid layer basically contains fructose and the other layer is glucose, which crystallizes by forming glucose monohydrate, with each glucose molecule fixing only one molecule of water; fructose is more soluble and stays in solution for a longer period of time (Yao et al., 2003; Gleiter et al., 2006). There are many factors that affect crystallization, such as composition, physicochemical parameters (moisture, water activity) and the range temperature (between 13–23 ºC this process

occurs faster) (Yoa et al., 2003; Conforti et al., 2006; Diminš et al., 2008). Impurities (dust, dirt), air bubbles, pollen grains and beewax particles have been reported to influence the nucleation of honey (Bhandari et al., 1999).

The rate of nucleation and crystal growth depends on temperature, with lower temperatures producing smaller crystal sizes, due to the limited mobility of the molecules (Bhandari et al., 1999; Conforti et al., 2006).

A study made by Bonvehi (1989) attempted to predict the crystallization of honey with respect to the glucose composition and correlated the coefficient of supersaturation (CS) of glucose in honey with crystallization. He defined the CS as the ratio of the concentration of solute (w/w) in water at a given temperature to the concentration of solute in saturated solution at the same temperature, with the value varing from 1.8 to 2.6 in honey. According to this author, honey with a CS < 1.8 will remain liquid for a long time, whereas honey with a value > 2.6 will crystallize very quickly.

Tabouret (1979) suggested an alternative method for the prediction of crystallization, based on the ratio between physicochemical parameters (equation 1.1).

$$Tabouret\ index = \left[\frac{(G/M)}{(1-a_w)^n}\right] \qquad (1.1)$$

Where, G is the glucose content (%, wet basis), M is the moisture content (%, wet basis), a_w is the water activity, and the power n depends upon the moisture of the honey (n = 1 for moisture > 17%, and n = 2 for moisture < 17%).

In table 1.8, some ratios and factors that may induce or predict of the potential for the crystallization phenomena in honeys are summarized.

Table 1.8 Various crystallization indicators used for honeys (from Bhandari et al., 1999).

Indicators	No crystallization	Fast Crystallization
Glucose/Water content ratio	< 1.70	> 2.16
Fructose/Glucose ratio	> 1.33	< 1.11
(Glucose/Water)/Fructose ratio	< 0.30	> 0.49
% Glucose	< 27.7	>35
CS	< 1.8	> 2.6
Tabouret index	< 9.8	>12.6

Cavia et al., (2007) studied the evolution of acidity, throughout 30 months, in honeys stored at room temperature and analyzed the influence of induced granulation on acidity evolution in Spanish honeys. None of the stored honeys showed free acidity values higher than the legal limits (50 meq/kg) and the acidity was constant for the initial 15 months. But storage and induced granulation had an effect on honey lactones; this parameter decreased after 20 months of storage, and less markedly in samples subjected to induced granulation.

1.9. Quality parameters

Honey as a food product must meet certain parameters of quality and freshness, which have been established by various regulatory agencies to ensure the final quality of this valuable and desired product. Among the International and National regulatory agencies, the Codex Alimentarius Commission (2001), the European Union (2001) and the Portuguese Norm-1307 (1983) can mentioned. Table 1.9 summarizes the physicochemical parameters with their respective limits, as established by the above mentioned agencies.

Table 1.9 Limits to physicochemical parameters established by different entities.

Physicochemical Parameters	**Limits Values**	**Considerations**	**Legislations**
Ash content	Max. 0.6 (%)	Nectar honey	[1, 2]
	Max. 1 (%)	Honeydew honey	
Diastase activity	Min. 8 (ºGothe)	In general	[1, 2, 3]
	Min. 3 (ºGothe)	Honey with lower enzyme content (*e.g.* citrus honeys) and an HMF content of not more than 15 (mg /kg).	
Electrical conductivity	Max. 0.8 (mS/cm)	Nectar honey	[2, 3]
	Min. 0.8 (mS/cm)	Honeydew honey	
		Exceptions: *Arbutus unedo, Erica, Eucaliptus, Tila* spp., *Calluna vulgaris, Leptospermum, Melaleuca* spp.	
HMF content	Max. 40 (mg/kg)	In general, after treatment and mixes	[1, 2, 3]
	Max. 15 (mg/kg)	Honey with lower enzyme content	
	Max. 80 (mg/kg)	Honeys from tropical countries and blends of these honeys	
Moisture content (MC)	Max. 21 (%)	In general	[1]
	Max. 20 (%)		[2, 3]
	Max 23 (%)	Exceptions: *Calluna*	
Reducing sugar	Min. 65 (%)	Nectar honey	[1]
(expressed fructose + glucose)	Min. 60 (%)	Nectar honey	[1, 2, 3]
	Min. 45 (%)	Honeydew honey, bends of honeydew with nectar honey	

Table 1.9 (Continued).

Physicochemical Parameters	**Limits Values**	**Considerations**	**Legislations**
Sucrose content	Max. 5 (%)	In general	[1, 2, 3]
	Max. 10 (%)	Exceptions: *Medicago sativa*, *Citrus* spp., *Robina pseudoacacia*, *Hedysarum*, *Banksia menziesii*, *Eucalyptus camaldulensis*, *Eucryphia lucida*, *Eucryphia milliganii*.	
	Max. 15 (%)	Exceptions: *Lavandula* spp., *Borago officinallis*.	
Free acid	Max. 50 (meq/kg)	In general	[1, 2, 3]
	Max. 80 (meq/kg)	Baker´s honey	[3]
Water insoluble solid	Max. 0.1 (g/100g)	In general	[1, 2, 3]
	Max. 0.5 (g/100g)	Pressed honey	

[1] Portuguese Norm-1307 (1983).

[2] Codex Alimentarius Commission (2001).

[3] European Union (2001).

CHAPTER 2

OBJECTIVES

"Spider caves" art ruprecht

Chapter Two: Objectives

2.1. General Objective

In recent years, the worldwide consumption of all kinds of honey has increased due the potentially beneficial compounds that it contains according to the floral origin. Improvements in taste and consumer habits are also important. Honey production is carried out throughout the country, but the highest concentration of hives is in the South area. Therefore, the main objective of this study is to increase the knowledge of the quality of honey from the Algarve area. In the present study several aspects were studied, they correspond to different steps of the work.

2.1.1. First stage:

Evaluation of effect of storage in some relevant quality parameters (physicochemical) and bioactive compounds present of honeys from the Algarve were studied after three year storage. All data presented for year 2006 has been extracting from previous study carried out by Figueira et al.

2.1.2. Second stage:

Characterization of physicochemical parameters and bioactive compounds of strawberry tree (*Arbutus unedo* L.) honey.

2.1.3. Third stage:

Comparison between physicochemical parameters and bioactive compounds of commercial honeys and artisanal strawberry tree honey.

2.1.4. Fourth stage:

Simple evaluation of the use of non-invasive methods as an alternative to the presently recommended method for the determination of the botanical origin of honey.

CHAPTER 3

EVALUATION OF THE CHANGES IN PHYSICOCHEMICAL QUALITY PARAMETERS AND BIOACTIVE COMPOUNDS DURING STORAGE OF HONEYS FROM THE ALGARVE AREA

"Spider caves" art ruprecht

Chapter Three: Evaluation of the changes in physicochemical quality parameters and bioactive compounds during storage of honeys from the Algarve area.

3.1. Introduction

Honey is a natural food produced by *Apis mellifera* bees from the nectar of blossoms (nectar honey) or from the secretions of living plants or excretions of plant sucking insects of the living part of plants (honeydew honey) that transform and combine with specific substances of their own, store and leave in the honey comb to ripen and mature; it is composed primarily by a mixture of sugars (85–95%) and water (16–18%) approximately, and minority compounds (proteins, phenolic compounds, free amino acids, organics acids, vitamins and minerals) (Silva et al., 2009; Kahnraman et al., 2010). The quality of the honey, available on the market differs on account of various factors like geographical, seasonal, floral source and storage conditions. The honey is considered relatively stable during storage, with only minor changes in some compounds and some physical and chemical properties; the speeds of these changes occur depend on conditions of storage and processing. Still, it is susceptible to physical and chemical changes during storage; for instance, it tends to darken and lose its aroma and flavor (White et al., 1963).

Freshly extracted honey is liquid, but during storage, sooner or later honey, will crystallize to a certain extent; the speed at which this phenomenon occurs depends on the relation of fructose/glucose (Cavia et al., 2007). Cristallized honeys are not popular with consumers and can only be marketed liquefied. Gentle heating is mostly used to liquefy crystallized honey (usually not more than 40–50 ºC), but this process can be detrimental to quality of honey (Castro-Vázquez, 2008).

Two common factors that determine the quality and freshness are hydroxymethylfurfural (HMF) and diastase activity (DA); the first is a cyclic aldehyde that is produced by degradation of fructose and the second is an indicator of the level of enzymatic activity (Silva et al, 2009; Tosi et al., 2008).

Several factors influence in the formation of HMF and decreases of diastase activity in honey: temperature and time of heating; storage conditions; use of metallic containers and the chemical properties of honey, which are related to the floral source from which the honey has been extracted, these include pH, total acidity, mineral content; however, no information on the correlation between chemical characteristics and either the HMF level of the honey or the diastase activity is available (Zappalà et al., 2005; Turhan, 2009; Gomes et al., 2010a). For both factors the limit accepted, according to the European Union (2001) and the Codex Alimentarius Commission (2001) are for HMF content < 40 mg/kg and for diastase activity ≥ 8 Gothe.

Several researchers (White, et al., 1964; Wang et al., 2004) have evaluated the effect of storage on the quality of honey from different sources through the determination of DA and HMF content. However, the impact of industrial processing and storage on the quality of honey is not well understood.

The overall objective of this first study, therefore, was to determine the effects of storage (3 years) on physicochemical parameters (ash content, electrical conductivity, pH, total acidity, colour, moisture content, proline content, total soluble solid, water activity, diastase activity and hydroxymethylfurfural content) and bioactive compounds (phenolic and flavonoid content) of honey.

3.2. Experimental

3.2.1. Honeys samples

Thirty one samples (table 3.1) harvested from different Algarve regions (south region of Portugal, the Sotavento area, figure 3.1), were collected directly from different beekeepers, between the months of July to December in 2006. The floral origin of honey samples was characterized directly by the beekeepers in relation to the location where the beehives were situated and the accessibility of plant food sources; basically the samples were classified into eight categories: *Arbutus*, *Erica*, *Cistus ladanifer*, *Rosmarinus*, *Palmae*, *Carduus*, *Ceratonia*, and *Citrus*.

Figure 3.1 Honey samples (n=31) obtained Algarve province of South Portugal from Sotavento (B-D) area.

All nectar honey samples were analyzed 2 weeks after recollection (fresh honeys, results 2006); later the samples were stored for three years in glass bottles in darkness at room temperature (20 ± 3°C) to minimize alterations until analyzed (storage honeys, results 2009).

Table 3.1 Honey samples analyzed.

Location	Nº Sample		Date of harvest	Kind of honey, main plant sources	Location fig. 3.1
Ameixal	9	Ameixial – Loulé	July, 21	Multifloral	B
(*n* = 3)	6	Benfarros-Loulé	September, 14	(*Arbutus*, *Ericaceae*)	
	31	Ximeno – Ameixial	December, 13		
Cachopo	12	Arrunhado- Cachopo	July, 25	Multifloral	B
(*n* = 9)	13	Azinhosa - Tavira	September, 1	(*Arbutus*, *Ericaceae*,	
	4	Barragem do Leitejo-Cachopo	September, 26	*Cistus*, *Rosemarius*)	
	3	Barranco do Caminho-Feiteira	October, 5		
	2	Curral dos Bois	October, 10		
	1	Feitoso-Cachopo	July, 22		
	26	Frunchosa de Cima	October, 12		
	11	Leitejo - Monte da Ribeira	July, 22		
	7	Urzal-Cachopo	July, 14		
Martin Longo	27	Alcarias Grandes	October, 12	Multifloral	B
(*n* = 7)	21	Malfrade	October, 11	(*Cistus*, *Rosemarius*,	
	8	Monte da Estrada-Martinlongo	July, 21	*Citrus*)	
	22	Odeleite	October, 11		
	29	Penteadeiras – Martinlongo	October, 19		
	20	Taipas	October, 11		
	10	Umbrias – Martinlongo	July, 21		
Sta. Catarina	16	Alcaria do Cume	September, 14	Multifloral	D
(*n* = 8)	32	Cova da Muda - S. Brás de Alportel	December, 14	(*Cistus*, *Rosemarius*)	
	33	Desbarato - S. Brás de Alportel	December, 14		
	28	Desbarato - Sta. Catarina	October, 19		
	18	Garrobo	October, 10		
	30	Malhada do Judei - Sta. Catarina	October, 19		
	19	Portela da Corcha	October, 10		
	17	São Brás	September, 20		
Tavira	25	Asseca	October, 12	Multifloral	D
(*n* = 4)	15	Luz de Tavira	September, 14	(*Palm*ae, *Carduus*,	
	24	Moncarapacho	October, 11	*Ceratonia*, *Citrus*)	
	23	Porto Carvalhoso	October, 11		

3.2.2. Physicochemical parameters

3.2.2.1. Ash content

The ash content was determined by placing 5–10 g of honey sample in a crucible in a muffle furnace for calcination (550 ºC x 6 h) until constant weight, with the precaution of including a previous caramelisation step on a heating plate to control the production of foams and sample losses. Measurements of ash were performed in triplicate and the mean value obtained used.

3.2.2.2. Electrical conductivity (EC)

The conductivity of the honey samples was determined in a 20% (w/w) solution made up with CO_2-free deionized and distilled water. It was measured at 20 ºC with a Crison conductimeter 525 (Cataluña, Spain), and the results were expressed as (mS/cm). The measurements were performed in triplicate for each sample and the mean was determined.

3.2.2.3. pH

The pH of the honey was measured in a solution of 5 g honey in 25 mL of CO_2-free distilled water, using a pH-meter Crison micropH-2001 (Cataluña, Spain). The instrument was calibrated with standard buffer solutions of pH 7 and pH 4, prior to measuring the pH of samples. Determinations were made in triplicate.

3.2.2.4. Total acidity

The total acidity was determined using the AOAC recommended method no. 962.12 (AOAC, 1990). Approximately 10 g of the honey samples were dissolved in 75 mL of CO_2-free distilled water. The solution was potentiometrically titrated to pH 8.6 by adding a 0.05 N NaOH solution.

Potentiometric determinations were performed with a pH-meter Crison micropH-2001 (Cataluña, Spain). The measurements were performed in triplicate for each sample and the mean results were expressed as (meq/kg).

3.2.2.5. Colour by colorimeter

Visual colour was measured using a DR LANGE spectro-colour d/8º LMG170 D65/10º version 2.0 colorimeter (Düsseldorf, Germany). The instrument was first calibrated with a black and white standard. The CIE-***Lab*** colour parameters used were $\boldsymbol{L}^*$ (lightness: $\boldsymbol{L}^*$ = 100 for white and 0 for black), $\boldsymbol{a}^*$ (redness/greenness axis: positive $\boldsymbol{a}^*$ is red and negative $\boldsymbol{a}^*$ is green), $\boldsymbol{b}^*$ (yellowness/blueness axis: positive $\boldsymbol{b}^*$ is yellow and negative $\boldsymbol{b}^*$ is blue) (Saxena, 2009; Bulut and Kilic, 2009).

3.2.2.6. Moisture content (MC)

The MC in honey was determined using an Abbe refractometer (Atago® 1T Abbe Refractometer, Tokyo, Japan). In general, the refractive index increases with the increase in the solid content. The refractive indices of honey samples were measured at room temperature (20 ± 3 ºC) and the readings were further corrected for temperature using a factor of 0.00023/ºC. MC was determined in triplicate and the percentage MC values corresponding to the corrected refractive index values were calculated using the Chataway table revised and updated (Codex Alimentarius Commission, 2001).

3.2.2.7. Total soluble solids (TSS)

Total soluble solids were determined by refractometry using an Abbe Atago® 1T (Tokyo, Japan) and results were expressed in ºBrix. All the measurements were performed at room temperature and the readings were corrected for a standard temperature of 20 ºC by adding the correction factor of 0.00023/ºC.

3.2.2.8. Proline content

The proline content was determined using the method described by Cha-um and Kirdmanee (2009). One gram of honey sample was dissolved in approximately 50 mL of distilled water. The solution was mixed with 1 mL aqueous sulfosalicylic acid (3% w/v) and filtered through filter paper (Whatman # 1). The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 mL of glacial acetic acid and 20 mL 6M H_3PO_4) and incubated at 95 ºC for 1 h. The reaction was terminated by placing the container in an ice bath. The reaction mixture was vigorously mixed with 2 mL toluene. After warming at 25 ºC, the chromophore was measured by Hitachi U-2000 UV/VIS (Tokyo, Japan) at 520 nm. The blank solution contained toluene. L-proline (0–40 μg/mL) was used as a standard to derive the calibration curve (linear).

3.2.2.9. Water activity (a_w)

The honey sample was placed inside the sample-holder, and used for determination of a_w using a water activity meter Rotronic-Hygrolab 3 (Rotronic AG, Bassersdorf, Switzerland), equipped with a temperature-controlled system which allows a temperature stable sampling environment; measurements were performed at a temperature of 25 ± 0.2 ºC. This measures the water activity of the sample based on its equilibrium relative humidity (ERH, %). The measurements were

performed in triplicate for each sample and the mean was determined. The relationship between a_w and ERH was calculated as follows as (equation 3.1, Saxena et al., 2009):

$$a_w = (ERH/100\%) \qquad (3.1)$$

3.2.2.10. Diastase activity after Schade

Diastase activity (DA) was measured with Schade, according to the International Honey Commission (Bodganov, 2002). The honey sample (5 g) was dissolved in 15 mL Milli-Q water and 5 mL acetate buffer solution (pH 5.3), transferred to a 50 mL volumetric flask containing 3 mL sodium chloride (0.029 g/mL) solution and diluted to volume. Using a volumetric pipette, 10 mL of honey solution were transferred into a 50 mL flask and placed in a 40 ºC water bath along with a second flask containing 10 mL of starch solution. After 15 min, 5 mL starch solution were added to the honey solution, mixed and timed. At periodic intervals (5, 10, 15, 20 min depending on the activity), for the first time after 5 min, 0.5 mL aliquots of the mixture were mixed with 5 mL diluted iodine solution and 20 mL Milli-Q water, vortexed and the absorption immediately measured at 660 nm using a UV/VIS spectrophotometer Hitachi U-200 (Tokyo, Japan) against a water blank. Using regression (without using data point at 0 min), lines were fitted to the absorption data and the diastase number was calculated from the time taken for the absorbance to reach a values of 0.235. The diastase number was calculated following the method of the International Honey Commission (Bogdanov, 2002); results were expressed (as Gothe degrees) as mL of 1% starch hydrolyzed by enzyme in 1 g of honey, in 1 h at 40 ºC.

3.2.2.11. Hydroxymethylfurfural (HMF) content

The HPLC method used was based on the method recommended by the International Honey Commission (Bogdanov 2002). Briefly, the honey samples (5 g) were diluted to 50 mL with Milli-Q water filtered using a 0.45 µm nylon membrane filter (Merck, Germany) and 20 µl injected into an HPLC system (Jasco, LG-1580-04 with PU-2080 plus Intelligent HPLC pump) equipped with a Photodiode Array Detector (JASCO, MD-2015 Plus Multiwavelength Detector). The HPLC column was a Merck Purospher Star RP-18e, 5µm (Merck, Germany). The HPLC included an isocratic mobile phase, 90% water and 10% methanol (Fisher Scientific, UK) at a flow rate of 0.7 mL/min. All solvents used were of HPLC grade. The detection wavelength was 285 nm. The HMF content of the sample was calculated by comparing the corresponding peak area of the sample and those of the standard solutions of HMF ($C_6H_6O_3$, Sigma-Aldrich, Germany) after correcting for the honey dilution. There was a linear relationship (R^2 = 0.9993) between the concentration and the area of the HMF peak (results were calculated as the mean of three values and expressed as mg/kg).

3.2.3. Bioactive compounds

3.2.3.1. Determination of total phenolic content (TPC)

The TPC was determined spectrophotometrically using the Folin-Ciocalteu Method (FCM) according to Meda et al., (2005) and Alvarez-Suarez et al., (2009). Approximately 5 g of honey were treated with 50 mL of distilled water, and the resulting solution filtered using a qualitative filter paper. The filtered solution (0.5 mL) was mixed for 5 min, with 2.5 mL Folin-Ciocalteu´s phenol reagent 0.2 N (Sigma-Aldrich, Germany) and then 2 mL of a 75 g/L Na_2CO_3 solution were added. Samples were incubated, in the dark, at room temperature for 2 h and their absorbance was read at 760 nm in a UV/VIS double beam spectrophotometer Hitachi U-2000 (Tokyo, Japan). The blank solution consisted of methanol, instead of honey. The total phenolic content was determined from a standard curve prepared using standard solutions, within the range of 0 to 150 µg/mL (R^2 = 0.9941). The mean of four readings was used, expressed as mg of gallic acid equivalents (GAE)/100g of honey.

3.2.3.2. Determination of total flavonoid content (TFC)

The TFC was determined according to Meda et al., (2005) and Alvarez-Suarez et al., (2009) with some modifications. Approximately 1 g of honey sample was dissolved in methanol to obtain a final concentration of 0.04 g/mL. 5 mL of each solution was then mixed with 5 mL of 2% $AlCl_3$. The mixture was left in the dark for 10 min, at room temperature. The absorbance was measured at a wavelength of 415 nm using a Hitachi U-2000 double beam UV/VIS spectrophotometer (Tokio, Japan). The blank sample consisted of 5 mL honey solution with 5 mL methanol without $AlCl_3$. The total flavonoid content was determined from a standard curve prepared using standard solutions, within the range of 0 to 50 µg/mL (R^2 = 0.9927). The mean of four readings was used, expressed as mg of quercitin equivalents (QE)/100 g of honey.

3.2.3.3. Determination of scavenging radical

The scavenging activity of honey samples for 2,2-diphenyl-2-picryl-hydrazyl (DPPH) was measured as described by Baltrušaitytė et al., (2007), Alvarez-Suarez et al., (2009) and Saxena et al (2009), with some modifications. Honey samples were dissolved in methanol with final concentration of 0.1 mg/mL. Briefly, 0.75 mL of each sample solution were mixed with 1.5 mL of DPPH in methanol (0.039 mg/mL), all mixtures were left for 25 min at room temperature in the dark; after this the absorbance was read at 517 nm (Abs_H). The blank sample consisted of 0.75 mL of methanol to which 1.5 mL of DPPH were added (Abs_B). Ascorbic acid was used as a positive control (0–70 µg/L). The radical scavenging activity was calculated as follows (equation 3.2):

$$\%\, Inhibition = \left[\frac{(Abs_B - Abs_H)}{Abs_B}\right] \cdot 100 \qquad (3.2)$$

3.2.3.4. Antioxidant capacity

The antioxidant capacity was evaluated as described by Meda et al., (2005). Approximately 1 g of honey sample was dissolved in methanol to obtain a final concentration of 0.04 mg/mL. Then 0.75 mL of each solution was then mixed with 1.5 of a 0.05 mg/mL solution of DPPH (Sigma-Aldrich, Germany) in methanol. The mixture was left in the dark for 15 min at room temperature. The absorbance was measured at a wavelength of 517 nm using a Hitachi U-2000 double beam UV/VIS spectrophotometer (Tokyo, Japan). The blank sample consisted of 0.75 mL of a honey solution with 1.5 mL of methanol. The antioxidant content was determined from a standard curve prepared using standard solutions, within the range of 0 to 12 µg/mL, (R^2 = 0.9948) for ascorbic acid and for quercitin of 0 to 8 µg/mL (R^2 = 0.9899). The mean of four values was calculated and expressed as mg of ascorbic acid equivalent antioxidant content (AEAC)/100 g of honey and mg of quercitin equivalent antioxidant content (QEAC)/100 g of honey.

3.2.4. Statistical analysis

The results were expressed as mean values ($\bar{x}$) and standard deviation (*SD*). The results obtained for all the parameters of the honey samples, both in 2006 and 2009, were analyzed using analysis of variance; in addition the and Student´s t-test was used to examine differences with fresh honey and honey storage for 3 years. The data analyses were carried out using Statgraphics® Centurion XV (StatPoint, Virginia, USA). Differences were considered significant at $p < 0.05$.

3.3. Results and discussion

3.3.1. Physicochemical parameters

The results for physicochemical parameters are shown in the following figures (3.2 to 3.13).

- ***Ash results***

The ash contents for both areas (figure 3.2) were relatively lower in comparison with nectar honeys for fresh honey and storage honey. Area B presents a mean value of 0.06% in 2006 and after storage there was a slight increment to 0.07%. In the case of area D, in 2006 it showed a mean value of 0.07%, which was incremented to 0.12% during storage. Normally, the nectar honeys have values of ash content below 0.60% (EU, 2001; Codex Alimentarius Commission, 2001), which were lower than the values found for honeydew. The mineral content of honey depends on various factors, namely the botanical origin, the geographical conditions, soil composition, environmental pollution and extraction of techniques (Conti, 2000; Nanda et al, 2003).

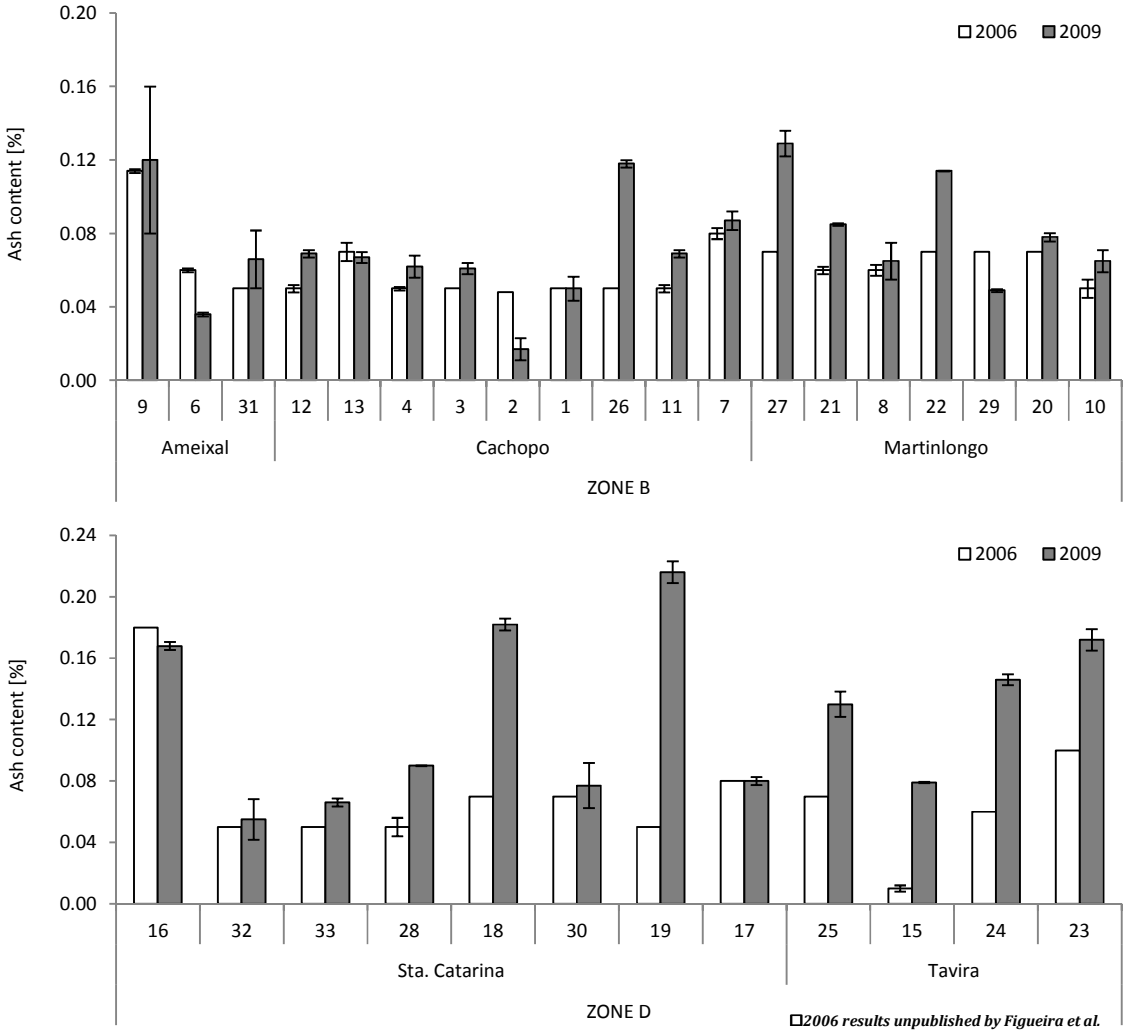


Figure 3.2 Results for ash content (%) after three years of storage for honey samples from areas B and D. See table 3.1 for list of sample number.

- ***Electrical conductivity (EC)***

The results obtained after three years of storage showed a decrease in values of conductivity (figure 3.3) for all samples (except sample 2, area B). These results contradicted what was established by several authors (Feás et al., 2010; Gomes et al., 2010a) where the increment of ash content, higher ionic level and organic acids, generated an increment in the electrical conductivity values. The conductivity measures all the ionizable organic and inorganic substances present in honey (Felsner et al., 2004).

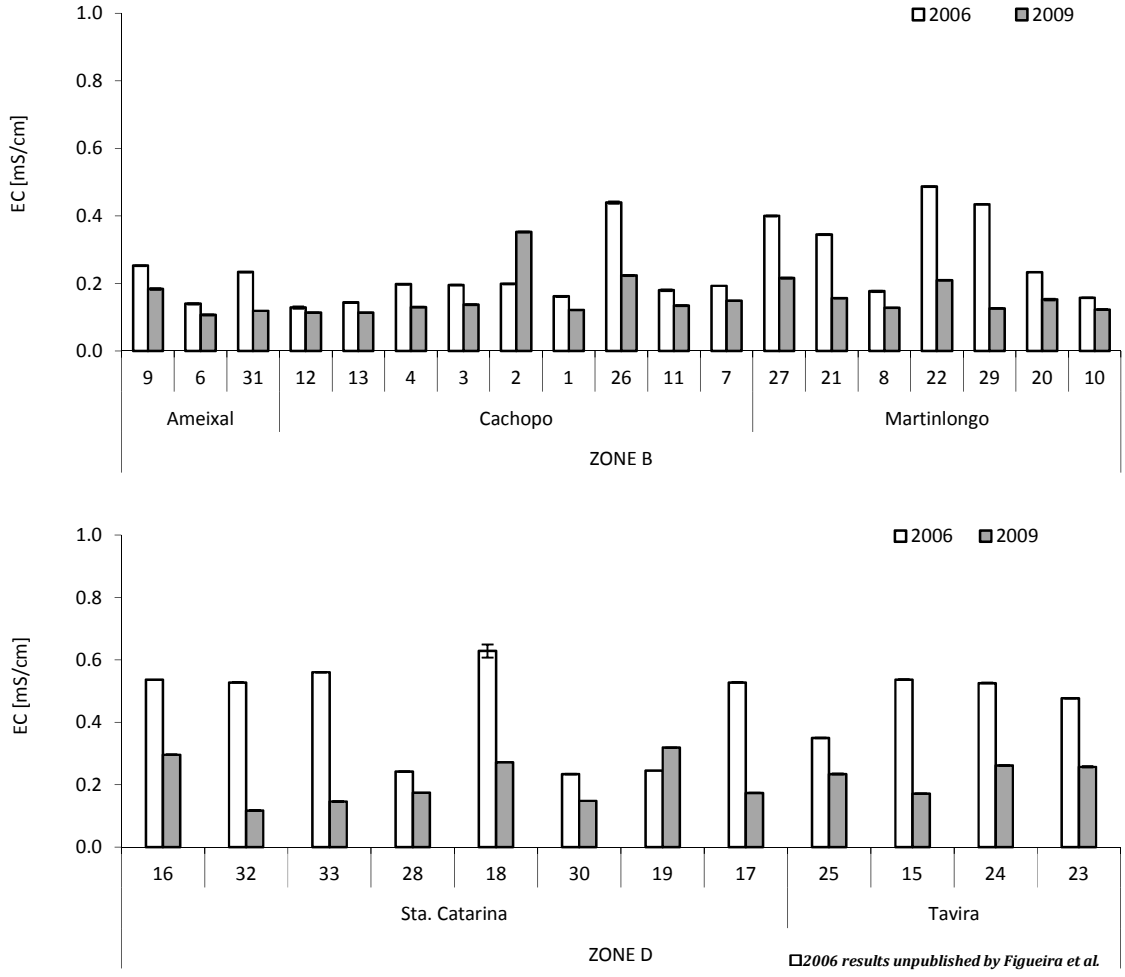


Figure 3.3 Results of electrical conductivity (mS/cm) after three years of storage for honey samples from areas B and D. See table 3.1 for list of sample number.

- ***pH***

For the honey samples analyzed initially (2006) mean pH values ranged from 3.61 (area B) and 3.59 (area D); after three years of storage the pH of samples were slight incremented for both areas, the mean pH value was 3.76 for area B and the mean value was 3.84 for area D (figure 3.4), these differences were, however, not statistically significant; similar results were also reported by Jimenez et al., (1994) who found that a non-significant increment in pH was observed after two years of storage at room temperature. The pH values were in accordance with the acceptable range for honey; similar results were also obtained by other authors for Portuguese honeys (Silva et al, 2009; Gomes et al, 2010a).

In general, honey is acidic by nature irrespective of its variable geographical origin. This parameter is of great importance during extraction and storage, as it influences the texture, stability and storage (Terrab et al., 2004a; Idris et al., 2011).

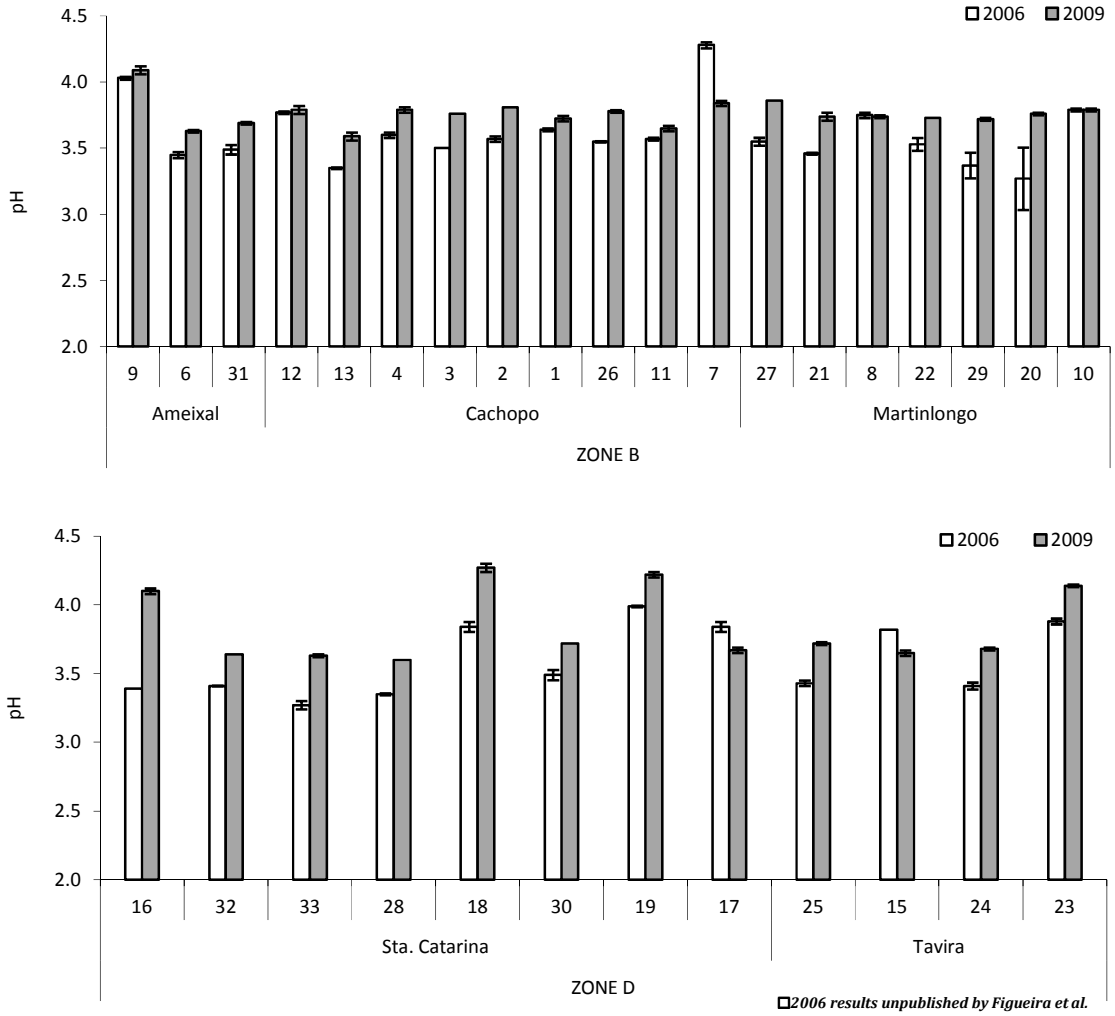


Figure 3.4 Results of pH after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

- ***Total acidity***

Total acidity mean values (figure 3.5) of honey samples were 17.88 meq/kg (area B) and 16.36 meq/kg (area D) in 2006; but after three years of storage all samples showed higher values (except samples 6 and 1, area B). These changes were statistically significant (p <0.05). However, none of the samples showed values exceeding the legal limit allowed (50 meq/kg) found the highest value for honey sample number 28 with 39.56 meq/kg after storage. This may be taken as an indication of quality of all honey samples under storage conditions. The acidity of honey is due to the presence of organic acids, particularly gluconic acid produced from nectar, during ripening, by glucose oxidase; in equilibrium with their lactones or esters, and inorganic ions, such as phosphate and chloride (Ajlouni and Sujirapinyokul, 2010; Kahraman et al., 2010).

Similar results were obtained by several authors, which reported that after storage all honeys samples increased their total acidity values (Jimenez et al,. 1994; Cavia et al., 2007; Castro-Vazquez et al., 2008, Qamer et al., 2009). These data illustrates the significant influence of floral type on the total acidity of honey. The variation in acidity among different honey types may be attributed also to variation due to harvest season (Singh and Bath, 1998).

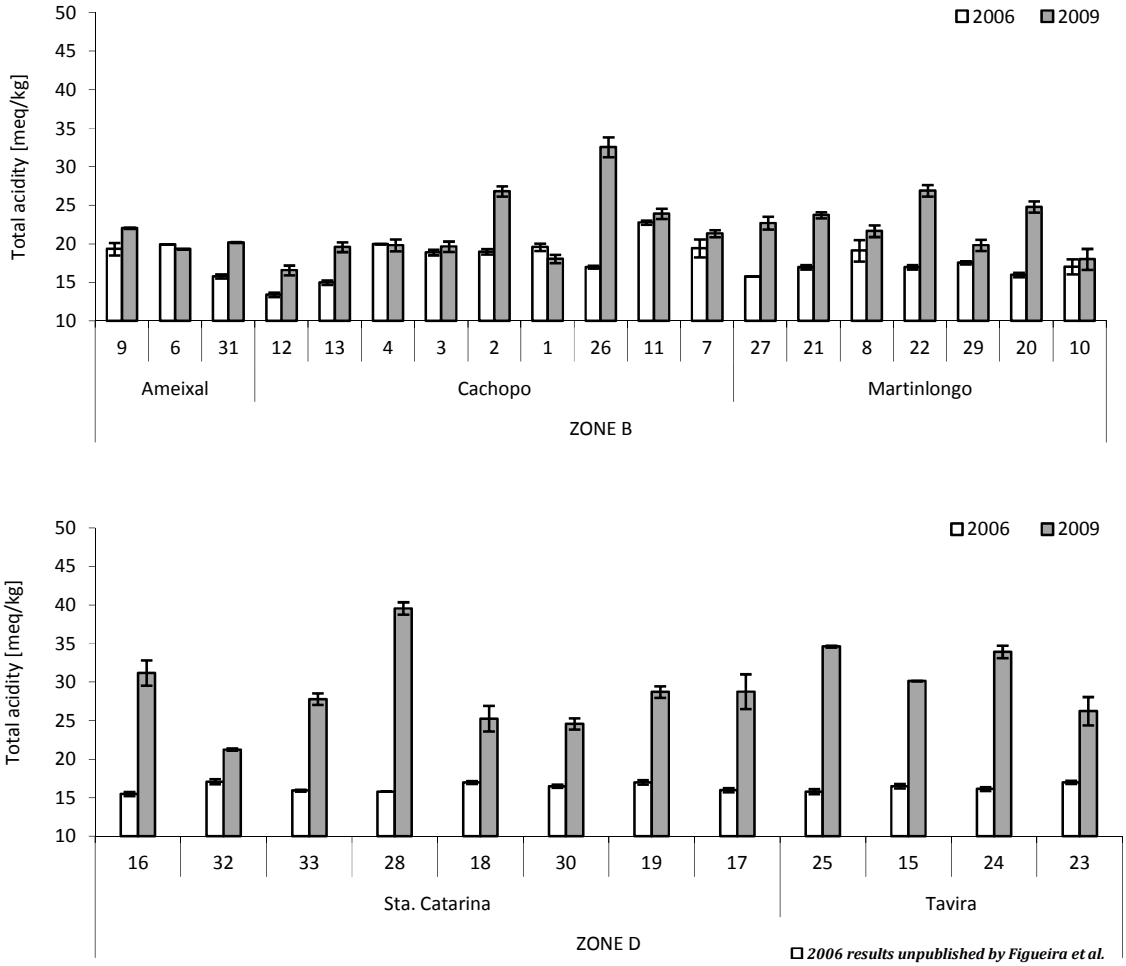


Figure 3.5 Results of total acidity (meq/kg) after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

- ***Colour***

All honeys were characterised using the visual observation CIE-*Lab* system. The differences in colour (Δ E) were determined using the equation (3.3):

$$\Delta E = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]} \qquad (3.3)$$

where:

$$\Delta \boldsymbol{L^*} = (L^*_{2006} - L^*_{2009});\ \Delta \boldsymbol{a^*} = (a^*_{2006} - a^*_{2009});\ \Delta \boldsymbol{b^*} = (b^*_{2006} - b^*_{2009})$$

The delta (Δ) E for honey samples obtained from Algarve is shown in figure 3.6. The parameters L^* (lightness) did not show significant differences after storage. In 2006 mean values of 31.35 (area B) and 30.38 (area D) were measured. These values were maintained in the similar range after storage, with mean values of 31.39 and 30.36, respectively being obtained. González-Miret et al., (2005) classified honey samples into two groups regarding their lightness value: light honey (citrus, rosemary, lavender, eucalyptus and thyme) with $L^* > 50$ and dark honeys (honeydew, heather, chestnut and avocado) with $L^* < 50$. Considering this classification all of the honey samples obtained in Algarve can be placed in the group of dark honeys, since both initially and after storage time they continued to maintain that rating of "dark" for both areas (B and D). However, it was generally observed that all honeys stored decreased their L^* values, which lead to some darkening of the samples, as expected. Similar results were obtained by Jiménez et al., (1994) after storage of honey for 24 months at different temperatures (4–7 ºC, 16–28 ºC); these samples showed darkening and this phenomena was increased with higher temperatures of storage.

Parameters a^* and b^* showed mean values for fresh honeys of -1.05 and 5.57 (area B), and -1.05 and 4.94 (area D); respectively. After three years, these parameters changed, showing some more redness (increment of the a^* value) as well as more yellow components (increments of the b^* value).

Difference in colour between fresh honeys was attributed to an increment of melanoidin compounds (Maillard reaction) which have a brown colour, producing an overall diming, and causing considerable changes in honey or by oxidation of polyphenols (quinines) (Jimenez et al., 1994; Gonzales et al., 1999). The rate of darkening has been related to the composition of honey and to the storage conditions (*e.g.* temperature, light, time) (White, 1964; Jiménez et al., 1994; Gonzales et al., 1999).

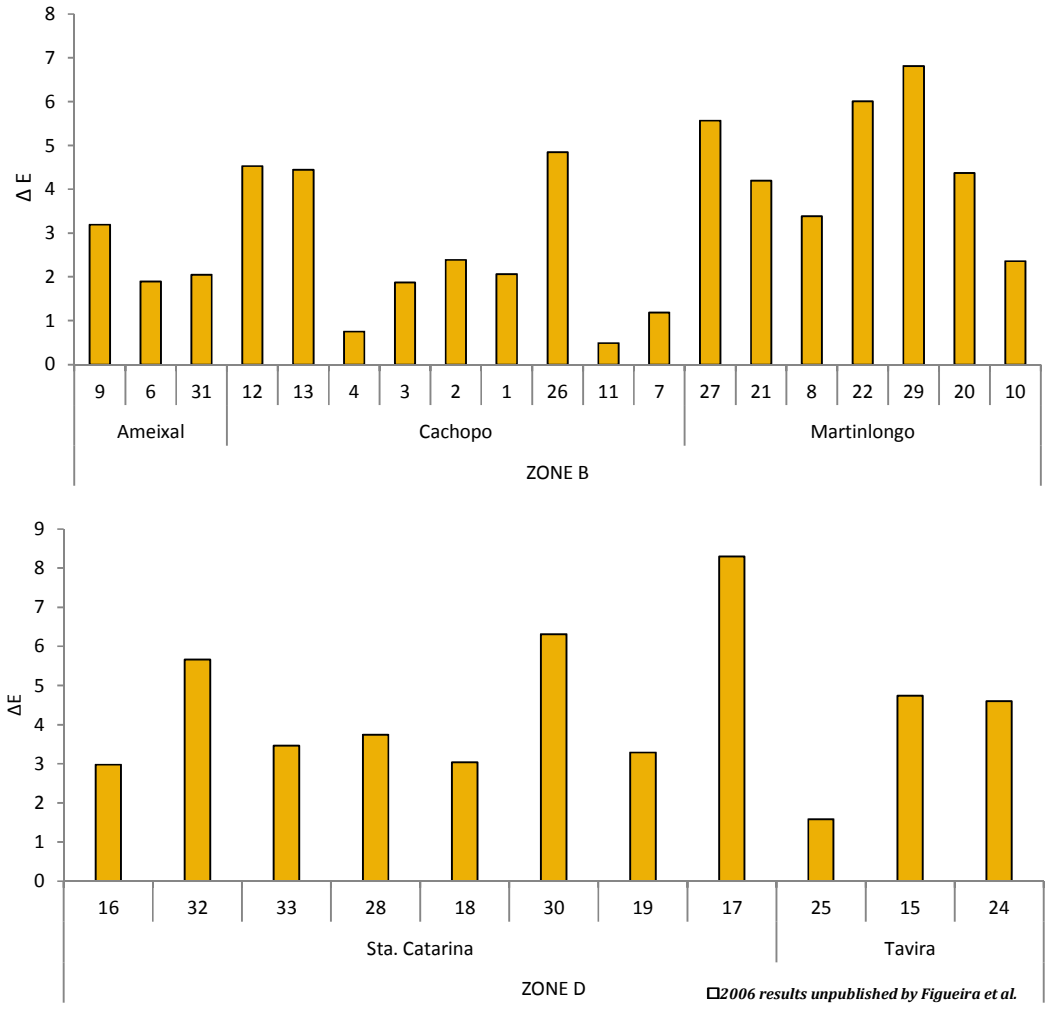


Figure 3.6 Results of differences of colour (Δ E) after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

- ***Moisture content (MC)***

Moisture in the analyzed honeys after three years (figure 3.7), presented a mean value approximately of 15.71% for honeys from area B and 15.74% for honeys from area D. In comparison with initial values, changes were minor; only the honey from sources of Palma presented a significant reduction in the values of moisture (Tavira samples: 25, 15, 24, and 23). The maximum amount of water content in honey is regulated for safety against fermentation. All samples showed mean values of less than 16.0% water, the maximum amount allowed by European Union (2001) and Portuguese Norm-1307 (1983) being 20% moisture. The water content of honey depends on various factors such as: the botanical origin, the harvesting season, the degree of maturity reached in the hive, the processing techniques and storage conditions (*e.g.* temperature, light, O_2, etc) (Finola et al., 2007).

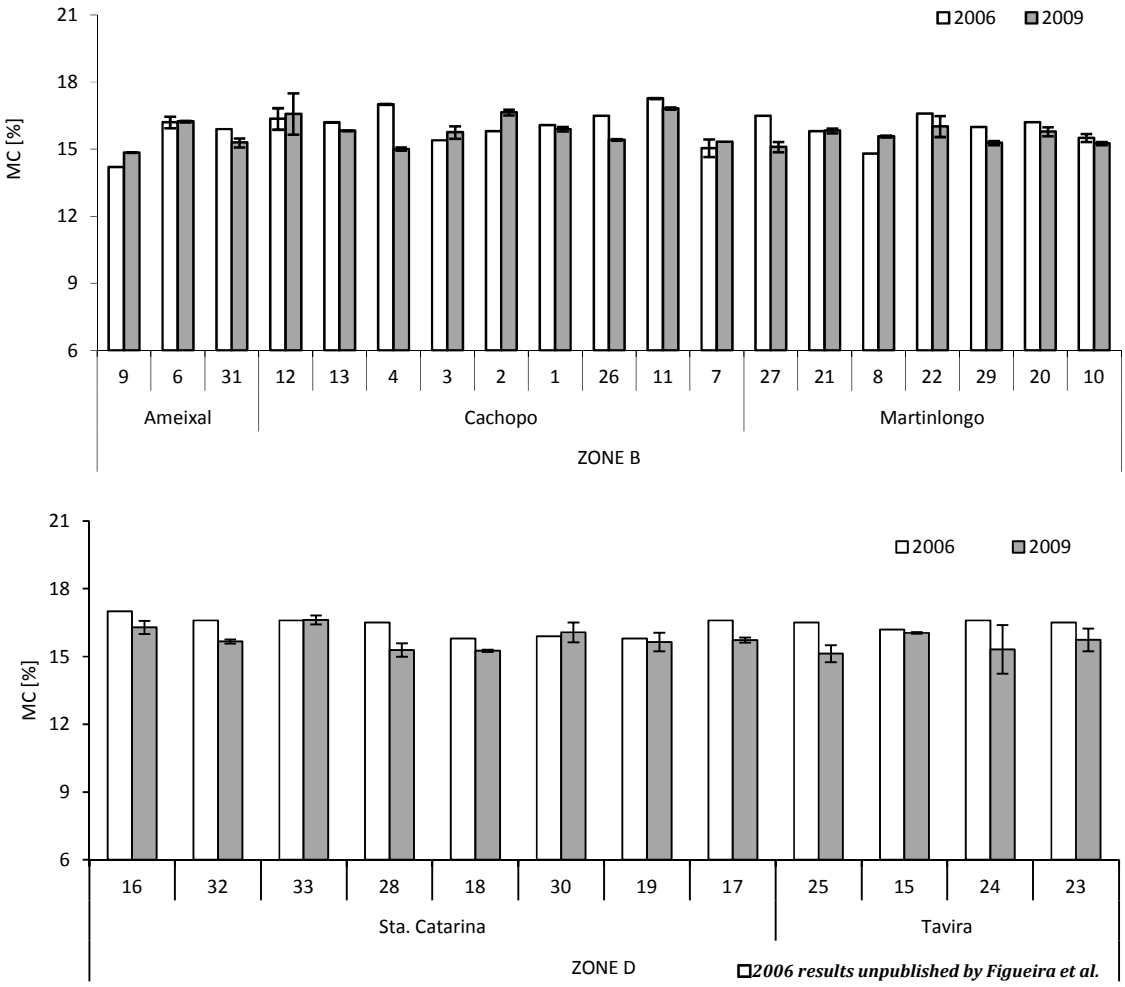


Figure 3.7 Results of moisture content (MC, %) after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

- ***Total soluble solids (TSS)***

The total soluble solids (figure 3.8) content showed mean values between from 82.2 ºBrix (for area B) and 81.7 ºBrix (area D) in 2006; after three years all the samples showed a slight increment in their values (not significant) 84.3 and 84.2 ºBrix, respectively; this phenomenon occurs due to a loss of water content, generating the concentration of the solids presents in honey.

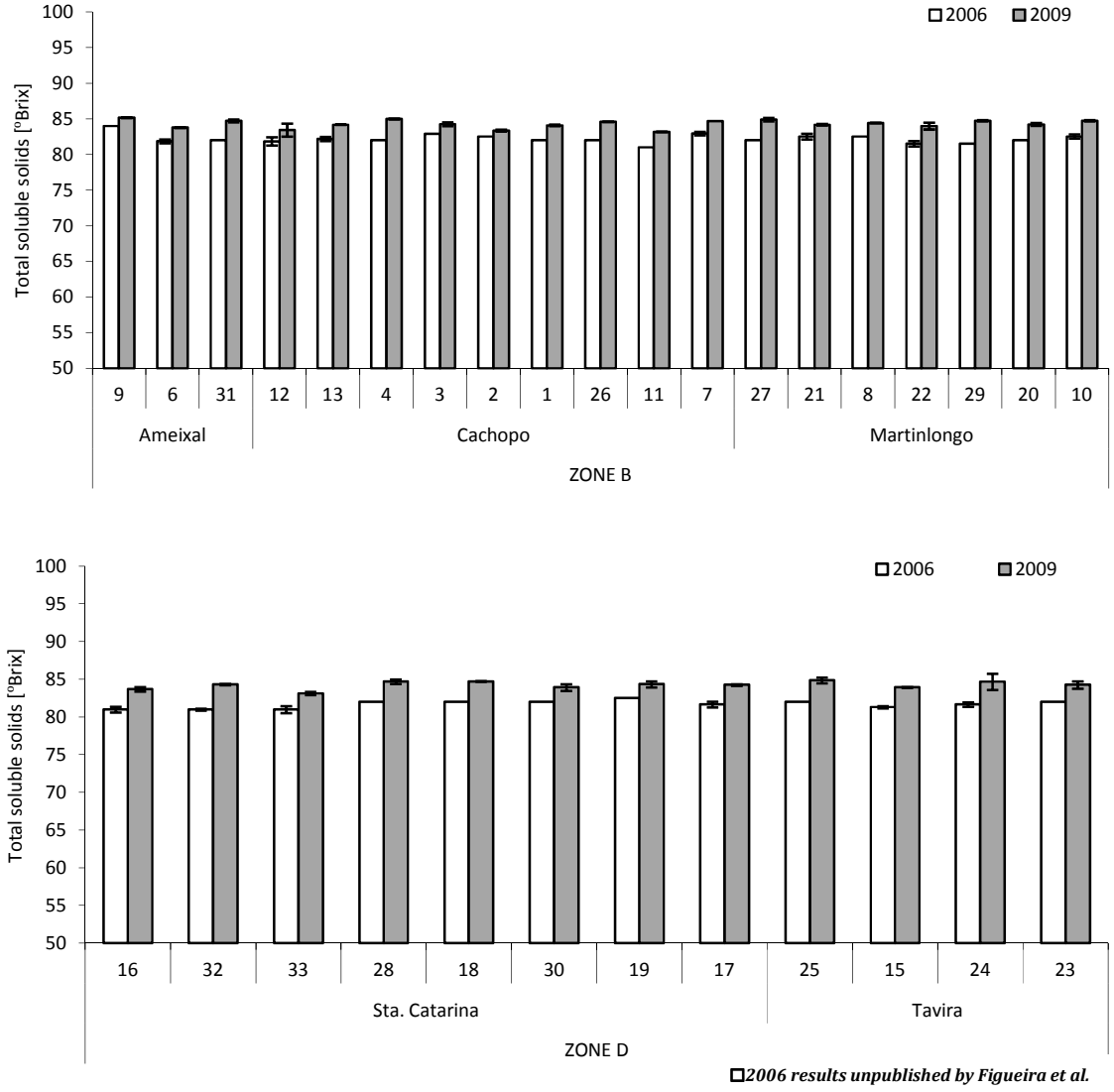


Figure 3.8 Results of total soluble solids (ºBrix) after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

It is important to remember that the MC and TSS content of honey are strictly linearly correlated: anomalous values may be a reliable index for adulterations, as suggested by Conti (2000), which has been confirmed in his study. When the correlation between both parameters was studied, the coefficient of determination observed for samples in 2006 (R^2 = 0.71) and in 2009 (R^2 = 0.98) showed a good relationship (figure 3.9).

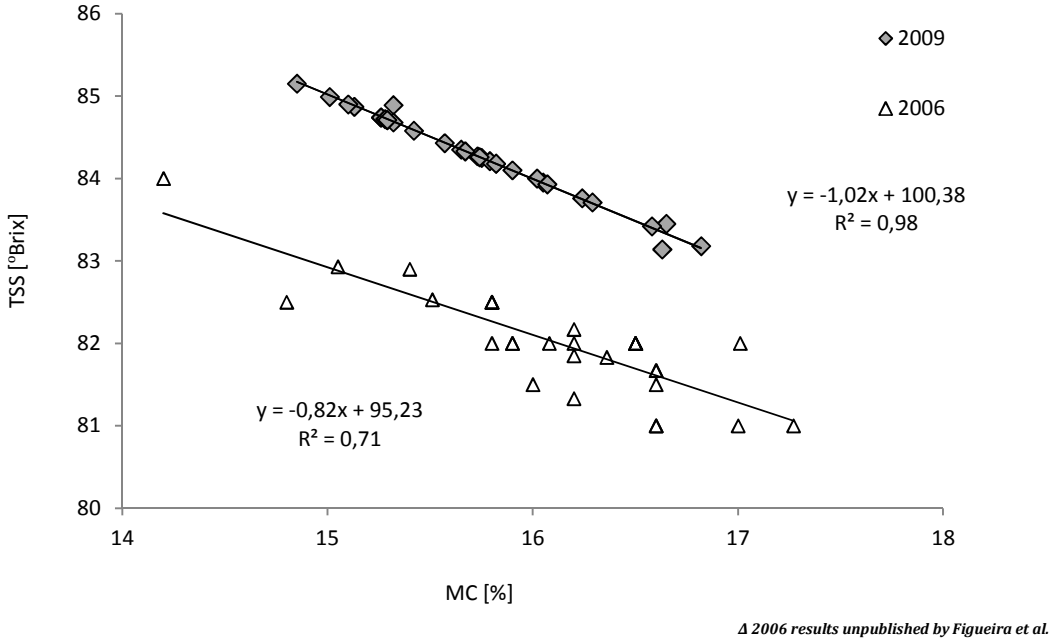


Figure 3.9 Linear relationship between moisture content (MC, %) and total soluble solids (TSS, ºBrix) for honey samples analyzed (2006 and 2009).

- ***Proline content***

Proline is the major amino acid present in honey (50–85% total). This amino acid has been suggested as an indicator of honey ripeness, as well as a parameter of quality. Normally the proline content in honey should be more than 180 mg/kg (Azeredo et al., 2003; Bogdanov, 2009). Results obtained for honeys studied in 2006 showed mean values for area B of 137.8 mg/kg and area D mean value of 212.2 mg/kg (figure 3.10); these lead to the suggestion that samples from area B did not presente a suitable maturity, since after three years all honeys showed values higher than 180 mg/kg; for area B, the mean value was 447.1 mg/kg and area D mean was 601.6 mg/kg; which means that honey stored with the passage of time was reaching maturity.

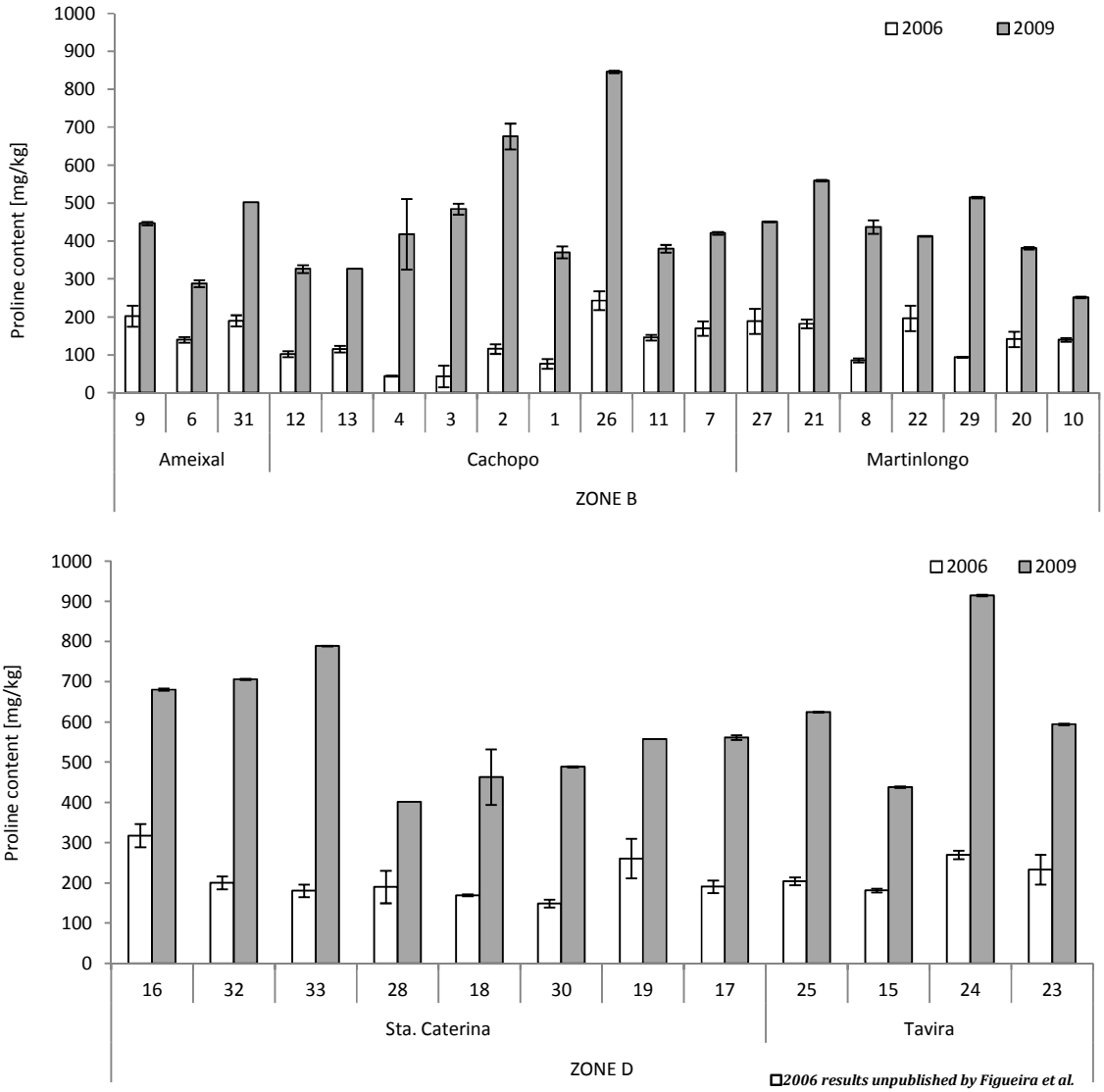


Figure 3.10 Results of proline content (mg/kg) after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

- ***Water activity (a_w)***

The water activity (figure 3.11) initially presented mean values of 0.52 (area B) and 0.54 (area D); after keeping the samples under storage conditions the water activity values were slightly lower than the initial (0.51 and 0.51, to those respectively). These values are in accordance with the acceptable range for honey and similar to those obtained in other studies (Gleiter et al., 2006). The a_w parameter is more important in the inhibition of microorganism development and enzyme activity. The osmophilic yeasts are only able to grow above minimal water activities of 0.6 and are specialists in surviving under conditions of high sugar concentrations. Such osmophilic yeasts (*Saccharomyces* spp.) are responsible for honey fermentation, resulting in the formation of ethyl alcohol and carbon dioxide. The alcohol can be further oxidised to acetic acid and water resulting in a sour taste (Saxena et al., 2009). Fermentation processes are indeed virtually annulled when the moisture content is <17.1%, while for higher moistures they are dependent on the number of osmotic yeasts (>1000/g of honey for 17.1% <moisture <18% and >10/g honey for 18.1< moisture <19%). The samples analyzed should be regarded as "safe" with respect to this phenomenon, showing lower values in $a_w \leq 0.57$ and moisture ≤ 17.3% (Lochhead, 1933; Conti, 2000).

There is a relationship, between moisture content (%) and water activity; the a_w is mainly determined by the presence of soluble chemical species (mainly fructose and glucose); the difference between the a_w of the different honey types is the result of its diverse sugar compositions (Gleiter et al., 2006). Similar results were found by others researchers (Abramovič et al., 2008; Chirife et al., 2006; Zamora et al., 2006).

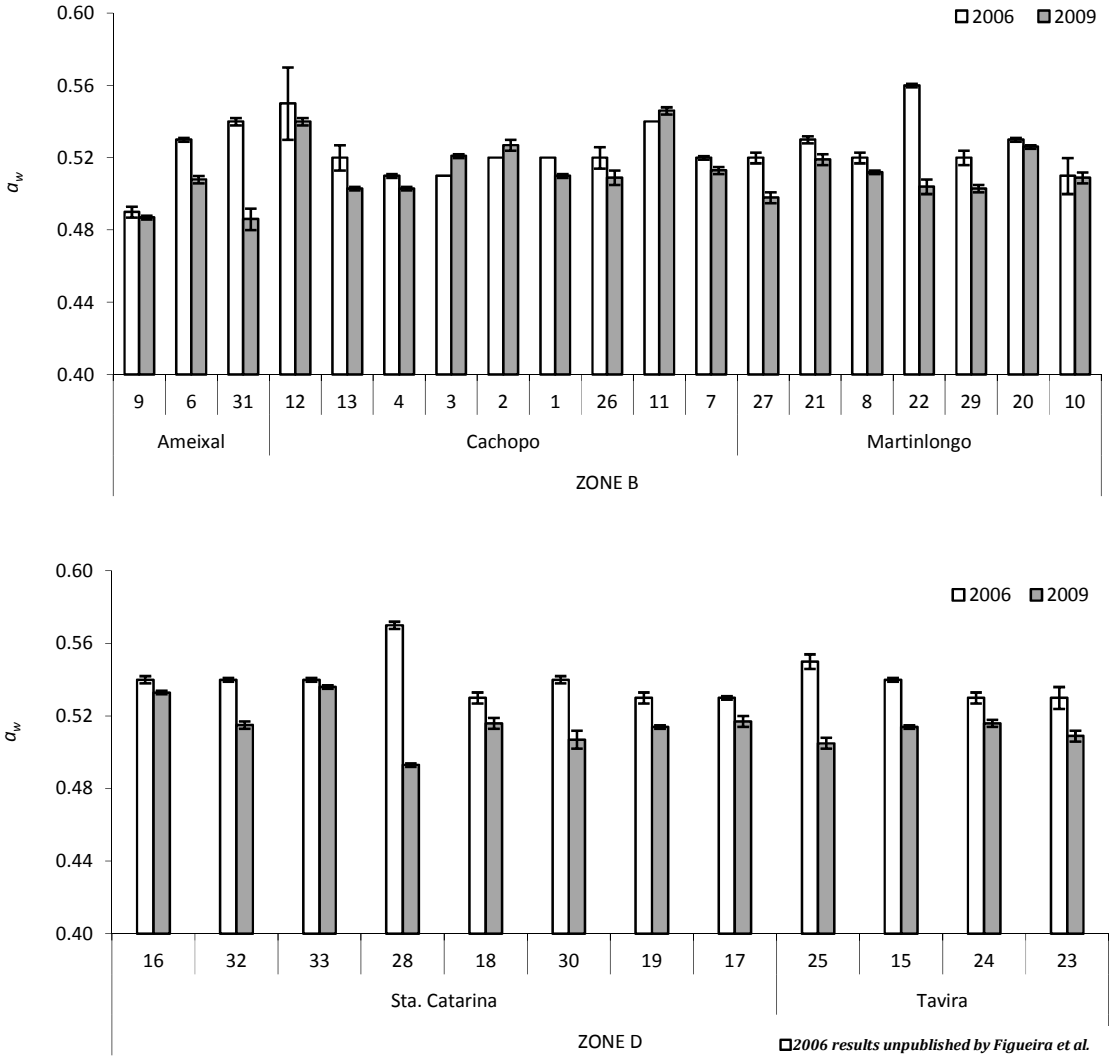


Figure 3.11 Results of water activity after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

- ***Diastase activity (DA)***

In the present study, the mean diastase activity (DA, figure 3.12) for the samples initially analyzed in 2006 was 17.78 Gothe (area B) and 14.39 Gothe (area D). The samples stored for three years showed values of 7.83 and 8.35 Gothe; these results showed a significant decrease in the enzyme activity. The average value obtained for area B is not within the limits established by the European Union (2001) and Codex Alimentarius Commission (2001) for diastase activity parameter which can not be less than 8 Gothe. On the other hand, some honeys (28, 18 and 23 samples) from area D had values below this limit. Similar results were obtained by Turhan (2009); the diastase activity in honey stored for one year at 20 ºC decreased from 14.6 to 10.7 Gothe. Also, Castro-Vázquez et al., (2008) and Qamer et al., (2009) observed that the diastase enzyme decreases its activity after storage. The diastase activity is closely related to its structure and can be modified by denaturation, brought about by heating or changes in pH. Tosi et al., (2008) and Ajlouni & Sujirapinyokul (2010) reported the effects of the thermal treatment on the activity of diastase and concluded that the activity of the enzyme decreased after subjecting samples to a heat treatment.

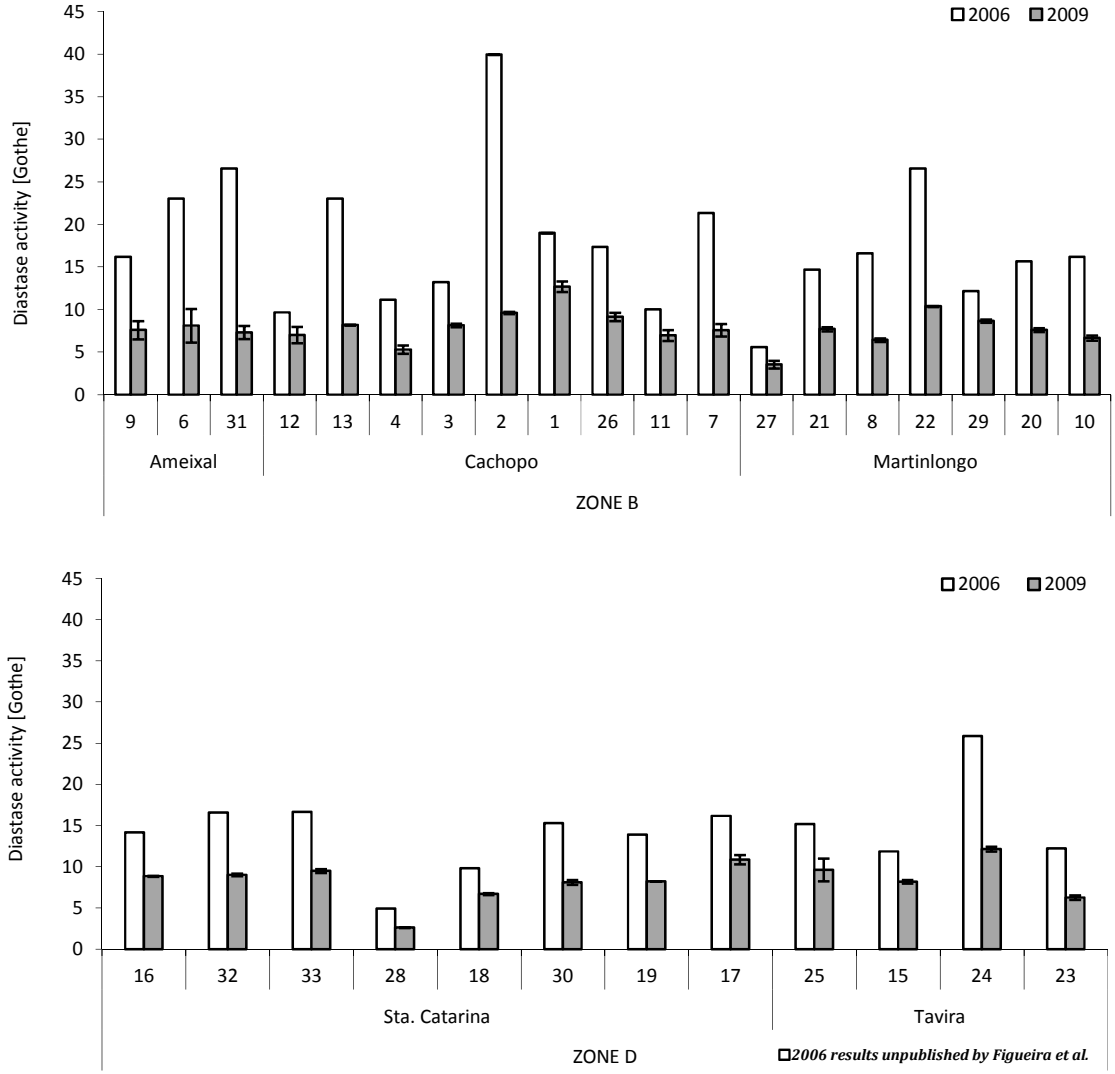


Figure 3.12 Results of diastase activity (Gothe) after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

- ***Hydroxymethylfurfural (HMF) content***

The results reported for hydroxymethylfurfural content (figure 3.13) in the honey samples analyzed showed values excessively high after storage, even surpassing the limit established by the European Union (2001) and Codex Alimentarius Commission (2001). The limit for this parameter is not more than 40 mg/kg. The initial HMF content for fresh honeys from area B showed a mean value of 6.59 mg/kg and area D was a mean value of 13.47 mg/kg; after three years of storage these values were significantly increased to 108.99 and 100.55 mg/kg, respectively. Only one sample (number 30 from area D) had a value lower than the limit (i.e., 16.8 mg/kg). Similar results were obtained by other authors (Turhan, 2009; Khalil et al., 2010a) who, after analyzing honey stored for 12–24 months, found that the HMF level increased progressively for all the samples and that this process can be favored by changes in pH and acidity of honey. Studies reported that HMF has genotoxic effects and mutagenic potential (Khalil et al., 2010a; Capuano and Fogliano, 2011). Several authors (Qamer et al., 2009; Turhan, 2009; Khalil et al., 2010a) have established a limit of storage and consumption time and be consumed for honeys, with recommendations for it not to be kept for more than eight and twelve months to guarantee the quality, freshness and safety of these.

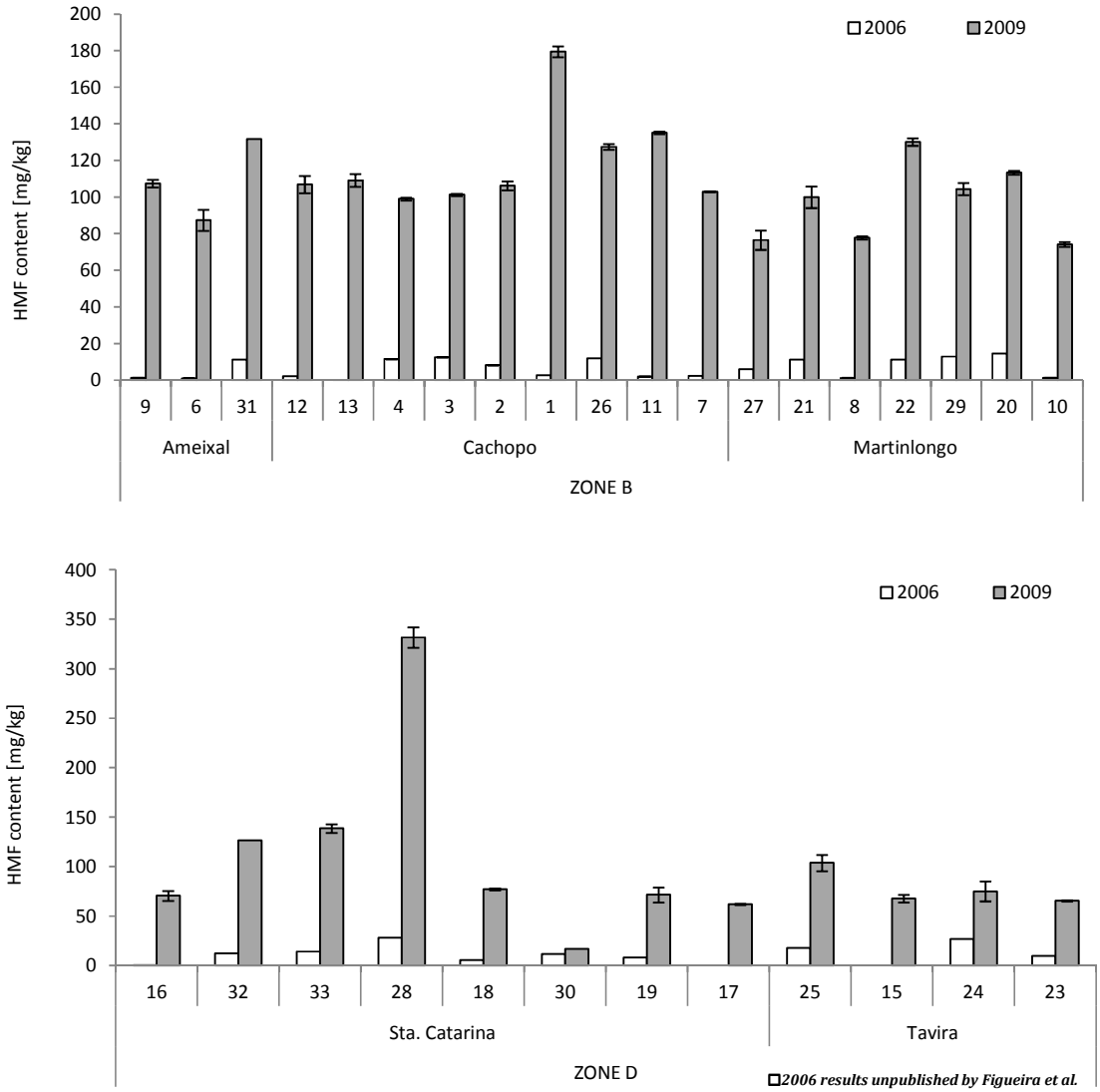


Figure 3.13 Results of hydroxymethylfurfural content (mg/kg) after three years of storage for honey samples from of areas B and D. See table 3.1 for list of sample number.

3.3.2. Bioactive compounds

Table 3.2 shows the results obtained for the total phenolic content as well as the total flavonoid content of honey samples immediately after collection (2006) and after three years of storage, according the collectivy area.

- ***Total phenolic content (TPC)***

For the TPC (table 3.2) the samples analyzed in 2006, for area B showed a range from 23.56 to 43.86 mg of gallic acid equivalent (GAE)/100g of honey (mean value 34.21 mg of GAE/100 g of honey) and area D a range 24.64 to 52.17 mg of GAE/100g of honey (mean value 38.15 mg of GAE/100 g of honey), those values were not significantly different ($p < 0.05$). The comparison of the total phenolic content of the honey samples upon three years of storage, for area B did not show changes (mean value 34.45 mg of GAE/100g of honey), but for samples from area D, was observed an increment of 8.03% in the mean value (mean value 41.48 mg of GAE/100g of honey). Some of the causes for this behaviour in the variability of values of phenolic content could be the consequence of the formation of compounds that could react as electron-donors, incrementing the real amount of phenol (Al-Mamary et al., 2002; Gheldof et al., 2002; Ferreira et al., 2009). Folin-Ciocalteu´s Method (FCM) is general for the estimation of the total phenolic content; other components such as peptides, vitamins C, E, carotenoids, sugars, amino acids, proteins, organic acid or enzymes (glucose oxidase, catalase) can also influence the results but the components that influence more, by increasing the total phenolic value are the products of Maillard reactions (melanoidins), because several investigations had demonstrated that those compounds presented an effect that is similar to a reducing agent (mixture of phosphotungstic acid and phosphomolibdic acid) (Alvarez-Suarez et al., 2009; Ferreira et al., 2009). Honey is very prone to Maillard reactions during storage or when subjected to thermal processing. These occur when sugars condense with free amino acids leading to the formation of brown melanoidins (Turkmen et al., 2006; Rufián-Henares and Morales, 2007).

Regarding the TPC of honey after three years of storage, when compared with other fresh honeys, not from different locations, the samples collected in 2006 in the Algarve region had a mean value for area B of 34.45 mg of GAE/100 g of honey and for a value of area D of 41.48 mg of GAE/100g of honey. These were levels similar to those observed by Ferreira et al., (2009), who analyzed different types of honey (light, amber and dark) obtained from the Northeast Portugal and found that they showed a range from 22.6 to 72.8 mg of GAE/100g honey. One other research, performed by Estevinho et al., (2008), who analyzed samples obtained from Northern Portugal presented values between 4.1–13.0 mg/100g of honey, but this range was for the extraction of phenolic compounds from honey samples that were funther analyzed by HPLC which may explain the lower values. The differences in concentration and type of phenolic substances in honey can be explained by the different geographical conditions, which are very variable, and it also depends on the floral origin of honey as they are the major factors responsible for its biological activity, including antioxidant and antimicrobial (Küçük et al., 2007).

Table 3.2 Comparison between total phenolic content (TPC; mg of GAE/100 g of honey) and total flavonoid content (TFC; mg of QE/100 g of honey) before (2006) and (2009) after three years of storage, for each honey sample obtained from different areas of South Portugal (Algarve). See table 3.1 for list of samples.

			TPC (mg of GAE/100g)		TFC (mg of QE/100g)	
			2006	**2009**	**2006**	**2009**
		Sample	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
AREA B (*n = 19*)	Ameixal	9	41.01 ± 1.3	37.44 ± 0.35*	1.34 ± 0.00	10.02 ± 0.71**
		6	32.2 ± 0.59	30.40 ± 0.25*	13.09 ± 2.29	7.56 ± 0.59**
		31	23.56 ± 0.00	31.93 ± 0.73*	14.57 ± 1.34	10.73 ± 1.45**
	Cachopo	12	32.36 ± 0.42	28.03 ± 1.32*	9.75 ± 0.49	7.65 ± 1.21
		13	30.46 ± 0.32	26.59 ± 0.53*	11.94 ± 1.11	10.21 ± 0.77
		4	33.70 ± 0.93	33.95 ± 0.72	13.09 ± 2.29	10.08 ± 0.97
		3	37.56 ± 0.53	40.98 ± 4.44	12.24 ± 0.60	9.92 ± 0.38**
		2	36.30 ± 0.83	48.61 ± 0.16*	15.45 ± 0.36	11.49 ± 0.30**
		1	42.20 ± 5.46	36.47 ± 0.28	9.60 ± 0.40	8.97 ± 0.59
		26	41.71 ± 0.43	52.06 ± 0.36*	16.70 ± 0.53	14.1 ± 1.00**
		11	33.73 ± 0.55	29.38 ± 0.13*	15.65 ± 0.96	9.76 ± 1.02**
		7	43.86 ± 0.97	34.60 ± 1.21*	18.31 ± 0.30	12.76 ± 4.84
	Martinlongo	27	29.30 ± 0.58	26.56 ± 0.20*	25.80 ± 0.84	10.48 ± 0.65**
		21	36.55 ± 1.53	26.98 ± 0.32*	14.15 ± 0.39	10.43 ± 1.23**
		8	35.95 ± 0.41	34.54 ± 0.51*	1.40 ± 0.03	8.90 ± 0.17**
		22	26.64 ± 1.69	33.38 ± 0.33*	18.18 ± 0.00	10.47 ± 0.11**
		29	38.78 ± 2.05	53.21 ± 0.72*	10.94 ± 0.72	10.77 ± 1.23
		20	24.72 ± 0.94	22.59 ± 0.40*	13.74 ± 0.26	12.44 ± 1.16
		10	29.45 ± 0.87	26.84 ± 0.10*	10.92 ± 0.05	8.95 ± 0.26**
		Mean	**33.57**	**33.68**	**12.99**	**10.38**
		S.D.	**5.90**	**7.61**	**5.54**	**1.63**
		Range	**23.56-43.86**	**22.59-53.21**	**1.34-25.80**	**7.56-14.12**
AREA D (*n = 12*)	Sta. Catarina	16	52.17 ± 0.73	49.42 ± 0.10*	18.71 ± 0.62	12.05 ± 1.00**
		32	34.55 ± 0.40	33.41 ± 0.37*	14.57 ± 1.34	10.52 ± 0.00**
		33	45.22 ± 0.40	37.31 ± 0.37*	14.57 ± 1.34	10.12 ± 1.11**
		28	48.67 ± 0.24	68.90 ± 1.04*	10.21 ± 0.42	20.37 ± 0.36**
		18	38.86 ± 2.86	46.46 ± 0.13*	15.72 ± 1.61	9.87 ± 0.73**
		30	24.64 ± 0.58	34.81 ± 0.08*	11.55 ± 0.13	9.45 ± 0.98**
		19	38.78 ± 2.05	53.21 ± 0.72*	3.19 ± 0.00	15.51 ± 0.22**
		17	42.66 ± 0.5	38.42 ± 0.56*	14.57 ± 1.34	13.45 ± 0.55**
	Tavira	25	35.61 ± 0.49	34.75 ± 0.25*	23.27 ± 1.09	12.08 ± 0.05**
		15	32.33 ± 0.05	30.86 ± 0.65*	11.26 ± 1.95	9.95 ± 0.20
		24	30.3 ± 2.46	32.89 ± 0.90	19.28 ± 0.19	11.02 ± 0.58**
		23	34.04 ± 0.26	37.30 ± 0.22*	12.72 ± 0.61	12.90 ± 1.33
		Mean	**38.15**	**41.48**	**14.14**	**12.27**
		S.D.	**7.94**	**11.13**	**5.10**	**3.11**
		Range	**24.64-52.17**	**30.86-68.90**	**3.19-23.27**	**9.45-20.37**

* *Results shown statistically significant differences ($p<0.05$) in 2006;* ***Results shown statistically significant differences ($p<0.05$) in 2009. The 2006 results unplubished by Figueira et al.*

- ***Total flavonoid content (TFC)***

The results for the TFC mg of quercitin equivalent (QE)/100g of honey of samples are presented in table 3.2. The samples analyzed in 2006 showed a range of 1.34 to 25.80 mg of QE/100 g of honey (mean value 12.99 mg of QE/100 g of honey) for area B; honey collected in area D showed a range of values from 3.19 to 23.27 mg of QE/100 g of honey (mean value 14.14 mg of QE/100 g of honey); comparing the results obtained from both areas there are no statistically significant differences ($p < 0.05$). After three years of storage, total flavonoid content of honeys harvested in areas B and D decreased of 25.14% to the mean value (mean value 10.38 mg of QE/100 g of honey) and 15.24% (mean value 12.27 mg of QE/100 g of honey), respectively. These values of the TFC were higher than those obtained by Al et al., (2009) who analyzed different honeys, obtaining values within a range of 0.91 to 2.42 mg of QE/100 g of honey (for *Acacia*), 4.7–6.98 mg of QE/100 g of honey (for *Tilia*) and 11.53–15.33 mg of QE/100 g of honey (for *Helianthus*), as well as Meda et al., (2005) who found a range of 0.17 to 8.35 mg of QE/100 g of honey (mean value 2.57 mg of QE/100 g of honey); Blasa et al., (2006) who obtained a range of 1.23–2.93 (mg of catequin equivalents (CEs)/100 g of honey) for multifloral honey and a range of 0.45 to 1.01 mg of CEs/100 g of honey, for unifloral honey samples from Italy. Results obtained by Ferreira et al., (2009) presented much higher values of flavonoid content: (from 123.62 to 587.42 mg/kg of honey) for samples from Northern Portugal. The TFC, was determined using a spectrophotometric method for the quantification of flavonoids with $AlCl_3$, which is specific for flavones and flavonols (Chang et al., 2002), which makes our results very trustable.

- ***Antioxidant capacity***

The antioxidant capacity (table 3.3) of the honeys analyzed showed for area B a mean value of 11.48 mg of quercitin equivalent antioxidant capacity (QEAC)/100g of honey and 2.39 mg of ascorbic acid equivalent antioxidant capacity (AEAC)/100g of honey; samples collected in area D showed values of 16.88 mg of QEAC/100g of honey and 4.02 mg of AEAC/100g of honey, respectively and were not significant different ($p < 0.05$).

The little differences between values can be explained by the different geographical conditions. The honey samples collected in area B (Ameixal, Cachopo and Martinlongo) were less exposed to weather conditions of the sea coast in comparison with area D (Sta. Catarina and Tavira). Phenolic contents of honey and consequently the overall antioxidant capacity depends on the floral sources used to collect honey, which predominance is dependent on seasonal and environmental factors. Therefore, difference in honey properties from same floral sources were expected since the composition of active compounds in honey from different locations should be different (Al-Mamary et al., 2002; Silici et al., 2010).

Meda et al., (2005) repoted the values of 12.94 mg of QEAC/100g of honey and 27.04 mg of AEAC/100g of honey for antioxidant capacity of multifloral honeys; also Saxena et al., (2009) analyzed Indian honeys, finding that their total antioxidant capacity ranged from 15 to 30 mg of AEAC/100g of honey. Thus our results are similar to those reported by these authors.

On the other hand, Silici et al., (2010) analyzed *Rhododendron* honey and reported that its total antioxidant capcity varied from 12.76 to 80.80 mg of AEAC/g honey; the honey analyzed from the South of the Algarve showed lower levels of antioxidant capacity with reference to mg of AEAC content.

Table 3.3 Results obtained for the antioxidant capacity (mg QEAC/100 g of honey; mg AEAC/100 g of honey) and of the DPPH scavenging (%) honey samples analyzed in 2009 according to the place of collection, after three years of storage. See table 3.1 for list of samples.

			Antioxidant capacity		
			[mg of QEAC/100g]	**[mg of AEAC/100g]**	**Inhibition DPPH [%]**
		Sample	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
AREA B (*n = 19*)	Ameixal	9	11.08 ± 0.69	2.31 ± 0.21	18.03 ± 1.99
		6	7.41 ± 1.51	1.23 ± 0.45	9.46 ± 0.36
		31	15.96 ± 1.04	3.56 ± 0.31	13.66 ± 3.37
	Cachopo	12	7.65 ± 0.14	1.46 ± 0.04	12.18 ± 1.79
		13	10.26 ± 1.13	1.97 ± 0.34	15.61 ± 1.97
		4	8.84 ± 1.36	1.65 ± 0.41	17.01 ± 1.27
		3	8.26 ± 1.20	1.47 ± 0.36	13.94 ± 3.07
		2	12.14 ± 0.75	2.53 ± 0.23	22.89 ± 1.99
		1	9.34 ± 1.85	1.59 ± 0.55	13.04 ± 0.00
		26	19.79 ± 1.48	4.85 ± 0.44	24.36 ± 2.00
		11	10.78 ± 1.43	2.18 ± 0.43	17.77 ± 1.08
		7	14.09 ± 0.20	3.06 ± 0.06	19.18 ± 0.36
	Martinlongo	27	15.37 ± 0.52	3.60 ± 0.15	21.05 ± 0.71
		21	12.38 ± 1.67	2.62 ± 0.50	15.85 ± 0.55
		8	8.85 ± 0.55	1.58 ± 0.16	14.71 ± 2.35
		22	17.67 ± 1.72	4.29 ± 0.52	20.36 ± 2.19
		29	15.25 ± 2.46	3.37 ± 0.74	14.54 ± 1.77
		20	7.15 ± 0.73	1.07 ± 0.22	17.27 ± 2.19
		10	5.80 ± 0.95	0.94 ± 0.29	14.21 ± 1.79
		Mean	**11.48**	**2.39**	**16.59**
		S.D.	**3.93**	**1.13**	**3.79**
		Range	**5.80-19.79**	**0.94-4.85**	**9.46-24.36**
AREA D (*n = 12*)	Sta. Catarina	16	21.81 ± 1.06	5.51 ± 0.32	27.28 ± 1.62
		32	17.65 ± 1.41	4.11 ± 0.42	16.29 ± 2.48
		33	17.65 ± 1.11	4.21 ± 0.33	18.55 ± 2.48
		28	19.59 ± 0.96	4.75 ± 0.29	23.56 ± 0.35
		18	19.46 ± 0.33	4.87 ± 0.10	25.00 ± 1.97
		30	14.96 ± 2.06	3.48 ± 0.62	18.42 ± 1.95
	Tavira	19	13.57 ± 2.96	3.05 ± 0.89	27.84 ± 1.46
		17	14.24 ± 3.49	3.24 ± 1.05	21.70 ± 1.62
		25	18.08 ± 0.52	4.41 ± 0.16	25.39 ± 1.64
		15	10.18 ± 1.23	2.15 ± 0.37	17.89 ± 1.26
		24	13.81 ± 2.02	3.06 ± 0.61	19.07 ± 1.09
		23	21.51 ± 2.01	5.38 ± 0.60	24.23 ± 2.92
		Mean	**16.88**	**4.02**	**22.10**
		S.D.	**3.54**	**1.03**	**3.96**
		Range	**10.18-21.81**	**2.15-5.51**	**16.29-27.84**

- ***DPPH scavenging***

With respect to the results of inhibition of DPPH analyses (table 3.3), these demonstrated that the most active radical scavengers were found in honeys collected in area D (range of 16.29 to 27.84%, a mean value 22.10%), followed by area B (range of 9.46 to 24.36%, a mean value 16.59%). The DPPH free radical has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Estevinho et al., 2008; Silici et al., 2010). Similar results were obtained by Pérez et al., (2007) to honey samples from Madrid (Spain), which showed a mean value of 20.7%; also Vela et al., (2007) analyzed honey from the North of Spain, obtaining higher values for DPPH (28.7 ± 16.6%). The higher values of DPPH, the higher the antioxidant capacity of the samples.

Several authors (Aljadi and Kamaruddin, 2004; Krpan et al., 2009; Saxena et al., 2009) have established a direct relationship between the antioxidant capacity of honey and the amount of bioactive compounds (*e.g.* phenols and flavonoids) present (Beretta et al., 2005; Blasa et al., 2006), along with other compounds also involved. This is consistent with our results because the area D showed higher total phenolic and flavonoid contents, which results in an increased antioxidant capacity and an inhibition of free radicals. The differences in values between both areas can be explained by the geographical conditions. Phenolic contents of honey, and consequently antioxidant capacity, depends on the floral sources used to collect honey, which predominance is dependent on seasonal and environmental factors. The majority of plants that are used by bees to collect honey biosynthesize antioxidants and may be used as a natural source of free radical scavenging compounds. Consequently, the bioactive compounds of vegetal origin can be transferred to honey (Baltrušaitytė, et al., 2007).

3.4. Conclusions

The following parameters were analyzed in all honey samples: pH, total acidity, ash content, EC, moisture and water activity, and were found to show a good level of quality, by meeting honeys´ European Legislation (EC Directive 2001/110) and Portuguese legislation (1983), after being kept under storage for three years, except for the hydroxymethylfurfural content and the diastase activity. It can be concluded that storage during a period of three years caused an evident loss of the quality and freshness of honeys. It must also be taken into account that the conditions of storage are very important (*e.g.* temperature, time, light) because the time of storage has made damages similar to those produced by thermal processing.

Regarding, the total phenolic and total flavonoid contents, the honey samples obtained from the South region (Algarve, Portugal) showed a relatively good amount in comparison with the reported in other studies cited; considering that samples of honeys have time of storage of three years in conditions wich ensured and minimized any alterations.

A moderate correlation was found between the antioxidant activity of honey and its total phenolic and total flavonoid contents, indicating that the antioxidant capacity of honey is mainly due to the presence of these components and interactions with other compounds. The little differences showed in antioxidant activity according to the areas of sampling, can be attributed to the fact that not all plant products bear the same phenolic composition and not all phenolics possess the same antioxidant capacity. Therefore, it is not the quantity but the quality of polyphenols, which serves as the major determinant of the antioxidant capacity of food.

CHAPTER 4

CHARACTERIZATION OF PHYSICOCHEMICAL PARAMETERS AND BIOACTIVE COMPOUNDS OF STRAWBERRY TREE (*Arbutus unedo* L.) HONEY

"Spider caves" art ruprecht

Chapter Four: Characterization of physicochemical parameters and bioactive compounds of the strawberry tree (*Arbutus unedo* L.) honey.

4.1. Introduction

The strawberry tree (*Arbutus unedo* L., Ericaceae) is a typical evergreen plant that thrives in the Iberian Peninsula and in the Mediterranean Basin, as well as in other regions with hot summers and mild rainy winters; it is native of Greece, Lebanon, Ireland, Turkey, Southern Europe and the Sardinian island (Italy). In Portugal this species is mainly implanted in the South (Algarve); "Medronho" is the local name of the strawberry tree (Celikel et al., 2008, Tuberoso et al., 2010; Gomes et al., 2010b; Oliveira et al., 2011). This plant is a perennial shrub that has an ornamental value (height about 7–9 m) due to its attractive fruits in the fall and winter, and pinkish-white flowers in the fall, this plant has strong resistance to hard environmental conditions (Celikel et al., 2008). The appearance of fruits is spherical with a diameter of about 2–3 cm, have an orange-red colour and are tasty only when fully ripe; they are characterized by an excessive sugar content (42–52%), are a good source of minerals, specially rich in calcium, vitamin C, and the total phenols have been estimated to be 14.6 mg/dried fruit, of which 1.01 mg/g are anthocyanins (*e.g*. cyanidin was identify as the main contributor to the red colour) (Özcan and Hacıseferoğulları, 2007; Pallauf et al., 2008). The bitter taste of fruits can be attributed to tannins, together with other phenolic compounds. Its fruits are processed into traditional products such as alcoholic beverages, jams, jellies and marmalades, and are also consumed as fresh fruit (Pallauf et al., 2008; Tuberoso et al., 2010; Oliveira et al., 2011).

The fruits are know in folk medicine as antiseptic, diuretic, and laxative, while the leaves are used as astringent, diuretic, urinary anti-septic, antidiarrheal, depurative and more recently in the theraphy of hypertension, diabetes and in the treatment of inflammatory diseases. Several studies showed that the leaf extract contains several phenolic compounds, like tannins, flavonoids, phenolic

glycosides, as well as α-tocopherol (Özcan and Hacıseferoğulları, 2007; Oliveira et al., 2009; Takrouni and Boussaid, 2010).

The flowers (a bit-like hot-air balloons), are produced in clusters (15–30 flowers) on red stems, are bell-shaped, lobes recurved, white or pink and nectar-scented. These flowers are significant sources of the nectar for bees, which can be used to transform into for strawberry tree honey (Celikel et al., 2008; Takrouni and Boussaid, 2010).

Honey is a natural substance produced by honeybees (*Apis mellifera* L.) from flower nectar or from honeydew, has a high nutritional and prophylactic-medicinal value. It is a source of readily available sugar, organic acids, some amino acids, macro- and microelements and biologically active substances (Juszczak and Fortuna, 2006). The composition of honey is influenced by the floral source, climate and environmental conditions, and beekeeper skills. Therefore, in every region of the World, the composition of honeys is different (Bhandari et al., 1999). Nowadays the current tendency is to define the differencing character of honeys from different sources in order to obtain a standard of quality and authenticity for this product allowing it to be competitive in the market (Castro-Vázquez et al., 2010).

Melissopalynological analysis, especially the identification and quantification of pollen grains in honey sediment, is the reference method used to determine the botanical origin of honey samples. This method shows various limitations depending on the beekeeping techniques (extraction, filtering, etc), size of the pollens, the flower morphology, which may affect nectar contamination in the flowers, and consequently the pollen grains in the honey sediment compared to its respective nectar. Besides, this technique is time consuming and requires special expertise and skill to identify each of the different pollens present in the honey samples (Cabras et al., 1999; Bianchi et al., 2005). Normally, honeys are classified as unifloral, when the pollen frequency of one plant is over 45%

(predominant pollen). In some cases, the botanical classification is carried out in a different manner when the pollen grains are "under- or over-represented" in relation to the nectar their flowers yields. For strawberry tree honey to be considered as unifloral it must show a frequency between 8–20% of pollen grains of *Arbutus unedo*, because of its under–represented sediment, due to low pollen content (Felsner et al., 2004; Von Der Ohe et al., 2004).

Some of the popular types of honey available in Portugal are of unifloral origin, which include heather (*Erica* sp.), rosemary (*Rosmarinus officinalis*), sunflower (*Helianthus annuus*) and orange (*Citrus* spp) are high in demand, because consumers prefer honeys clear yellow with a sweet flavour. A small amount of strawberry tree honey is made in the South of Portugal. This kind of honey is a low artisanal production from which very little is known in terms of physicochemical and bioactive properties, both at local and at international level. It is moderately appreciated by local consumers in comparison with other honeys; due to its distinct fragrance and bitter aftertaste, this honey is known locally as "bitter honey" (Andrade et al., 1999; Martins et al., 2008). The sensory characteristics are easily recognizable: it is amber-colored when liquid and beige-brown when crystallized; the smell is intense, characteristic, ripe, and similar to that of coffee; the taste is slightly sweet initially and decidedly bitter and astringent later (Tuberoso et al., 2010). This kind of honey is also produced in the Sardinian island where it is widely appreciated, with market prices being from 4 to 8 times higher than those of a sweet honey. Because it has been shown that it is a good source of bioactive compounds (antioxidant and antimicrobial compounds), consumers are willing to pay a high value for this product (Bianchi et al., 2005; Tuberoso et al., 2010; Rosa et al., 2011). The number of customers that attach a great importance to the origin of food products as an indicator of quality is remarkable. Many consumers seek high quality products with a clear regional identity of the provenance areas, therefore for the apiculture industry it is in their best interest to offer honeys with specific geographical characteristics, bioactive compounds and superior quality (Castro-Vázquez et al., 2010).

Honey is considered as a natural product part of traditional medicine since ancient times and is used as a food preservative and sweetening agent (Ferreira et al., 2009; Feás et al., 2010). The beneficial role of honey is partially attributed to its bioactive compounds, such as antioxidants. The amount and type of this antioxidants depends largely upon the floral source/variety of the honey and diverse researches have demonstrated a correlation between its antioxidant activity and total phenolic content (Aljadi and Kamaruddin, 2004; Ferreira et al., 2009; Biesaga and Pyrzynska, 2009; Brudzynski and Miotto, 2011b, 2011c; Rosa et al., 2011). The phenolic compounds are considered as beneficial for human health, since they decrease the risk of degenerative diseases by reducing oxidative stress and inhibiting macromolecular oxidation. It has been demonstrated that they possess free radical-scavenging and metal-chelating activity in addition to their reported anticarcinogenic properties (Antony et al., 2002; Pyrzynska and Biesaga, 2009; Morais et al., 2011).

The strawberry tree honeys made in Portugal have been little studied, in comparison with famous Sardinian honeys, and scientific data for this type of honey is rare in the literature. The aim of this work is to allow a better knowledge of the characterization of physicochemical parameters and bioactive compounds of strawberry tree honey produced in the Algarve and, if possible, to increment its demand.

4.2. Experimental

4.2.1. Honey samples

The analysis of the strawberry tree (*Arbutus unedo* L.) artisanal honey samples (figure 4.1) were harvested in the January of 2010 in the South of Portugal (specifically inside São Brás de Alportel) directly from a local beekeeper. Analysis of each sample was carried out in triplicate for each test. All samples were obtained by centrifugation, unblended with other honeys and unpasteurized. Honey samples (n = 7) were stored at room temperature (20 ± 3 ºC) until analysis.

Figure 4.1 Photography of a strawberry tree honey sample obtained from the South of Portugal.

4.2.2. Melissopalynology (pollen analysis)

The honey unifloral types were determined by pollinic analysis after acetolysis (Louveaux, et al., 1978; Lieux, 1980; Hesse and Waha, 1989; Von Der Ohe et al., 2004). This work was carried at the laboratories of the Centro de Química de Vila Real (CQVR) at Universidade de Trás-os-Montes e Alto Douro (UTAD), with the help of Engineer Miguel Maia. A 20 g sample of honey was dissolved with 40 mL of warm distilled water (40 ºC). The honey solution was stirred, transferred to centrifuge tubes and centrifuged for 15 min at 5000 rpm. The supernatant liquid was decanted to the last drop by tilting the tube at a 45º angle. Ten mL of acetolysis solution (1 mL of H_2SO_4 to 9 mL of $C_4H_6O_3$), were added to the remaining sediment in each tube and the tubes were placed in a water-bath at 70 ºC for 10 min. These were centrifuged again for 10 min at 4500 rpm, all of the supernatant liquid removed and a drop of liquefied Kaiser´s glycerol gelatin (Merck, Darmstadt, Germany) mixtured with the sediment (pollen grains). The entire sediment was placed on a slide and spread out over an area of approximately 20 x 20 mm. For each sample, at least 800 pollen grains were counted. The pollen grains were grouped by pollinic types, according to Valdés et al., (1987).

4.2.3. Physicochemical parameters

4.2.3.1. Reducing sugars

Reducing sugars were determined using 3,5-dinitrosalicyllic acid (DNSA). A 1 mL of the honey solution (1 mg/mL) was mixed with equal amount of DNSA solution and incubated in a boiling water bath for 10 min (Saxena et al., 2009). The mixture was allowed to cool to ambient temperature, mixed with 7.5 mL of distilled water and the absorbance was measured at 540 nm using a UV-VIS spectrophotometer Hitachi U-2000 (Tokyo, Japan). A standard glucose stock solution, within the range of 0 to 1000 µg/mL, (R^2 = 0.9945), was used as a standard curve.

4.2.3.2. Optical rotation (OR)

OR was measured polarimetrically using the method recommended by the International Honey Comission (Bogdanov, 2002). Approximately 12 g of honey sample (corresponding to about 10 g of dry substance/100 mL) were clarified first with Carrez I reagent (10 mL, for 30 s), and then with Carrez II (10 mL, for 30 s). Distilled water was then added to a final volume of 100 mL and left for 24 h. The solution was filtered and read out in an Atago® Polax-D polarimeter (Tokyo, Japan). The optical rotation $(\alpha)_D^{20}$, expressed as the angle of rotation of the polarized light at the wavelength of the sodium D line (λ = 589.3 nm) at 20 ºC, was calculated using equation (4.1):

$$(\alpha)_D^{20} = (\alpha \cdot 100)/(L \cdot p) \qquad (4.1)$$

Where: $\boldsymbol{\alpha}$ = angular rotation determined; $\boldsymbol{L}$ = length in decimeters of the polimeter tube; $\boldsymbol{p}$ = mass, expressed as grams of dry honey.

4.2.3.3. Colour by absorbance

The absorbance of a solution of 50% honey in water (w/v) was read at 635 nm in a 1 cm cell, in a double beam UV/VIS spectrophotometer Hitachi U-2000 (Tokyo, Japan) (Ferreira et al., 2009). The honeys were classified according to the Pfund scale, after conversion of the absorbance values, using the following equation (4.2) and values were classified according table 4.1 (White, 1984):

$$(mm\ Pfund) = -38.70 + (371.39 \cdot \Delta A_{635}) \qquad (4.2)$$

Table 4.1. Honey colour expressed in absorbance and Pfund scale (from Naab et al., 2008).

Honey colour	Absorbance	Pfund scale (mm)
Water white	0.104–0.125	< 8
Extra white	0.125–0.148	9–17
White	0.148–0.195	18–34
Extra light amber	0.195–0.238	35–50
Light amber	0.238–0.333	51–85
Amber	0.333–0.411	86–114
Dark amber	> 0.411	> 114

4.2.3.4. Diastase activity with Phadebas

The diastase activity was measured using the Phadebas amylase test tablets purchased from Magle (Lund, Sweden), according to the International Honey Commission (Bogdanov, 2002). An insoluble blue dyed cross-linked type of starch was used as the substrate (Tosi et al., 2008). This is hydrolysed by the enzyme, yielding blue water-soluble fragments, which were determined spectrophotometrically at 620 nm. The absorbance of the solution, which is directly proportional to the diastase activity of the sample, was determined using a Hitachi U-2000 double beams UV/VIS spectrophotometer (Tokyo, Japan). Diastase activity was obtained from the absorbance measurements by using the equations:

$$DN = (28.2 \cdot \Delta A_{620}) + 2.64 \qquad (4.3)$$

and

$$DN = (35.2 \cdot \Delta A_{620}) - 0.46 \qquad (4.4)$$

For either high (8 to 40 diastase units) or low (up to 8 diastase units), respectively. Eqs. (4.3) and (4.4) are suggested by the International Honey Commission (Bogdanov, 2002). Diastase activity was referred to as diastase number (DN) in the Schade scale, which corresponds to the Gothe scale number, or to g of starch hydrolysed per hour at 40 ºC, per 100 g of honey.

4.2.3.5. Viscosity

The rheological properties were determined using a rotational rheometer Brookfield DV-II (Brookfield Engineering Labs Inc., Stoughton, Massachusetts, USA) with a system of coaxial cylinders (cup diameter 19.10 mm, bob diameter 9.39 mm and 24.23 mm length). The presence of air bubbles and crystals can affect the viscosity measuring of honey. Prior to measurement approximately 15 g of each honey sample were warmed at a temperature of 45 ºC during at least three hours, to dissolve the crystals. Then the samples were placed in the measuring element of the rheometer and thermostated (water bath) to reach the desired temperature of measurement. The determination of viscosity was performed at different shear rates, ranging from 1–50 rpm, and temperatures ranging from 20 to 40 ºC (at 5 ºC intervals).

The other methods for the determination of physicochemical parameters: ash content, electrical conductivity, pH, total acidity, colour by colorimeter, moisture content, total soluble solids, water activity and HMF content were performed as described in Chapter 3, section 3.2.2.

4.2.4. Bioactive compounds

The determination if bioactive compounds (total phenolic and total flavonoid contents, antioxidant activity and scavenging radical) were performed as in Chapter 3, section 3.2.3.

4.3. Statistical analysis

The data analysis for the results obtained for strawberry tree artisanal honey samples of all parameters (physicochemical and bioactive compounds), were carried out employing Statgraphics® Centurion XV (StatPoint, Virginia, USA) using analysis of variance and Student´s t-test used to examine differences between Italian and Portuguese honey´s. The results are expressed as mean value ($\bar{x}$) and standard deviation (*SD*). Differences were considered significant at $p < 0.05$.

4.4. Results and discussion

4.4.1. Pollen analysis

The results of microscopic analysis of the honeys analyzed are summarized in figure 4.2. Thirty one pollen types were found in the samples. Pollen from *Echium plantagineum, Ceratonia siliqua, Cistaceae, Arbutus unedo, Lavandula stoechas, Reseda luteola* and *Citrus* spp. types were present in all of them (86%). *Arbutus unedo* L. was the predominant pollen in all samples, accounting for more than 20% of the pollen. Von Der Ohe et al., (2004) established that for a honey to be considered a strawberry tree honey classified as unifloral, this sample must show a range of pollen grains between 8 to 20% of this type of flower (lower pollen content under-represented, percentages of pollen grains < 45%) (Andrade et al., 1999; Persano Oddo et al., 2004; Bianchi et al., 2005; Morais et al., 2011). Therefore, all samples correspond to unifloral *Arbutus unedo* L. honey (with a mean pollen content of 31.96%; figure 4.3). Similar results were also obtained by Tuberoso et al., (2010). Some samples showed secondary pollens (range 16–45% pollen content) these were *Cistaceae* (20.45%) and *Lavandula stoechas* (15.05%). Other important minor pollens (range 3–15%) found were *Ceratonia siliqua* (5.37%), *Cytisus scoporius* (5.11%), *Echium plantagineum* (4.52%), *Citrus* spp. (3.92%) and *Reseda luteola* (3.92%). The minor pollen (< 3%) was composed by *Pistacia terebinthus* (1.98%), *Olea europea* (1.87%), *Calendula arrensis* (1.53%), *Myrtus* spp (1.36%) and *Rosmarinus officinalis* (0.85%).

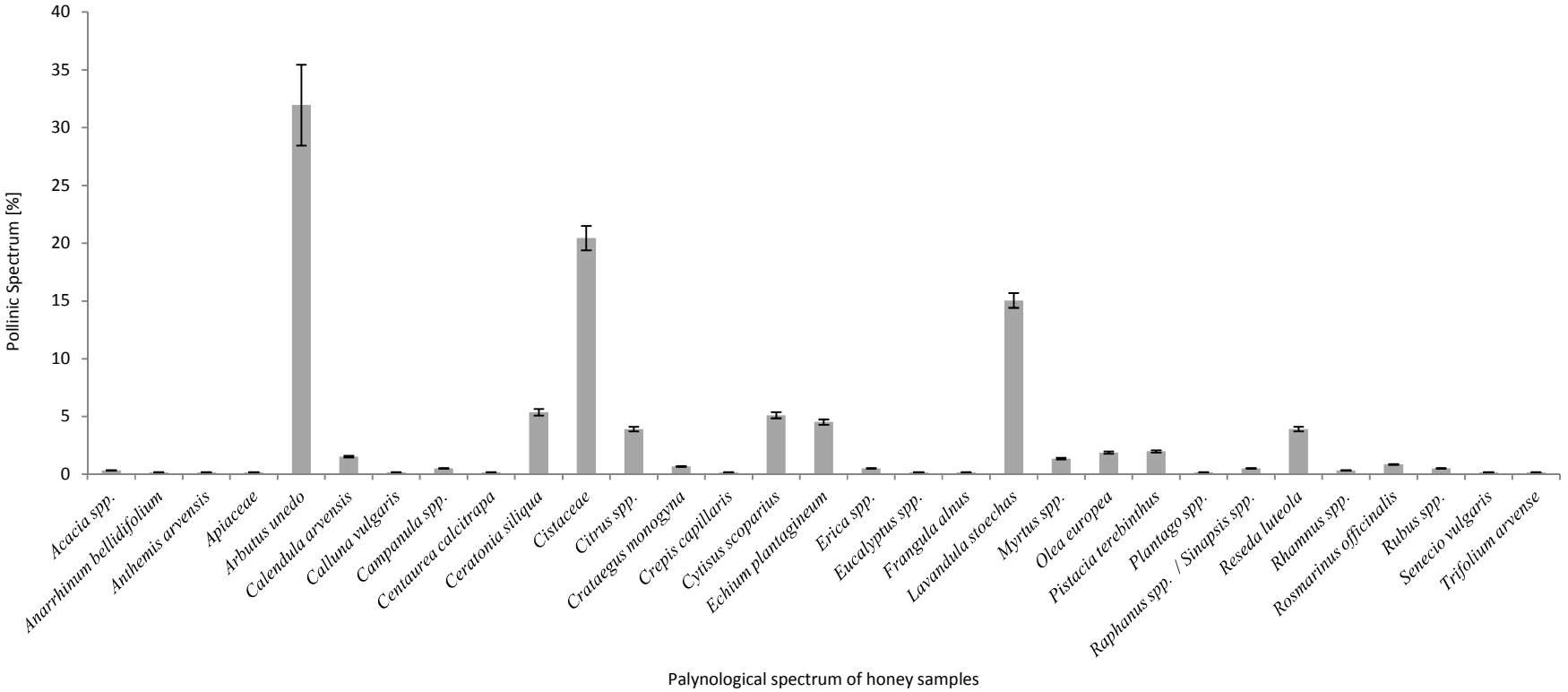


Figure 4.2 Palynological spectrum of the strawberry tree honey samples analyzed.

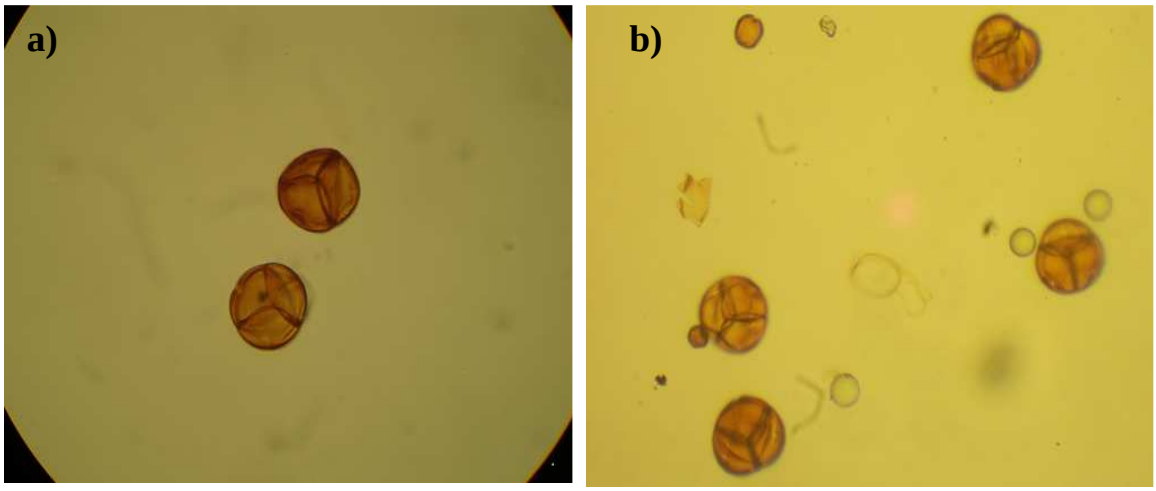


Figure 4.3 Photography of pollens *Arbutus unedo* L. from flowers (a) and honey (b) at 40x under light microscopy.

4.4.2. Physicochemical parameters

The following tables (from 4.2 to 4.7) shows the results obtained for each physicochemical parameter analyzed ($\bar{x}$ ± *SD*, range). All honey samples have values within the legal parameters established by international regulations (EU, 2001; Codex Alimentarius Commission, 2001), except for diastase activity (> 8 Gothe degrees).

- ***Ash content and electrical conductivity***

The ash content for of the honey samples analyzed shows a mean value of 0.58% and the electrical conductivity (EC) value was 0.64 mS/cm (table 4.2). In comparison with results obtained by Persano Oddo et al., (1995) for strawberry tree honey from Italy, values of the ash content were 0.32 ± 0.05% (significantly differences $p < 0.05$) and the EC was 0.74 ± 0.07 mS/cm (not significantly differences $p < 0.05$), respectively. Generally, the ash content of nectar honey is ≤ 0.6% when compared to honeydew honey or blends of honeydew where this value is ≥ 1.2%; on the other hand, the electrical conductivity for nectar honey is ≤ 0.8 mS/cm and for honeydew ≥ 0.8 mS/cm (EU, 2001; Codex Alimentarius Commission, 2001). The EC of honey is due to presence of minerals, organic acids, protein, sugar composition, and is a good tool to

distinguish between floral and honeydew honey, according to the present standards (Saxena et al, 2009; Kaškonienė et al., 2010b). Some nectar honeys, for example strawberry tree (*Arbutus unedo*), bell heather (*Erica*), eucalyptus (*Eucaliptus*), lime (*Tila* spp.), ling heather (*Calluna bulgaris*), manuka (*Leptospermum*) and tea tree (*Melaleuca* spp.) can show higher values of EC (Codex Alimentarius Commission, 2001; EU, 2001; Bogdanov, 2002). These two parameters have a linear relationship ($EC = [0.066 \cdot Ash\ content] + 0.56$) with increased ash contents being accompanied by an increase of EC (R^2 = 0.740; figure 4.4). This has been reported by several authors (Sancho et al. 1991a, 1991b; Feás et al., 2010; Gomes et al., 2010a). The ash content gives a direct measure of the inorganic residue after carbonization, while the electrical conductivity measures all the ionizable organic and inorganic substances (Felsner et al., 2004; Silva et al., 2009; Feás et al., 2010). The mineral content depend of various factors such botanical origin, geographical conditions, soil composition, environmental pollution and extraction techniques (Conti, 2000; Nanda et al., 2003).

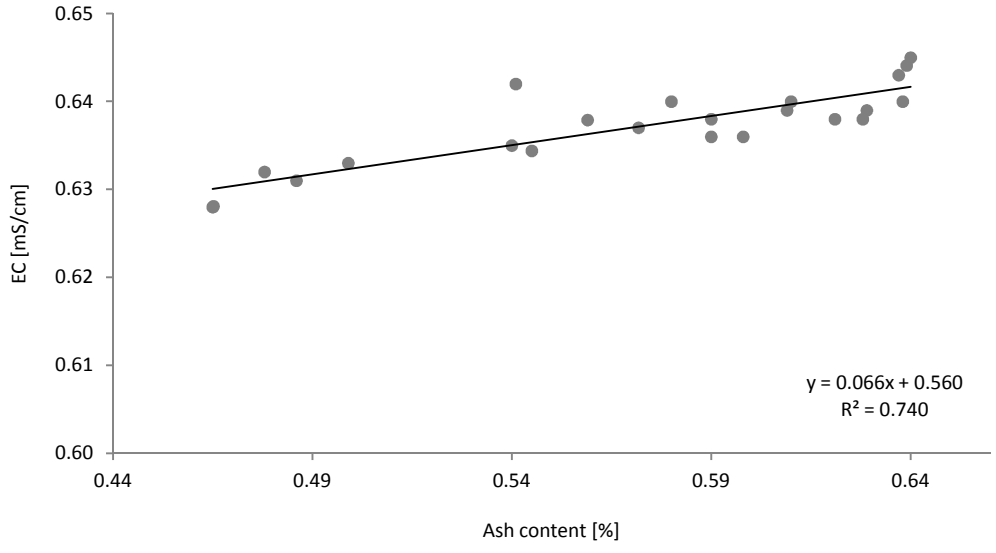


Figure 4.4 Linear regression of ash content (%) and electrical conductivity (EC; mS/cm) for the strawberry tree honey.

Table 4.2 Results for the ash content (%) and electrical conductivity (EC, mS/cm) determined in strawberry tree honey from the South of Portugal.

Parameters	**Range [min - max]**	$\bar{x} \pm SD$
Ash content (%)	0.465–0.640	0.58 ± 0.10
EC (mS/cm)	0.628–0.645	0.64 ± 0.01

- ***Moisture content (MC)***

The MC for the strawberry tree honeys analyzed was close to the limits (but did not exceeded 20%) established by EU (2001) and Codex Alimentarius Commission (2001), samples showed a mean value of 19.87% (table 4.3). This parameter is an important one to be considered for shelf-life. For high values of moisture it is possible to obtain fermentation by osmophilic yeast, reducing the overall quality of the honeys. This also indicates a premature extraction or extraction under high humidity conditions. Osmophilic yeast need high sugar concentrations and are able to grow to a minimal water for high sugar concentration and are to able to grow to a minimal water activity until 0.6 (Feás et al., 2010). The author Persano Oddo et al., (1995) found mean moisture of 18.9 ± 1.9% for the Italian honeys on this study (not significantly differences p <0.05). The water content depends on the botanical origin of honey, atmospheric conditions, the degree of maturity reached in the hive, the season of production, human manipulation, the processing techniques and storage conditions (*e.g.* temperature, light, O_2) and so it influences the quality of honey (Finola et al., 2007).

- ***Total soluble solids (TSS)***

TSS content of the honeys samples showed a mean value of 78.35 ºBrix (table 3.3) variations in total soluble solids are found to be dependent on climate and floral source amongst other factors. There is a strictly linear relationship: $TSS = (-0.914 \cdot Moisture\ content) + 96.46$ (figure 4.5) between moisture and total soluble solids (Silva et al., 2009). Conti (2000) confirmed that anomalous values may be a reliable index for adulterations.

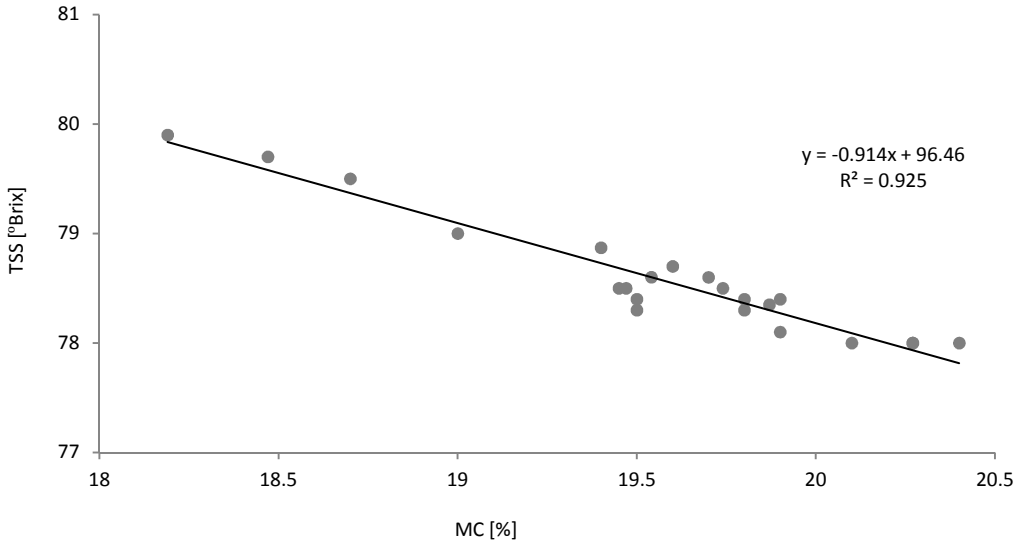


Figure 4.5 Linear relationship between moisture content (MC, %) and total soluble solids (TSS, ºBrix) for strawberry tree honey samples.

- ***Water activity (a_w)***

For the water activity of the strawberry tree honey an average value of 0.65 was found (table 4.3); normally the water activity is within a range of 0.5–0.6 (Gleiter et al., 2006; Chirife et al., 2006; Zamora et al., 2006; Abramovič et al., 2008; Gomes et al., 2010a). The a_w parameter is more important in the inhibition of microorganism development and enzyme activity; this is mainly determined by the presence of soluble chemical species (mainly fructose and glucose); the differences between water activity values of the different honey types is the result of its diverse sugar composition (Gleiter et al., 2006). The osmophilic yeasts are only able to grow above minimal water activities of 0.60 and are specialists in surviving under conditions of high sugar concentrations. *Saccharomyces* spp. is responsible for generating honey fermentation (Saxena et al., 2009).

Table 4.3 Results for the moisture content (MC, %), total soluble solids (TSS, º Brix), water activity and reducing sugars (mg/100 g of honey) determined in strawberry tree honey from the South of Portugal.

Parameters	**Range [min - max]**	$\bar{x} \pm SD$
MC (%)	18.19–20.27	19.87 ± 0.57
TSS (ºBrix)	78.0–79.9	78.35 ± 0.49
a_w	0.647–0.649	0.65 ± 0.00
Reducing sugars (mg/100 g of honey)	70.84–78.38	73.64 ± 4.12

- ***Reducing sugars content***

The reducing sugars in strawberry tree honey was 73.64 ± 4.12 mg/100 g of honey (table 4.3), similar results were also found by Persano Oddo et al., (1995) for strawberry tree Italian honey with a mean 69.3 ± 3.1 (not significantly differences p <0.05). Standards of EU (2001) and Codex Alimentarius Commission (2001) require the following: for nectar honey must be ≥ 60 g/100g of honey, in honeydew honey must be ≥ 45 g/100g of honey. Reducing sugars, which include mainly glucose and fructose, are the major constituents of honey (Kaškonienė et al., 2010b). The carbonyl groups of reducing sugars can react with free amino residues from aminoacids/proteins generating melanoidin compounds, which are formed at several stages during the Maillard reaction, including Amadori compounds to amino reductoes or reductones and the formation of polymers. These compounds have a brown coloration and have been demonstrated to possess a strong antioxidant activity, therefore they contribute to a product with more active compounds (Viuda-Martos et al., 2008; Ferreira et al., 2009; Brudzynski and Miotto, 2011b, 2011c; Jerković et al., 2011).

- ***Optical rotation (OR)***

Regarding the optical rotation $(\alpha)_D^{20}$, all samples present a laevorotatory behavior (negative values) characteristic of nectar honeys, showing an average value of -6.06º (table 4.4). Normally the honey that shows this OR has a higher fructose content, but the overall optical rotation

depends on the concentration of the various sugars presents (Dinkov et al., 2004; Diminš et al., 2008). Similar results were also obtained by Persano Oddo et al., (1995), who found similar mean values of -13 ± 1.8º for Italian strawberry tree honey (significantly differences p <0.05).

Table 4.4 Results for the optical rotation (OR; $(\alpha)_D^{20}$), colour by colorimeter (L^*, a^*, b^*) and absorbance (mm Pfund) determined in strawberry tree honey from the South of Portugal.

Parameters	**Range [min - max]**	$\bar{x} \pm SD$
OR $(\alpha)_D^{20}$	-6.37 – -5.50	-6.06 ± 0.36
Colour by colorimeter		
L^*	31.84–51.72	42.03 ± 10.05
a^*	-0.48 – -0.1	-0.27 ± 0.10
b^*	8.11–16.48	12.42 ± 3.91
Colour by absorbance (mm Pfund)	18.49–29.26	23.07 ± 5.56

- ***Colour***

The colour of the samples was classified according to their absorbance (table 4.4) as white, with a mean value of 23.07 mm Pfund, which was lighter than the samples studied by Persano Oddo et al., (1995), who classified their honeys as light amber (70 ± 10 mm Pfund; significantly differences p <0.05). Honey samples, when analyzed with a colorimeter, showed values of L^* = 42.03 (lightness), a^* = -0.27 (greenness), and b^* = 12.42 (yellowness), with this last component being the one that mostly influenced in the colour, in general. According to the classification by González-Miret et al., (2005), strawberry tree honeys can be placed in the groups of dark honey (L^* < 50).

Colour parameters depend on various factors, with their mineral content being an important one. Light honeys usually have low ash contents, while dark honeys generally have higher ash contents (Gomes et al., 2010a). Sometimes the melanoidins (Maillard reaction) concentrations which have a brown colour also plays an important role to some foods (Jimenez et al., 1994; Gonzales et al., 1999).

- ***pH***

The pH of the analyzed honey samples had an average value of 4.52 (table 4.5). This parameter is of a great importance during extraction and storage, since acidity can influence the texture, stability, and shelf-life of honey (Terrab et al., 2004a; Feás et al., 2010; Idris et al., 2011). This value is in accordance with the acceptable range for nectar honey (from 3.2 to 4.5); significantly differences ($p < 0.05$) results were also obtained by Persano Oddo et al., (1995) for strawberry tree honey (from 4.0 to 4.4, a mean 4.2 ±0.1).

Table 4.5 Results for the pH and total acidity (meq/kg) determined in strawberry tree honey from the South of Portugal.

Parameters	**Range [min - max]**	$\bar{x} \pm SD$
pH	4.47–4.57	4.52 ± 0.05
Total acidity (meq/kg)	24.63–29.16	26.76 ± 2.28

- ***Total acidity***

The total acidity of all samples were within the International legislation (Codex Alimentarius Commission, 2001; EU, 2001), which required a value of $\leq$ 50 meq/kg. Honey samples studied in the present work showed a mean value of 26.76 meq/kg (table 4.5). Strawberry tree honey obtained from Italy showed a range of values from 27.3 to 53.4 meq/kg, with a mean 39.6 ± 8.3 meq/kg (Persano Oddo et al., 1995, not significantly differences p <0.05). The presence of organic acids, particularly gluconic acids produced from nectar during ripening by glucose oxidase, in equilibrium with their lactones or esters, and inorganic ions, such as phosphate and chloride, contribute to the acidity of honey (Ajlouni and Sujirapinyokul, 2010; Kahraman et al., 2011). High acidity can be indicative of fermentation, since the yeasts and molds are capable of developing in an acidic environment and do not grow well in alkaline media (Silva et al., 2009).

- ***Diastase activity (DA)***

The diastase activity and hydroxymethylfurfural content are widely recognized as parameters indicating the freshness and/or overheating of honey (Gomes et al., 2010a; Khalil et al., 2010).

For DA, samples of honey have showed a mean value of 3.58 Gothe (table 4.6). This enzymatic activity is much lower than the limits established by International regulation, which should not be less than 8 Gothe degrees (Codex Alimentarius Commission, 2001; EU, 2001). However, similar results (not significantly differences p <0.05) were also obtained by Persano Oddo et al., (1990; 1995), with the honeys analyzed showing a range of 0 to 9.2 with a mean of 5.2 ± 2.8 Gothe. The activity depends upon geographic and floral origins of the product, as well as on its freshness (Gomes et al., 2010a). Normally the thermal treatment (*e.g.* pasteurization) decreases the diastase activity (Castro-Vázquez et al., 2008; Tosi et al., 2008; Qamer et al., 2009; Turhan, 2009; Ajlouni and Sujirapinyokul, 2010).

Table 4.6 Results for the diastase activity (Gothe) and hydroxymethylfurfural content (mg/kg) determined in strawberry tree honey from the South of Portugal.

Parameters	**Range [min - max]**	$\bar{x} \pm SD$
DA (Gothe)	3.4–3.7	3.58 ± 0.13
HMF content (mg/kg)	13.46–17.44	16.11 ± 2.30

- ***Hydroxymethylfurfural (HMF) content***

For the HMF content of the honeys analyzed an average value of 16.11 mg/kg was obtained (table 4.6). Persano Oddo et al., (1995) who found the HMF level for Italian honeys with a mean of 4.4 ± 3.2 mg/kg (significantly differences p <0.05). The Codex Alimentarius Commission (2001) and EU (2001) established the maximum HMF level allowed in honey as 40 mg/kg, with the following exceptions: 80 mg/kg for honey from countries with tropical temperatures, and 15 mg/kg for honey with a low enzymatic level, respectively. Several factors influence the formation of HMF in honey. These factors may include the use of metallic

containers, the physicochemical parameters (pH, total acidity, mineral content) of honey itself, which are related to the floral source from which the honey has been extracted, the humidity and from thermal and/or photochemical stress. The hydroxymethylfurfural formation results from the acid-catalyzed dehydration of hexose sugars, with fructose being particularly susceptible (Spano et al., 2006; Khalil et al., 2010; Capuano and Fogliano, 2011).

Taking into account the results obtained for the above physicochemical parameters: ash content, electrical conductivity, pH, optical rotation, reducing sugars; and so as for melissopalynological analysis, showed clearly shows that all honey samples studied were nectar honeys.

- ***Viscosity***

Table 4.7 shows of the results for the viscosity (Pa s) and the activation energy (kJ/mol) of strawberry tree honey at different temperatures. The viscosity is an important factor that concerns both processing parameters of honey production (*e.g.* velocity of centrifuging or filtering) and its sensory properties perceived by consumers (Witczak et al., 2011). The rheological properties of honey depend on many factors, such as its composition and temperature, but the major is the water content. Also, the composition of individual sugars, and the amount and type of colloids are important (Abu-Jdayil et al., 2002; Juszczk and Fortuna, 2006).

Table 4.7 Results for the viscosity (Pa s) and activation energy (E_a, kJ/mol) of strawberry tree honey at different temperatures.

	Temperature (º C)				
Parameters	**20**	**25**	**30**	**35**	**40**
η (Pa s)	30.11	15.66	8.67	3.48	1.45
R^2	0.998	0.997	0.998	0.998	0.999
E_a (kJ/mol)	115.24				
R^2	0.988				

For the strawberry tree honey studied, the behaviour flow was adjusted with the Newtonian model at different temperatures (figure 4.6). The model was : $\tau = \eta\dot{\gamma}$ (equation 4.5); where $\tau$is shear stress (Pa), η is the viscosity (Pa s) and $\dot{\gamma}$ is shear rate (s^{-1}); this was found to be the best-fit model for the flow curves at different temperatures. Similar results were reported by others authors after analysis of diverse types of honeys (*e.g.* acacia, buckwheat, linden, multifloral, citrus, apple, honeydew) (Mossel et al., 2000; Zaitoun et al., 2001;Abu-Jdayil et al., 2002; Sopade et al., 2002; Lazaridou et al., 2004; Juszczak and Fortuna, 2006; Recondo et al., 2006; Yanniotis et al., 2006).

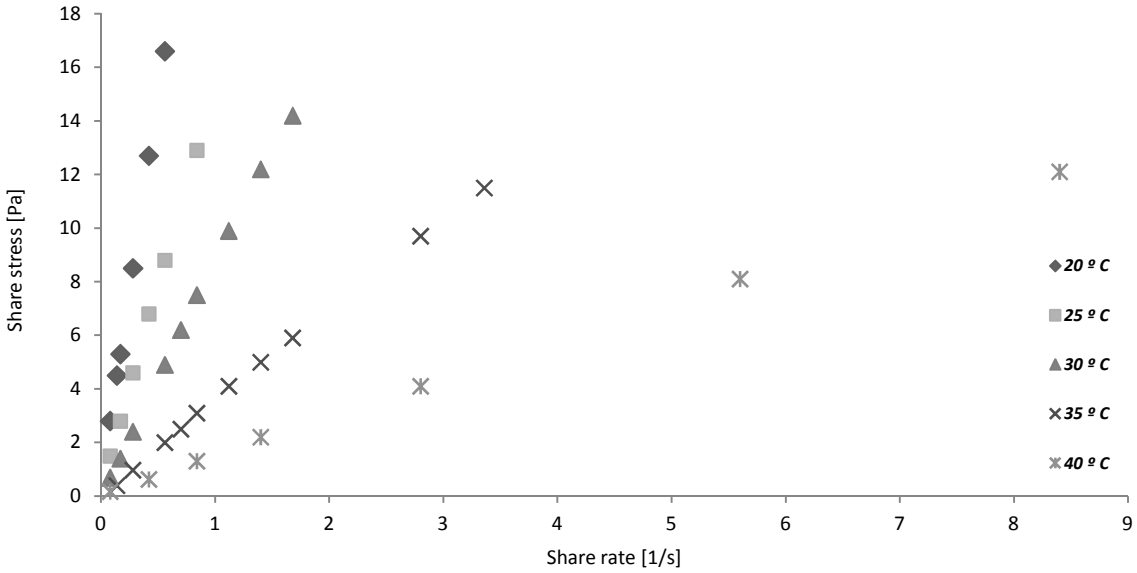


Figure 4.6 Flow curves of strawberry tree honey at different temperatures.

Several authors have reported that types of honey showed a Non-Newtonian behaviour, this flow can be generated by crystal presence, non-sugar compounds, colloidal material and presence of high-molecular compounds (*e.g.* proteins or dextran) (Mossel et al., 2003; Gómez-Díaz et al., 2004; Ahmed et al., 2007; Juszczk and Fortuna, 2006; Smanalieva and Senge, 2009; Gómez-Díaz et al., 2009).

The viscosity (figure 4.7) of strawberry tree honeys decreased with temperature rising; this effect was adjusted using Arrhenius model. The decrease of viscosity is a result of less molecular friction and diminishing of hydrodynamic forces decreased (Mossel et al., 2003; Recondo et al., 2006; Gómez-Díaz et al., 2009). The activation energy (E_a = 115.24 kJ/mol) was slightly, but similar values were obtained by Juszczk and Fortuna (2006) for nectar-honeydew (E_a = 105.25 kJ/mol) and linden honeys (E_a = 103.58 kJ/mol); and Mossel et al., (2000) found values for Australian honey between 66.32 to 124.48 kJ/mol. This parameter (E_a) reflects the sensitivity of viscosity to temperature changes; higher values means that the viscosity is relatively more sensitive to a temperature change (Lazaridou et al., 2004).

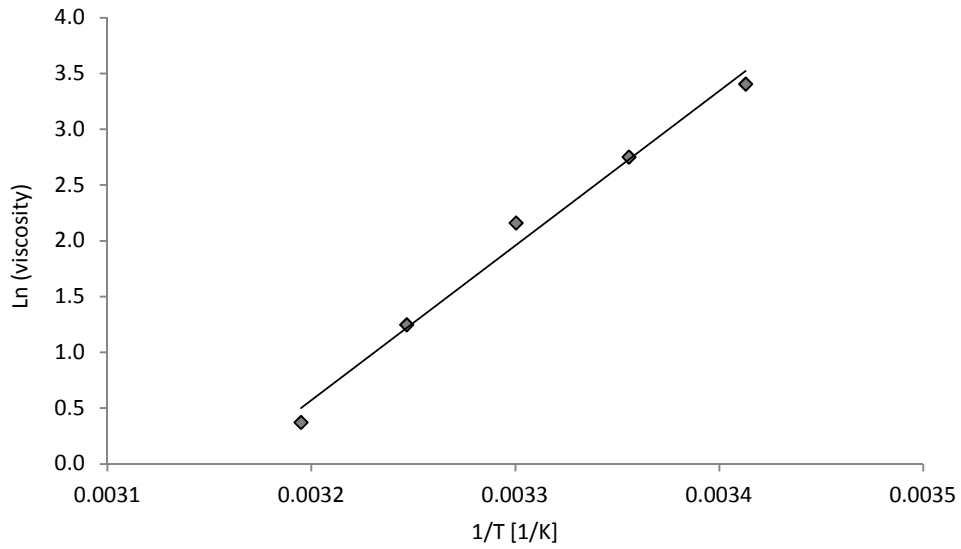


Figure 4.7 Arrhenius model fit for strawberry tree honeys.

4.4.3. Bioactive compounds

Table 4.8 shows the results obtained for the total phenolic content and total flavonoid content of the strawberry tree honey samples.

- ***Total phenolic content (TPC)***

This kind of honey has shown a high amount of TPC, 94.47 mg of gallic acid equivalents (GAE)/100 mg of honey (table 4.8). A similar level was also found by Rosa et al., (2011) who, for honey of the same floral origin found values of 97.2 mg of GAE/100 mg of Italian honey. Slightly lower results were obtained by Beretta et al., (2005) who found commercial strawberry tree honey samples with 78.96 mg of GAE/100 g of honey; when compared with other honeys from the North of Portugal analyzed by Ferreira et al., (2009) the results obtained for the phenolic content of rosemary (with 22.61 mg of GAE/100 g of honey); viper´s bugloss (with 40.62 mg of GAE/100 g of honey) and heather (with 72.77 mg of GAE/100 g of honey), the strawberry tree honey had the highest values. Significant higher values were also found for different unifloral honeys from Slovenia (acacia, lime, chestnut, fir, spruce and forest) were found by Bertoncelj et al., (2007). Similarly, as above, high phenolic contents were found in other studies (Meda et al, 2005; Al et al., 2009; Lachman et al., 2010; Silici et al., 2010).

The variance values of phenolic content could be the consequence of the formation of compounds that could react as electron-donors, incrementing the real amount of phenol (Al-Mamary et al., 2002; Gheldof et al., 2002; Ferreira et al., 2009). Folin-Ciocalteu´s method (FCM) is general for the estimation of the total phenolic content; other components such as peptides, vitamins C, E, carotenoids, sugars, amino acids, proteins, organic acids or enzymes (glucose oxidase, catalase); can also influence the results but the components that influence more, by increasing the total phenolic value are the product of Maillard reaction (melanoidins), because several investigations had demonstrated that those compounds presented an effect that is similar to a reducing agent (mixture of phosphotungstic acid and phosphomolibdic acid)

(Alvarez-Suarez et al., 2009; Ferreira et al., 2009). Honey is very prone to Maillard reactions during storage or when subjected thermal processing. These occur when sugars condense with free amino acids leading to the formation of brown melanoidins (Turkmen et al, 2006; Rufián-Henares and Morales, 2007).

Table 4.8 Results for the total phenolic content (TPC, mg of GAE/100 g of honey) and total flavonoid content (TFC, mg QE/100 of honey) of the strawberry tree honey samples.

Parameters	**Range [min - max]**	$\bar{x} \pm SD$
TPC (mg of GAE/100 g of honey)	91.7–96.1	94.47 ± 2.37
TFC (mg of QE/100 g of honey)	4.09–5.77	5.33 ± 0.63

- ***Total flavonoid content (TFC)***

TFC for strawberry tree honey was 5.33 mg of QE/100 g of honey (table 4.8). This value is lower than those found by Pichichero et al., (2009) after analyzing honeys from different floral sources from Italy; those honeys showed a range of flavonoid contents from 6.73 mg quercitin equivalents (QE)/100 g of honey for acacia (*Robinia pseudoacacia*) to 21.16 mg of QE/100 g of honey for savory (*Satureja hortensis*). One kind of honey (sulla, *Hedysarum* sp.) showed the lowest TFC value 4.18 mg of QE/100 g of honey. As for the strawberry tree samples, they showed similar results to the reported for lime honeys, found by Al et al., (2009), which samples showed ranges between 0.91–2.42 mg of QE/100 g of honey (for *Acacia*), 4.70–6.98 mg of QE/100 g of honey (for *Tilia*); similar results were also found for honeys from Burkina Faso, 0.17–8.35 mg of QE/100 g of honey (Meda et al., 2005); Blasa et al., (2006) obtained a range of 1.23–2.93 mg of catequin equivalents (CEs)/100 g of honey. In comparison with Portuguese honeys, rosemary (12.4 mg of CEs/100 g of honey), viper´s bugloss (34.3 mg of CEs /100 g of honey) and heather (58.7 mg of CEs /100 g of honey), the strawberry tree honey TFC was significantly lower (Ferreira et al., 2009). Flavonoids inhibit auto-oxidation reactions and have a scavenging effect on free radicals, by different mechanisms (Isla et al., 2011).

Most of the phenolic compounds present in honey are in the form of flavonoids (quercitin, kaempherol, pinocembrin, etc) whose concentrations depend on various factors, including plant

species used by the bees, health of the plant, geographical origin and climatic characteristics of the production local, where the floral origin is the major factor responsible for biological activity, including antioxidant and antimicrobial, because the bioactive compounds of vegetal origin can be transferred to honey (Küçük et al., 2007; Baltrušaitytė et al., 2007; Viuda-Martos et al., 2008; Pichichero et al., 2009). Several researches on the phenolic fraction established homogentisic acid (2,5-dihydroxypenylacetic acid; HGA) as a useful marker to assess the botanical origin of strawberry tree honey. It has been demonstrated that HGA is the most abundant phenolic compounds in this type of honey, representing approximately 50–60% of the total phenols and certainly provides an important contribution to the high antioxidant and antiradical activities of strawberry tree honey (Cabras et al., 1999; Scanu et al., 2005; Tuberoso et al., 2010; Rosa et al., 2011).

- ***DPPH scavenging***

The strawberry tree honey obtained from the South of Portugal showed a mean value of DPPH scavenging of 43.46% (table 4.9). Similar results were found for Saxena et al., (2009) for commercial Indian honey samples that showed ranged from 44 to 71% for DPPH scavenging activity. The strawberry tree samples analyzed showed a higher value of scavenging activity in comparison with samples studied by Beretta et al., (2005). This author found values for this property near 1.63 mg/mL for DPPH scavenging of commercial strawberry tree honey, which showed clearly that kind of honey had higher radical-scavenging activity. Rosa et al., (2011) found values of the scavenging ability on DPPH radicals of 4.8 mmol trolox equivalent antioxidant capacity (TEAC)/kg of honey for strawberry tree honeys produced in Italy. Samples of the Portuguese honeys studied by Ferreira et al., (2009) showed significant higher scavenging activities of: 106.7, 130.5 and 168.9 mg/mL for heather, viper´s bugloss and rosemary, respectively. The DPPH free radical has the adventage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Estevinho et al., 2008; Silici et al., 2010).

The higher values of DPPH, the higher the antioxidant capacity of the honey samples. Several authors (Aljadi and Kamaruddin, 2004; Krpan et al., 2009; Saxena et al., 2009) have established a direct relationship between the antioxidant capacity of honey and the amount of bioactive compounds (*e.g.* phenol and flavonoids) present, along with other compounds also involved (Beretta et al., 2005; Blasa et al., 2006).

Table 4.9 Results for the DPPH scavenging (%) and antioxidant capacities (mg of AEAC/100 g of honey and mg of QEAC/100g of honey) of the strawberry tree honey samples.

Parameters	**Range [min - max]**	$\bar{x} \pm SD$
DPPH scavenging (%)	40.28–45.20	43.46 ± 4.86
Antioxidant capacity		
(mg of AEAC/100 g of honey)	18.01–18.25	18.15 ± 0.12
(mg of QEAC/100 g of honey)	9.64–9.78	9.72 ± 0.07

- ***Antioxidant capacity***

The antioxidant capacity of strawberry tree honey showed values of 18.15 mg of ascorbic acid equivalents antioxidant capacity (AEAC)/100 of honey and 9.72 mg of quercitin equivalents antioxidant capacity (QEAC)/100 g of honey (table 4.9). These values were similar to those obtained by different researchers, for example: Meda et al., (2005) for multifloral honey (4.27–17.30 mg of AEAC/100 g and 10.20–37.87 mg of QEAC/100 g of honey) and Saxena et al., (2009) who found values between 15.1 to 29.5 mg of AEAC/100 g of honey for Indian honeys. Many studies have demonstrated that honey serves as a source of natural antioxidants, which are effective in reducing the risk of heart diseases, cancer, immune-system decline, cataracts, different inflammatory process, etc. Among the mechanisms of action suggested are: free radical sequestration, hydrogen donation, metallic ion chelation, or their ability to act as a substrate for radicals such as superoxide and hydroxyl have been suggested (Viuda-Martos et al., 2008). The antioxidant activity of honey has been strongly correlated with the content of phenolic and flavonoid compounds, but they are not solely responsible for this activity as there are other constituents such as vitamins, carotenoids, melanoidins, enzymes, organic acids,

peptides and possibly other minor compounds possessing antioxidant capacity (Estevinho et al., 2008; Rosa et al., 2011).

In general, it can be established that this kind of honey has the highest antioxidant activity, when compared with other kinds of honey samples, for example: sulla, chestnut, chicory, dandelion, eucalyptus, citrus, acacia, etc. (Beretta et al., 2005; Rosa et al., 2011). On the other hand, *abbamele* (a typical product originally from Sardinia, obtained from the recuperation of this kind of honey from combs) have antioxidant activities comparable to those of well-known products such as red wines and vegetables; therefore the most bioactive compounds present in this product come directly from the original source *Arbutus unedo* L. honey (Spano et al., 2008; Jerković et al., 2011).

4.5. Conclusions

For the first time, it was possible to characterize the physicochemical parameters of strawberry tree honey from the Algarve. After the comparison made between strawberry tree honey obtained from the South of Portugal with strawberry tree honey from Italy, it was found that all a the physicochemical parameters are within the limits established by International regulations and have shown no significant differences among themselves, confirming that honey composition is highly dependent on weather and environmental conditions; except of colour parameter that clearly was different for the Portuguese honey, which was lighter (extra white) than the Italian honey (light amber); besides of the ash content, optical rotation, pH and HMF content.

This study can generate an increase in the commercial value of this artisanal honey, also provides information related to the bioactive compounds present in this type of honey, hoping to rise preference (higher demand) from the local consumers. Therefore, this kind of honey should, like in Italy, where it is very appreciated and consumed in spite of high commercial value, be better promoted.

CHAPTER 5

COMPARISON BETWEEN COMMERCIAL HONEYS AND STRAWBERRY TREE (*Arbutus unedo* L.) HONEY IN PHYSICOCHEMICAL PARAMETERS AND BIOACTIVE COMPOUNDS

"Spider caves" art ruprecht

Chapter Five: Comparison between commercial honeys and strawberry tree (*Arbutus unedo* L.) honey in physicochemical parameters and bioactive compounds.

5.1. Introduction

In the previous chapter strawberry tree honey was studied and the main properties analyzed revealed that it is indeed interesting honey product that deserves better promotion as a quality product for both its unique bioactivity and for its physicochemical properties.

Commercial honey has to obey to a series of parameters regulated by National and International bodies. Within those limits commercial honey appear in supermarket shelf with one major concern, which is to sell as much as possible. Consumers normally prefer honey that are light in colour and show no sign of cristallization. Honey is a natural food produced by honeybee that transform and combine this with specific substances that leave it in honeycomb to ripen and mature. It is composed primarily by mixture of carbohydrates, mainly fructose (38%) and glucose (30%) and water (16–18%) approximately, with other minor components (*e.g.* proteins, free amino acids, organic acids, phenolic compounds, vitamins and minerals) (Pires et al., 2009; Kahraman et al., 2010) this proportions give enough margin for honey adulteration and for unclear product descriptions on the label. In the present chapter a comparative study with commercial honey is presented.

Honey composition depends on the plants visited by the bees, which can produce nectar or honeydew, and also on the climatic, environmental conditions and processing. The European Union (2001) establishes that the floral source, together with the regional, territorial or topographical origin should be specified on the label of the product, and encourages the use of analytical procedures to determine the authenticity of honeys (Feás et al., 2010; Wei et al., 2011). The methods most used and widely recognized by legislators are physicochemical and pollen analysis (melissopalynology). These have been used to characterize honey samples in order to distinguish their source kind or floral origin, as well as to control its quality (Popek,

2002; Martins et al., 2008; Pires et al., 2009; Feás et al., 2010). One other methodology that can determine or able to distinguish the botanical origin and to identify and quantify certain deviation, from purity (*e.g.* fermentation, impurities, off-odours and flavours) is the sensory evaluation (Piana et al., 2004). It is also an essential tool that allows you to evaluate market trends and provides comprehensive and informative data about the human perception of product quality (Stolzenbach et al., 2011).

Unifloral honeys, predominantly from a single botanical source, are in higher demand from the consumer, which means that they also have a higher commercial value for the producers. Therefore, the characterization of honeys is necessary in order to improve our response to consumer demands. In general, unifloral honeys are more expensive than multifloral ones. Some unifloral honeys are more appreciated than others, due to their organoleptic characteristics or their beneficial properties (Martins et al., 2008; Silva et al., 2009; Feás et al., 2010).

Several researches have shown that some minority compounds in honey act as bioactive compounds, which have antimicrobial and antioxidant properties. Some of these compounds are enzymes (glucose oxidase, catalase), flavonoids, ascorbic acid, phenolic acids and carotenoids (Al et al., 2009; Ferreira et al., 2009; Gomes et al., 2010a; Silici et al., 2010).

An increase in consumption at a global level, over the last few years can be attributed to the general increase in living standards and a higher interest in natural and beneficial health products. The honey of World commerce varies greatly in quality, and its quality is assed largely on the basis of colour, flavor and lately by the presence of compounds related to health benefits (Al et al., 2009; Saxena et al., 2009; Silva et al., 2009; Silici et al., 2010).

Several types of honey are produced in Portugal, where honey production is a traditional practice well implanted in several regions (Pires et al., 2009). In 2010 the volume of production of honey in Portugal was 7436 tons with the major type of honey produced being french

lavender (40%), multifloral (30%), sunflower (10%), eucalyptus (10%), heather (5%) and orange blossom (5%) (Instituto Nacional Estatistica, Portugal, 2010; Federação Nacional dos Apicultores de Portugal, 2011).

In the South of Portugal a bitter honey is produced in a small scale from the typical strawberry tree in this area. "Medronho" is the local name of the strawberry tree (*Arbutus unedo* L.). This plant is a small evergreen shrub, spread throughout the Mediterranean. The "medronho" tree is largely appreciated by the red spherical fruits that are used principally in preparations of alcoholic beverages (Tuberoso et al., 2010; Oliveira et al., 2011). This type of honey is not very appreciated by the consumer, is known locally as "bitter honey" because of its flavor being slightly bitter than the typical honeys and dark colour; the Portuguese consumers prefers heather honeys (*Erica* sp.) and they believe that this type of honey is superior to other types produced locally (Andrade et al., 1999; Martins et al., 2008; Feás et al., 2010).

The goal of the present study was to compare the strawberry three honey samples widely consumed nationwide which concerns it's in physicochemical parameters as well as the presence of bioactive compounds. Besides, the preference level was also evaluated by a sensory panel, in order to establish the degree of preference of the strawberry tree honey as compared with commercial honeys known and most consumed in Portugal.

5.2. Experimental

5.2.1. Honey samples

Four kinds of unifloral commercial honeys samples (nectar honey, figure 5.1) were purchased from a local shop. All the samples were kept in their original packages and left at room temperature until further analysis. The expiration date was the same for all honeys (December 2012). The four commercial brands of Portuguese honey samples were coded as follows *Arbutus* (strawberry tree, B-1), *Helianthus* (sunflower, G-1), *Citrus* (orange blossom, L-1) and *Lavender* (french lavender, R-1). The classifications of honey were performed by honey producers. All analyses were carried out in triplicate. The data used for the characterization of artisanal strawberry tree (was coded as MJ), has been reported in Chapter 4, section 4.2.

Strawberry tree honey (B-1)

Sunflower honey (G-1)

Orange blossom honey (L-1)

French lavender honey (R-1)

Figure 5.1 Types of commercial honey samples analyzed.

5.2.2. Melissopalynology (pollen analysis)

The determination of the honey´s floral source was performed according to the described in Chapter 4, section 4.2.2.

5.2.3. Physicochemical parameters

The methods for determination of physicochemical parameters (ash content, electrical conductivity, moisture content, total soluble solids, water activity, reducing sugars, optical rotation, pH, total acidity, colour by colorimeter and absorbance, diastase activity with Schade and HMF content) were performed as described in Chapter 3, section 3.2.2 and Chapter 4, section 4.2.3.

5.2.4. Bioactive compounds

The determination if bioactive compounds (total phenolic and total flavonoid contents, antioxidant activity) were performed as described in Chapter 3, section 3.2.3.

5.2.5. Sensory descriptive analysis

5.2.5.1. Panel selection

The panel consisted of 15 untrained subjects, comprising 4 men and 11 women (22 – 45 years of age), who regularly participate in the sensory evaluation of foods. The panellists were mainly the members of staff and students of the Food Engineering Department, who had good experience in odour and flavour profiling of a number of food products and had previously, participated in other panels to evaluate honey.

5.2.5.2. Sample preparation

The different types of honeys were poured into transparent glass cups (15 g) and closed with a sheet of plastic. These were coded with three-digit random numbers and served at room

temperature. Water and crackers were provided for the panellists mouth-rinsing between samples.

5.2.5.3. Evaluation

The sensory descriptive analysis was carried out in two stages. First stages, five attributes to evaluate honey were selected: colour, aroma, viscosity, crystal presence and flavor. Each parameter was scored using a five levels scale (table 5.1). The second stages the preference and acceptability of panellists between honeys was evaluated, according to the hedonic scale (nine levels) presented in table 5.1. A set of 5 samples (B-1, G-1, L-1, R-1 and MJ) were evaluated using two sensory replicates assessed across 2 days and the presentation order was changed. Sessions were performed in a testing room with 10 individual booths that were separated from the area where the samples were prepared.

Table 5.1. Attributed evaluated in sensorial analysis with their respective scale.

	Attribute	**Scale**
First stage	Colour	According to the Pfund scale (annexes figure 9.4.1): 1 = extra white 2 = white 3 = extra light amber 4 = light amber 5 = amber
	Aroma	1 = none → 5 = intense
	Viscosity	1 = very liquid → 5 = gummy
	Crystal presence	1 = grainy → 5 = soft
	Flavour	1 = bitter → 5 = sweet
Second stage	Preference/Acceptability	According hedonic scale: 1 = disliked enormously 2 = disliked very 3 = disliked moderately 4 = disliked slightly 5 = not like or dislike 6 = like slightly 7 = like moderately 8 = like very 9 = like enormously

5.2.6. Statistical analysis

The results obtained for four commercial honey samples and artisanal honey to all parameters (physicochemical and bioactive compounds), were analyzed using analysis of variance (ANOVA) and Student´s t-test used to examine differences between diverse types of honey. The results are expressed as mean values ($\bar{x}$) and standard deviation (*SD*). Differences were considered significant at $p < 0.05$. The data analyses were carried out using Statgraphics® Centurion XV (StatPoint, Virginia, USA).

5.3. Results and discussions

5.3.1. Pollen analysis

The results of the microscopic analysis of the commercial honey samples analyzed are summarized in table 5.2. Unifloral status generally refers to the presence of a single pollen type in quantities higher than 45% of the total pollen content in the spectrum; but there are some exceptions (Bianchi et al., 2005; Valle et al., 2007), and some of the samples studied fit the exception criteria. According to Von Der Ohe et al., (2004) to consider a honey as unifloral from orange blossom, then the sample must have a *Citrus* pollen content between 2–42%, similarly strawberry tree origin, for which an *Arbutus* pollen content between 8–20%. On the other hand, some flowers can contain pollen which can be under-represented in some cases, in this included sunflower honey with should be present between 12–92% of *Helianthus* pollen content and also the french lavander honey origin which must have present some pollen belonging to the *Lamiaceae* family which comprise: *Rosmarinus* (with 10–57% of this type of pollen), *Lavandula latifolia* (15–42%) or *Lavandula* x *intermedia* (1–20%).

The analysis of the data in table 5.2, shows that we can classify the honeys as: sample L-1 (with 30.2% of *Citrus* spp. pollen, figure 5.2a) was classified as citrus honey; sample G-1 (with 24.6% of *Helianthus annuus* pollen, figure 5.2b) was classified as sunflower honey; sample R-1 (with 54.2% of *Lavandula stoechas* pollen, figure 5.2c) was classified as french lavander honey and the last sample B-1 (with 20.4% of *Arbutus unedo*) was classified as monofloral strawberry tree honey (Pérez-Arquillué et al., 1995; Piazza and Persano Oddo, 2004; Persano Oddo et al., 2004). The results obtained after pollen analysis confirmed the identities of the honey sources indicated by the producers in the labels.

Table 5.2 Results of pollen analyzed for four commercial honey (B-1, G-1, L-1 and R-1) samples analyzed.

Honey sample	Pollinic spectrum (%)
B-1	***Arbutus unedo* (20.4%; S),** *Lavandula stoechas* (13.1%), *Cistaceae* (10.4%), *Reseda luteola* (9.4%), *Citrus* spp. (7.8%), *Ceratonia siliqua* (7.8%), *Echium plantagineum* (6.3%), *Olea europea* (5.7%), *Erica* sp. (5.2%), *Rosmarinus officinalis* (3.1%), *Crataegus monogyna* (2.6%), *Senecio vulgaris* (2.1%), *Calendula arvensis* (1.5%), *Anarrhinum bellidifolium* (1.0%), *Trifolium arvense* (1.0%), *Crepis capillaris* (1.0%), *Cytisus scoparius* (0.5%), *Anthemis arvensis* (0.5%).
L-1	***Citrus* spp. (30.2%; S),** *Echium plantagineum* (11.4%), *Rubus* spp. (7.0%), *Cytisus scoparius* (6.8%), *Pistacia terebinthus* (5.1%), *Olea europea* (4.8%), *Reseda luteola* (4.4%), *Quercus* spp.(4.0%), *Lavandula stoechas* (2.2%), *Crataegus monogyna* (2.0%), *Raphanus* spp./ *Sinapsis* spp.(1.7%), *Eucalyptus* spp. (1.5%), *Rosmarinus officinalis* (1.1%), *Erica* sp. (1.1%), *Acacia* spp. (1.0%), *Oxalis corniculata* (0.6%).
G-1	***Helianthus annuus* (24.6%; S)**, *Echium plantagineum* (14.1%), *Pistacia terebinthus* (6.9%), *Lavandula stoechas* (5.4%), *Olea europea* (5.3%), *Quercus* spp. (5.1%), *Rubus* spp. (3.5%), *Rosmarinus officinalis* (3.0%), *Eucalyptus* spp. (3.0%), *Citrus* spp. (2.9%), *Crepis capillaries* (1.3%), *Campanula* spp. (1.1%), *Senecio vulgaris* (1.1%), *Raphanus* spp. / *Sinapsis* spp. (0.9%), *Lotus creticus* (0.7%), *Reseda luteola* (0.7%), *Crataegus monogyna* (0.5%).
R-1	***Lavandula stoechas* (54.2%; D),** *Echium plantagineum* (8.0%), *Rubus* spp. (5.6%), *Cytisus scoparius* (5.5%), *Campanula* spp. (2.9%), *Myrtus* spp. (1.7%), *Olea europea* (1.5%), *Acacia* spp. (1.4%), *Eucalyptus* spp. (1.4%), *Rhamnus* spp. (1.3%), *Quercus* spp. (1.3%), *Senecio vulgaris (*0.9%).

- *Predominant pollen (D) > 45% of the pollen*
- *Secondary pollen (S): 16–45 %*
- *Important minor pollen (M): 3–15 %;*
- *Minor pollen (T): less than 3%.*

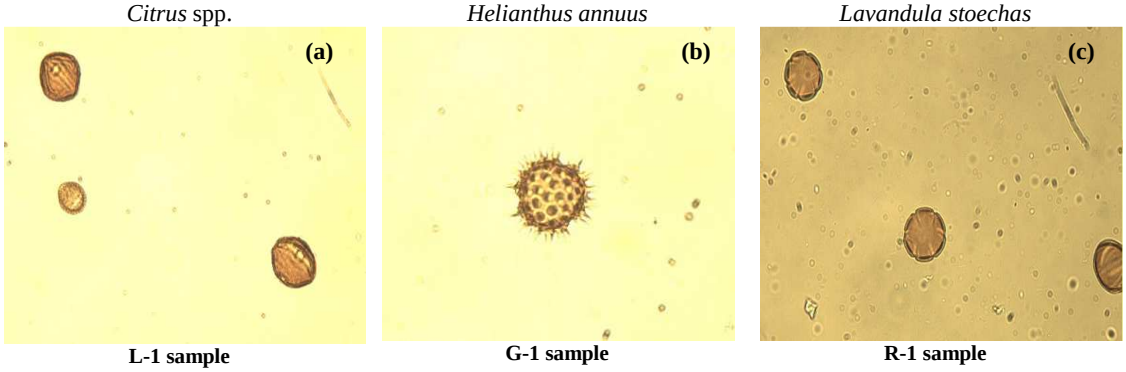


Figure 5.2 Photography of the microscopy at 40x of pollen present in the commercial honey samples analyzed (*Citrus* spp. (a), *Helianthus annuus* (b) and *Lavandula stoechas* (c), respectively).

5.3.2. Physicochemical parameters results

Visually, none of the commercial honey samples showed any signs of deterioration (fermentation), or granulation before initiating the physicochemical determinations. Only sample MJ (artisanal strawberry tree honey) showed a small amount of little crystals (figure 4.1, chapter 4). The following tables (from 5.3 to 5.9) show the mean values obtained for each physicochemical parameter analyzed in each honey sample.

- ***Colour***

The colour by colorimeter (table 5.3) measured and the corresponding values of L^*, a^* and b^* of honey obtained. Only sample B-1 showed the presence of red components (positive a^*). The rest of the samples (G-1, L-1, R-1 and MJ) had green and yellow components (negative a^* and positive b^*, respectively); however, honey samples G-1 and MJ showed a more marked influence of the yellow components. For the lightness (L^* value) of the samples B-1, G-1, L-1 and R-1 showed the lower values (< 33) and do not have any significant differences; but the darker sample was B-1 (L^* = 29.09). On the other hand, the artisanal strawberry tree honey (MJ) showed they higher lightness (L^* = 42.03). The colour of the honeys was also determined by absorption (table 4.3), where were classified according to the Pfund scale (White, 1984). Two samples were classified as dark amber (> 114 mm Pfund) these were honeys B-1 (mean value 173.2 mm Pfund) and G-1 (mean value = 145.6 mm Pfund). Samples L-1 (mean value 44.9 mm Pfund) and R-1 (mean value 76.6 mm Pfund) were classified as light extra amber (30–50 Pfund mm) and light amber (50–80 Pfund mm), respectively. Honey MJ was classified as white (18–34 Pfund mm) with a mean value of 23.0 mm Pfund.

Table 5.3 Results for the colour parameters (colorimeter and absorbance) of the commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ).

	Honey samples				
Parameters	B-1	G-1	L-1	R-1	MJ
Colour by colorimeter					
L^*	29.09 ± 1.38	31.07 ± 0.29	32.62 ± 0.18	32.69 ± 1.60	42.03 ± 10.05
a^*	1.55 ± 0.32^{a}	-0.07 ± 0.29^{b}	-1.17 ± 0.15^{c}	-1.00 ± 0.15^{c}	-0.27 ± 0.10^{b}
b^*	3.57 ± 1.76^{d}	10.01 ± 0.47^{e}	4.77 ± 0.20^{d}	6.30 ± 0.86^{d}	12.42 ± 3.91^{e}
Colour by absorbance (mm Pfund)	173.9 ± 1.9	145.6 ± 2.8	44.9 ± 2.4	76.6 ± 3.1	23.07 ± 5.56
	dark amber	*dark amber*	*light extra amber*	*light amber*	*white*

Results with letters shown significant differences ($p < 0.05$) for each parameter.

A comparison of the colour parameters of the two honeys with the same floral origin (B-1 and MJ samples) showed that these were completely different. The colour in honey depends on various factors, with their mineral content being an important one. Light-colored honeys usually have low ash contents (below 0.1%), while dark-colored honeys generally have higher ash contents (Gomes et al., 2010a); our results were very different in some aspects. For example found that the honey classified as white (MJ) was the one showing the higher ash content.

- ***Moisture content (MC)***

MC varied from 15.49% (R-1 sample) to 18.19% (B-1 sample) for commercial honeys (table 5.4). The maximum amount of water content in honey is regulated for safety against fermentation by molds (*Penicillium* and *Mucors*) that reduce its quality. All samples contained mean values lower than the 20%, the value allowed by the European Union (2001) and Codex Alimentarius Commission (2001). The water content of honey depends on the harvest season, of the maturity reached in the hive, moisture content of the original plant, the processing techniques and storage conditions. A high moisture (> 21%) honey indicates a premature extraction or an extraction under high humidity conditions (Feás et al., 2010). The strawberry tree honeys (B-1 and MJ) had a higher of water contents, 18.19% and 19.87%, probably because they are winter honeys; this could accelerate crystallization which, if fact, had began in the

artisanal strawberry tree samples, at this level of water content two phases start to appear and the lower one, which is richer in glucose content starts to crystallization (Gomes et al., 2010a).

Table 5.4 Results for the moisture content (MC, %), total soluble solids (TSS, ºBrix) and water activity of the commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ).

	Honey samples				
Parameters	B-1	G-1	L-1	R-1	MJ
MC (%)	18.19 ± 0.11^{a}	16.68 ± 0.44^{b}	16.96 ± 0.12^{b}	15.49 ± 0.33^{c}	19.87 ± 0.57^{d}
TSS (ºBrix)	79.87 ± 0.06	81.30 ± 0.44	80.93 ± 0.12	82.60 ± 0.10	73.64 ± 4.12
a_w	0.620 ± 0.004^{e}	0.564 ± 0.000^{f}	0.592 ± 0.000^{g}	0.568 ± 0.000^{h}	0.65 ± 0.00^{i}

Results with letters shown significant differences ($p < 0.05$) for each parameter.

- ***Total soluble solids (TSS)***

Total soluble solids content (table 5.4) of the commercial honey samples analyzed showed mean values between 79.87 ºBrix (B-1) and 82.60 ºBrix (R-1). The moisture and TSS are strictly correlated; high values of total soluble solids correspond to a loss of MC; an anomalous correlation may be a reliable index of adulteration (Conti, 2000; Silva et al., 2009). The differences in these two parameters can be attributed to harvesting season, the beehive handling practices applied by beekeepers and the degree of maturity reached in the hive (Feás et al., 2010; Silva et al., 2009).

- ***Water activity (a_w)***

Water activity is one of the most important parameters in microorganism development inhibition and enzyme activity. The osmophilic yeasts are only able to grow above a minimal water activity of 0.60 and are specialists to survive in conditions of high sugar concentrations. Such osmophilic yeasts (*Saccharomyces* spp.) are responsible for causing honey fermentation, resulting in the formation of ethanol and carbon dioxide. The alcohol can be further oxidized to acetic acid and water resulting in a sour taste (Saxena et al., 2009). The commercial honeys analyzed showed values between 0.564 (G-1 sample) and 0.620 (B-1 sample; table 5.4).

Fermentation processes are indeed virtually annulled when the moisture content is < 17.1%, while for higher values they are dependent on the number of osmotic yeasts (> 1000/g of honey for 17.1 < moisture < 18% and > 10/g of honey for 18.1 < moisture < 19%). The four honeys analyzed should be regarded as safe with respect to this phenomenon, since they showed lower values of $a_w \leq 0.62$ and moisture ≤ 18.20% (Conti, 2000).

- ***Electrical conductivity (EC)***

The electrical conductivity represents a parameter increasingly used in routine honey quality control, and can be considered as a valid criterion for the determination of honey´s botanical origin or, more specifically, for the differentiation between nectar honey or honeydew. The International legislations (EU, 2001; Codex Alimentarius Commission, 2001) establishes the limits for the EC values as a function of the kind of honey, normally nectar honey (with some exceptions), should have values below 0.8 mS/cm and honeydew values higher than 0.8 mS/cm. The EC values of the honeys analyzed (table 5.5) ranged between 0.113 to 0.481 mS/cm (R-1 and B-1 samples, respectively). Electrical conductivity of honey may be explained by taking into account the ash and acid contents, which reflects the presence of ions, organic acids and proteins; the higher their contents, the higher is the resulting conductivity. Results obtained for the samples in this study were in accordance to Codex Alimentarius Commission (2001) that establishes a maximum value for nectar honey (< 0.8 mS/cm).

Table 5.5 Results for the electrical conductivity (mS/cm) and ash content (%) of the commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ).

	Honey samples				
Parameters	B-1	G-1	L-1	R-1	MJ
EC (mS/cm)	0.481 ± 0.006^{a}	0.334 ± 0.004^{b}	0.216 ± 0.001^{c}	0.113 ± 0.001^{d}	0.64 ± 0.01^{e}
Ash content (%)	0.300 ± 0.006^{f}	$0.149 \pm 0.007^{g*}$	$0.108 \pm 0.001^{*}$	$0.049 \pm 0.008^{h*}$	0.58 ± 0.10^{i}

Results with letters shown significant differences (p < 0.05) for each parameter.

- ***Ash content***

One other parameter used for this purpose is also the ash content. Both parameters (electrical conductivity–ash) depend on the mineral content of the honey: ash gives a direct measure of the inorganic residue after carbonization, while the electrical conductivity measures all the ionizable organic and inorganic substances (Pires et al., 2009; Silva et al., 2009; Gomes et al., 2010a).

The commercial honeys analyzed showed ash contents (table 5.5) ranging between 0.049% (R-1 sample) and 0.30% (B-1 sample). Ash values obtained were below 0.60%, as expected for nectar honeys (Codex Alimentarius Commission 2001; EU 2001). The increase in ash content of the honey from the South region was accompanied an the increase of electrical conductivity, as previously reported by Sancho et al., 1991b; 1992; Silva et al., (2009), Pires et al., (2009), Feás et al., (2010) and Gomes et al., (2010a). For the honeys analyzed obtained a linear relationship $(EC = 1.433 \cdot ash\ content + 0.069)$ with a coefficient of determination R^2 = 0.952 (figure 5.4).

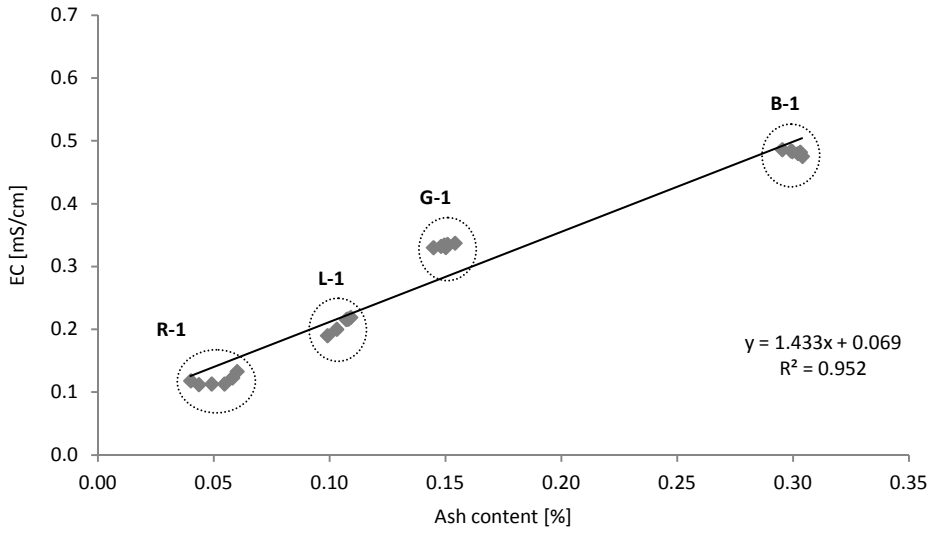


Figure 5.4 Linear regression of ash content (%) and electrical conductivity (mS/cm) of commercial honey (B-1, G-1, L-1 and R-1) analyzed.

The *Arbutus* honeys (B-1 and MJ) showed significant differences between ash content and electrical conductivity. The values obtained for these parameters are dependent on geographical conditions, soil composition, environmental pollution and extraction techniques (Nanda et al, 2003; Conti, 2000).

- ***Optical rotation (OR)***

The commercial honeys analyzed (table 5.6) presented values between -14.72º (B-1) to -12.50º (L-1), these values are not significantly different ($p < 0.05$), so a nectar honey origin could be established for all samples (levorotarory behaviour) have even MJ honey (value = -6.06º), since honeydew and some adulterated honeys, normally have dextrorotatory optical activity. This is a consequence of a normally higher percentage of fructose (~38%), which shows a negative specific rotation over glucose; the overall value for the OR is a result of the values of the different honey sugars present. The honeydew honey is characterized by higher concentrations of oligosaccharides, mainly the trisaccharides melozitose and raffinose, which usually are not found in nectar honey (Dinkov, 2003; Nozal et al., 2005). Measurements of specific rotation are currently used in Greece, Italy and UK to distinguish the botanical origin (nectar honey or honeydew) and adulteration of honey (Nanda et al., 2003). Similar results were obtained by Šarič et al., (2008) after the analysis of different unifloral Croatian honeys, when all the samples showed a levorotatory behavior.

Table 5.6 Results for the optical rotation (α_D^{20}), reducing sugar (mg/100 g of honey) of commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ).

	Honey samples				
Parameters	B-1	G-1	L-1	R-1	MJ
OR (α_D^{20})	-14.72 ± 2.63	-14.14 ± 0.62	-12.5 ± 0.42	-13.22 ± 0.28	-6.06 ± 0.36
Reducing sugars (mg/100g of honey)	71.17 ± 3.88[a]	78.89 ± 3.51[b*]	73.51 ± 2.12[c*]	72.00 ± 1.64[a]	73.64 ± 4.12[c]

Results with letters shown significant differences ($p < 0.05$) for each parameter.

- ***Reducing sugars***

For reducing sugars (fructose and glucose), for nectar honey can not be less than 60 g/100 g of honey, except for honeydew honeys, for which it is ≥ 45 g/100 g of honey (EU, 2001; Codex Alimentarius Commission, 2001). All the commercial honeys showed mean values higher than the legislation limit, as shown in table 5.6. These levels were also observed by other authors in Portuguese honeys (Andrade et al., 1999; Pires et al., 2009; Gomes et al., 2010a).

- ***pH***

Our commercial samples presented a pH that ranged from 3.75 (L-1) to 4.27 (B-1); these results (table 5.7) are in accordance to what is with the acceptable for nectar honeys. Similar values were also obtained for other Portuguese honeys (Silva et al., 2009; Feás et al., 2010).

The low pH of honey inhibits the presence and growth of microorganisms, since most bacteria grow in a neutral and mildly alkaline environment, while yeast and moulds are capable of developing in an acidic environment (pH 4.0–4.5) and do not grow well in alkaline media (Khalil et al., 2010). The pH values of honey are of great importance during extraction and storage, since acidity can influence the texture, stability, and shelf-life of honey (Ajlouni and Sujirapinyokul 2010).

Table 5.7 Results for the pH and total acidity (meq/kg) of commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ).

	Honey samples				
Parameters	B-1	G-1	L-1	R-1	MJ
pH	4.27 ± 0.03^{a}	3.90 ± 0.04^{b}	3.75 ± 0.01^{c}	3.99 ± 0.09^{b}	4.52 ± 0.05^{d}
Total acidity (meq/kg)	17.36 ± 0.870^{e}	14.15 ± 0.56^{f}	20.36 ± 0.55^{g}	7.47 ± 0.82^{h}	26.76 ± 2.28^{i}

Results with different letters shown significant differences ($p < 0.05$) for each parameter.

- ***Total acidity***

None of the samples studied exceeded the limit proposed by the Codex Alimentarius Commission (2001), which is no more than 50 meq/kg. For the honey samples in this study total acidity (table 5.7) ranged from 7.47 to 20.36 meq/kg (R-1 and L-1, respectively). High free acidity values may indicate the fermentation of sugars (fructose or glucose) by yeasts, which contribute to the level of free acidity in honey.

- ***Viscosity***

Regarding viscosity, all commercial honeys analyzed showed a Newtonian behaviour at different temperatures (table 5.8). These results were similar to those found by several other authors (Yanniotis et al., 2006; Recondo et al., 2006; Smanalieva and Senge, 2009; Cohen and Weihs, 2010).

Table 5.8 Viscosity (η, Pa s) and activation energy (E_a, kJ/mol) of the commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ) at different temperatures.

	η (Pa s) Honey samples				
Temperature (ºC)	**B-1**	**G-1**	**L-1**	**R-1**	**MJ**
20	23.86	52.76	33.00	57.70	30.11
25	11.73	21.44	12.94	26.67	15.66
30	6.69	11.46	7.13	14.63	8.67
35	3.24	6.42	3.99	7.73	3.48
40	1.68	3.59	2.42	4.26	1.45
Ea (kJ/mol)	100.51	100.51	97.83	98.43	115.24
R^2	0.998	0.993	0.989	0.999	0.988

All honey samples fit to the Arrehenius model, as they showed linearity of the Ln η with 1/temperature (1/K), and decreased with an increment of temperature. Cohen and Weihs (2010) reported that the activation energy (E_a) decreased with the increment of the moisture content; this trend was also found for all the honeys under study. The E_a was observed reflects the sensitivity of viscosity to temperature changes; higher values mean that the viscosity is relatively more sensitive to a temperature change (Lazaridou et al., 2004). Therefore, in the honeys analyzed, the sensitivity increased in the following order: MJ > B-1, G-1 > R-1 > L-1 (table 5.8). The decrease in viscosity is due to a lower molecular friction as hydrodynamic forces decrease (Mossel et al., 2003; Recondo et al., 2006; Gómez-Díaz et al., 2009).

- ***Hydroxymethylfurfural (HMF) content***

HMF and diastase activity (DA) are widely recognized as parameters for the evaluation of honey freshness and/or overheating (Ajlouni and Sujirapinyokul, 2010; Kahraman et al., 2010; Khalil et al., 2010).

Two of the commercial honey samples (B-1, 61.31 mg/kg and G-1, 68.13 mg/kg) showed levels of HMF higher than the allowed limits of 40 mg/kg (Codex Alimentarius Commission, 2001; EU 2001), which are indicative of temperature abuse during processing and/or bad storage conditions (*e.g.* temperature) (Ajlouni and Sujirapinyokul, 2010; Khalil et al., 2010). Samples L-1 and R-1 had values of 16.7 and 24.52 mg/kg (table 5.9), both within the limits established by legislations.

Fresh honey is usually heated in order to facilitate processing. However, excessive treatment leads to the formation of HMF and reduces honey quality. Other factors that influence the formation of HMF in honey during storage conditions are: the use of metallic containers and the physicochemical properties (pH, total acidity, and mineral content), the presence of organic acids and low water activities also favours HMF production (Ajlouni and Sujirapinyokul, 2010).

From the results of the HMF content, it can be concluded that honey samples B-1 and G-1 were subjected to heating or pasteurization during the production process, to facilitate the handling process or to prolong their stability against possible fermentations; possible the conditions of storage were also not controlled. On the other hand, the artisanal strawberry tree honey (MJ) showed a high degree of freshness, with a mean value of 16.11 mg/kg.

Table 5.9 Results for the hydroxymethylfurfural (mg/kg) and diastase activity (Gothe) of the commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ).

	Honeys				
Parameters	B-1	G-1	L-1	R-1	MJ
HMF (mg/kg)	61.31 ± 3.30^{a}	68.13 ± 1.63^{b}	16.7 ± 2.79^{c}	24.52 ± 1.19^{d}	16.11 ± 2.30^{e}
DA(Gothe)	8.01 ± 0.28	7.56 ± 0.02	10.68 ± 0.04	7.60 ± 0.01	3.58 ± 0.13

Results with letters shown significant differences ($p < 0.05$) for each parameter.

- ***Diastase activity (DA)***

Diastase is a natural enzyme of honey; its level depends upon the geographical and floral source of the product; it decreases when the honey is subjected to certain thermal conditions or over time (Babacan and Rand, 2005). Honey samples G-1 (mean value of 7.56 Gothe) and R-1 (mean value of 7.60 Gothe), showed the lower values of the diastase activity (table 5.9) with respect to the limit established by the International Honey Commission (Bogdanov, 2002), the European Union (2001) and the Codex Alimentarius Commission (2001); which states that it must not be less than or equal to 8 Gothe. Legislations have established the minimum diastase activity value of 3 Gothe, for honeys with natural low enzyme contents. In honeys with a DA lower than 8 and higher than or equal to 3, the HMF content must not be higher than 15 mg/kg. If DA is equal to or higher than 8, the HMF limit is 60 mg/kg (Tosi et al., 2008). Under this criterion of low diastase activity, only honey R-1 was found to be within the limits, but the HMF content was higher than 15 mg/kg; therefore this sample did not have the quality and freshness established by regulations.

Aljouni and Sujirapinyokul (2010) reported the effects of the thermal treatment in the diastase activity of the honey samples, and concluded that the enzyme activity decreased after subjecting the samples to a heat treatment.

After analyzing the physicochemical parameters of all the commercial honeys; it may be concluded that only sample L-1 was within the limits of freshness and quality established for nectar honey. For the rest of the samples (B-1, G-1 and R-1), they were found to be off the limits established by regulations for the HMF content and diastase activity, which are indicative of temperature abuse during processing and/or bad conditions of storage. Clearly, the legislations is not being enforced, may be, due to the difficulty or capacity of the authorities to verify quality and authenticity.

Simple methods and preferably faster ones would be required. A protected denomination of origin (POD) denomination would probably help to assure quality.

5.3.3. Bioactive compounds results

The results obtained for all of the honey samples analyzed for the total phenol and total flavonoid content are shown in the table 5.10.

- ***Total phenolic content (TPC)***

The TPC of the four commercial honeys showed values between 22.11 and 91.83 mg of gallic acid equivalents (GAE)/100 g of honey for samples R-1 and B-1, respectively (table 5.10). Similar results were obtained by Ferreira et al., (2009) for three unifloral honeys obtained from the Northeast of Portugal, which showed values between 22.62 and 72.78 mg of GAE/100 g of honey, with the darker honeys being the ones with higher concentrations. Other researches, for example by Al et al., (2009) for Romanian unifloral honey and honeydew showed values between 2 and 125 mg of GAE/100 g of honey; as well as Meda et al., (2005) which found that floral honeys and multifloral honeys showed a higher total phenolic range of 32.59–114.75 mg of GAE/100g of honey. Jasicka-Misiak et al., (2012) found that Polish unifloral honeys had values of phenolic contents between 59.9 to 121.4 mg of GAE/100 g of honey for heather honeys. Estevinho et al., (2012) after analyzing organic honeys from North of Portugal obtained values of phenol content between 67.8 to 69.8 mg/100 g of honey. The concentration and type of phenolic substances present depends on the floral source of honey, these are the major factors responsible for biological activity of honey; it also depends on seasonal and environmental factors; processing may also have an effect on honey composition and antioxidant capacity (Al et al., 2009; Lachman et al., 2010; Silici et al., 2010). It is important to note that Folin-Ciocalteu´s method (FCM) is a general method for the estimation of the TPC; is method of determination can include other compounds such as: peptides, vitamins C and E, carotenoids, sugars, amino acids, enzymes and melonoidins compounds (Maillard reaction products) that would increase the real value (Rufián-Menares and Morales, 2007; Alvarez-Suarez et al., 2009; Ferreira et al., 2009; Brudzynski and Miotto 2011b, 2011c).

The samples with same floral origin (B-1 and MJ) showed similar values (91.83 and 97.47 mg of GAE/100 g of honey), which may lead to the conclusion that honeys from *Arbutus* source will have higher amounts, of phenolic contents. In comparison with other types, this honey has a higher concentration of phenols, this is a characteristics that is not been addressed commiently in commercial terms.

Table 5.10 Results of total phenolic content (TPC, mg of GAE/100 g of honey) and total flavonoid content (TFC, mg of QE/100 g of honey) of the commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal strawberry tree honey (MJ).

	Honey samples				
Parameters	B-1	G-1	L-1	R-1	MJ
TPC (mg of GAE/100 g of honey)	91.83 ± 0.61^{a}	39.54 ± 0.18^{b}	27.37 ± 0.06^{c}	22.11 ± 0.13^{d}	97.47 ± 2.37^{a}
TFC (mg of QE/100 g of honey)	$4.49 \pm 0.57^{e*}$	4.31 ± 0.06^{e}	2.67 ± 0.88^{f}	2.62 ± 0.69^{f}	$5.33 \pm 0.63^{e*}$

** Samples without significant differences ($p < 0.05$) for the same parameters*

- ***Flavonoid content (TFC)***

The TFC (table 5.10) of all commercial honeys showed values from 2.62 to 4.49 mg of quercitin equivalents (QE)/100 g of honey for samples R-1 and B-1, respectively. These results are similar to those obtained by Meda et al., (2005) for several types of honey and Al et al., (2009). Normally, the values they have obtained research goes from 0.17 to 8.35 mg of QE/100g of honey (*e.g.* multifloral) and from 0.91 to 15.33 mg of QE/100 g of honey (*e.g.* acacia and sunflower), respectively. Normally, the TFC is determined by using the HPLC method (Yao et al., 2004a; 2004b; Ferreira et al., 2009); with this methodology Yao et al (2004b) and Tomás-Barberán et al., (2001) found that total flavonoid content for Australian sunflower honey and Portuguese rosemary honey to range between 1.79 and 3.41 mg/100 g of honey, respectively. In the honey analyzed a spectrophotometric method was used for the quantification of flavonoids with $AlCl_3$, which is specific for flavones and flavonols, and showed the real content of total

flavonoids present in the honey (Chang et al., 2002). Several researchers (Ferreira et al., 2009; Estevinho et al., 2012) used standard solutions of catequin to determine the flavonoid content, found values for Portuguese honeys with values (from the North), between 12.3–58.7 mg of catequin equivalents (CEs)/100 g of honey and 49.4–56.3 mg of CEs/100 g of honey, respectively.

- ***Antioxidant capacity***

Regarding the antioxidant capacity results (table 5.11), the commercial honeys analyzed by as exhibited lower values in comparison with other studies (Meda et al., 2005; Saxena et al., 2009). The samples in this study showed antioxidant capacities in the range of 3.02–21.49 mg of ascorbic acid equivalents antioxidant capacity (AEAC)/100 g of honey and 1.45–11.51 mg of quercitin equivalents antioxidant capacity (QEAC)/100 g of honey (L-1 and B-1, respectively), with the sample with higher capacity being sample B-1 (strawberry tree), which coincides with the honey sample that showed the higher value for the total phenolic and total flavonoid contents. Components in honey responsible for its antioxidant effect are mostly phenolic compounds (Viuda-Martos et al., 2008; El Gharras, 2009; Pyrzynska and Biesaga, 2009; Lachman et al., 2010). In one other study, Silici et al., (2010) analyzed the antioxidant capacity of samples from *Rhododendran* honey obtained from Turkey, with results ranging from 12.76 to 80.80 mg of AEAC/100 g of honey, which are higher than those from our study. Also, Meda et al., (2005) analyzed different honeys obtained from Burkina Faso, which showed values from 4.27 (multifloral) to 33.34 mg of QEAC/100 g of honey from *Vitellaria*.

Table 5.11 Results of antioxidant content of commercial honey (B-1, G-1, L-1 and R-1) analyzed and artisanal honey (MJ).

	Honey				
Parameters	B-1	G-1	L-1	R-1	MJ
Antioxidant capacity					
(mg of AEAC/100 g of honey)	21.49 ± 0.11^{a}	6.05 ± 0.09^{b}	3.02 ± 0.33^{c}	3.74 ± 0.05^{d}	18.15 ± 0.12^{e}
(mg of QEAC/100 g of honey)	11.51 ± 0.06^{f}	3.06 ± 0.05^{g}	1.45 ± 0.18^{h}	1.83 ± 0.03^{h}	9.72 ± 0.07^{i}
DPPH scavenging (%)	30.80 ± 3.60^{j}	26.27 ± 2.65^{j}	19.73 ± 3.13^{k}	21.84 ± 1.70^{k}	43.46 ± 4.86^{l}

** Samples without significant differences ($p < 0.05$) for the same parameters.*

A highly linear correlation (figure 5.5) was found between the antioxidant capacity for two determinations (ascorbic acid and quercitin equivalent antioxidant), radical scavenging (DPPH), and TPC, with a regression of R^2 = 0.965, R^2 = 0.964 and R^2 = 0.948, respectively, which indicated that the phenolic compounds were the implicated in the antioxidant effects and radical scavenging activity; this results are in accordance with the findings of other authors, whilst Al et al., (2009) found a correlation of R^2 = 0.94 for the antioxidant activity and TPC. Beretta et al., (2005) found a correlation of 0.918 between DPPH and phenol content, whilst Aljadi and Kamaruddin (2004) obtained a R^2 = 0.75.

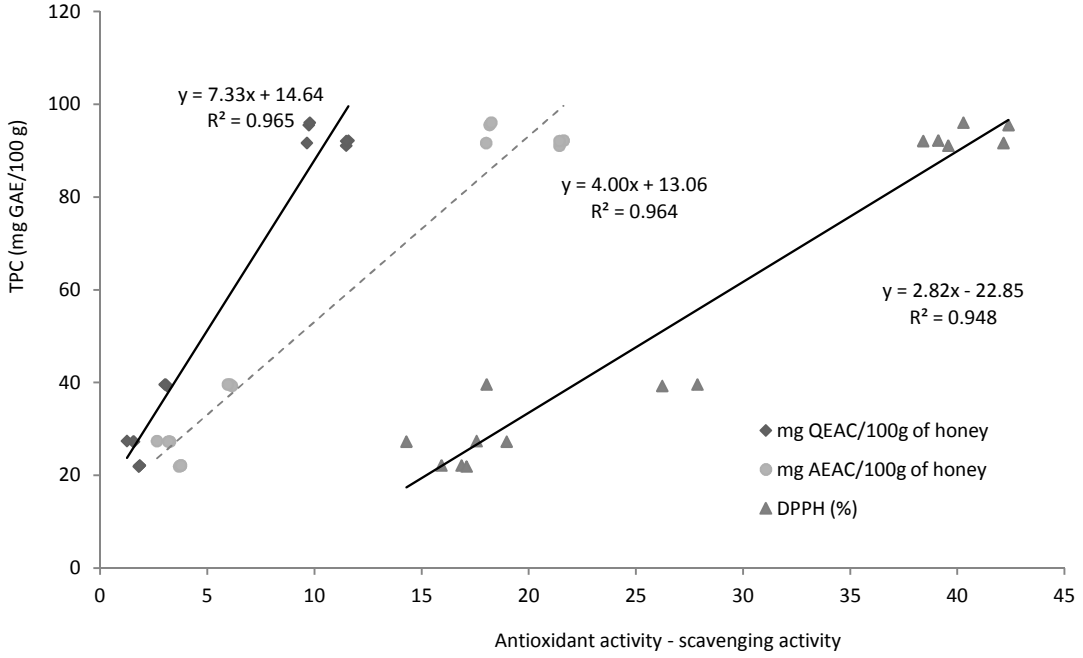


Figure 5.5 Relationship between antioxidant activity (mg of QEAC/100 g and mg of AEAC/100g) and radical scavenging (%) and total phenolic content (TPC, mg of GAE/100 g) for commercial honey (B-1, G-1, L-1 and R-1) and artisanal strawberry tree honey (MJ).

Many authors (Aljadi and Kamaruddin 2004; Ferreira et al., 2009; Lachman et al., 2010) had established that the higher antioxidant activities were found in darker honey samples as well as in honey with higher water contents; this can also be observed in sample B-1 (dark amber according to the Pfund scale; 21.49 mg of AEAC/100 g of honey, 11.51 mg of QEAC/100 g of honey and 18.19% moisture), which presents this behavior in comparison with other commercial samples.

Studies have very clearly indicated that not all plant products bear the same antioxidant capacity. It is not the quantity but the quality of the polyphenolic compounds, some of which do not possess functional antioxidant properties, which serves as the major determinant of the antioxidant capacity of food. This may explain the possible differences between the honey samples analyzed (Saxena et al., 2009).

The strawberry tree honeys (samples B-1 and MJ) have shown highest values for bioactive compounds (phenols and flavonoids) as well as antioxidant activity. The comparison between these two samples with the same floral origin had similar values of bioactive compounds and antioxidant capacities. However, the honey B-1 had slightly higher values; this difference can be explained by a higher concentration of melanoidin compounds, which have demonstrated to also have an antioxidant capacity (Wang et al., 2011, Brudzynski and Miotto, 2011b, 2011c).

Melanoidins are brown polymers which generate darkening with the increment of its concentration; the formation of these polymers is generated during thermal processing or storage time (Antony et al., 2002). Turkmen et al., (2006) and Bertoncelj et al., (2007) demonstrated that dark honeys showed a higher antioxidant capacity. Therefore, the commercial honey (B-1) showed a higher antioxidant activity most likel because it was to subjected thermal processing, generating changes in the colour parameters (browning), an increment in the HMF content (above limit), and a higher of bioactive compounds concentration and antioxidant

capacity (Turkmen et al., 2006); where compared with artisanal strawberry tree honey, where there was browning and the hydroxymethylfurfal content was below the legal limit.

5.3.4. Sensorial results

The results are summarized into the spider-web diagrams (figures 5.6 and 5.7) with of the scores of five attributes: colour, aroma, viscosity, crystal presence and flavor are showed.

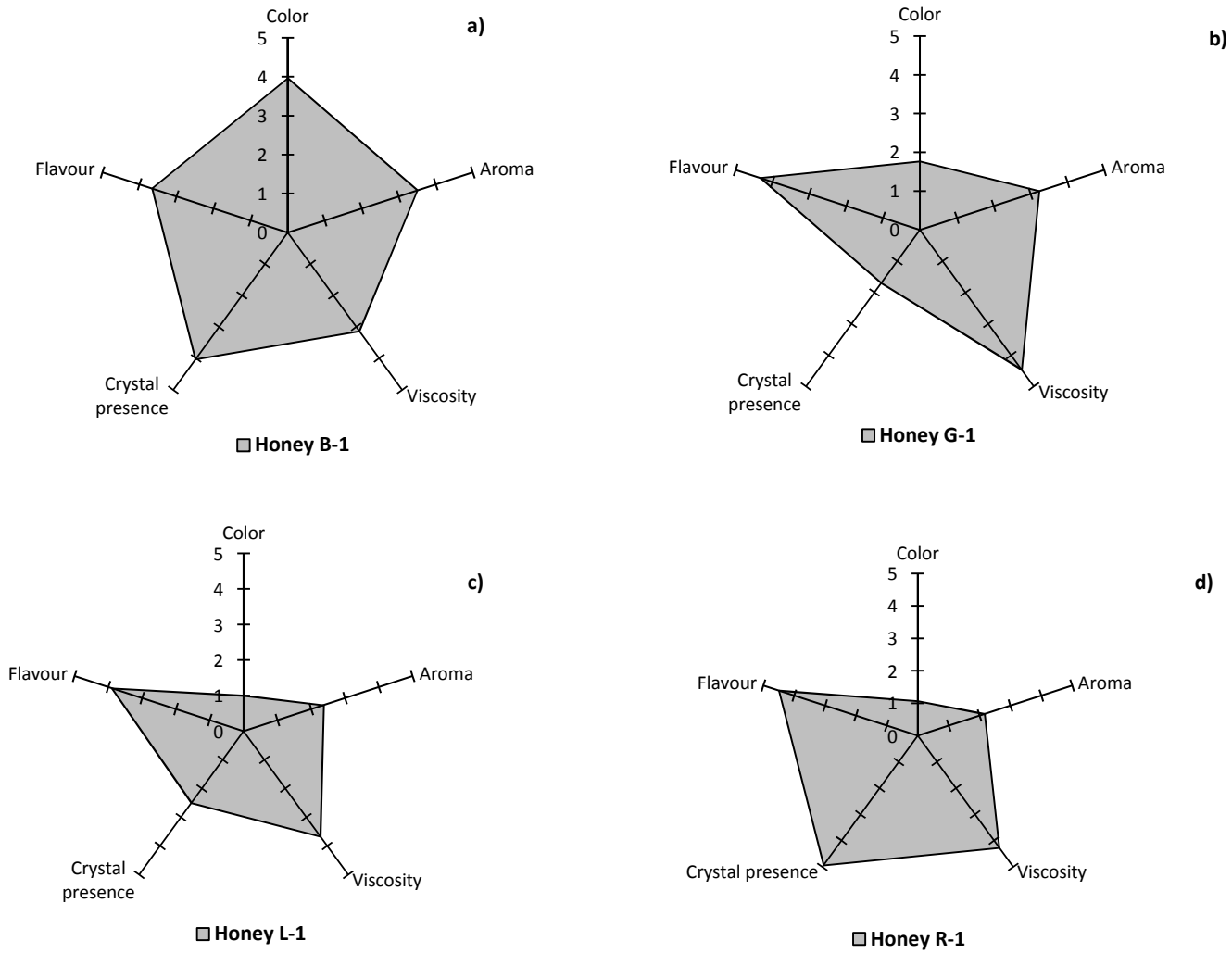


Figure 5.6 Spider-web diagrams of commercial honey samples (B-1, G-1, L-1 and R-1) showing the five attributes evaluated.

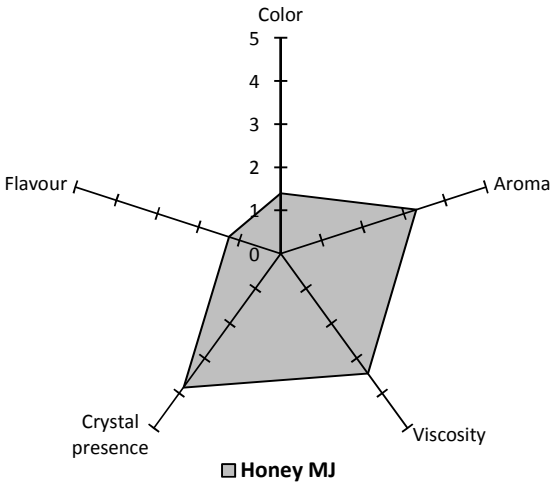


Figure 5.7 Spider-web diagram of artisanal strawberry tree honey (MJ) showing the five attributes evaluated.

After of analysis of variance (ANOVA) between groups. We can conclude that all honey were significantly different $p < 0.05$.

Honey B-1 (commercial strawberry tree, figure 5.6a) was characterized by an amber colour, moderate aroma intensity ($\bar{x}$ ± SD; 3.5 ± 1.1) detected by the panellists, for viscosity was more gummy (3.1 ± 0.8) than liquid, for crystal presence was mild (4.0 ± 0.9) and the flavor has more sweet influence (3.7 ± 0.9) than bitter. Some comments written by panelists, were that this sample had caramel, smoke, burned and toffee notes in the taste and odor; these are typical products generated by thermal processing.

Honey G-1 (commercial sunflower, figure 5.6b) was characterized by a between extra white to white colour (1.8 ± 0.5); with a slightly intensive aroma (3.2 ± 1.3), a more gummy viscosity (4.5 ± 0.7), slightly grainy crystal presence (1.7 ± 0.8), and the flavor was moderatly sweeter (4.3 ± 0.8).

Honey L-1 (commercial orange blossom, figure 5.6c) was characterized by an extra white colour by all panellists (1 ± 0), the aroma was slightly perceived (2.4 ± 1.2), the viscosity was lightly gummy (3.7 ± 1.0), with a slightly soft crystal presence (2.5 ± 1.0), and slightly bitter flavor (3.9 ± 1.0).

Honey R-1 (commercial french lavender, figure 5.6d) was characterized by an extra white colour (1.1 ± 0.3), a sçightly perceived aroma (2.2 ± 1.1), a slightly gummy viscosity (4.3 ± 0.9), a virtually absent crystal presence (4.9 ± 0.4), and a highly influenced sweet taste flavor (4.5 ± 0.5).

Honey MJ (artisanal strawberry tree, figure 5.7) was characterized by an extra white colour (1.4 ± 0.7), an aroma that was moderately intense (3.3 ± 1.3), a middle viscosity between liquid to gummy (3.4 ± 0.9), crystal a slightly soft crystal presence (3.8 ± 0.8) and an extremely bitter flavor (1.3 ± 0.6). This sample is locally denominated as “bitter honey” in Algarve (South Portugal).

Figure 5.8 shows the overall preference/acceptability of the panellists. Preferences were as following: honey coded was R-1(7.7 ± 1.6) > G-1 (6.1 ± 1.7) > B-1 > L-1 (5.3 ± 1.9). The last appreciated place was MJ, with the lowest score (2.3 ± 1.2).

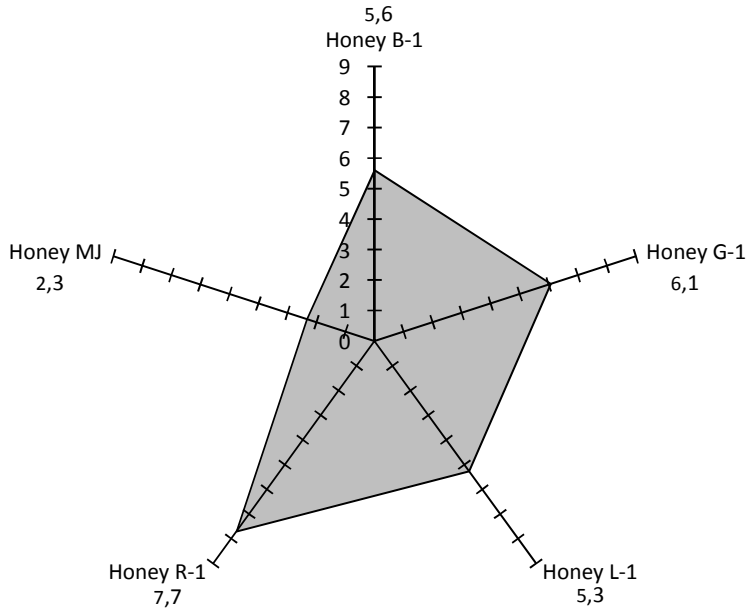


Figure 5.8 Spider-web diagrams of preference/acceptability by panallists of the different commercial honeys (B-1, G-1, L-1 and R-1) and artisanal strawberry tree honey (MJ).

Honeys B-1 and MJ had the same floral origin (strawberry tree/*Arbutus unedo*), but showed a significantly differences in the preference/acceptability by the panellist, which may be explained by the fact that the commercial honey (B-1) had been thermally processed generating compounds that might mask the bitter taste intensity (Galán-Soldevilla et al., 2005).

Anupama et al., (2003) reported a good correlation between sensory and physicochemical parameters. In our study, with the sensory analysis it was possible to identify some compounds (aroma and taste), that were thermally generated during temperature abuse or bad conditions of storage (Castro-Vázquez et al., 2008), and only could be determined with physicochemical characterization (hydroxymethylfurfural content and diastase activity).

5.4. Conclusions

The commercial samples of honey B-1 and R-1 did not fit within the European legislation standards relative to diastase activity and hydroxymethylfurfural content, respectively. Honey G-1 shows higher values for both parameters (DA and HMF), which are not compliant with current regulations, reflecting inadequate sample manufacture and/or storage conditions; the other physicochemical parameters are within the limits established by legislation; therefore only honey L-1 is within the limits established by the regulations (EU, 2001 and Codex Alimentarius Commission, 2002).

From the other results obtained in this study such as: electrical conductivity, ash content and optical rotation and pollen analysis it can be established that the four commercial samples were nectar honeys, which coincides with the information provided in the label.

The present study also demonstrated that honeys from the botanical *Arbutus unedo* floral origin (B-1 and MJ) contains an important source of phenolic compounds (phenols and flavonoids), showing a substantial antioxidant capacity in comparison with the remaining honeys (G-1, L-1 and R-1), which are best known and consumed locally.

Through the use of sensorial analysis it was possible to identify some compounds (aroma and flavor) generated by thermal processing. Preferences were established, between the honey samples used in this stady. The ranking of the honeys is in accordance to local consuming habits.

Taking into account the results obtained for the bioactive compounds, in *Arbutus unedo* honey, it is possible to add value to this type of honey, generating a higher consumer and preference; since it was proved that it represents a good source of bioactive compounds, which may be directly consumed.

Another aspect, eventually less obvious, is that bitter honey should not be competing for the same market. It should be treated as an exquisite product with highly relevant bioactivity and commercialized as a gourmet product.

CHAPTER 6

NON-INVASIVE DETERMINATION OF HONEY BOTANICAL ORIGIN: AN APPROACH BASED IN CHEMOMETRICS AND FUSION OF E-TONGUE AND OPTICAL SPECTROSCOPY

"Spider caves" art ruprecht"

Chapter Six: Non-invasive determination of honey botanical origin: an approach based in chemometrics and fusion of e-tongue and optical spectroscopy.

6.1 Introduction

Honey is generally considered one of the most appreciable natural products. The composition depends of various factors, one of the most important being its botanical source, which give to honeys unique and individual organoleptic characteristics, physicochemical properties and bioactivy. Modern consumers, seek high quality products with a clear regional identify, with certification of the provenance areas and floral origin (Castro-Vázquez et al., 2010; Wei et al., 2010). The traditional technique used to identify honey botanical origin is the melissopalynological method (Von Der Ohe et al., 2004; Stanimirova et al., 2010), that is, the analysis and identification of pollens contained in honey (Iglesias et al., 2004; Bianchi et al., 2005; Maia et al., 2005). Nevertheless, this method is not being absolutely conclusive, is extremely tedious, has some limitations, is time-consuming and requires trained analysts (Escriche et al., 2011b). Alternative methods to establish honey origin are currently under active research (Cotte et al., 2003; Nozal et al., 2005; Bertelli et al., 2007; Wei and Wang, 2011). Therefore several studies have turned up efforts to use non-invasive techniques to determine composition, quality, geographical and botanical origin of honeys (Dias et al; 2008; Aliferis et al., 2010; Wei and Wang, 2011). New techniques are being widely used for quality control as well determination of physicochemical parameters in the field of foods analysis. These techniques have been called as non-invasive techniques, because they do not generate transformation and/or destruction of the sample. Further advantages include short analysis time, easy or even no necessary previous preparation of the sample to analyze (Gallardo et al., 2005; Cavaco et al., 2011). Some of the non-invasive methods used to the determination or classification of honeys are spectrophotometry (Qiu et al., 1999; García-Alvarez et al., 2002; Batsoulis et al., 2005; Bertelli et al., 2007), mass spectrometry (Aliferis et al., 2010; Bertoncelj et al., 2011), emission spectroscopy and electronic sensing (Wei et al., 2010; Major et al., 2011).

Near-Infrared (NIR) Spectroscopy is based on the electromagnetic absorption by organic compounds in the wavelength range of 780 nm to 2500 nm (Qiu et al., 1999; Osborne, 2006; Woodcock et al., 2007; Cavaco et al., 2009). This technique has been applied to food and agricultural industries. NIR spectroscopy has been used to analyze chemical composition and physical properties to diverse foodstuffs (*e.g.* grains, meat, fruits, milk, beer, and dairy products) and to detect adulteration of various food ingredients (Osborne, 2006; Bertinelli et al., 2007). Finally, some studies have used this method for the determination of chemical composition in honeys. Qiu et al., (1999) determined some chemical composition (moisture, fructose, glucose, sucrose, maltose, free acidity, lactone and HMF content) and the results obtained showed that this method was feasible for rapid analysis of the water content and sugar composition with good correlation coefficients. García-Alvarez et al., (2000; 2002) analyzed the fructose, glucose, moisture and polarimetric parameters with NIR transflectance spectroscopy, for determination of the sugars (fructose/glucose), water content, and polarimetric parameters. This technique provided good tools for the prediction of the values for most quantities. However, it was not a suitable technique for quantitative determination of sucrose content in honeys samples with lower content (< 3% of sucrose). Batsoulis et al., (2005) investigated the Fourier Transform (FT)-Raman spectroscopic technique for the determination of fructose and glucose content in honey samples. The sugars content was measured by the HPLC classical method and by the FT-Raman method and the results showed similar values for the two methods.

The electronic sensing refers to the capability of reproducing human senses using sensor arrays and pattern recognition systems that can be analyzed with appropriate statistical software. (Benedetti et al., 2004; Cavaco et al., 2011); for example: electronic nose (e-nose) and electronic tongue (e-tongue) (Escuder-Gilabert and Peris, 2010). The e-nose is can detect odours or flavours; basically the instruments consist of head space sampling, sensor array and pattern recognition modules. The more commonly used sensors include metal-oxide-semiconductor devices, conducting polymers,

quartz crystal microbalance and surface acoustic wave devices (Ampuero et al., 2004; Cavaco et al., 2011). On the other hand, the e-tongue can be defined as a "multisensory system analysis based on chemical sensor arrays and a suitable pattern recognition method" (Escuder-Gilabert and Peris, 2010; Wei et al., 2010). The information on taste substances is transduced into an electrical signal, which is input to a pattern recognition algorithm. Sensors are entered directly into solution without any preparation. There are three types of e-tongue, which are based on potentiometric electrodes, impedance spectroscopy, amperometric, conductimetric and voltammetric electrodes (Scampicchio et al., 2008; Winquist, 2008; Escriche et al., 2011b). Several works presented investigation using e-tongue for the determination and classification of botanical origin of honey. In the research by Dias et al., (2010) an array with 20 all-solid-state potentiometric electrodes with polymeric membranes, was constructed and evaluated for differentiation of commercial honeys with different pollen profiles. The chemometric tools used were principal components analysis (PCA) and linear discriminant analysis (LDA). It was found that the e-tongue had a reasonable efficiency (84% and 72% of success in calibration and cross-validation, respectively) for classification of monofloral honey samples according to melissopalynology. Wei and Wang (2011) used six voltammetric electrodes (gold, silver, platinum, palladium, tungsten, and titanium) to classify seven kinds of monofloral honey. The databases were analyzed with PCA, discriminant function analysis (DFA) and cluster analysis (CA). The results showed that signal obtained with different electrodes, after of analysis with PCA, DFA and CA were able to classify 100% of honeys. Wei et al., (2009) used an e-tongue with seven potentiometric chemical sensors to classify eight honeys of different botanical and geographical origin. The data were analyzed by PCA, CA and artificial neural network (ANN). It was found that all honey samples were discriminated with good result by PCA (97.4%), CA (90%) and ANN (93.7%) with respect to the identification of the botanical origin, in the other hand to determine of the geographical origin, the ANN was the best method (95%) for the determination of the geographical origin.

Other important aspect related with the use of these new techniques is the mathematical analysis of the signal obtained from electric or optical sources. These signals are usually constituted by a wealth of data, distributed among dozens to thousands of variables. To deal with this problem several methods of multivariate analysis are used; which involves the use of mathematic and statistical techniques to extract information from complex data sets. The data is usually analyzed in the form of a matrix, the rows being the samples and the columns being the measured variables (one variable may be, in an example with spectrophotometry, the absorbance at 600 nm the next variable the absorbance at 601 nm). The analysis is performed on the whole matrix (that is, on all variables at the same time) and not just at a single component. This allows to untangle all the complicated interactions between the constituents and understand their combined effects on the whole matrix. Nowadays, the application of supervised pattern recognition and multivariate statistical techniques, like PCA, LDA, ANN, partial least square (PLS) or discriminant analysis (DAs), provides the possibility of analyzing the entire food sample matrix and to make a classification (Corbella and Cozzolino, 2006; 2008; Major et al., 2011).

Multivariate analysis may be applied either to destructive, non-destrutive or a combination of both types of measurements. Any set of samples and measurement variables may be used to perform prediction and/or classification models. For example Bentabol et al., (2011) differentiated among nectar and suspected honeydew honey using physicochemical parameters (moistures, water activity, electronic conductivity, color, hydroxymethylfurfural, acidity, pH, proline, diastase and invertase activities), sugar composition (fructose, glucose, maltose, isomaltose, trehalose, turanose and melezitose) and pollen analysis. The results obtained showed a high percentage of correct nectar and suspected honeydew honey classifications (96.1% after cross-validation) by applying multivariate data.

There is also recent investigation on the problem of sensor fusion. This question arises when one tries to combine the data coming from different sensors into one single matrix for analysis. The simple concatenation of data is rarely the best approach. There are then the so called *n-way* methods, a branch of multivariate analysis that deals with this kind of problems. One of the most used methods in this context is the multy-way PCA (MPCA, Namikos et al., 1994).

The goal of the research described in this chapter was to investigate the potential of non-invasive techniques to determine the botanical origin of four kind honeys from South Portugal. The techniques used were the electronic tongue, the NIR/Vis spectrophotometry and UV/Vis spectrophometry. These were used together with chemometrics, specifically PCA, which is one of the most fundamental techniques in chemometrics, used alone or as a first stage in a more complex analysis. We have also investigated some possibilities of performing the PCA analysis in different ways. This included analyzing the fit coefficients to the data instead of analyzing the data by itself. This procedure has shown a spectacular result in the case of e-nose, although the same effect could not be retrieved for the optical spectra. Finally, we have tried a new approach to the analysis of data fusion, through a variation of MPCA.

6.2 Experimental

6.2.1 Honey samples

The analysis was performed on four kinds of honeys with different floral origin (samples studied in Chapter 5); which were orange blossom honey (*Citrus* spp.), french lavender honey (*Lavander*), sunflower honey (*Helianthus*) and strawberry tree honey (*Arbutus*). The honey samples were coded using its name in Portuguese: citrus was named "Lar", french lavender was named "Ros", sunflower was named "Gir" and to strawberry tree was named "Med". Prior to use, samples were kept at room temperature (20 ± 3 ºC).

6.2.2 Electronic tongue (e-tongue) measurements

The e-tongue developed in the electronics laboratory included four working electrodes (figure 6.1), and a reference electrode (gold). The four working electrodes consisted of small plates (length 10 mm, width 5 mm) made of aluminum (Al), gold (Au), platinum (Pt, small basket) and indium thin oxide (ITO). All electrodes were embedded in composite material placed around the reference electrode (figure 6.1). The electrodes were connected to an Agilent 4284A Precision LCR Meter (Santa Clara, CA, USA). The honey samples were diluted in deionised water to obtain a solution with a final concentration of 1 g/mL. All samples were assayed in a volume of 30 mL, which allowed the four electrodes to be fully immersed. Each electrode was set at a voltage of 50 mV and was frequency scanned from 20 to 2000 Hz. The data conveyed by the e-tongue contained two physically independent variables: capacitance, expressed in nanofaradio (nF), and conductance, expressed in microSiemens (μS). We have also used a rerived quantity, which is the ratio conductance/(2 π log frequency) and designated hereafter by (G/w). All measures were carried out at room temperature 20 ± 3 ºC. Prior to each sample measurement the sensor was conditioned in

honey in order to obtain stable sensor responses. The sensors were rinsed with deionized water after each analysis.

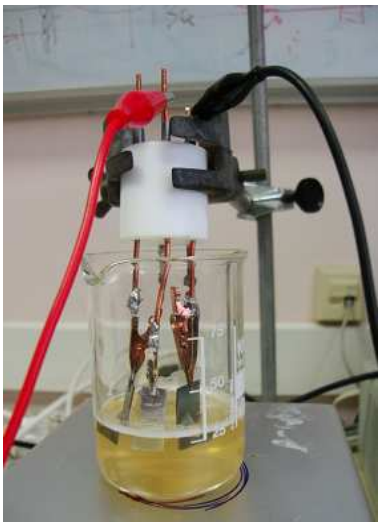


Figure 6.1 Photography of places of the sensors e-tongue.

6.2.3 UV-Vis Spectroscopy measurements

Honeys were diluted with deionised water to obtain a final concentration of 0.17 g/mL (w/v). Absorbencies of the honey solutions were analyzed in 1 cm path–length quartz cell. The spectroscopic measurements were taken with an UV-Visible Spectrophotometer (Cary 50 Bio, Varian, Australia). The absorbance spectrum profile of honey solutions was scanned in the range of wavelengths between 200 and 400 nm. The measurements were made in triplicate for each honey.

6.2.4 Vis-NIR Spectroscopy measurements

Honey samples were diluted with distilled water to 70 ºBrix (Kelly et al., 2004; Woodcock et al., 2007) prior to the measures. The samples were also warmed in a water bath to a maximum of 50 ºC with the aim of melting the crystals.

The honey samples were then placed into 1 mL plastic disposable cuvettes (1 cm wide). The absorption spectra were taken with a Vis-NIR spectrometer (USB4000-VIS-NIR Spectrometer, Ocean Optics, Dunedin, FL, USA) with an average resolution of 1.4 nm (optical resolution imposed by the entrance slit of 25 µm). Spectra spanned the wavelength range from 345.26 to 1036.70 nm in a total of 3648 data points with an average separation of about 0.19 nm. The first and the last points showed pronounced instrumental oscillations and were removed from the analysis. We have then kept only the range from 403.85 to 1032.2 nm, in a total of 3347 data points. All the measurements were taken in the transmittance configuration, by placing the cuvettes in a specific support cell (Ocean Optics, USA). Light from a tungsten halogen source (HL-2000-FHSA, Ocean Optics, Dunedin, FL, USA) was sent to the sample through an optical fiber and the light re-emerging from the sample collected by a second fiber and sent to the spectrometer. All Vis/NIR measurements were carried out in the dark at room temperature 20 ± 3 ºC. The measurements were performed in triplicate for each honey solution against a blank of distilled water. For acquisition, processing and calibration, specific software was used (Spectra Suite, Ocean Optics, Dunedin, FL, USA).

6.2.5 Data analysis

The data analysis obtained with the diverse techniques (e-tongue, UV-Vis spectroscopy and Vis-NIR spectroscopy) were performed with the aid of Dr. Rui Guerra and Dra. Ana Cavaco. The analysis of the results through PCA was carried out using the program Matlab R2010b (The MathWorks, Inc, Natick, USA) and the program PLS Toolbox version 6.0.1 (Eigenvector Research, Inc, Wenatchee, WA, USA), which is Matlab toolbox.

6.3 Results and discussion

Principal Components Analysis (PCA) is one of the most used multivariate techniques. PCA is applied to a set of measurements that are mathematically transformed in order to relate common factors, to reduce the dimensionality of multivariate date and to provide a quick preview of the data structure. It is a way of identifying patters in what seems, at first, scattered dispersed data (Lazarevic et al., 2012).

In practical terms it differs from classical physicochemical characterization in the way that it is not looking or treating a specific property easily identified. In short, PCA analyses a large number of signals, that are statistically treated to obtain a final dominant picture where the main factors of variability are identified and sequenced by importance. These main factors are called principal components and correspond to transformations on the initial variables. The new variables allow to condensate the behavior of the large number of initial variables in a small number of principal components. Hence, the first principal component (PC1) is the variable explaining the larger part of the variability, the second component (PC2) comes next in importance, and so on. A problem containing thousands of variables, as may arise in spectroscopy, may be reduced to less than tem PC's.

6.3.1 Electronic tongue (e-tongue) data

The structure of the e-tongue data is depicted in the following form (figure 6.2).

Al	Pt	Au	ITO	Al	Pt	Au	ITO	Al	Pt	Au	ITO	**4 electrodes**
1…21	1…21	1…21	1…21	1…21	1…21	1…21	1…21	1…21	1…21	1…21	1…21	**21 frequencies**
Capacitances				**Conductances**				**G/w**				**3 measures**

Figure 6.2 Structure of the e-tongue data.

The e-tongue measurements gave a total of 252 variables per honey sample (in total there were 13 honey samples: 3 sunflower, 3 orange blossom, 4 strawberry tree, 3 french lavender). This number corresponds to 3 readings (capacities, conductances and G/W) x 4 electrodes (Al, Pt, Au and ITO) x 21 frequencies (from 20 to 2000 Hz, in intervals with logarithmic spacing). Therefore were obtained 3 x 4 x 21 = 252 variables.

The figures 6.3 (a, b, c) show one example of the measures obtained for the 13 honey samples with the electronic tongue using the Aluminum electrode. The plots show the capacitance, conductance and G/W, respectively.

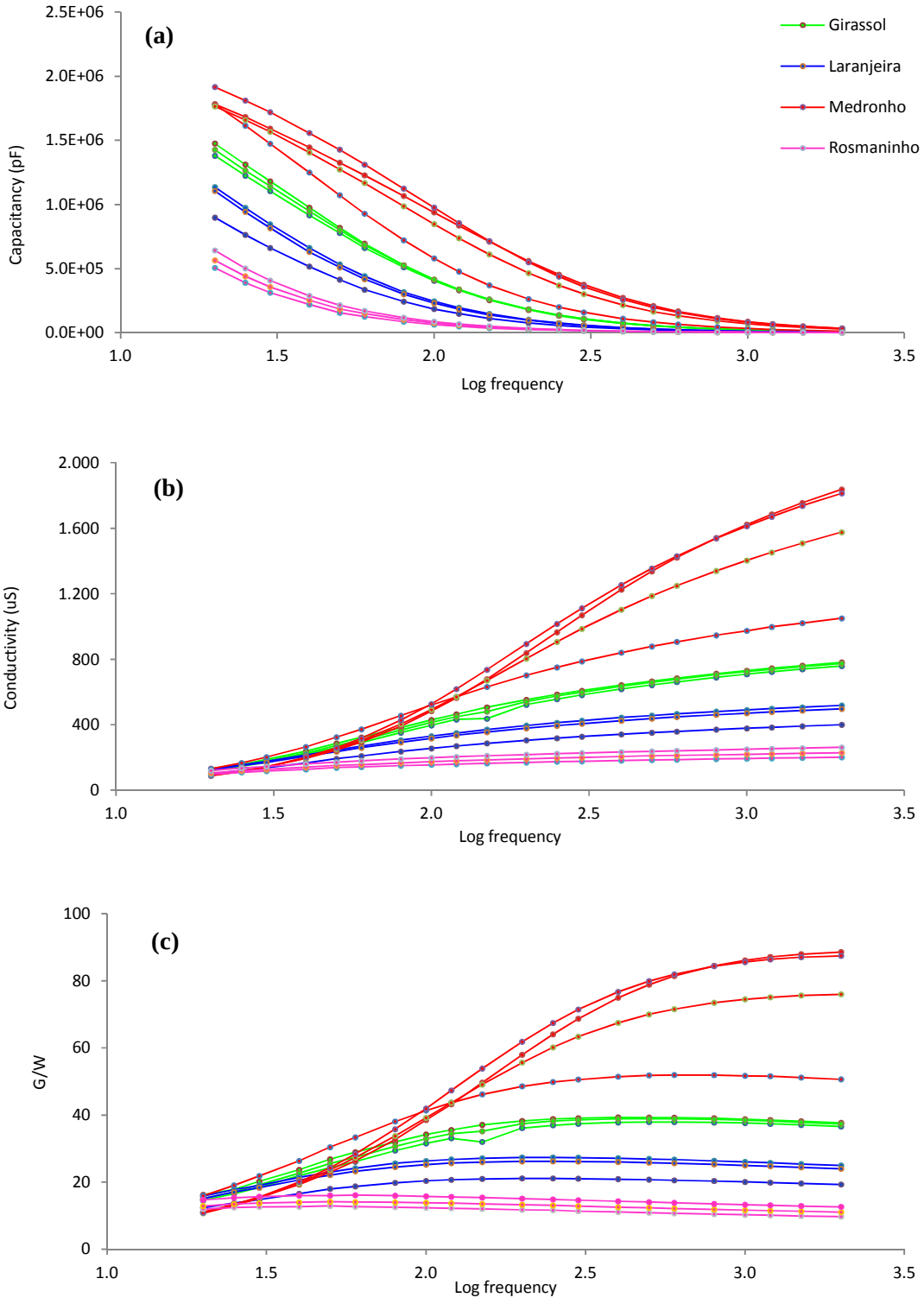


Figure 6.3 Example of the plots of the 3 readings (capacity, **a**; conductivity **b**; G/W, **c**) obtained with e-tongue for 13 all honey samples (○sunflower; ○orange blossom; ○strawberry tree and ○french lavender) using the Aluminum electrode.

In the previous figures it is clear that the four kinds of honey have different behaviours: strawberry tree ("Medronho") has always the higher values and then in descent orders there are sunflower ("Girassol"), orange blossom ("Laranjeira") and french lavender ("Rosmaninho"). The remaining electrodes (Pt, Au and ITO) showed the same behaviour, particularly the clearer separation of the honey samples with higher frequencies.

At first sight it seems possible to separate the samples just by a simple visual inspection of the figures. However, it is necessary statistical foundation. For this reason was applied a PCA analysis for all measurements obtained with e-tongue.

Prior to any PCA analysis there is a step called *preprocessing*. In this step the data is transformed by some mathematical operation with the aim of improving the global quality of the results obtained through PCA. The most common preprocessing procedures include filtering (to remove noise present in the signals) and derivation (in order to remove simple background effects). There are more sophisticated preprocessing techniques, but not deal with them here. For the analysis of results were considered three different types of preprocessing:

- Option 1: were having assumed the measures as they were. In other words, this means that were not performed any preprocessing. This also means that the shifts were accepted as meaningful (and not due to offset effects).
- Option 2: were having removed the effect of the offset and constant/linear background by the application of derivatives to the raw data.
- Option 3: were having fitted the shape of the curves by physically meaningful mathematical expression and apply the PCA procedure to the new set of variables (the fitting parameters) describing the shape of the curves. This method would have the advantage of being more robust against instrumental offset drifts.

In practice, the consistent operation of the e-tongue must rely on the calibration of the instrument with a known substance. This procedure is necessary to compensate for any instrumental offset drift or background. As explained above, offset may be eliminated by the use of derivatives. However, complex background effects (variable with frequency) are not fully eliminated by derivation and hence calibration against a known standard is the only way to take them into account. The bottom line here is that any of the preprocessing options described above does not eliminate the need of a rigorous calibration.

6.3.1.1 PCA on the electronic tongue (e-tongue)

The data matrix to PCA analysis for electronic tongue consisted of 13 rows (honey samples) and 252 columns (variables). In this step the matrix was supplied as:

Orange blossom ("Laranjeira")	**Strawberry tree ("Medronho")**	**French Lavender ("Rosmaninho")**	**Sunflower ("Girassol")**
Line/sample 1: Lar L1	Line/sample 4: Med MJ	Line/sample 8: Ros R1	Line/sample 11: Gir G1
Line/sample 2: Lar Bravura	Line/sample 5: Med Brito	Line/sample 9: Ros Brito	Line/sample 12: Gir Brito2011
Line/sample 3: Lar Brito	Line/sample 6: Med Pb2009	Line/sample 10: Ros SerraMel	Line/sample 13: Gir Brito2014
	Line/sample 7: Med Pb2010		

The figure 6.4 shows a two dimensional PCA plot four kinds of honey samples: Ros (french lavender), Lar (orange blossom), Gir (sunflower) and Med (strawberry tree) measurements performed by the e-tongue. The most important direction is the first, and referred to as "Principal Component 1" (PC1). Next, in importance, comes obviously the "Principal Component 2" (PC2).

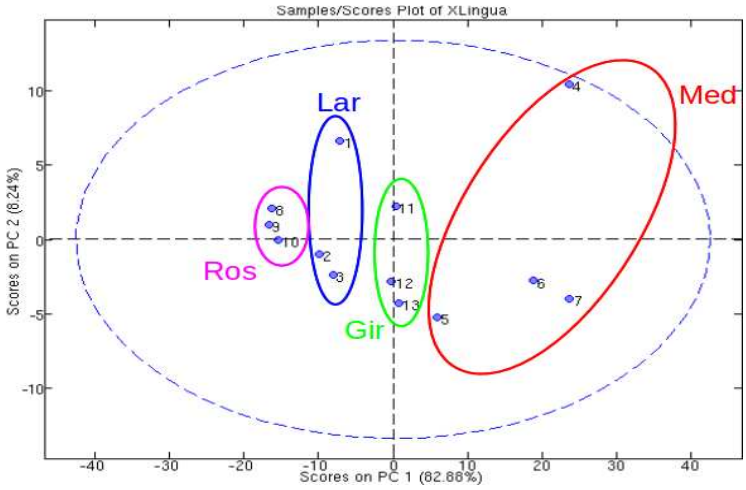


Figure 6.4 PCA scores plot for the e-tongue raw data of orange blossom (Lar ---); strawberry tree (Med ---); frech lavender (Ros ---); sunflower" (Gir ---) measurements. The number of honey samples is N=13 and each point represents one sample.

The first two principal components (PC1 and PC2) represent 91.12% of the total variation between samples measurements. As shown in figure 6.4, the PCA analysis groups the honey samples into four distinct clusters (Ros, Lar, Gir and Med) according to their botanical origin. The discrimination was made essentially by the first component (PC1–82.88% of the variance). It may be seen that the high frequencies variables in G/W and the capacitance with gold electrode were the most important contributions to PC1 (plot of PC1 loading; chapter 9, figure 9.6.1). The Med (strawberry tree, four honey samples) and Ros (french lavender, three honey samples) align along this direction in opposite sides, the first loading positively while the second loads negative. On the other side, Lar (orange blossom, three honey samples) and Gir (sunflower, three honey samples) have basically zero loading along PC1. On the other side, the second component (PC2–8.24% of the variance) does not allow for any kind of discrimination (figure 6.4). The Ros cluster seems to be the most homogeneous, while the Med cluster was the most heterogeneous. In the latter case some samples were disposed in the plot much apart, meaning that they have different electrical characteristics in spite of pertaining to the same group.

Since the measurements span a large range and there are several orders of magnitude (specifically for capacity values), was applied logarithm to the initial raw data matrix. That is, if X is the matrix of the raw data, then have simply taken:

$$X_1 = \log(X) \qquad (6.1)$$

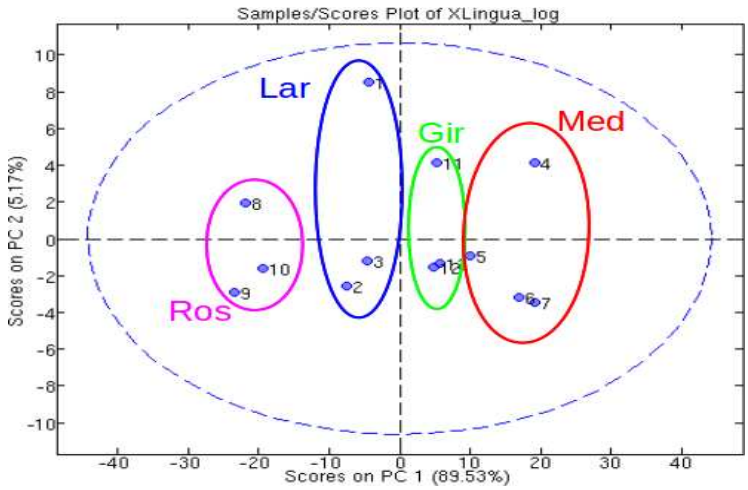


Figure 6.5 PCA scores plot for the logarithm of the e-tongue raw data matrix. Orange blossom (Lar ---); strawberry tree (Med ---); frech lavender (Ros ---); sunflower" (Gir ---). The number of honey samples is N=13 and each point represent one sample.

The result of this mathematic operation on the PCA plot is shown in figure 6.5. The conclusions are essentially the same as before (figure 6.4), although the plot becomes a little more compact (especially Med cluster); this indicates that the dispersion observed in the raw data matrix was overestimated and due to the large dynamic range of the measurements. The new PCA plots represent 94.70% of the total variance between honey samples measurements, PC1 (89.53%) and PC2 (5.17%); maintaining four clusters.

Derivation of the raw matrix usually leads to best results by removing any constant offset present in the signal. However, this process has the disadvantage of generating a new matrix with a relative higher content of noise. Hence the new matrix is:

$$X_2 = \frac{dX}{df} \qquad (6.2)$$

where X is the raw data matrix and f stands for frequency.

In order to perform the derivation it was necessary to make a pre-processing of the raw data, included an interpolation of the data to a set of equally spaced frequency points (from 20 to 2000 Hz in steps of 5 Hz).

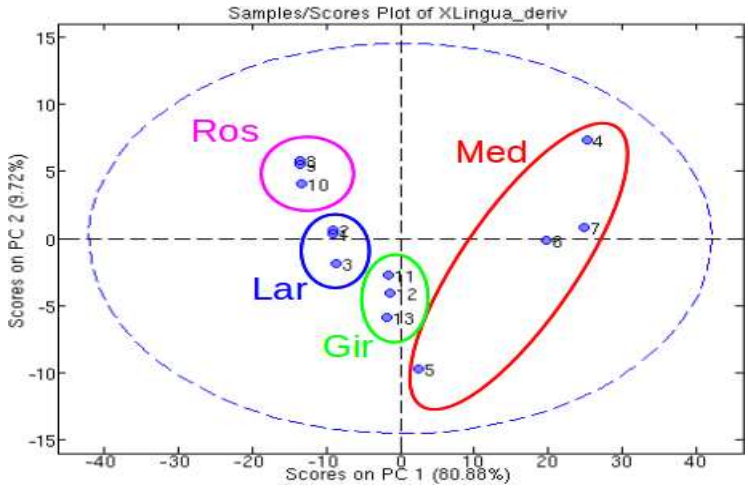


Figure 6.6 PCA scores plot for the derivative of the e-tongue data. Orange blossom (Lar ---); strawberry tree (Med ---); frech lavender (Ros ---); sunflower" (Gir ---) measurements. The number of honey samples N=13 is and each point represents one sample.

The new structure of the PCA plots (figure 6.6) now is more elaborate than before (figures 6.5 and 6.4). Recall that in the raw data analysis the discrimination was afforded by PC1 only. Now the PC2 (9.72% of the variance) plays some role. For example Ros (french lavender) and Gir (sunflower)

may be distinguished better by PC2, because of opposite loading in PC2. In whole, the analysis based on the derivative seems more robust than the analysis based on the raw data. The new role for PC2 may be understood as follows: the analysis of raw data was dominated by the offset. Therefore, the process of derivation removes any constant offset and hence the PCA will look for sources of variability other than offset. This means that PC2 will bring new information which allows better classification between samples.

In whole, this section demonstrates that the e-tongue data contains more information than simply offset. And what is more, removing the offset conveys a more convincing separation of honey types through PCA. The next step will be explore this fact in more detail and will be able to further improve the PCA clustering of honey types.

Was proceed then to another way of looking into the results, which consists of using data transformed by curve fitting. This means that we have fitted analytical expressions to the curves and used the fit parameters as "shape indicators". Then these fit parameters were analyzed with PCA rather than the raw data by itself.

The first model used for the capacitance may be found on the classical paper Pething and Kell (1987) and Kuang and Nelson (1998):

$$C(\omega) = C_{\infty} + \frac{C_S - C_{\infty}}{1 + w^2\tau^2} \qquad (6.3)$$

where $w = 2\pi f$ is the angular frequency (f is the frequency), τ is a relaxation time, C_s is the steady state capacitance, that is, the capacitance taken at $f = 0$, and C_∞ is the capacitance taken at frequencies much larger than $1/(2\pi f)$.

The expression above is derived from basic physical principles. However, there are in the literature some empirical deviations from this basic law, developed in order to achieve a better adjustment with the experimental data. One of the widest spread approximations is the Cole equation 6.4, in which the exponent 2 is replaced by (1-α) (α being an empirical fit parameter) in the expression of the permittivity. Following the same trend, were have used the following expression for the fits:

$$C(\omega) = C_\infty + \frac{C_S - C_\infty}{1+(w\tau)^\alpha} \qquad (6.4)$$

The second model for conductance was, again according Pethig and Kell (1987) and Kuang and Nelson (1998),

$$G(\omega) = G_S + (G_\infty - G_S) \cdot \frac{w^2\tau^2}{1+w^2\tau^2} \qquad (6.5)$$

where G stands for conductance and the subscripts have the same meaning as before (G_∞ = conductance at frequencies $1/(2\pi f)$ and G_s = conductance at $f = 0$). Again, were having adopted an empirical variation of this expression:

$$G(\omega) = G_S + (G_\infty - G_S) \cdot \frac{(w\tau)^\alpha}{1+(w\tau)^\alpha} \qquad (6.6)$$

Finally, the third model for relation G/W. Here were adopted a purely empirical expression, on the basis of the shape and not on any physical assumption. It turned out that the better fitting function was of the type:

$$G/W\ (\omega) = \frac{\omega^D}{A+B\omega+C\omega^2} \qquad (6.7)$$

Here all the parameters are purely empirical, which means that they do not bear any physical significance. These fits were applied successively to the 13 honey samples for each electrodes (Al, Pt, Au and ITO) and for each type of measure (capacitance, conductivity and G/W). Hence, each dataset of 21 frequencies was substituted by simply 4 fit parameters. The initial 252 variables were replaced by 48 variables, as shown in figure 6.7.

Al	Pt	Au	ITO	Al	Pt	Au	ITO	Al	Pt	Au	ITO	**4 electrodes**
1…4	1…4	1…4	1…4	1…4	1…4	1…4	1…4	1…4	1…4	1…4	1…4	**4 fit coefficients**
Capacitances				**Conductances**				**G/w**				**3 measures**

Figure 6.7 The structure of the e-tongue data when described by the coefficients of the fits.

The new matrix for the PCA has with 13 rows (honeys samples) and 48 variables (4 fit parameters x 3 measures x 4 electrodes). This matrix was again submitted to the PCA procedure and the result showed in figure 6.8.

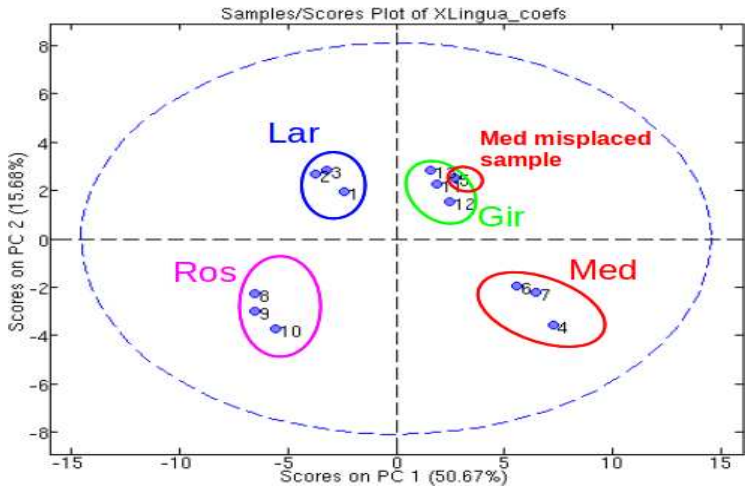


Figure 6.8 PCA scores plot for the fit coefficients of the e-tongue data. Orange blossom (Lar ---); strawberry tree (Med ---); frech lavender (Ros ---); sunflower" (Gir ---).

This new PCA plot provides better separation between the clusters (66.35% of total variance):

- each kind of honey samples occupies one quadrant
- Lar (orange blossom honey) is in the negative region of PC1 and positive region of PC2.

- Ros (french lavender honey) is in the negative regions of the both (PC1 and PC2).
- Gir (sunflower honey) is in the positive regions to both (PC1 and PC2).
- Med (strawberry tree honey) is in the positive region of PC1 and negative regions of PC2.

This means that the samples may be distinguished by the combination of PC1 (50.67% of variation) and PC2 (15.68% of variation). These results are better than the ones obtained through the raw data analysis (figure 6.4), where the classification was based only on PC1. In the PCA on the fit coefficients both PC1 and PC2 are important which increases the discrimination ability of the analysis. As stated already above, in the case of the raw data analysis, the PCA translates quantitatively the impression one has by direct inspection of the curves, that is: the honey types may be distinguished by the offset of the curves. Hence, the PC1 of the raw data has basically the meaning of offset. On the other side the fit coefficients matrix has intrinsic information about the shape of the curves and PC1 was more related with shape, not with offset. Hence, this PCA analysis becomes more robust and better allows classification of the honey samples.

Looking specifically at the scores plot it is possible to see that the samples within each kind of honey became closer to each other. Indeed, the plot shows (figure 6.8) less dispersion among samples of the same class. However, not everything is in place. The sample number 5 (identified in the plot by “Med misplaced”) is closer to the Gir (sunflower) cluster than Med (strawberry tree).

Looking back at figure 6.3, it is possible to identify one of the red curves (strawberry tree) with shape similar to the green ones (sunflower). From the viewpoint of the electrical properties this sample is actually behaving like a sunflower sample.

The conclusions of the tests with the e-tongue show that the analysis of the e-tongue data in the raw form was not the best approach. The raw data was dominated by an offset between the different curves (capacity, conductivity and relation G/W) which may be meaningful but which was not the best way to discriminate among different kinds of honey. In other words, looking directly into the data it was not the correct way to classify the honey types, although it seems easy and appealing at the first sight (figure 6.4). The reason for this was that the border between kinds of honey was not clearly defined. However, the values of the curves where the transitions take place were not obvious. On the other side, there was always the possibility of having extraneous offsets introduced by the instrumentation and consequent misinterpretation of the results. Hence, it is highly advisable to have a means of classifying the samples independent of offset or, at least, the most independent as possible.

The first attempt was made in this direction by introducing the derivative preprocesses. As is well known, the derivative removes constant offset and may improve the robustness of the analysis. This happened indeed. The derivative analysis yielded better clustering of the honey types in the PCA plot (figure 6.6).

Looking further into the classification based on the shape of the curves, were tried a new preprocessing. This was based on performing fits to the curves with semi-empirical expressions and four fitting parameters; then we have executed the PCA procedure on the matrix of the fitting parameters. This method proved to give the best results, providing the best clustering in the PCA plot and achieving a separation of the clusters according to the botanical origin honeys (figure 6.8).

Several researches have used the electronic tongue technique to determination of the botanical classification of honey. Dias et al., (2008) analyzed 52 commercial honey samples obtained from different regions of Portugal with multi-sensor through 20 sensors with voltage measurements. The

results obtained demonstrated that e-tongue has a reasonable efficiency for classification of honey of the most common three kinds (*Erica*, *Echium* and *Lavandula*). Wei et al., (2009) used e-tongue method with seven potentiometric chemical (taste) sensors to classify eight kinds of honeys of different floral origin and five kinds of Acacia honeys of different geographical origin. The results showed that e-tongue with PCA could classify and identify different honey samples. Major et al., (2011) used a commercial electronic tongue (αAstree, Alpha M.O.S) for botanical classification and physicochemical characterization on 12 honey samples (5 Acacia, 4 honeydew and 3 chestnuts). The e-tongue was comprised of seven potentiometric chemical sensors. Botanical classification was performed by PCA (79.57% of total variance), Artificial Neural Network (ANN), Canonical Correlation Analysis (CCA) and physicochemical characterization by ANN. After multivariate analysis the e-tongue technique showed the potential as a tool rapid honey classification and characterization. Other study, Wei et al., (2011) used a voltametric e-tongue based on multifrequency large amplitude pulses to classify monofloral honey of seven kinds of floral origin. The e-tongue was composed by six electrodes (Au, Ag, Pt, Pd, W and Ti) and with the help of multivariate analysis it was possible to discriminate the seven kinds of honey of different floral origin. The last research was by Escriche et al., (2012), who evaluated the effectiveness of a potentiometric electronic tongue, made of various metals (Au, Ag, Cu) and metallic compounds (Ag_2O, AgCl, Ag_2CO_3, Cu_2O) to the differentiation of honey in three states (raw, liquefied and pasteurized). The potentiometric values do not seem suitable for discrimination between state honeys but showed efficiency to classify honey with different floral origin (PCA 85% of total variance).

6.3.2 UV-Vis spectroscopy data

The absorption spectra taken in the UV spanned the wavelength range from 200 to 400 nm, in a total of 201 data points with an average separation of about 1 nm. The raw data for 35 honey samples were shown in figure 6.9:

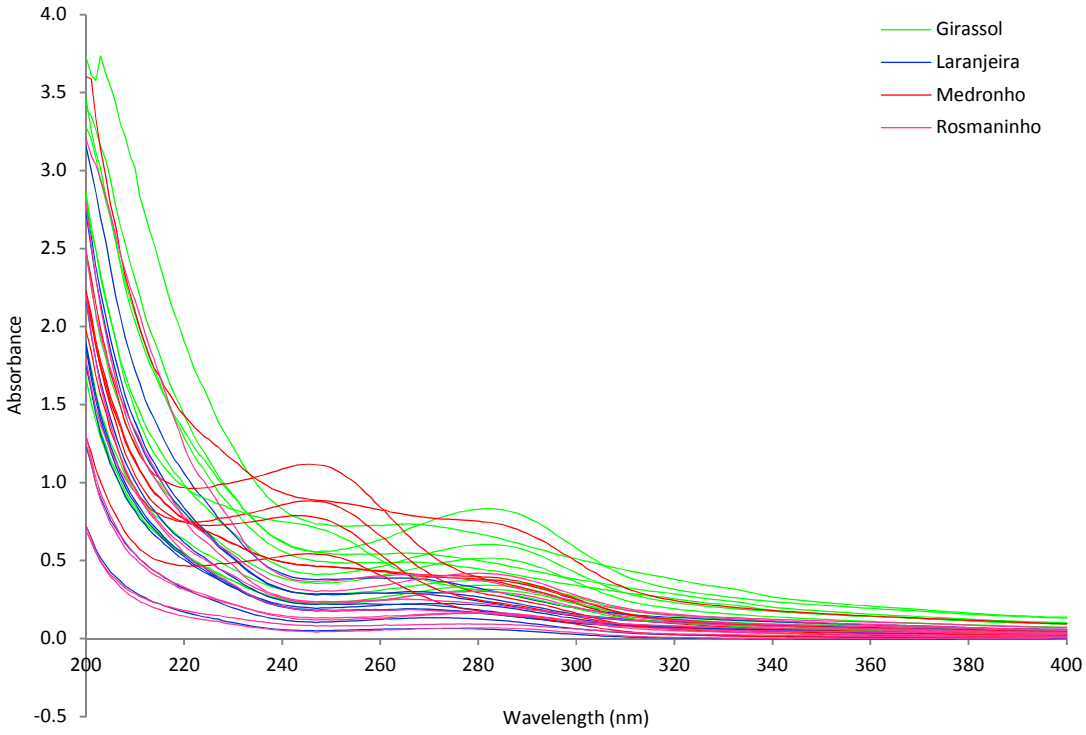


Figure 6.9 Data points for UV-Vis Spectroscopy. The honey type is identified by a color scheme, Girassol (---, sunflower); Laranjeira (---, orange blossom); Medronho (---, strawberry tree); Rosmaninho (---, french lavender).

The curves show an increase of absorption towards the UV (200–300 nm). There is a more or less pronounced intermediate peak (310–400 nm) and then the absorption tends to zero. It should be noted that the concentrations of the samples used in UV and Vis-NIR spectroscopies were very different (much lower in the UV measurements, 0.17 g/mL) and hence the absolute values of absorbance in this section and in the Vis-NIR section cannot be compared directly.

6.3.2.1 PCA on the UV-Vis spectroscopy

The honey samples for the UV-Vis analysis were the same of the e-tongue, but were have made some additional replicate measurements (a replicate measurement is a measurement performed on the same type of honey, but extracting the sample from a different bottle/production batch). Hence, the input for the PCA analysis is a matrix of 35 rows (honey samples) and 201 columns (variables = absorbance at each measured wavelength). In this step the matrix is supplied as is. The lines were attributed as follows:

Orange blossom ("Laranjeira")	**Strawberry tree ("Medronho")**	**French Lavender ("Rosmaninho")**	**Sunflower ("Girassol")**
[class 1] Lines/samples 1-3: Lar L1 (3 replicates)	[class 4] Line/sample 10-12: Med MJ (3 replicates)	[class 8] Line/sample 18-20: Ros R1 (3 replicates)	[class 11] Line/sample 27-29: Gir G1 (3 replicates)
[class 2] Line/sample 4-6: Lar Bravura (3 replicates)	[class 5] Line/sample 13-15: Med Brito (3 replicates)	[class 9] Line/sample 21-23: Ros Brito (3 replicates)	[class 12] Line/sample 30-32: Gir Brito2011 (3 replicates)
[class 3] Line/sample 7-9: Lar Brito (3 replicates)	[class 6] Line/sample 16: Med Pb2009 (1 replicate)	[class 10] Line/sample 24-26: Ros SerraMel (3 replicates)	[class 13] Line/sample 33-35: Gir Brito2014 (3 replicates)
	[class 7] Line/sample 17: Med Pb2010 (1 replicate)		

Between square parentheses indicated the class number used in the e-tongue analysis. For example: the first 3 samples were all Lar L1 (orange blossom honey) and in the e-tongue analysis the sample #1 was precisely Lar L1. The same applies to the remaining classes.

The application of the PCA procedure to all honey samples produces the plot shown in figure 6.10. It may be observed some dispersion, even among the replicates of the same kind of honey. The dispersion in the UV was larger than in the Vis-NIR spectra, as we shall see in the next sections. It

is possible that the compounds that absorb in the UV were more sensitive to storage conditions than those that absorb in the Vis/NIR. Hence, bad storage conditions alter more significantly the UV spectra; for example increment of the melanoidins content (product of Maillard reaction) that are brown compounds or oxidations of polyphenols (Jimenez et al., 1994; Gonzales et al., 1999).

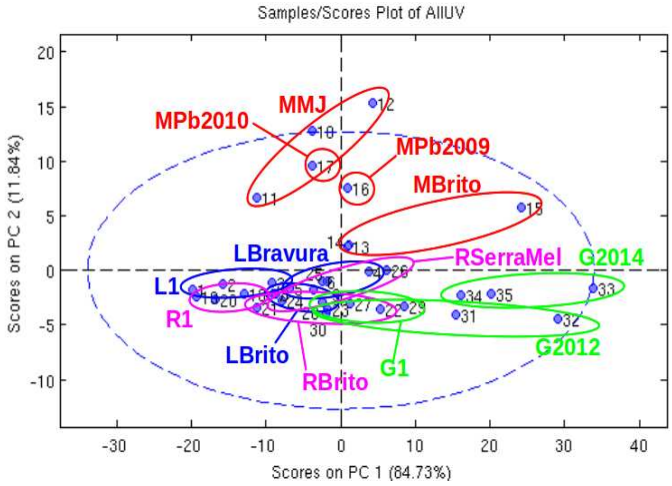


Figure 6.10 PCA on the raw UV-Vis data and all the samples. Orange blossom honey (Lar ---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

The PCA (96.57% of total variance) plot of UV-Vis shows clearly that there is no obvious distinction between orange blossom (Lar) and french lavender (Ros); these clusters are mainly located in negative region of both quadrants (PC1 and PC2). However, sunflower (Gir) and strawberry tree (Med) were reasonably separated from the other classes. The strawberry tree honey was mainly located in positive region for PC1 and PC2.

In order to perform a comparison with the results obtained with electronic tongue was created a new matrix of 13 rows (honey samples) and 201 columns (variables). The 13 rows were the averages of the replicates. The PCA plot of the new matrix (13 x 201) is shown in figure 6.11.

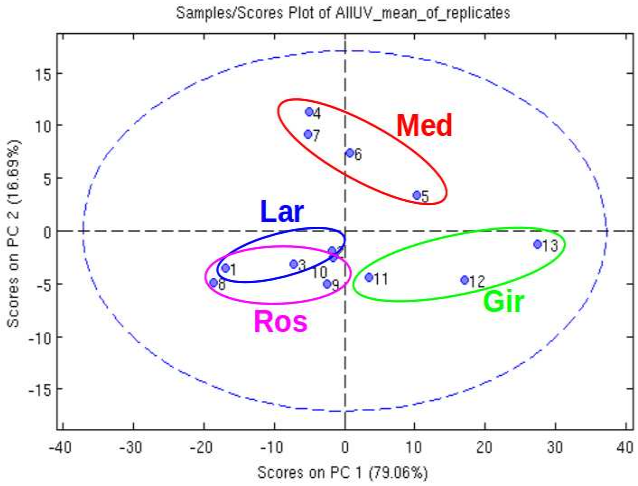


Figure 6.11 PCA on the raw UV-Vis data and one the average on the replicates. Orange blossom honey (Lar ---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

The first two components (PC1 and PC2) represent 97.75% of the total variance between all samples measurements. With the new matrix the separation of the Med and Gir clusters is clearer; on the other hand for the other two clusters (Lar and Ros) this separation is not evident (figure 6.11).

Following the same steps done in the e-tongue analysis, were have applied next the derivative preprocess in order to remove any constant slope background or offset. Were have calculated the first and second derivatives of the spectral data using the Savitsky-Golay method (Kelly et al., 2004; Woodcock et al., 2007) filter with width of 21 points for each side, polynomial order 2 and derivative order 1; with this procedure one obtains smooth curves. After this pretreatment a new PCA plots were (90.03% of total variance, figure 6.12) for the first and second derivatives. This plot was very similar to the one obtained with the raw data (figure 6.11). Hence, the same problem remains it is not possible to clearly distinguish the difference between orange blossom honey (Lar) and french lavender honey (Ros).

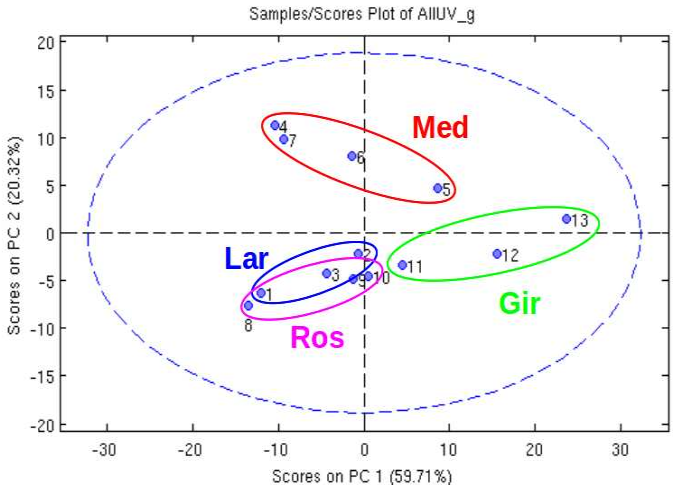


Figure 6.12 PCA plot for the first derivative of the UV-Vis spectroscopy data. Orange blossom honey (Lar---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

From the second derivate was obtained a new PCA plot (61.47% of total variance, figure 6.13) that does not seem to improve the PCA results obtained with the first derivative. On the contrary, there is a clear increment of the dispersion to Gir cluster, probably due to the noise that comes with the derivative operation.

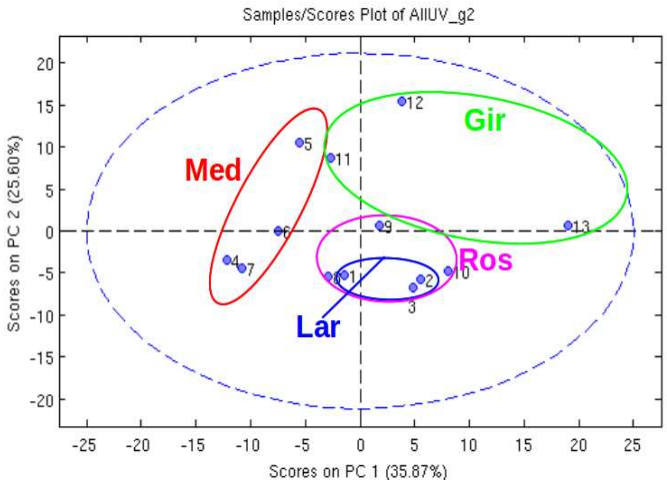


Figure 6.13 PCA plot for the second derivative of the UV-Vis spectroscopy data. Orange blossom honey (Lar---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

Following the steps taken for electronic tongue, were have preceded to the parametrization of the UV data curves through curve fitting.

All the curves show a tendency to increase in the low wavelength range (220–290 nm), but the suggested peak is not observed in spectra, lying below 200 nm (figure 6.9). Hence, this general tendency was assumed as a baseline. This baseline was fitted to the first 10 points and to the last 50 points. The shape of the fitting curve was:

$$y(x) = \frac{1}{A + Bx + Cx^2 + Dx^3} \qquad (6.8)$$

The choice of this function does not have any underlying physical assumption. It was chosen simply because it adjusted well to all spectra. To ease the process of fitting we have made all the fits in reduced variables *x*, such that:

$$x_i = \frac{\lambda_i - \lambda_1}{\lambda_{201} - \lambda_1}, i = 1 \ldots 201 \qquad (6.9)$$

where the λ_i are the 201 measured wavelengths. In the end the variables were transformed back to the original wavelength scale. The next figure shows the results obtained after the first step of baseline fitting. This is an example for one spectrum (figure 6.14),

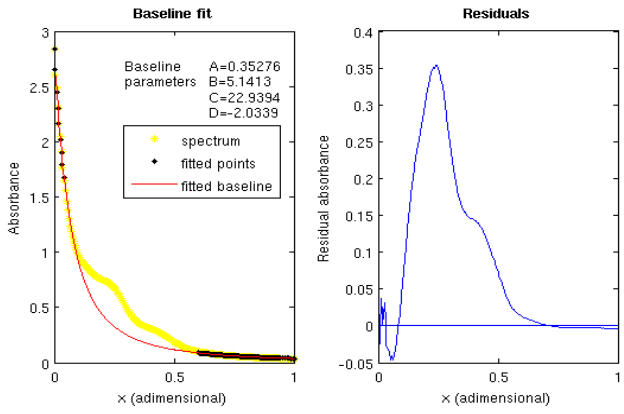


Figure 6.14 Plot of parametrization of the UV data for one sample (left). The initial data is shown in yellow, the points used for the fit in black and the baseline fit in red. Plot of the residual spectrum (right).

The left plot shows the original spectrum in yellow. The points used to perform the fit are shown in black and the fit to the baseline is shown as a red line. On the right were plot the residual, that is, the initial spectrum minus the baseline:

$$residual = spectrum - baseline \tag{6.10}$$

The residual shows one positive peak and one negative, smaller peak. In the second step of the fit we adjust Gaussians to the positive and negative peaks:

$$peak(\lambda) = \frac{A}{\sigma\sqrt{2\pi}} \exp\left[-(\lambda-\mu)^2 / 2\sigma^2\right], \tag{6.11}$$

This process was repeated three times (baseline + 2 stages of peak fitting) to each curve. Therefore each curve was then parametrized by a total of 4 (baseline) + 12 (Gaussians) = 16 parameters (one example in figure 6.15); with this procedure we have obtained a new matrix to use in PCA that has then the following dimension: 13 (honey samples) rows and 16 (variables) columns.

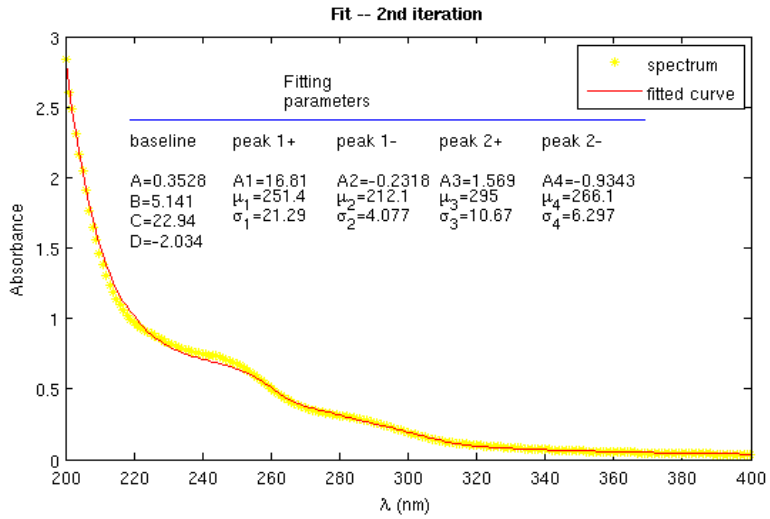


Figure 6.15 Example of the parametrization of one UV-Vis spectrum (baseline + two stages of peak fitting) with 16 fit parameters.

The results to new PCA plot after parametrization of the UV curve is shown in figure 6.16.

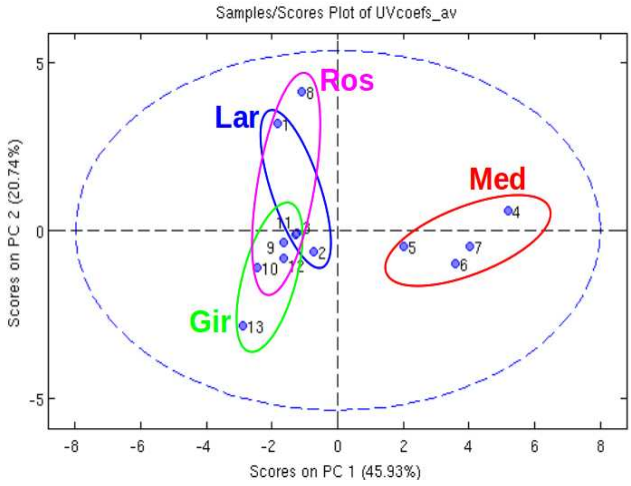


Figure 6.16 PCA plot for parametrization of UV-Vis spectrum. Each point represents one sample. Orange blossom honey (Lar---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

The result is disappointing when compared to the results obtained from the electronic tongue (figure 6.8). The parametrization of the curve was a worse approach than using the first and second derivate transformation (figures 6.12 – 6.13) and or even raw data average (figure 6.11). An example is the Gir (sunflower) cluster which is now partially merged with Lar (orange blossom) and Ros (french lavender) clusters. Only the Med (strawberry tree) cluster is positively identified. There is, however, a new and positive point: the criterion to identify Med cluster is PC1>0. This is a strong condition, since PC1 (45.93% of variance) is the most important component. On the other side, the scores plot obtained for the first derivative of the spectra suggest a criterion PC2>0 (20.32% of variance), which is not so strong (figure 6.16). Hence, if the main goal was to separate strawberry tree honey from the other types of honey the parametrization approach is the best choice. For a general application however, it seems that the first derivative is the best approach, and we will keep it.

6.3.3 Vis-NIR spectroscopy data

The absorption spectra taken in the Vis-NIR spanned the wavelength range from 345.26 to 1036.70 nm, in a total of 3648 data points with an average separation of about 0.19 nm. The first and the last points showed pronounced instrumental oscillations and were removed from the analysis. Therefore were have used the range from 403.85 to 1032.2 nm, in a total of 3347 data points. The raw data are depicted in figure 6.17:

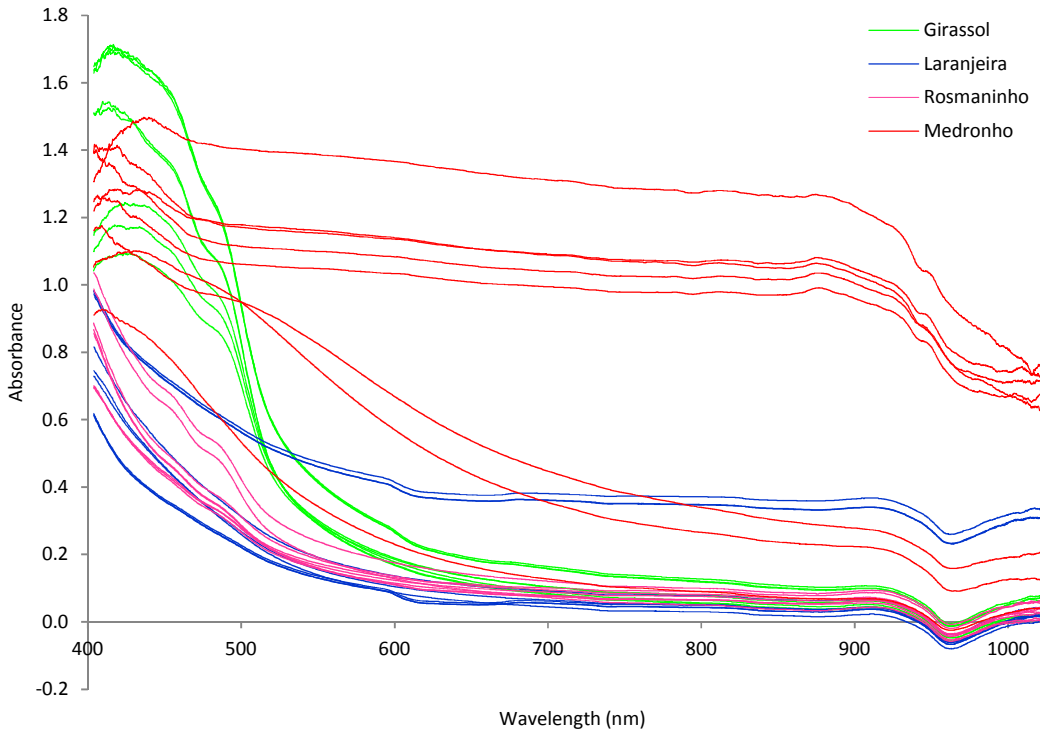


Figure 6.17 Vis/NIR spectra from 403.85 to 1032.2 nm (total 3347 points). The honey type is identified by a color scheme, Orange blossom honey (Laranjeira ---); strawberry tree honey (Medronho---); french lavender honey (Rosmaninho ---) and sunflower honey (Girassol ---).

All the curves show more intense absorption in the blue (420–440 nm), suggesting even more absorption in the UV. The blue peaks were large and decrease to an approximately constant background. The exception to this trend comes from the Medronho (strawberry tree honey) spectra,

which were characterized by high plateaus across the entire visible region (400–750 nm). It is possible that the origin of these plateaus is scattering rather than absorption. Indeed, if the Medronho honey samples would have more or larger suspended particulates, than the extinction of the beam due to scattering effects would increase dramatically. The next result of this effect would be an observable increase in absorbance, although not caused by absorption itself.

The other interesting aspect was the dip observed in the spectra around 960 nm. In some cases the absorbance goes negative, indicating that the blank (water) absorbs more than honey in this wavelength. To understand why, it is useful to look at plot of the absorption coefficient of water (figure 6.18) obtained from the data of Hale and Querry (1973) and Palmer and Williams (1974).

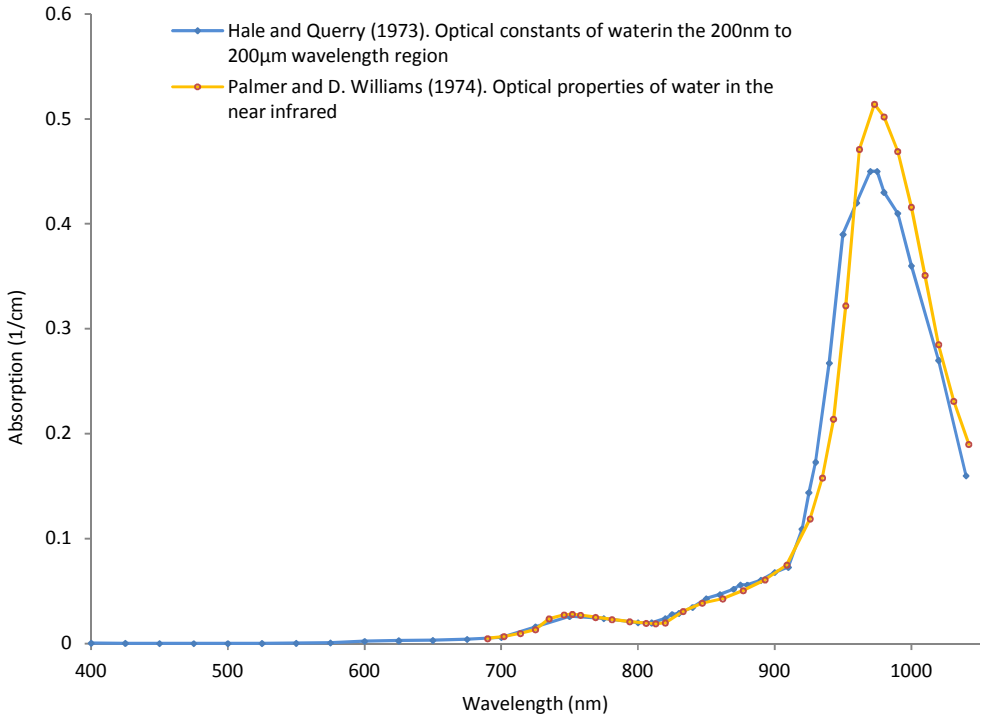


Figure 6.18 Data of Hale and Querry (1973) for Vis-NIR (400–1040 nm) and Palmer and Williams (1974) for Vis-NIR (690–1042 nm) measurements of water.

The figure 6.18 was obtained from the data of Hale and Querry (1973) and Palmer and Williams (1974). The absorption by water has a pronounced local peak around 970–975 nm (absorption = 0.45 cm^{-1}) and 973 nm (absorption = 0.514 cm^{-1}), respectively (data experimental in annexes table 9.6.2). The organic compounds of honey should not have the same behavior in this wavelength and hence the absorption of honey decreases relatively to that of water around 960–970 nm.

6.3.3.1 PCA on the Vis-NIR spectroscopy

The honey samples for the Vis-NIR analysis were the same used in the UV-Vis analysis, but with some additional replicate measurements (the replicates are not exactly the same of the UV-Vis). Hence, the input for the PCA analysis was a matrix of 34 rows (honey samples) times 3347 columns (variables = absorbance at each measured wavelength). In this step the matrix is supplied as is. The lines were attributed as follows:

Orange blossom ("Laranjeira")	**Strawberry tree ("Medronho")**	**French Lavender ("Rosmaninho")**	**Sunflower ("Girassol")**
[class 1] Lines/samples 1-3: Lar L1 (3 replicates)	[class 4] Line/sample 10-12: Med MJ (3 replicates)	[class 8] Line/sample 18-20: Ros R1 (3 replicates)	[class 11] Line/sample 26-28: Gir G1 (3 replicates)
[class 2] Line/sample 4-6: Lar Bravura (3 replicates)	[class 5] Line/sample 13-15: Med Brito (3 replicates)	[class 9] Line/sample 21-23: Ros Brito(3 replicates)	[class 12] Line/sample 29-31: Gir Brito2011 (3 replicates)
[class 3] Line/sample 7-9: Lar Brito (3 replicates)	[class 6] Line/sample 16: Med Pb2009 (1 replicate)	[class 10] Line/sample 24-25: Ros SerraMel (2 replicates)	[class 13] Line/sample 32-34: Gir Brito2014 (3 replicates)
	[class 7] Line/sample 17: Med Pb2010 (1 replicate)		

Between square parentheses were indicated the class number used in the e-tongue analysis. For example: the first 3 samples were all Lar L1 (orange blossom honey) and in the e-tongue analysis the sample #1 was precisely Lar L1. The same applies to the remaining classes.

The application of the PCA (99.10% of total variance) procedure to all honey samples produces the plot shown in figure 6.19. As expected, replicates of the same floral origin honey stay close to each other. The exception was Med Brito (samples 13, 14 and 15), where it can be seen that honey sample 13 was far away from the other two. It was also clear that there is no clear distinction between Lar (orange blossom) and Ros (french lavender). However, Gir (sunflower) and Med (strawberry tree) were reasonably separated from the other classes.

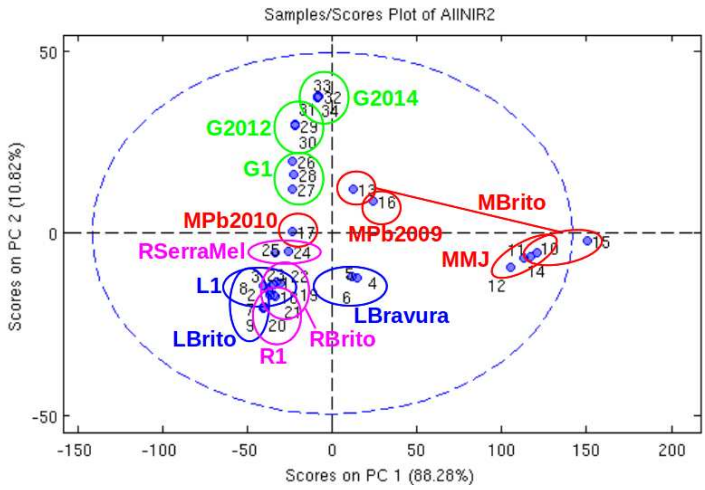


Figure 6.19 PCA on the raw Vis-NIR data and all the samples. Orange blossom honey (Lar ---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

In order to perform a comparison with the results obtained with the e-tongue and UV-Vis results, were have performed the average over the replicates and obtain the same 13 classes used in the previous analysis. The PCA plot to new matrix is shown in figure 6.20.

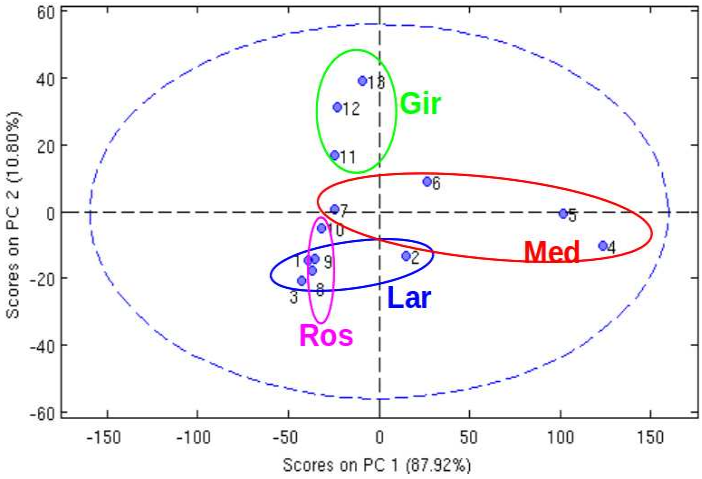


Figure 6.20 PCA on the raw Vis-NIR data and averages of the replicates. Orange blossom honey (Lar ---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

The result obtained by the new PCA (98.72% of total variance) plot after average of the replicates shows basically basically the same information as the previous PCA plot (figure 6.19). The Lar and Ros clusters were not distinguishable, Gir cluster was clearly different from the others and Med cluster had a large dispersion: especially 6 and 7 samples.

In the remaining of this section, were analyzed only the data consisting on the average of the replicates, because were have seen that the information is basically the same with or without averaging.

Following the steps taken in the e-tongue and UV-Vis data, we presented nest the results obtained after derivative preprocessing. The derivatives were obtained through the application of the first and second derivative Savitzky-Golay filter (Kelly et al., 2004; Woodcock et al., 2007) with width of 50 points for each side, polynomial order 2 and derivative order 1. After this pretreatment the PCA was run and the plot obtained is shown in figure 6.21 to first derivate.

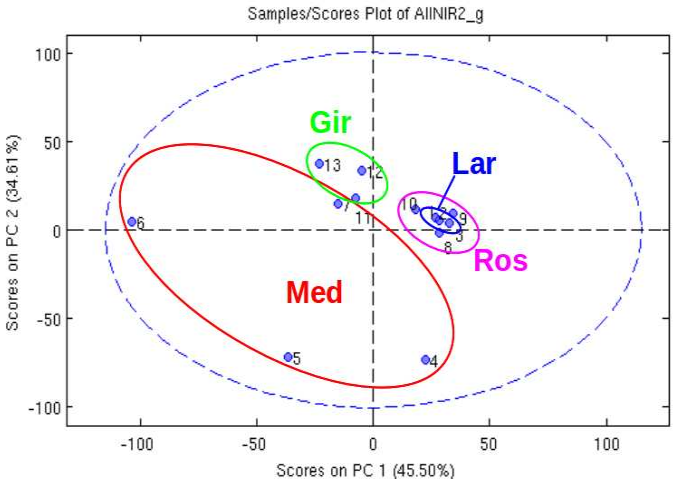


Figure 6.21 PCA plot for the first derivative of the Vis-NIR spectroscopy data. Orange blossom honey (Lar ----); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

The PCA plot for the second derivative is shown in figure 6.22. The dispersion of the clusters is reduced the separation between Med and Gir improved. Hence, the new PCA (63.69% of total variance) plot is slightly better than the average raw data analysis and the first derivative analysis (figure 6.20 and 6.21, respectively). However, the impossibility of separating the Lar cluster and Ros cluster is still obvious.

Best results were obtained by Woodcock et al., (2007) after using this technique at the wavelength range of 1100–2498 nm, to confirm the geographical origin of the different honey samples. This study identified and classified honey from different countries (Ireland, Mexico, Spain, Argentine, Czech Republic and Hungary) with analysis of data performed PCA plots (96% of total variance). Ruoff et al., (2006) used the Fourier transform-NIR technique to evaluate the authentication of eight unifloral and polyfloral honey, with the help of chemometric evaluations. The results established that NIR spectroscopy combined with chemometrics offers a promising approach for the

authentication of certain unifloral honeys and to problems related with the determination of the composition of polyfloral honeys.

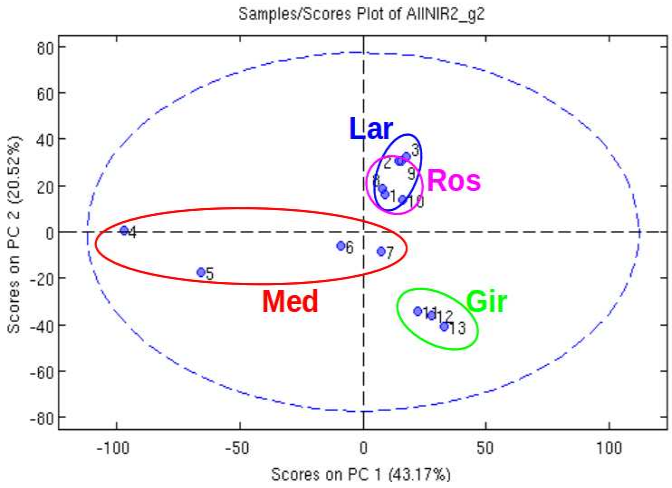


Figure 6.22 PCA plot for the second derivative of the Vis-NIR spectroscopy data. Orange blossom honey (Lar ---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

Finally were proceed to the last step obtain parametrized curves and the corresponding fitting coefficients to be used as the PCA matrix.

First of all, a baseline was determined. This baseline corresponds to the linear fit of data between 750 and 850 nm, where a linear slope was observed for all the spectra. The baseline is hence of the form:

$$baseline = m\lambda + b, \qquad (6.12)$$

where λ represents the wavelength.

The linear fit was then extrapolated across the entire wavelength range and was used as baseline. Figure 6.23 shows an example for orange blossom honey. The baseline is represented in the insert, as the black straight line.

After this first step, the baseline was subtracted from the spectrum. The remaining curve shows prominent peaks on the left and on the right. These peaks are fitted by Gaussian curves of the form:

$$peak(\lambda) = \frac{A}{\sigma\sqrt{2\pi}} \exp\left[-(\lambda-\mu)^2 / 2\sigma^2\right], \qquad (6.13)$$

where A is the amplitude, μ is the central wavelength of the peak and σ the standard deviation.

Figure 6.23, shows the peaks that are superimposed on the background. The most important peak is on the left and it is fitted by a first Gaussian. In the insert this Gaussian is represented in red line. Usually one Gaussian alone is not enough, and a second, auxiliary Gaussian is used to improve the fit. This second Gaussian is represented by the magenta line in the inserts. Finally, the dip in the NIR part of the spectrum (related to the shape of the water absorption curve, as explained before) is fitted by a third Gaussian. The latter is represented in blue in the insert of the figure.

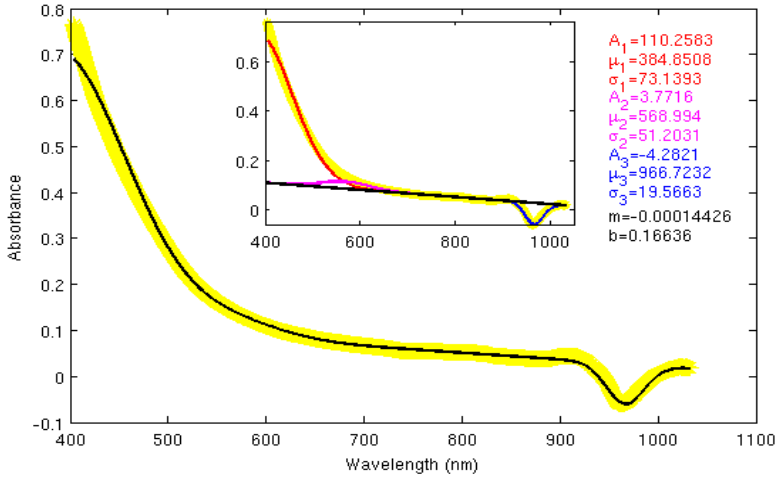


Figure 6.23 Fit for one of the samples of orange blossom. Experimental data (---) and fitted curve (---). In the insert: experimental data (---), baseline (---), Gaussian 1 (---), Gaussian 2 (---) and Gaussian 3 (---). The fitting coefficients are written in the same color code.

From the previous explanation it is now clear that the Vis-NIR curves were parameterized by eleven coefficients (slope and ordinate at the origin, *m* and *b*; and the 3 amplitudes, 3 center wavelengths and 3 standard deviations from the Gaussians). The new matrix for use in the PCA has then 13 rows (honey samples) and 11 columns (variables). Were have added a twelfth variable, which is the squared sum of errors *sse*:

$$sse = \sum (y_i - Y_i)^2 \qquad (6.14)$$

where y_i represents the experimental data and Y_i represents the fitted curve. The sum runs over all the 3347 variables.

Applying the PCA algorithm to this new matrix one obtains the result depicted in figure 6.24.

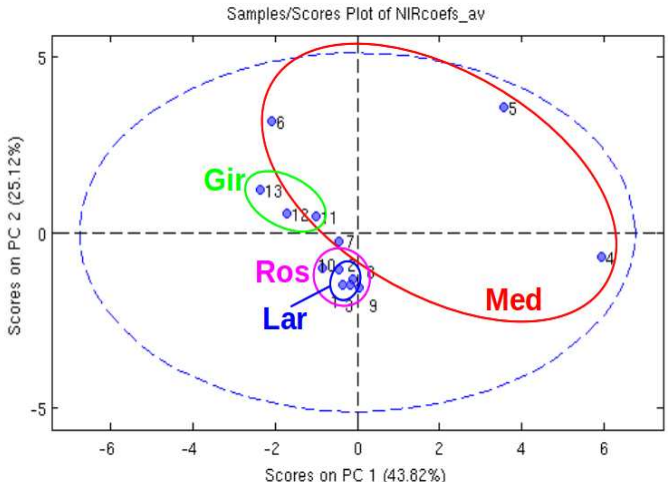


Figure 6.24 PCA plot obtained through the Vis-NIR fitting coefficients; total of 13 samples with 12 variables. Orange blossom honey (Lar ---); strawberry tree honey (Med ---); french lavender honey (Ros ---) and sunflower honey (Gir ---).

Contrary to the results obtained with the e-tongue, and similarly to the UV-Vis results, the parametrization of the VIS-NIR curves did not yield better PCA (68.94% of total variance) results than the derivative approach. This was probably due to the fact that the Vis-NIR (and also the UV-Vis) spectra are complex curves, and a simple parametrization scheme such as the one employed here, fails to capture all the important features. More robust fitting procedures should be developed in a future work.

It was also interesting to note that the parametrization approach yielded worst results for the optical spectroscopic techniques (UV and NIR) and better for the electrical spectroscopic technique (e-tongue). However, one must have in mind that the electrical spectroscopic technique is actually conveying three types of curves for each sample (capacitance, conductance and G/w) and each of the optical techniques was conveying only one curve (the absorbance). The electrical spectroscopy data benefits from the beginning from more "contrast" between the samples due to its three-fold

nature. In this perspective it would be more enlightening to compare the e-tongue data with the aggregate of UV-Vis and Vis-NIR data. One could then understand this ensemble as the spectroscopic data, with two output curves (UV and Vis-NIR). In order to create that ensemble we have simply joined the UV fit parameters matrix [13 (honey samples) x 16 (4 baseline + 12 Gaussians)] with the Vis-NIR fit parameters matrix [13 (honey samples) x 12 (11 coefficients + *sse*)], creating a matrix for the spectroscopic fit parameters (13 x 28). The new PCA analysis on this matrix resulted in the scores plot shown in figure 6.25.

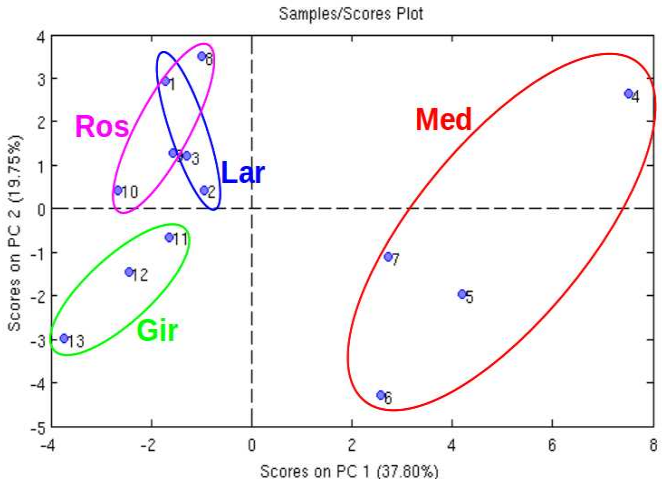


Figure 6.25 PCA plot join of UV-Vis and Vis-NIR spectrum data for the 13 honey samples and 28 variables.

This scores plot was indeed much better than any of the scores plotted obtained from the individual UV and Vis-NIR data. The separation between Med (strawberry tree honey) cluster, in the positive region of PC1, and the other classes was very clear (negative region PC1), as for the Gir (sunflower) cluster of honey samples. There was still an overlap between Ros (french lavender honey) and Lar (orange blossom honey) clusters. Anyway, this was the best scores plot obtained so far from spectroscopic data.

This last result shows clearly that the specificity of the PCA (57.55% of total variance) clustering improves with the addition of independent sources of information. The final goal will be to merge all the information available, and this will be the subject of the last section.

6.4 PCA analysis performed with sensor fusion

The next goal is the merging of all the information available and be able to extract the best of it.

The first approach was what one could describe as "brute force" approach: simply merge all the available matrices and apply PCA to this global matrix. This approach is shown schematically in figure 6.26.

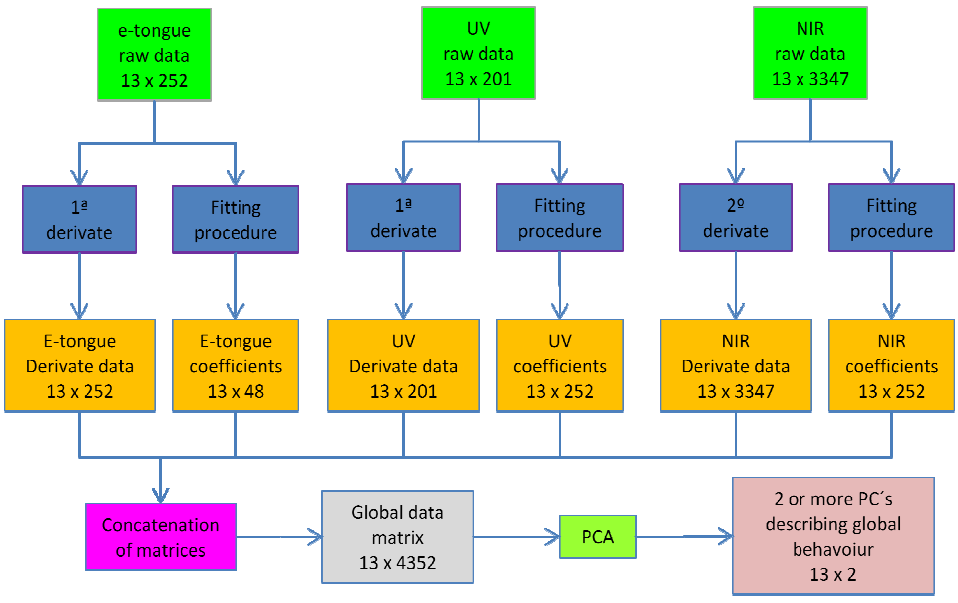


Figure 6.26 Schematic diagram for the fusion of the data coming from the three techniques (e-tongue, UV-Vis and Vis-NIR) – the "brute force" approach.

In this approach we have used all the fitting parameters matrices and the most promising "spectra-like" matrices (for example, for the Vis-NIR analysis that the second derivative yielded best results

than raw data and first derivative data). The result was a global matrix with 13 lines (samples) and 4352 columns (variables). One again a PCA was applied on this matrix and the scores plot is shown in figure 6.27.

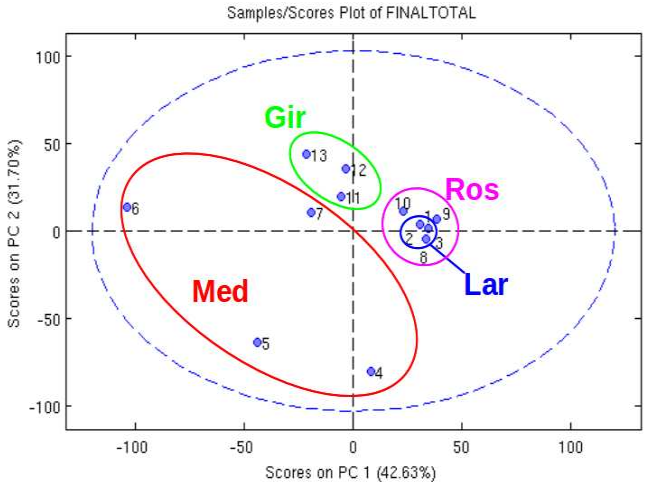


Figure 6.27 PCA plot of "brute force" according diagram schematic in figure 6.25.

The result was essentially the same as obtained in previous PCA scores plots. And it was not one of the best: the dispersion on Med (strawberry tree honey) cluster was large; so large indeed that sample 7 should be classified as sunflower honey (Gir cluster). The overlap between Lar (orange blossom honey) and Ros (french lavender honey) clusters assume the worst possible form, with the Lar ellipse totally surrounded by the Ros one.

In brief, the brute force approach did not yield good results. One of the main reasons for this is easy to understand: the dimensionality of the several contributions (that is, of each matrix) is very different. The Vis-NIR second derivative spectra contribute alone with 3347 of the 4352 variables. So, the PCA analysis was biased towards the NIR contribution, and explains mainly the variability associated with this contribution. So, it is important to place all the contributions on the same level, that is, with the same dimensionality.

In the case of Vis-NIR derivative data, we could pick, for example, 1 in 200 points, obtaining reduced spectra of about 60 points. But then some questions would be raised. For example, it was not obvious what should be the good choice of dimensionality: 60 points will be sufficient? or less would be better? And dropping 199 points out of 200 would surely lead to loss of information - most probably, the most important wavelengths would be lost.

Hence, an arbitrary reduction of dimensionality is not a good answer to the problem. In the following, was proposed an approach that solves the problem of dissimilar dimensionalities while keeping the fundamental information of each contribution.

The graphical description of the proposed approach is depicted in figure 6.28.

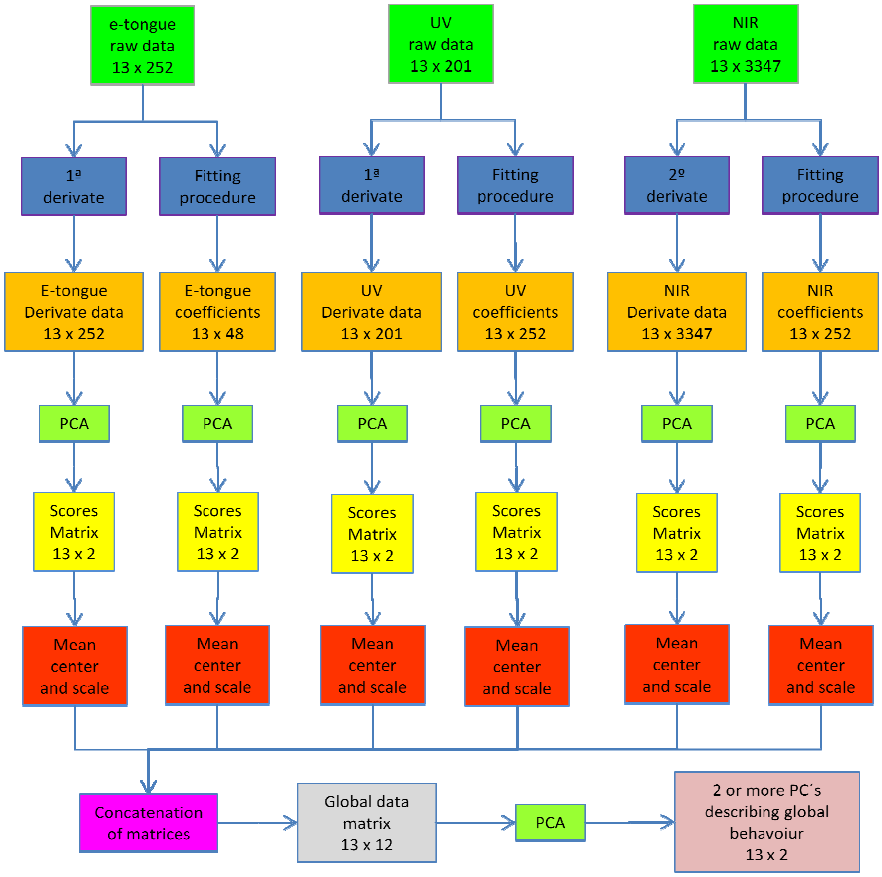


Figure 6.28 Schematic diagram for the new approach proposed for data fusion.

The idea is simple: each contribution (each matrix) is analyzed by PCA separately. The output of each PCA analysis is a 13 x 2 scores matrix (generally 13 x N, where N is the number of principal components, but we retain only the first two). The first two components of each contribution retain its essential information. And after the PCA all contributions are represented by the same number of variables: the two principal components. The second step is the so called group scaling: each scores matrix is scaled independently. The third and last step is the merging of all the scores matrices (instead of merging the entire matrices as before). The global matrix obtained in this way has 12 variables, two per contribution. After a final PCA procedure on this global matrix one obtains a scores plot describing the global behavior of the samples under e-tongue, UV and Vis-NIR measures. The method described is essentially a variation of MPCA (Multi-way Principal Component Analysis). The MPCA scores plot is shown in figure 6.29.

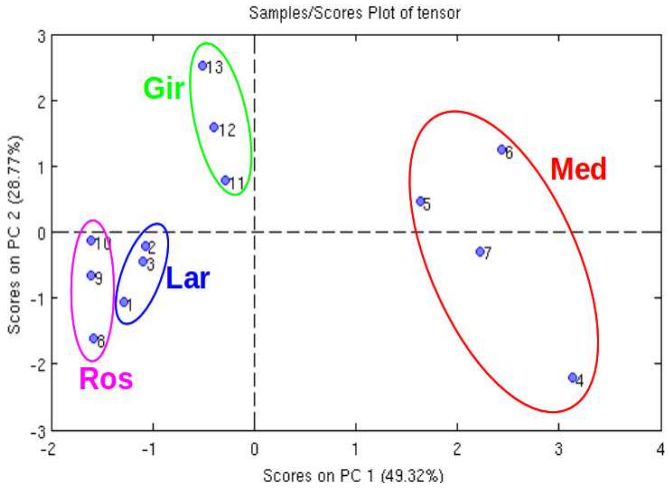


Figure 6.29 MPCA plot with new approach according to figure 6.28.

The clusters are finally fully separated. The separation between the Ros and Lar clusters is not as obvious as in the PCA scores plot obtained with the fitting coefficients of the e-tongue data. However, all the samples were in the right slots. The Med cluster appears to be very different from the other honey types, which was materialized by the distance between the Med ellipse and the

other three ellipses. It was also clear that there is a significant dispersion within the Med (strawberry tree honey) group. This means that the strawberry tree honey samples were not uniform and show a large variation from sample to sample. French lavender honey (Ros) and orange blossom honey (Lar) were the closest types, as indicated by the proximity of the respective ellipses. One of the reasons causing this similarity may be the fact that both honeys are produced in the spring with partial overlap of the production periods (the peak production for both occurs around April). On the other side, sunflower (Gir) is a summer variety and hence has properties that differ clearly from those of spring honeys. Hence, the Gir ellipse appears clearly separated from Ros and Lar. It is also worth notice that Med is an autumn production, a fact which stresses again the difference between the honey types.

The PCA scores plot (figure 6.8) obtained with the fitting coefficients of the e-tongue data seems more appealing than the final plot obtained through MPCA. Should we expect a better plot with MPCA than that obtained for a particular PCA? First of all, it is important to say that the PCA scores plot obtained with the fitting coefficients of the e-tongue data gives a relatively weak criterion for PC2. This is because PC2 in this case only explains about 15.68% of the observed variability (figure 6.8). This means that the appealing visual aspect of the plot is based on a weak foundation. The PC2 for the MPCA plot, on the other hand, explains about 28.77% of the variability, that is, almost the double of the PCA plot. And the percentage of variability explained by both PC1's were similar and close to 50%. Put in other words, the PCA plot of the e-tongue fitting coefficients (figure 6.8), while appealing, only explains about 66.35% of the whole variability. The MPCA scores plot explains about 78% (figure 6.29). This means that the MPCA plot is more reliable. Furthermore, the PCA plot failed to classify correctly one of the strawberry tree honey samples (Med cluster). So, the MPCA appears as the most reliable method to classify the samples.

Now, what were the more important variables in the MPCA prediction? In order to see that one must look into the loadings plot. The loadings plot gives the loading each variable has along the first and second principal components. It is depicted in figure 6.30.

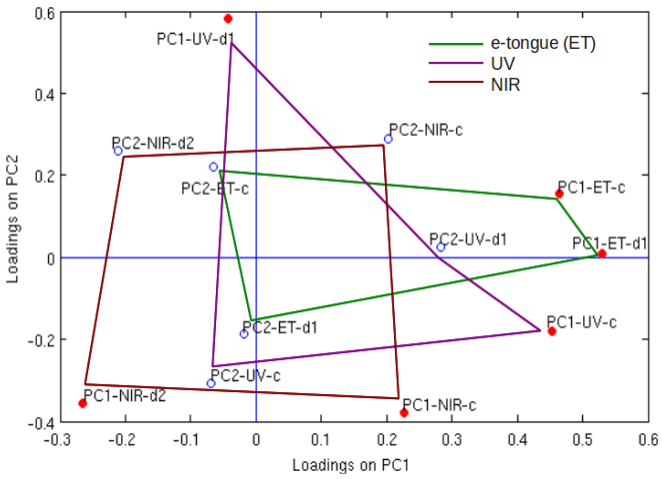


Figure 6.30 Loadings plot for the MPCA results.

Each one of the 12 MPCA variables was labeled according to the following convention: (PC1/PC2)-(ET/UV/NIR)-(c/d1/d2)

where: (PC1/PC2) = identification of the principal component (1 or 2);

(ET/UV/NIR) = identification of the method: e-tongue (ET), UV-Vis spectroscopy (UV) or NIR-Vis spectroscopy (NIR),

(c/d1/d2) = identification of the type of data transformation: fit coefficients (c), first derivative (d1) or second derivative (d2).

In order to improve the readability of the plot (figure 6.30), red dots identify variables associated with PC1 (in PCA). The variables farther away from the origin have more weight in the definition of the PC's in MPCA. Hence PC1-ET-d1 was most important variable loading positively in PC1, followed closely by PC1-ET-c and PC1-UV-c. On the other side, PC1-NIR-d2 was the variable loading more negatively on PC1. This variable was also loading heavily PC2, together with PC1-NIR-c (negative load) and PC1-UV-d1 (positive load). In general the red dots were distributed in the outer part of the plot, while the open circles remain mainly in the inner part. This means that the PC1 related variables were more important in the definition of PC1 and PC2, and this was expected.

Another way of analyzing the plot (figure 6.30) was to draw a polygon joining the four points corresponding to each diagnostic. The green polygon joins the e-tongue points; the violet polygon joins the UV-Vis points and the dark red polygon joins the Vis-NIR points. From here was seen that the e-tongue data is mostly associated with the PC1 (in MPCA) because the polygon was stretched in the horizontal direction. The PC2, on the other side is mainly defined by the optical diagnostics. The Vis-NIR contribution was always the most important in the negative loadings, both for PC1 and PC2.

As a final remark was compared the output of MPCA with the output of another popular method of classification, which is the generation of dendrograms through clustering methods. For this purpose were have used the PLS toolbox cluster function together with the Ward's method for measuring the samples distance. This function was applied to the same 13 x 12 matrix used in the last step of MPCA. The result is shown in figure 6.31.

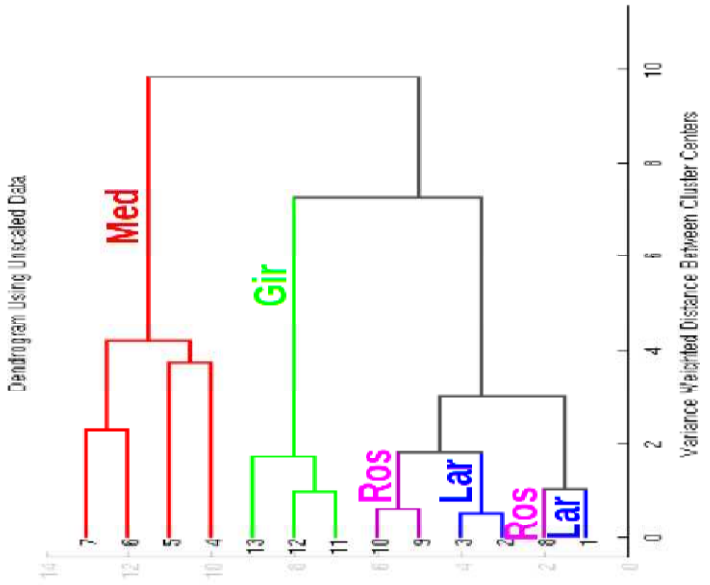


Figure 6.31 Dendrograms.

It is clear in the dendrogram that the Med and Gir clusters have been clearly identified, but that Ros and Lar were not fully separated. Looking back into the MPCA scores plot (figure 6.29), shown that sample 1 is very close to the Ros cluster. Therefore, both methods are in agreement here. However, there is one difference between the classifications for Med. The dendrogram pairs samples 4 and 5, but in the MPCA scores plot (figure 6.29) the samples 5, 6 and 7 are very different from sample 4. Globally both methods gave approximately equal results. However, one must stress that the basis for the clustering technique was the previous PCA processing that led to the ultimate 13 x 12 matrix. It is then more appropriate to consider this classification technique as a hybrid between PCA and clustering.

6.5 Conclusions

We evaluated three non invasive techniques together with PCA, a fundamental tool in chemometric analysis. Were established that these techniques have a high potential as easy alternative or complementary methods directed to the classification and identification of honeys of different floral origin. These methods are fast and reliable in comparison with the melissopalynology (analysis pollen) which has a great demand in the time necessary to prepare and analyzed the samples. But it is clear that this study using the e-tongue, UV-Vis and Vis-Nir spectroscopy has to be expanded with more samples and more kinds of honey. Nevertheless, the results obtained so far are sufficiently encouraging as a starting point for the development of new methods for the quality control and in the honey industry. Their use could bring several advantages: low cost, continuous monitoring, non-destructive assays: simple sample preparation procedures, no chemicals involved and no additional costs in skilled manpower.

CHAPTER 7

GENERAL CONCLUSIONS

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Chapter Seven: General Conclusions

7.1 Conclusions

- The conditions of storage have a high impact in the freshness of honey. After more of than year of shelf life; the parameters that define freshness (hydroxymethylfurfural content and diastase activity), were wide away from the limits established by International regulations, although most of the other parameters remained remarkably unchanged. Therefore the optimum period for storage to honey is less than twelve months, but the time of shelf-life can be increment maintained controlled conditions of storage (*e.g.* temperature, light).

- The physicochemical characterization and determination of bioactive compounds in strawberry tree (*Arbutus unedo* L.) honey, obtained directly from the producer, was within of limits established by International regulations, and showed a very good source of antioxidant compounds (phenolic compounds) similarly to what is reported for homologous Italian honey. The results found can be used to upturn demand for this exquisite type of honey. Ideally, strawberry tree honey should be establishing as a typical regional product with added commercial value.

- For commercial honey samples currently marketed in supermarket, some were not within the limits of freshness parameters (hydroxymethylfurfural and diastase activity) established by regulations. This demonstrates a lack of concern from those who produce and market honey, as well as the will or capacity of authorities to control the quality of this very valuable product – honey.

- Non-invasive techniques (*e.g.* electronic tongue and spectroscopy analysis), are a important and complementary tools, fast, effective and which allows to establish and determine of the botanical origin of honey with good correlation. These new types of analysis, in the short time can, in due time, replace in the first instance the tedious and complex melissopalynological analysis. Although this was a very exploratory project, it was crystal clear the two types of honey, including the strawberry tree honey, can be easily differentiated by these techniques.

- Strawberry tree honey, due to its properties, has the potential to become a reference product in the region and since its commercial value can be increased up to four times, as indicated by the value of strawberry tree from Italy, it is less likely to find it adulterated in supermarket shelves. A proper recognition should come through certification and creation of a protected denomination of origin (PDO).

CHAPTER 8

REFERENCES

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Chapter Eight: References

8.1 References

1. Abramovič, H., Jamnik, M., Burkan, L., Kač, M. (2008). Water activity and water content in Slovenian honeys. *Food Control*, 19, 1086-1090.
2. Abu-Jdayil, B., Ghzawi, A. A., Al-Malah, K. I. M., Zaitoun, S. (2002). Heat effect on rheology of light- and dark-colored honey. *Journal of Food Engineering*, 51, 33-38.
3. Accorti, M., Piazza, M. G., Persano-Oddo, L. (1987). Electric conductivity and ashes content of honey. *Apiacta*, 22, 19-20.
4. Ahmed, J., Prabhu, S. T., Raghavan, G. S. V., Ngadi, M. (2007). Physico-chemical, rheological, calorimetric and dielectric behavior of selected Indian honey. *Journal of Food Engineering*, 79, 1207-1213.
5. Ajlouni, S., Sujirapinyokul, P. (2010) Hydroxymethylfurfuraldehyde and amylase contents in Australia honey. *Food Chemistry*, 119, 1000-1005.
6. Al, M. L., Daniel, D., Moise, A., Bobis, O., Laslo, L., Bogdanov, S. (2009). Physico-chemical and bioactive properties of different floral origin honeys from Romania. *Food Chemistry*, 112, 863-867.
7. Aliferis, K. A., Tarantilis, P. A., Harizanis, P. C., Alissandrakis, E. (2010). Botanical discrimination and classification of honey samples applying gas chromatography/mass spectrometry fingerprinting of headspace volatile compounds. *Food Chemistry*, 121, 856-862.
8. Alissandrakis, E., Daferera, D., Tarantilis, P. A., Polissiou, M., Harizanis, P. C. (2003). Ultrasound-assisted extraction of volatile compounds from citrus flowers and citrus honey. *Food Chemistry*, 82, 575-582.
9. Alissandrakis, E., Tarantilis, P. A., Harizanis, P. C., Polissiou, M. (2005a). Evaluation of four isolation techniques for honey aroma compounds. *Journal of the Science of Food and Agriculture*, 85, 91-97.
10. Alissandrakis, E., Tarantilis, P. A., Pappas, C., Harizanis, P. C., Polissiou, M. (2011). Investigation of organic extractives from unifloral chestnut (*Castanea sativa* L.) and eucalyptus (*Eucalyptus globules* Labill.) honeys and flowers to identification of botanical marker compounds. *Food Science and Technology*, 44, 1042-1051.
11. Aljadi, A. M., Kamaruddin, M. Y. (2004). Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chemistry*, 85, 513-518.
12. Al-Mamary, M., Al-Meeri, A., Al-Habori, M. (2002). Antioxidant activities and total phenolics of different types of honey. *Nutrition Research*, 22, 1041-1047.

13. Alvarez-Suarez, J. M., Tulipani, S., Romandini, S., Vidal, A., Battino, M. (2009). Methodological aspects about determination of phenolic compounds and In Vitro evaluation of antioxidant capacity in the honey: A review. *Current Analytical Chemistry*, 5, 293-302.
14. Alvarez-Suarez, J. M., Tulipani, S., Díaz, D., Estevez, Y., Romandini, S., Giampieri, F., Damiani, E., Astolfi, P., Bompadre, S., Battino, M. (2010). Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. *Food and Chemical Toxicology*, 48, 2490-2499.
15. Anam, O. O., Dart, R. K. (1995). Influence of metal ions on hydroxymethylfurfural formation in honey. *Analytical Proceedings Including Analytical Communications*, 32, 515-517.
16. Andrade, P. B., Amaral, M. T., Isabel, P., Carvalho, J. C. M., Seabra, R. M., Proença da Cunha, A. (1999). Physicochemical attributes and pollen spectrum of Portuguese heather honey. *Food Chemistry*, 66, 503-510.
17. Anklam, E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry*, 63 (4), 549-562.
18. Ampuero, S., Bogdanov, S., Bosset, J. O. (2004). Classification of unifloral honeys with an MS-based electronic nose using different sampling modes: SHS, SPME and INDEX. *European Food Research and Technology*, 218, 198-207.
19. Antony, S. M., Han, I. Y., Rieck, J. R., Dawson, P. L. (2002). Antioxidative effect of Millard reaction products added to Turkey meat during heating by addition on honey. *Journal of Food Science*, 67 (5), 1719-1724.
20. Anupama, D., Bhat, K. K., Sapna, V. K. (2003). Sensory and physico-chemical properties of commercial samples of honey. *Food Research International*, 36, 183-191.
21. AOAC, 1990. In: K. Helrich (Ed.), Official methods of analysis (15th Ed.). Arlington, VA. USA: *Association of Official Analytical Chemists*.
22. Azeredo, L., da C., Azereso, M. A. A., de Souza, S. R., Dutra, V. M. L. (2003). Protein content and physicochemical properties in honey samples of *Apis mellifera* of different floral origins. *Food Chemistry*, 80, 249-254.
23. Babacan, S., Pivarnik, L. F., Rand, A. G. (2002). Honey amylase activity and food starch degradation. *Journal of Food Science*, 67 (5), 1625-1630.
24. Babacan, S., Rand, A. G. (2005). Purification of Amylase from honey. *Journal of Food Science*, 70 (6), C413-418.
25. Babarinde, G. O., Babarinde, S. A., Adegbola, D. O., Ajayeoba, S. I. (2011). Effects of harvesting methods on physicochemical and microbial qualities of honey. *Journal of Food Science Technology*, 48 (5) 628-634.

26. Ball, D. W. (2007). The chemical composition of honey. *Journal of Chemical Education*, 84 (10), 1643-1646.
27. Baltrušaitytė, V., Venskutonis, R. P., Čeksterytė, V. (2007). Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chemistry*, 101, 502-514.
28. Barhate, R. S., Subramanian, R., Nandini, K. E., Hebbar, U. H. (2003). Processing of honey using polymeric microfiltration and ultrafiltration membranes. *Journal Food and Engineering*, 60, 49-54.
29. Bath, P. K., Singh, N. (1999). A comparison between *Helianthus annuus* and *Eucalyptus lanceolatus* honey. *Food Science and Technology*, 67, 389-397.
30. Bath, P. K., Singh, N. (2001). Effect of microwave heating on hydroxymethylfurfural formation and browning in *Helianthus annuus* and *Eucalyptus lanceolatus* honey. *Food Science and Technology*, 38, 366-368.
31. Batsoulis, A. N., Siatis, N. G., Kimbaris, A. C., Alissandrakis, E. K., Pappas, C. S., Tarantilis, P. A., Harizanis, P. C., Polissiou, M. G. (2005). FT-Raman Spectroscopic simultaneous determination of fructose and glucose in honey. *Journal of Agricultural and Food Chemistry*, 53, 207-210.
32. Benedetti, S., Mannino, S., Sabatini, A. G., Marcazzan, G. L. (2004). Electronic nose and neural network use for the classification of honey. *Apidologie*, 35, 1-6.
33. Bentabol, A., Hernández, Z., Rodríguez, B., Rodríguez, E., Díaz, C. (2011). Differentiation of blossom and honeydew honeys using multivariate analysis on the physicochemical parameters and sugar composition. *Food Chemistry*, 126, 664-672.
34. Beretta, G., Granata, P., Ferrero, M., Orioli, M., Facino, R. M. (2005). Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. *Analytica Chimica Acta*, 533, 185-191.
35. Bertelli, D., Plessi, M., Sbatini, A. G., Lolli, M., Grillenzoni, F. (2007). Classification of Italian honeys by mid-infrared diffuse reflectance spectroscopy (DRIFTS). *Food Chemistry*, 101, 1565-1570.
36. Bertoncelj, J., Doberšek, U., Jamnik, M., Golob, T. (2007). Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. *Food Chemistry*, 105, 822-828.
37. Bertoncelj, J., Polak, T., Kropf, U., Korošec, M., Golob, T. (2011). LC-DAD-ESI/MS analysis of flavonids and abscisic acid with chemometric approach for the classification of Slovenian honey. *Food Chemistry*, 127, 296-302.
38. Bianchi, F., Careri, M., Musci, M. (2005). Volatile norisoprenoids as markers of botanical origin of Sardinian strawberry-tree (*Arbutus unedo* L.) honey: Characterization of aroma

compounds by dynamic headspace extraction and gas chromatography-mass spectrometry. *Food Chemistry*, 89, 527-532.

39. Biesaga, M., Pyrzynska, K. (2009). Liquid chromatography/tandem mass spectrometry studies of the phenolic compounds in honey. *Journal of Chromatography A*, 1216, 6620-6626.
40. Bhandari, B., D´Arcy, B., Kelly, C. (1999). Rheology and crystallization kinetics of honey: present status. *International Journal of Food Properties*, 2 (3), 217-226.
41. Blasa, M., Candiracci, M., Accorsi, A., Piacentini, M. P., Albertini, M. C., Piatti, E. (2006). Raw *Millefiori* honey is packed full of antioxidants. *Food Chemistry*, 97, 217-222.
42. Bogdanov, S. (1997). Nature and origin of the antibacterial substances in honey. *Lebensmittel-Wissenschaft und-Technologie*, 30, 748-753.
43. Bogdanov, S. (2002). International Honey Commission. *Swiss Bee Research Centre*, 1-62.
44. Bogdanov, S., Ruoff, K., Oddo, L. P. (2004). Physico-chemical methods for the characterisation of unifloral honeys: a review. *Apidologie*, 35, S4-S17.
45. Bogdanov, S. (2009). The Book of Honey. *Bee Product Science*.
46. Bonvehí, J. S. (1989). Estudio de la validez de los indices que predicen la cristalizacion en la miel. *Revista de Agroquimica y Tecnologica de Alimentos*, 29, 47-62.
47. Bonvehí, J. S., Coll, F. V. (1995). Characterization of Citrus honey (*Citrus* spp.). Produced in Spain. *Journal of Agricultural and Food Chemistry*, 43, 2053-2057.
48. Bonvehí, J. S., Torrentó, M. S., Raich, J. M. (2000). Invertase activity in fresh and processed honeys. *Journal of the Science of Food and Agriculture*, 80, 507-512.
49. Brudzynski, K., Kim, L. (2011a). Storage-induced chemical changes in active components of honey de-regulate its antibacterial activity. *Food Chemistry*, 126, 1155-1163.
50. Brudzynski, K., Miotto, D. (2011b). Honey melanoidins: Analysis of the compositions of the high molecular weight melanoidins exhibiting radical-scavenging activity. *Food Chemistry*, 127, 1023-1030.
51. Brudzynski, K., Miotto, D. (2011c). The relationship between the content of Miallard reaction-like products and bioactivity of Canadian honeys. *Food Chemistry*, 124, 869-874.
52. Bulut, L., Kilic, M. (2009). Kinetics of hydroxymethylfurfural accumulation and color change in honey during storage in relation to moisture content. *Journal of Food Processing and Preservation*, 33, 22-32.
53. Cabras, P., Angioni, A., Tuberoso, C., Floris, I., Reniero, F., Guillou, C, Ghelli, S. (1999). Homogentisic acid: a phenolic acid as a marker of strawberry-tree (*Arbutus unedo*) honey. *Journal of Agricultural and Food Chemistry*, 47, 4064-4067.

54. Čajka, T., Hajšlová, J., Cochran, J., Holadová, K., Klimánková, E. (2007). Solid phase microextration-comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry for the analysis of honey volatiles. *Journal of Separation Science*, 30, 534-546.
55. Capuano, E., Fogliano, V. (2011). Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. *LWT-Food Science and Technology*, 44, 793-810.
56. Castro-Vázquez, L., Díaz-Maroto, M. C., Pérez-Coello, M. S. (2007). Aroma composition and new chemical markers of Spanish citrus honeys. *Food Chemistry*, 103, 601-606.
57. Castro-Vázquez, L., Díaz-Maroto, M. C., González-Viñas, M. A., De la Fuente, E., Pérez-Coello, M. S. (2008). Influence of storage conditions on chemical composition and sensory properties of citrus honey. *Journal of Agricultural and Food Chemistry*, 56, 1999-2006.
58. Castro-Vázquez, L., Díaz-Maroto, M. C., González-Viñas, M. A., Pérez-Coello, M. S. (2009). Differentiation of monofloral citrus, rosemary, eucalyptus, lavender, thyme and heather honeys base on volatile composition and sensory descriptive analysis. *Food Chemistry*, 112, 1022-1030.
59. Castro-Vázquez, L., Díaz-Maroto, M. C., de Torres, C., Pérez-Coello, M. S. (2010). Effect of geographical origin on the chemical and sensory characteristics of chestnut honeys. *Food Research International*, 43, 2335-2340.
60. Cavaco, A. M., Pinto, P., Antunes, D., Marques da Silva, J., Guerra, R. (2009). 'Rocha' pear firmness predicted by a Vis/NIR segmented model. *Postharvest Biology and Technology*, 51, 311-319.
61. Cavaco, A. M., Miguel G., Antunes, D., Guerra, R. (2011). Determination of Geographical and botanical origin of honey: from sensory evaluation to the state of the art of non-invasive Technology. In G., Bondurand & H., Bosch (Eds), Honey: Production, Consumption and Health Benefits (1-40), Nova Science Publishers, Inc.
62. Cavia, M. M., Fernández-Muiño, M. A., Alonso-Torre, S. R., Huidobro, J. F., Sancho, M. T. (2007). Evolution of acidity of honeys from continental climates: Influence of induced granulation. *Food Chemistry*, 100, 1728-1733.
63. Ceballos, L., Pino, J. A., Quijano-Celis, C. E., Dago, A. (2010). Optimization of HS-SPME/GC-MS method for determination of volatile compounds in some Cuban unifloral honeys. *Journal of Food Quality*, 33, 507-528.
64. Celikel, G., Demirsoy, L., Demirsoy, H. (2008). The strawberry tree (*Arbutus unedo* L.) selection in Turkey. *Scientia Horticulture*, 118, 115-119.

65. Chang, C. C., Yang, M. H., Wen, H. M., & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorometric methods. *Journal of Food and Drug Analysis*, 10, 178-182.
66. Cha-um, S., Kirdmanee, C. (2009). Proline accumulation «, photosynthetic abilities and growth characters of sugarcane (*Saccharum officinarum* L.) Plantlets in response to iso-osmotic salt and water-deficit stress. *Agricultural Sciences in China*, 8 (1), 51-58.
67. Cheftel, J., Cuq, J., Lorient, D. (1989). Proteinas Alimentarias (pp. 37 – 47). Ed. Acribia, Zaragoza, España.
68. Chen, L., Mehta, A., Berenbaum, M., Zangerl, A., Engeseth, N. J. (2000). Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. *Journal of Agricultural and Food Chemistry*, 48, 4997-5000.
69. Chirife, J., Zamora, M. C., Motto, A. (2006). The correlation between water activity and % moisture in honey: Fundamental aspects and application to Argentine honeys. *Journal of Food Engineering*, 72, 287-292.
70. Ciulu, M., Solinas, S., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., Spano, N., Sanna, G. (2011). RP-HPLC determination of water-soluble vitamins in honey. *Talanta*, 83, 924-929.
71. Codex Alimentarius Commission. (2001). Revised Codex Standard for Honey, Codex STAN 12-1981, Rev. 1 (1987), Rev. 2.
72. Cohen, I., Weihs, D. (2010). Rheology and microrheology of natural and reduced-calorie Israeli honeys as a model for high-viscosity Newtonian liquids. *Journal of Food Engineering*, 100, 366-371.
73. Conforti, P. A., Lupano, C. E., Malacalza, N. H., Arias, V., Castells, C. B. (2006). Crystallization of honey at -20 ºC. *International Journal of Food Properties*, 9, 99-107.
74. Conte, L. S., Miorini, M., Giomo, A., Bertacco, G., Zironi, R. (1998). Evaluation of some fixed components for unifloral honey characterization. *Journal of Agricultural and Food Chemistry*, 46, 1844-1849.
75. Conti, M. (2000). Lazio region (central Italy) honeys: a survey of mineral content and typical quality parameters. *Food Control*, 11, 459-463.
76. Cooper, R. A., Molan, P. C., Harding, K. G. (2002). The sensitivity to honey of Gram-positive cocci of clinical significance isolated from wounds. *Journal of Applied Microbiology*, 93, 857-863.
77. Corbella, E., Cozzolino, D. (2006). Classification of the floral origin of Uruguayan honeys by chemical and physical characteristics combined with chemometrics. *Food Science and Technology*, 39, 534-539.

78. Corbella, E., Cozzolino, D. (2008). Combining Multivariate analysis and pollen count to classify honey samples accordingly to different botanical origins. *Chilean Journal of Agricultural Research*, 68, 102-107.
79. Cotte, J. F., Casabianca, H., Chardon, S., Lheritier, J., Grenier-Loustalot, M. F. (2003). Application of carbohydrate analysis to verify honey authenticity. *Journal of Chromatography A*, 1021, 145-155.
80. Cotte, J. F., Casabianca, H., Chardon, S., Lheritier, J., Grenier-Loustalot, M. F. (2004a). Chromatographic analysis of sugars applied to the characterization of monofloral honey. *Analytical and Bioanalytical Chemistry*, 380, 698-705.
81. Cotte, J. F., Casabianca, H., Giroud, B., Albert, M., Lheritier, J., Grenier-Loustalot, M. F. (2004b). Characterization of honey amino acids profile using high-pressure liquid chromatography to control authenticity. *Analytical and Bioanalytical Chemistry*, 378, 1342-1350.
82. Cuevas-Glory, L. F., Pino, J. A., Santiago, L. S., Sauri-Duch, E. (2007). A review of volatile analytical methods for determining the botanical origin of honey. *Food Chemistry*, 103, 1032-1043.
83. De la Fuente, E., Sanz, M. L., Martínez-Castro, I., Sanz, J., Ruiz-Matute, A. I. (2007). Volatile and carbohydrate composition of rare unifloral honeys from Spain. *Food Chemistry*, 105, 84-93.
84. De la Fuente, E., Ruiz-Matute, A. I., Valencia-Barrera, R. M., Sanz, J., Martínez Castro, I. (2011). Carbohydrate composition of Spanish unifloral honeys. *Food Chemistry*, 129 (4), 1483-1489.
85. De la Rosa, L. A., Alvarez-Parrilla, E., Moyers-Montoya, E., Villegas-Ochoa, M., Ayala-Zavala, J. F., Hernández, J., Ruiz-Cruz, S., González-Aguilar, G. A. (2011). Mechanism for the inhibition of Apple juice enzymatic browning by Palo Fierro (desert ironweed) honey extract and other natural compounds. *Food Science and Technology*, 44, 269-276.
86. Devillers, J., Morlot, M., Pham-Delègue, M. H., Doré, J. C. (2004). Classification of monofloral honeys based on their quality control data. *Food Chemistry*, 86, 305-312.
87. Dias, L. A., Peres, A. M., Vilas-Boas, M., Rocha, M. A., Estevinho, L. (2008). An electronic tongue for honey classification. *Microchimica Acta*, 163, 97-102.
88. Diminš, F., Küka, P., Küka, M., Čakste, I. (2006). The criteria of honey quality and its changes during storage and thermal treatment. *LLU Rakstí*, 16 (311), 73-78.
89. Diminš, F., Küka, P., Küka, M., Čakste, I. (2008). Content of carbohydrates and specific rotation angle of honey. *Foodbalt*, 121-125.

90. Dinkov, D. (2003). A scientific note on the specific optical rotation of three honeys types from Bulgaria. *Apidologie*, 34, 319-320.
91. Dinkov, D., Jelyazkova, I., Russev, V., Vachin, I. (2004). Specific optical activity and 5-hydroxymethyl-2-furaldehyde content in honey from bee colonies fed up with sugar solution and isosweet 77555 P. *Bulgarian Journal of Veterinary Medicine*, 7 (1), 57-62.
92. El Gharras, H. (2009). Polyphenols: food sources, properties and applications – a review. *International Journal of Food Science and Technology*, 44, 2512-2518.
93. Escriche, I., Kadar, M., Juan-Borrás, M., Domenech, E. (2011a). Using flavonoids, phenolic compounds and headspace volatile profile for botanical authentication of lemon and orange honeys. *Food Research International*, 44, 1504-1513.
94. Escriche, I., Kadar, M., Domenech, E., Gil-Sánchez, L. (2011b). A potentiometric electronic tongue for the dicrimination of honey according to the botanical origin. Comparison with traditional methodologies: Physicochemical parameters and volatile profile. *Journal of Food Engineering*, 109, 449-456.
95. Escuder-Gilabert, L., Peris, M. (2010). Review: Highlights in recent applications of electronic tongues in food analysis. *Analytica Chimica Acta*, 665, 15-25.
96. Escuredo, O., Silva, L. R., Valentão, P., Seijo, M. C., Andrade, P. B. (2012). Assessing *Rubus* honey value: Pollen and phenolic compounds content and antibacterial capacity. *Food Chemistry*, 130, 671-678.
97. Estevinho, L., Pereira, A. P., Moreira, L., Dias, L. G., Pereira, E. (2008). Antioxidant and antimicrobial effects of phenolic compounds extracts of Northeast Portugal honey. *Food and Chemical Toxicology*, 46, 3774-3779.
98. Estevinho, L. M., Feás, X., Seijas, J. A., Vázquez-Tato, M. P. (2012). Organic honey from *Trás-Os-Montes* region (Portugal): Chemical, microbiological and bioactive compounds characterization. *Food and Chemical Toxicology*, 50, 258-264.
99. European Union (EU). (2001). Council Directive 2001/110 relating to honey. Official Journal of the European Communities.
100. European Community, Regulations (EC/510/2006). E.C. (2006).
101. Fallico, B., Zappalà, M., Arena, E., Verzera, A. (2004). Effects of conditioning on HMF content in unifloral honeys. *Food Chemistry*, 85, 305-313.
102. Feás, X., Pires, J., Iglesias, A., & Estevinho, M. L. (2010). Characterization of artisanal honey produced on the Northwest of Portugal by melissopalynological and physico-chemical data. *Food and Chemical Toxicology*, 48, 3462-3470.

103. Federação Nacional dos Apicultura de Portugal. (2010, November, 11). General format. Retrieved from http://www.fnap.pt/index.php
104. Felsner, M. L., Cano, C. B., Bruns, R. E., Watanabe, H. M., Almeida-Muradian, L. B., Matos, J. R. (2004). Characterization of monofloral honeys by ash contents through a hierarchical design. *Journal of Food Composition and Analysis*, 17, 737-747.
105. Ferreira, I. C. F., Aires, E., Barreira, J. C. M., Estevinho, L. M. (2009). Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. *Food Chemistry*, 114, 1438-1443.
106. Ferreres, F., Tomás-Barberán, F. A., Soler, C., García-Viguera, C., Ortiz, A., Tomás-Lorente, F. (1994). A simple extractive technique for honey flavonoid HPLC analysis. *Apidologie*, 25, 21-30.
107. Finola, M. S., Lasagno, M. C., Marioli, J. M. (2007). Microbiological and chemical characterization of honeys from central Argentina. *Food Chemistry*, 100, 1649-1653.
108. Galán-Soldevilla, H., Ruiz-Pérez-Cacho, M. P., Serrano Jimenéz, S., Jodral Villarejo, M., Bentabol Manzanares, A. (2005). Development of a preliminary sensory lexicon for floral honey. *Food Quality and Preference*, 16, 71-77.
109. Gallardo, J., Alegret, S., Del Valle, M. (2005). Application of a potentiometric electronic tongue as a classification tool in food analysis. *Talanta*, 66, 1303-1309.
110. Gallina, A., Stocco, N., Mutinelli, F. (2010). Karl Fischer titration to determine moisture in honey: a new simplified approach. *Food Control*, 21, 942-944.
111. García-Alvarez, M., Huidobro, J. F., Hermida, M., Rodríguez-Otero, J. L. (2000). Major components of honey analysis by Near-Infrared Transflectance Spectroscopy. *Journal of Agricultural and Food Chemistry*, 48, 5154-5158.
112. García-Alvarez, M., Ceresuela, S., Huidobro, J. F., Hermida, M., Rodríguez-Otero, J. L. (2002). Determination of polarimetric parameters of honey by Near-Infrared Transflectance Spectroscopy. *Journal of Agricultural and Food Chemistry*, 50, 419-425.
113. Gheldof, N., Wang, X. H., Engeseth, N. J. (2002). Identification and quantification of antioxidant components of honeys from various floral sources. *Journal of Agricultural and Food Chemistry*, 50, 5870-5877.
114. Gidamis, A. B., Chove, B. E., Shayo, N. B., Nnko, S. A., Bangu, N. T. (2004). Quality evaluation of honey harvested from selected areas in Tanzania with special emphasis on hydroxymethyl furfural (HMF) levels. *Plant Foods for Human Nutrition*, 59, 129-132.
115. Gleiter, R. A., Horn, H., Isengard, H. D. (2006). Influence of type and state of crystallization on the water activity of honey. *Food Chemistry*, 96, 441-445.

116. Gomes, S., Dias L. G., Moreira, L. L., Rodrigues, P., & Estevinho, L. (2010a). Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. *Food and Chemical Toxicology*, 48, 544-548.
117. Gomes, F., Simões, M., Lopes, M. L., Canhoto, J. M. (2010b). Effect of plant regulators and genotype on the micropropagation of adult tree of *Arbutus unedo* L. (strawberry tree). *New Biotechnology*, 27 (6), 882-892.
118. Gómez-Díaz, D., Navaza, J. M., Quintáns-Riveiro, L. C. (2004). Viscosimetric study of multifloral honeys with specific guarantee "Miel de Galicia". *Ciencia y Tecnología Alimentaria*, 4 (4), 234-239.
119. Gómez-Díaz, D., Navaza, J. M., Quintáns-Riveiro, L. C. (2009). Effect of temperature in the viscosity of honey. *International Journal of Food Properties*, 12, 396-404.
120. Gonzales, A. P., Burin, L., Buera, M. P. (1999). Color changes during storage of honeys in relation to their composition and initial color. *Food Research International*, 32, 185-191.
121. González-Miret, M. L., Terrab, A., Hernanz, D., Fernández-Recamales, M. A., Heredia, F. J. (2005). Multivariate correlation between color and mineral composition of honeys and by their botanical origin. *Journal of Agricultural and Food Chemistry*, 53, 2574-2580.
122. Goodall, I., Dennis, M. J., Parker, I., Sharman, M. (1995). Contribution of high performance liquid chromatographic analysis of carbohydrates to authenticity testing of honey. *Journal of Chromatography A*, 706, 353-359.
123. Gupta, J. K., Kaushik, R., Joshi, V. K. (1992). Influence of different treatments, storage temperature and period on some physic-chemical characteristics and sensory qualities of Indian honey. *Journal of Food Science and Technology*, 29, 84-87.
124. Hale, G. M., Querry, M. R. (1973). Optical constants of water in the 200 nm to 200 µm wavelength region. *Applied Optics*, 12, 555-563.
125. Hebbar., U. H., Nandini, K. E., Lakshmi, M. C., Subramanian, R. (2003). Microwave and infrared heat processing of honey and its quality. *Food Science and Technology Research*, 9, 49-53.
126. Hermosín, I., Chicón, R. M., Dolores, M. C. (2003). Free amino acid composition and botanical origin of honey. *Food Chemistry*, 83, 263-268.
127. Hesse, M., Waha, M. (1989). A new look at the acetolysis method. *Plant Systematics and Evolution*, 163, 147-152.
128. Idris, Y. M. A., Mariod, A. A., Hamad, S. I. (2011). Physicochemical properties, phenolic contents and antioxidant activity of Sudanese honey. *International Journal of Food Properties*, 14, 450-458.

129. Iglesias, M. T., De Lorenzo, C., Polo, M. C., Martín-Álvarez, P. J., Pueyo, E. (2004). Usefulness of amino acid composition to discriminate between honeydew and floral honey. Application to honeys from a small geographic area. *Journal of Agricultural and Food Chemistry*, 52, 84-89.
130. Instituto Nacional Estatistica Portugal (2011). Estatistica Agricolas 2010. In INE, I. P., Lisboa, Portugal (pp. 55-98).
131. Irish, J., Blair, S., Carter, D. A. (2011). The antimicrobial activity of honey derived from Australian flora. *Plos One*, 6 (3), e18229, 1-9.
132. Isengard, H. D., Schultheiß, D., Radović, B., Anklam, E. (2001). Alternatives to official analytical methods used for the water determination in honey. Food Control, 12, 459-466.
133. Isengard, H. D., Schultheiß, D. (2003). Water determination in honey-Karl Fischer titration, an alternative to refractive index measurements? *Food Chemistry*, 82, 151-154.
134. Isla, M. I., Craig, A., Ordoñez, R., Zampini, C., Sayago, J., Bedascarrasbure, E., Alvarez, A., Salomón, V., Maldonado, L. (2011). *Food Science and Technology*, 44, 1922-1930.
135. Jasicka-Misiak, I., Poliwoda, A., Dereń, M., Kafarski, P. (2012). Phenolic compounds and abscisic acid as potential markers for the floral origin of two Polish unifloral honey. *Food Chemistry*, 131, 1149-1156.
136. Jerković, I., Mastelić, J., Marijanović, Z., Klein, Ž., Jelić, M. (2007). Comparison of hydrodistillation and ultrasonic solvent extraction for the isolation of volatile compounds from two unifloral honeys of *Robinia pseudoacacia* L. and *Castanea sativa* L. *Ultrasonics Sonochemistry*, 14, 750-756.
137. Jerković, I., Kasum, A., Marijanović, Z., Tuberoso, C. I. G. (2011). Contribution to the characterization of honey-based Sardinian product *abbamele*: Volatile aroma composition, honey marker compounds and antioxidant activity. *Food Chemistry*, 124, 401-410.
138. Jimenéz, M., Mateo, J. J., Huerta, T., Mateo, R. (1994). Influence of the storage conditions on some physicochemical and mycological parameters of honey. *Journal of the Science and Food Agriculture*, 64, 67-74.
139. Johnston, J. E., Sepe, H. A., Miano, C. L., Brannan, R. G., Alderton, A. L. (2005). Honey inhibitions lipid oxidation in ready-to-eat ground beef patties. *Meat Science*, 70, 627-631.
140. Junzheng, P., Changying, J. (1998). General rheological model for natural honeys in China. *Journal of Food Engineering*, 36, 165-168.
141. Juszczak, L., Fortuna, T. (2006). Rheology of selected Polish honeys. *Journal of Food Engineering*, 75, 43-49.

142. Kabbani, D., Sepulcre, F., Wedekind, J. (2011). Ultrasound-assisted liquefaction of rosemary honey: Influence on rheology and crystal content. *Journal of Food Engineering*, 107, 173-178.
143. Kahraman, T., Buyukunal, S. K., Vural, & Altunatmaz S. S. (2010). Physico-chemical properties in honey from different regions of Turkey. *Food Chemistry*, 123 (1), 41-44.
144. Kamal, M. A., Klein, P. (2011). Determination of sugars in honey by liquid chromatography. *Saudi Journal of Biological Sciences*, 18, 17-21.
145. Kaškonienė, V., Venskutonis, P. R. (2010a). Floral markers in honey of various botanical and geographic origins: a review. *Comprehensive Reviews in Food Science and Food Safety*, 9, 620-634.
146. Kaškonienė, V., Venskutonis, P. R., Čeksterytė, V. (2010b). Carbohydrate composition and electrical conductivity of different origin honeys from Lithuania. *Food Science and Technology*, 43, 801-807.
147. Kelly, J. F. D., Downey, G., Fouratier, V. (2004). Initial study of honey adulteration by sugar solutions using Midinfrared (MIR) Spectroscopy and chemometrics. *Journal of Agricultural and Food Chemistry*, 52, 33-39.
148. Kenjerić, D., Mandić, M. L., Primorac, L., Čačić, F. (2008). Flavonoid pattern of sage (*Salvia officinalis* L.) unifloral honey. *Food Chemistry*, 110, 187-192.
149. Khalil, M. I., Salaiman, S. A., & Gan, S. H. (2010a). High 5-hydroxymethylfurfural concentrations are found in Malaysian honey samples stored for more than one year. *Food and Chemical Toxicology*, 48, 2388-2392.
150. Khalil, M. I., Salaiman, S. A., Boukraa, L. (2010b).Antioxidnat properties of honey and its role in preventing health disorder. *The Open Nutraceuticals Journal*, 3, 6-16.
151. Krauze, A., Krauze, J. (1991). Changes in chemical composition of stored honeydew honeys. *Acta Alimentaria Polonica*, 17 (2), 119-125.
152. Kropf, U., Korošec, M., Bertoncelj, J., Ogrinc, N., Nečemer, M., Kump, P., Golob, T. (2010). Determination of the geographical origin of Slovenian black locust, lime and chestnut honey. *Food Chemistry*, 121, 839-846.
153. Krpan, M., Marković, K., Šarić, G., Skoko, B., Hruškar, M., Vahčić, N. (2009). Antioxidant activities and total phenolics of acacia honey. *Czech Journal of Food Sciences*, 27, S245-S247
154. Kuang,W., Nelson, S. O. (1998). Low-frequency dielectric properties of biological tissues: A review with some new insights. *Transactions of the ASAE* 41(1), 173-184.
155. Küçük, M., Kolayli, S., Karaoğlu, S., Ulusoy, E., Baltacı, C., Candan, F. (2007). Biological activities and chemical composition of three honeys of different types from Anatolia. *Food Chemistry*, 100, 526-534.

156. Lachman, J., Kolihová, D., Miholová, D., Košata, J., Titěra, D., Kult, K. (2007). Analysis of minority honey components: Possible use for the evaluation of honey quality. *Food Chemistry*, 101, 973-979.
157. Lachman, J., Orsák, M., Hejtmánková, A., & Kovářova, E. (2010). Evaluation of antioxidant activity and total phenolics of selected Czech honeys. *Food Science and Technology*, 43, 52-58.
158. Laude, V. T., Naegel, L. Y., Horn, H. (1991). The physicochemical properties of some Philippine honeys. *Apidologie*, 22 (4), 371-380.
159. Lazarević, K. B., Andrić, F., Trifković, J., Tešić, Ž., Milijković-Opsenica, D. (2012). Characterisation of Serbian unifloral honeys according to their physicochemical parameters. *Food Chemistry*, 132, 2060-2064.
160. Lazaridou, A., Biliaderis, C. G., Bacandritsos, N., Sabatini, A. G. (2004). Composition, thermal and rheological behaviour of selected Greek honeys. *Journal of Food Engineering*, 64, 9-21.
161. Lee, H., Churey, J. J., Worobo, R. W. (2008). Antimicrobial activity of bacterial isolates from different floral sources of honey. *International Journal of Food Microbiology*, 126, 240-244.
162. Lieux, M. H. (1980). Acetolysis applied to microscopical honey analysis. *Grana*, 19, 57-61.
163. Lochhead, A. G. (1933). Factors concerned with the fermentation of honey. *Zentralbl Bakteriol Parasitenkd u Infektiosnkr Hyg Abt* II 88, 296-302.
164. Louveaux, J., Maurizio, A., Vorwohl, G. (1978). Methods of melissopalynology. *Bee World*, 51 (3), 125-138.
165. Lynn, E. G., Englis, D. T., Milum, V. G. (1936). Effect of processing and storage on composition and color of honey. *Food Research and Application*, 2, 13-18.
166. Maia, M., Russo-Almeida, P. A., Pereira, J. O. (2005). Caracterização do espectro polínico dos méis do Alentejo (Portugal). *Silva Lusitana*, 13 (1), 95-103.
167. Major N., Marković, K., Krpan, M., Šarić, G., Hruškar, M., Vahčić, N. (2011). Rapid honey characterization and botanical classification by an electronic tongue. *Talanta*, 85 (1), 569-574.
168. Martins, R. C., Lopes, V. V., Valentão, P., Carvalho, J. C. M., Isabel, P., Amaral, M. T., Batista, M. T., Andrade, P. B., & Silva, B M. (2008). Relevant principal component analysis applied to the characterization of Portuguese heather honey. *Natural Product Research*, 22 (17), 1560-1582.
169. Mateo, R., Bosh-Reig, F. (1997). Sugar profiles of Spanish unifloral honeys. *Food Chemistry*, 60 (1), 33-41.

170. McKibben, J., Engeseth, N. J. (2002). Honey as a protective agent against lipid oxidation in ground Turkey. *Journal of Agricultural and Food Chemistry*, 50, 592-595.
171. Meda, A., Lamien, C. E., Romito, M., Millogo, J., & Nacoulma, O.G. (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavening activity. *Food Chemistry*, 91, 571-577.
172. Molan, P. (1992). The antibacterial activity of honey. *Bee World*, 73, 5-28.
173. Mora, M. I., Marioli, J. M. (2001). Honey carbohydrate analysis by HPLC, with electrochemical detection, using NI-CR alloy electrode. *Journal of Liquid Chromatography and Related Technologies*, 24 (5), 711-720.
174. Morais, M., Moreira, L., Feás, X., Estevinho, L. M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology*, 49 (5), 1096-1101.
175. Moreira, R. F. A., De Maria, C. A. B., Pietroluongo, M., Trugo, L. C. (2010). Chemical changes in the volatile fractions of Brazilian honeys during storage under tropical conditions. *Food Chemistry*, 121, 697-704.
176. Mossel, B., Bhandari, B., D'Arcy, B., Caffin, N. (2000). Use of an Arrhenius model to predict rheological behaviour in some Australian honeys. *Food Science and Technology*, 33, 545–552.
177. Mossel, B., Bhandari, B., D´Arcy, B., Caffin, N. (2003). Determination of viscosity of some Australian honeys based on composition. *International Journal of Food Properties*, 6 (1), 87-97.
178. Mundo, M. A., Padilla-Zakour, O. I., Worobo, R. W. (2004). Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. *International Journal of Food Microbiology*, 97, 1-8.
179. Murphy, M., Cowan, C., Henchion, M., O´Reilly, S. (2000). Irish consumer preferences for honey: A conjoint approach. *British Food Journal*, 102, 585-597.
180. Naab, O. A., Tamame, M. A., Caccavari, M. A. (2008). Palynological and physicochemical characteristics of three unifloral honey types from central Argentina. *Spanish Journal of Agricultural Research*, 6 (4), 566-576.
181. Nagai, T., Sakai, M., Inoue, R., Inoue, H., Suzuki, N. (2001). Antioxidative activities of some commercially honeys, royal jelly, and propolis. *Food Chemistry*, 75, 237-240.
182. Nagai, T., Inoue, R., Kanamori, N., Suzuki, N., Nagashima, T. (2006). Characterization of honey from different floral sources. Its functional properties and effects of honey species on storage of meat. *Food Chemistry*, 97, 256-262.

183. Nagai, T., Inoue, R., Suzuki, N., Nagashima, T. (2009). Alpha-amylase from persimmon honey: purification and characterization. *International Journal of Food Properties*, 12, 512-521.
184. Nanda V, Sarkar B. C, Sharma H. K., & Bawa A. S. (2003). Physico-chemical properties and estimation of mineral content in honey produced from different plants in Northern India. *Journal of Food Composition and Analysis*, 16, 613-619.
185. Nomikos, P., MacGregor, J. F. (1994). Monitoring batch processes using multi-way principal components analysis. *American Institute of Chemical Engineers Journal*, 40 (8), 1361-1376.
186. Nozal, M. J., Bernal, J. L., Toribio, L., Alamo, M., Diego, J. C., Tapia, J. (2005). The use of carbohydrate profiles and chemometrics in the characterization of natural honeys of identical geographical origin. *Journal of Agricultural and Food Chemistry*, 53, 3095-3100.
187. Oliveira, I., Coelho, V., Baltasar, R., Pereira, J. A., Baptista, P. (2009). Scavenging capacity of strawberry (*Arbutus unedo* L.) leaves on free radicals. *Food and Chemical Toxicology*, 47, 1507-1511.
188. Oliveira, I., Guedes de Pinho, P., Malheiro, R., Baptista, P., Pereira, J. A. (2011). Volatile profile of *Arbutus unedo* L. fruits through ripening stage. *Food Chemistry*, 128, 667-673.
189. Orantes-Bermejo, F. J., Torres, C. F. P. (2009). Evolution of invertase activity in honey from *Castanea sativa* and *Rosmarinus officinalis* collected in Granada. *ARS Pharmaceutica*, 50 (3), 124-128.
190. Osborne, B. G. (2006). Near-Infrared Spectroscopy in Food Analysis. *Encyclopedia of Analytical Chemistry*. Meyers R. A. (Ed), ISBN 0471 97670 9 (1-14). Wiley & Sons Ltd.
191. Ouchemoukh, S., Schweitzer, P., Bey, M. B., Djoudad-Kadji, H. (2010). HPLC sugar profiles of Algerian honeys. *Food Chemistry*, 121, 561-568.
192. Özcan, M., Arslan, D., Ceylan, D. A. (2006). Effect of inverted saccharose on some properties of honey. *Food Chemistry*, 99, 24-29.
193. Özcan, M. M., Hacıseferoğulları, H. (2007). The Strawberry (*Arbutus unedo* L.) fruits: Chemical composition physical properties and mineral content. *Journal of Food Engineering*, 78, 1022-1028.
194. Pajuelo, A. G. (2004). Mieles de España y Portugal. Conocimiento y cata. (pp. 1-151). Montagud Editores S.A. (Ed), Barcelona, Spain. ISBN: 84-7212-109-7.
195. Pallauf, K., Rivas-Gonzalo, J. C., del Castillo, M. D., Cano, M. P., de Pascual-Teresa, S. (2008). Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. *Journal of Food Composition and Analysis*, 21, 273-281.

196. Palmer, K. F., Williams, D. (1974). Optical properties of water in the near infrared. *Journal of the Optical Society of America*, 64, 1107-1110.
197. Peréz, R. A., Iglesias, M. T., Pueyo, E., González, M., De Lorenzo, A. C. (2007). Amino acid composition and antioxidant capacity of Spanish honeys. *Journal of Agricultural and Food Chemistry*, 55, 360-365.
198. Peréz-Arquillúe, C., Conchello, P., Ariño, A., Juan, T., & Herrera, A. (1994). Quality evaluation of Spanish rosematy (*Rosmarinus officinalis*) honey. *Food Chemistry*, 51, 207-210.
199. Peréz-Arquillúe, C., Conchello, P., Ariño, A., Juan, T., & Herrera, A. (1995). Physicochemical attributes and pollen spectrum of some unifloral Spanish honeys. *Food Chemistry*, 54, 167-172.
200. Perez-Locas, C., Yaylayan, V. A. (2008). Isotopo labeling studies on the formation of 5-(Hydroxymethyl)-2-furaldehyde (HMF) from sucrose by pyrolysis-GC/MS. *Journal of Agricultural and Food Chemistry,* 56, 6717-6723.
201. Persano Oddo, L., Baldi, E., Accorti, M. (1990). Diastatic activity in some unifloral honeys. *Apidologie*, 21, 17-24.
202. Persano Oddo, L., Piazza, M. G., Sabatini, A. G., Accorti, M. (1995). Characterization of unifloral honeys. *Apidologie*, 26, 453-465.
203. Persano Oddo, L., Piazza, M. G., Pulcini, P. (1999). Invertase activity in honey. *Apidologie*, 30, 57-65.
204. Persano Oddo, L., Piana, L., Bogdanov, S., Bentabol, A., Gotsiou, P., Kerkvliet, J., Martin, P., Morlot, M., Ortiz Valbuena, A., Ruoff, K., Von Der Ohe, K. (2004). Botanical species giving unifloral honey in Europe. *Apidologie*, 35, S82-S93.
205. Pething, R., Kell, D. B. (1987). The passive electrical properties of biological systems: their significance in physiology, biophysics and biotechnology. *Physics in Medicine and Biology*, 32, 933-970.
206. Piana, M. L., Persano-Oddo, L., Bentabol, A., Bruneau, E., Bogdanov, S., Guyot-Declerck, C. (2004). Sensory analysis applied to honey: state of the art. *Apidologie*, 35, S26-S37.
207. Piazza, M. G., Persano-Oddo, L. (2004). Bibliographical review of the main European unifloral honeys. *Apidologie*, 35, S94-S111.
208. Pichichero, E., Canuti, L., Canini, A. (2009). Characterization of the phenolic and flavonid fractions and antioxidant power of Italian honeys of different botanical origin. *Journal of the Science of Food and Agriculture*, 89, 609-616.

209. Pires, J., Estevinho, M. L., Feás, X., Cantalapiedra, J., Iglesias, A. (2009). Pollen spectrum and physico-chemical attributes of heather (Erica sp.) honey of north Portugal. *Journal of the Science Food and Agriculture*, 89, 1862-1870.
210. Pontes, M., Marques, J. C., Câmara, J. S. (2007). Screening of volatile composition from Portuguese multifloral honeys using headspace solid-phase microextration-gas chromatography-quadrupole mass spectrometry. *Talanta*, 74, 91-103.
211. Popek, S. (2002). A procedure to identify a honey type. *Food Chemistry*, 79, 401-406.
212. Portuguese Norm NP-1307:1983 (Ed. 10)- Mel – Definição, classificação e caractetísticas.
213. Přidal, A., Vorlová, L. (2002). Honey and its physical parameters. *Czech Journal of Animal and Science*, 47 (10), 439-444.
214. Pyrzynska, K., Biesaga, M. (2009). Analysis of phenolic acids and flavonids in honey. *Trends in Analytical Chemistry*, 28 (7), 893-902.
215. Qamer, S., Muzaffar, N., Ali, S. S., Shakoori, A. R. (2009). Effect of storage on various honey quality parameters of unifloral sidder honey from Pakistan. *Pakistan Journal of Zoology*, 41 (4), 313-316.
216. Qiu, P. Y., Ding, H. B., Tang, Y. K., Xu, R. J. (1999). Determination of chemical composition of commercial honey by Near-Infrared Spectroscopy. *Journal of Agricultural and Food Chemistry*, 47, 2760-2765.
217. Radovic, B. S., Careci, M., Mangia, A., Musci, M., Gerboles, M., Anklam, E. (2001). Contribution of dynamics headspace GC-MS analysis of aroma compounds to authenticity testing of honey. *Food Chemistry*, 72, 511-520.
218. Rasmussen, C. N., Wang, X. H., Leung, S., Andrae-Nightingale, L. M., Schmidt, S. J., Engeseth, N. J. (2008). Selection and use of honey as an antioxidant in a French salad dressing system. *Journal of Agricultural and Food Chemistry*, 56, 8650-8657.
219. Recondo, M. P., Elizalde, B. E., Buera, M. P. (2006). Modeling temperature dependence of honey viscosity and of related supersaturated model carbohydrate systems. *Journal of Food Engineering*, 77, 126-134.
220. Risner, C. H., Kiser, M. J., Dube, M. F. (2006). An aqueous high-performance liquid chromatographic procedure for the determination of 5-hydroxymethylfurfural in honey and other sugar-containing materials. *Journal of Food Science*, 71 (3), C179-C184.
221. Rosa, A., Tuberoso, C. I. G., Atzeri, A., Melis, M. P., Bifulco, E., Dessì, M. A. (2011). Antioxidant profile of strawberry tree honey and its marker homogentisic acid in several models of oxidative stress. *Food Chemistry*, 129, 1045-1053.

222. Rufián-Henares, J. A., Morales, F. J. (2007). Functional properties of melanoidins: In vitro antioxidant, antimicrobial and antihypertensive activities. *Food Research International*, 995-1002.
223. Ruiz-Matute, A. I., Brokl, M., Soria, A. C., Sanz, M. L., Martínez-Castro, I. (2010). Gas chromatographic-mass spectrometric characterisation of tri- and tetrasaccharides in honey. *Food Chemistry*, 120, 637-642.
224. Ruoff, K., Luginbühl, W., Bogdanov, S., Bosset, J. O., Estermann, B., Ziolko, T, Amadò, R. (2006). Authentication of the botanical origin of honey by Near-Infrared spectroscopy. *Journal of Agricultural and Food Chemistry*, 54, 6867-6872.
225. Ruoff, K., Luginbühl, W., Kilchenmann, V., Bosset, J. O., Von der Ohe, K., Von der Ohe, W., Amadò, R. (2007). Authentication of the botanical origin of honey using profiles of classical measurands and discriminant analysis. *Apidologie*, 38, 438-452.
226. Rybak-Chmielewska, H., Szczęsna, T. (2003). Determination of saccharides in multifloral honeybee means of HPLC. *Journal of Apicultural Science*, 47 (2), 93-101.
227. Rybak-Chmielewska, H. (2007a). Changes in the carbohydrate composition of honey undergoing during storage. *Journal of Apicultural Science*, 51 (1), 39-47.
228. Rybak-Chmielewska, H. (2007b). High performance liquid chromatography (HPLC) study of sugar composition in some kinds of natural honey and winter stores processed by bees from starch syrup. *Journal of Apicultural Science*, 51 (1), 23-37.
229. Sánchez, M. P., Huidobro, J. F., Mato, I., Muniategui, S., Sancho, M. T. (2001). Evolution of Invertase activity in honey over two years. *Journal of Agricultural and Food Chemistry*, 49, 416-422.
230. Sancho, M. T., Muniategui, S., Huidobro, J. F., Simal, J. (1991a). Correlation between the electrical conductivity of honey in humid and in dry matter. *Apidologie*, 22, 221-227.
231. Sancho, M. T., Muniategui, S., Huidobro, J. F., Simal, J. (1991b). Relationships between electrical conductivity and total sulphated ash contents in Basque honeys. *Apidologie*, 22, 487-494.
232. Sancho, M. T., Muniategui, S., Sánchez, P., Huidobro, J. F., Simal-Lozano, J. (1992a). Evaluating soluble and insoluble ash, alkalinity of soluble and insoluble ash and total alkalinity of ash in honey using electrical conductivity measurements at 20 ºC. *Apidologie*, 23, 291-297.
233. Sancho, M. T., Muniategui, S., Huidobro, J. F., Lozano, J. S. (1992b). Aging of honey. *Journal of Agricultural and Food Chemistry*, 40, 134-138.

234. Sancho, M. T., Huidobro, J. F.,Mato, I., Muniategui, S., Sancho, M. T. (2001). Evolution of invertase activity in honey over two years. *Journal of Agricultural and Food Chemistry*, 49, 416-422.
235. Sanz, M. L., Del Castillo, M. D., Corzo, N., Olano, A. (2003). 2-Furoylmethyl amino acids and hydroxymethylfurfural as indicators of honey quality. *Journal of Agricultural and Food Chemistry*, 51, 4278-4283.
236. Sanz, M. L., González, M., De Lorenzo, C., Sanz, J., Martínez-Castro, I. (2004). Carbohydrate composition and physicochemical properties of artisanal honeys from Madrid (Spain): occurrence of *Echium* sp honey. *Journal of the Science of Food and Agriculture*, 84, 1577-1584.
237. Sanz, M. L., González, M., De Lorenzo, C., Sanz, J., Martínez-Castro, I. (2005). A contribution to the differentiation between nectar honey and honeydew honey. *Food Chemistry*, 91, 313-317.
238. Sanz-Cervera, S., Sanz-Cervera, M. M. (1994). Humedad, cenizas y conductividad eléctrica de mieles de la Rioja. *Zubía*, 12, 143-158.
239. Šarič, G, Matkovič, D, Hruškar, M, Vahčič, N. (2008). Characterisation and classification of Croatian honey by physicochemical parameters. *Food Technology and Biotechnology*, 46 (4), 355-367.
240. Saxena, S., Guatam, S., Sharma, A. (2009). Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chemistry*, 118 (2), 391-397.
241. Scampicchio, M., Ballabio, D., Arecchi, A., Cosio, S. M., Mannino, S. (2008). Amperometric electronic tongue for food analysis. *MicrochimicaActa*, 163, 11-21.
242. Scanu, R., Spano, N., Panzanelli, A., Pilo, M. I., Piu, P., Sanna, G., Tapparo, A. (2005). Direct chromatographic methods for the rapid determination of homogentisic acid in strawberry tree (*Arbutus unedo* L.) honey. *Journal of Chromatography A*, 1090, 76-80.
243. Schramm, D. D., Karim, M., Schrader, H. R., Holt, R. R., Cardetti, M., Keen, C. L. (2003). Honey with high levels of antioxidant can provide protection to healthy human subjects. *Journal of Agricultural and Food Chemistry*, 51, 1732-1735.
244. Semkiw, P., Skowronek, W., Skubida, P. (2008). Changes in water content of honey during ripening under controlled condition. *Journal of Apicultural Science*, 52 (1), 57-63.
245. Serrano, S., Villarejo, M., Espejo, R., Jodral, M. (2004). Chemical and physical parameters of Andalusian honey: classification of *Citrus* and *Eucalyptus* honeys by discriminant analysis. *Food Chemistry*, 87, 619-625.

246. Singh, N., Bath, P. K. (1998). Relationship between heating and hydroxymethylfurfural formation in different honey types. *Journal of Food Science and Technology*, 35, 154-156.
247. Silici, S., Sagdic, O., Ekici, L. (2010). Total phenolic content, antiradical, antioxidant and antimicrobial activities of Rhododendron honeys. *Food Chemistry*, 121, 238-243.
248. Silva, L. R., Videira, R., Monteiro, A. P., Valentão, P., & Andrade, P.B. (2009). Honey from Luso region (Portugal): Physicochemical characteristics and mineral contents. *Microchemical Journal*, 93 (1), 73-77.
249. Smanalieva, J., Senge, B. (2009). Analytical and rheological investigation into selected unifloral German honey. *European Food Research and Technology*, 229, 107-113.
250. Snowdon, J. A., Cliver, D. O. (1996). Microorganisms in honey. *International Journal of Food Microbiology*, 31, 1-26.
251. Sopade, P. A., Halley, P., Bhandari, B., D´Arcy, B., Doebler, C., Caffin, N. (2002). Application of the Williams-Landel-Ferry model to the viscosity-temperature relationship of Australian honeys. *Journal of Food Engineering*, 56, 67-75.
252. Soria, A. C., Martínez-Castro, I., Sanz, J. (2008). Some aspects of dynamic headspace analysis of volatile components in honey. *Food Research International*, 41, 838-848.
253. Spano, N., Casula, L., Panzanelli, A., Pilo, M. I., Piu, P. C., Scanu, R., Tapparo, A., Sanna, G. (2006). An RP-HPLC determination of 5-hydroxymethylfurfural in honey. The case of strawberry tree honey. *Talanta*, 68, 1390-1395.
254. Spano, N., Ciulu, M., Floris, I., Panzanelli, A., Pilo, M. I., Piu, P. C., Scanu, R., Sanna, G. (2008). Chemical characterization of a traditional honey-based Sardinian product: *Abbamele. Food Chemistry*, 108, 81-85.
255. Stanimirova, I., Üstün, B., Cajka, T., Riddelova, K., Hajslova, J., Buydens, L. M. C., Walczak, B. (2010). Tracing the geographical origin of honeys based on volatile compounds profiles assessment using pattern recognition techniques. *Food Chemistry*, 118, 171-176.
256. Stolzenbach, S., Byrne, D. V., Bredie, W. L. P. (2011). Sensory local uniqueness of Danish honeys. *Food Research International*, 44, 2766-2774.
257. Subramanian, R., Hebbar, H. U., Rastogi, N. K. (2007). Processing of honey: a review. *International Journal of Food Properties*, 10, 127-143.
258. Swallow, K. W., Low, N. H. (1990). Analysis and quantitation of the carbohydrates in honey using High-Performance Liquid Chromatography. *Journal of Agricultural and Food Chemistry*, 28, 1828-1832.
259. Tabouret, T. (1979). Role de láctivite de léau dans la cristallisation du miel. *Apidologie*, 10, 314-358.

260. Takrouni, M. M., Boussaid, M. (2010). Genetic diversity and population's structure in Tunisian strawberry tree (*Arbutus unedo* L.). *Scientia Horticulturae*, 126, 330-337.
261. Taormina, P. J., Niemira, B. A., Beuchat, L. R. (2001). Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. *International Journal of Food Microbiology*, 69, 217-225.
262. Terrab, A., Heredia, F. J. (2004a). Characterization of avocado (*Persea americana* Mill) honeys by their physicochemical characteristics. *Journal of the Science of Food and Agricultural*, 84, 1801-1805.
263. Terrab, A., Recamales, A. F., Hernanz, D., Heredia, F. J. (2004b). Characterisation of Spanish thyme honeys by their physicochemical characteristics and mineral contents. *Food Chemistry*, 88, 537-542.
264. Terrab, A., González-Miret, L. Y., Heredia, F. J. (2004c). Colour characterization of thyme and avocado honeys by diffuse reflectance spectrophotometry and spectroradiometry. *European Food Research and Technology*, 218, 488-492.
265. Thrasyvoulou, A., Manikis, J., Tselios, D. (1994). Liquefying crystallization honey with ultrasonic waves. *Apidologie*, 25, 297-302.
266. Tomás-Barberán, F. A., Martos, I., Ferreres, F., Radovic, B. S., Anklam, E. (2001). HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys. *Journal of the Science of Food and Agricultural*, 81, 485-496.
267. Torres, A., Garedew, A., Schmolz, E., Lamprecht, I. (2004). Calorimetric investigation of the antimicrobial action and insight into the chemical properties of "angelita" honey – a product of the stingless bee *Tetragonisca angustula* from Colombia. *Thermochimica Acta*, 415, 107-113.
268. Tosi, E., Ciappini, M., Ré, E., Lucero, H. (2002). Honey thermal treatment effects on hydroxymethylfurfural content. *Food Chemistry*, 77, 71-74.
269. Tosi, E., Martinet, R., Ortega, M., Lucero, H., Re, E. (2008). Honey diastase activity modified by heating. *Food Chemistry*, 106, 883–887.
270. Tuberoso, C. I., Bifulco, E., Caboni, P., Cottiglia, F., Cabras, P., Floris, I. (2010). Floral markers of Strawberry tree (*Arbutus unedo* L.) honey. *Journal of Agricultural and Food Chemistry*, 58, 384-389.
271. Turhan, I., Tetik, N., Karhan, M., Gurel, F., Tavukcuoglu, H. R. (2008). Quality of honeys influenced by thermal treatment. *Food Science and Technology*, 41, 1396-1399.
272. Turhan, K. (2009). Effects of thermal treatment and storage on hydroxymethylfurfural (HMF) content and diastase activity of honeys collected from middle Anatolia in Turkey. *Innovations in Chemical Biology*, doi: 10.1007/978-1-4020-6955-0, 233-239.

273. Turkmen, N., Sari, F., Poyrazoglu, E. S., Velioglu, Y. S. (2006). Effects of prolonged heating on antioxidant activity and color of honey. *Food Chemistry*, 95, 653-657.
274. Valdés, B., Diez, M. J., Fernandez, I. (1987). Atlas polinico de Andalucia Occidental. Instituto de Desarrollo Regional Nº43. Universidad de Sevilla. EXCMA Diputation de Cadiz.
275. Valle, A., Andrada, A., Aramayo, E., Gil, M., Lamberto, S. (2007). A melissopalynological map of the south and southwest of the Buenos Aires Province, Argentina. *Spanish Journal of Agricultural Research*, 5 (2), 172-180.
276. Vela, L., De Lorenzo, C., Pérez, R. A. (2007). Antioxidant capacity of Spanish honeys and its correlation with polyphenol content and other physicochemical properties. *Journal of the Science of Food and Agriculture*, 87, 1069-1075.
277. Vit, P., Gutiérrez, M. G., Titěra, D., Bednař, M., Rodríguez-Malaver, A. J. (2008). Czech honey categorized according to their antioxidant activity. *Acta Bioquímica Clínica Latinoamericana*, 42 (2), 237-244.
278. Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., Pérez-Álvarez, J. A. (2008). Functional properties of honey, propolis, and royal jelly. *Journal of Food Science*, 73 (9), 117-124.
279. Von Der Ohe, W., Oddo, L. P., Piana, M. L., Morlot, M., Martin, P. (2004). Harmonized methods of melissopalynology. *Apidologie*, 35, S18-25.
280. Vorlová, L., Přidal, A. (2002). Invertase and diastase activity in honeys of Czech provenience. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis Sborník Mendelovy Zemědělské a Lesnické Univerzity V Brně*, 8 (5), 57-65.

281. Wahdan, H. A. L. (1998). Causes of the antimicrobial activity of honey. *Infection*, 1, 26-31.
282. Wang, X. W., Gheldof, N., Engeseth, N. J. (2004). Effect of processing and storage on antioxidant capacity of honey. *Journal of Food Science*, 69 (2), 96-101.
283. Wang, H., Qian, H, Yao, W. (2011). Melanoidins produced by the Maillard reaction: Structure and biological activity. *Food Chemistry*, 128, 573-584.
284. Wei, Z., Wang, J., Liao, W. (2009). Technique potential for classification of honey by electronic tongue. *Journal of Food Engineering*, 94, 260-266.
285. Wei, Z., Wang, J., Wang, Y. (2010). Classification of monofloral honeys from different floral origins and geographical origins based on rheometer. *Journal of Food Engineering*, 96, 469-479.
286. Wei, Z., Wang, J. (2011). Classification of monofloral honeys by voltammetric tongue with chemometrics method. *Electrochimica Acta*, 56, 4907-4915.

287. Weryszko-Chmielewska, E., Chwil, M. (2007). Characterization of floral nectary and nectar of common bugloss (*Anchusa officinalis* L.). *Journal of Apicultural Science*, 51 (2), 25-29.
288. Weston, R. J., Brocklebank, L. K. (1999). The oligosaccharide composition of some New Zealand honeys. *Food Chemistry*, 64, 33-37.
289. Weston, R. J. (2000). The contribution of catalase and other natural products to the antimicrobial activity of honey: a review. *Food Chemistry*, 71, 235-239.
290. White, J. W. (1956). The composition of Honey. Tenth International Congress of Entomology, Montreal, Canada, 57-66.
291. White, J. W., Riethofm M. L., Kushnir, I. (1961). The effect of storage on carbohydrates, acidity and diastase content. *Journal of Food Science*, 26 (1), 63-71.
292. White, J. W., Subers, M. H., Kushnir, I. (1963). How processing and storage affect honey quality. *Gleanings in Bee Culture*, 91 (7), 422-425.
293. White, J. W., Kushnir, I., Subers, M. H. (1964). Effect of storage and processing temperatures on honey quality. *Food Technology*, 18 (4), 153-156.
294. White, J. W. (1979). Methods for determining carbohydrates, hydroxymethylfurfural, and proline in honey: collaborative study. *Journal of the Association of Official Analytical Chemists*, 62 (3), 515-526.
295. White, J. W. (1984). Instrumental color classification of honey: Collaborative study. *Journal of the Association of Official Analytical Chemists*, 67, 1129-1131.
296. White, J. W. (1994). The role of HMF and diastase assays in honey quality evaluation. *Bee World*, 75 (3), 104-117.
297. Winquist, F. (2008). Voltametric electronic tongues – basic principles and applications. *Microchimica Acta*, 163, 3-10.
298. Witczak, M., Juszczak, L., Galkowska, D. (2011). Non-Newtonian behavior of heather honey. *Journal of Food Engineering*, 104, 532-537.
299. Woodcock, T., Downey, G., Kelly, D., O`Donnell, C. (2007). Geographical classification of honye samples by Near-Infrared Spectroscopy: A feasibility study. *Journal of Agricultural and Food Chemistry*, 55, 9128-9134.
300. Wunderlin, D. A., Pesce, S. F., Amé, M. V., Faye, P. F. (1998). Decomposition of hydroxymethylfurfural in solution and protective effect of fructose. *Journal of Agricultural and Food Chemistry*, 46, 1855-1863.
301. Yanniotis, S., Skaltsi, S., Karaburnioti, S. (2006). Effect of moisture content on the viscosity of honey at different temperatures. *Journal of Food Engineering*, 72, 372-377.

302. Yao, L., Bhandari, B. R., Datta, N., Singanusong, R., D´Arcy, B. R. (2003). Crystallisation and moisture sorption properties of selected Australian unifloral honeys. *Journal of the Science of Food and Agricultural*, 83, 884-888.
303. Yao, L., Jiang, Y., Singanusong, R., D´Arcy, B., Datta, N., Caffin, N., Raymont, K. (2004a). Flavonoids in Australian *Melaleuca*, *Guioa*, *Lophostemon*, *Banksia* and *Helianthus* honeys and their potential for floral authentication. *Food Research International*, 37, 166-174.
304. Yao, L., Jiang, Y., Singanusong, R., D´Arcy, B., Singanusong, R., Datta, N., Caffin, N., Raymont, K. (2004b). Quantitative high-performance liquid chromatography analyses of flavonoids in Australian Eucalyptus honeys. *Journal of Agricultural and Food Chemistry*, 52, 210-214.
305. Zaitoun, S., Ghzawi, A. A., Al-Malah, K. I. M., Abu-Jdayil, B. (2001). Rheological properties of selected light colored Jordanian honey. *International Journal of Food Properties*, 4 (1), 139-148.
306. Zalibera, M., Staško, A., Šlebodová, A., Jančovičová, V., Čermáková, T., Brezová, V. Antioxidant and radical-scavenging activities of Slovak honeys – An electron paramagnetic resonance study. *Food Chemistry*, 110, 512-521.
307. Zamora, M. C., Chirife, J., Roldán, D. (2006). On the mature of the relationship between water activity and % moisture in honey. *Food Control*, 17, 642-647.
308. Zappalà, M., Fallico, B., Arena, E., Verzera, A. (2005). Methods for the determination of HMF in honey: a comparison. *Food Control*, 16, 273-277.

CHAPTER 9

ANNEXES

"Spider caves" art ruprecht

Chapter Nine: Annexes

9.1Annexes to Chapter 3

Table 9.3.1 Results for physicochemical parameters of honeys analyzed in 2006 and 2009.

			Ash [%]				**EC [mS/cm]**				**pH**			
			2006		2009		2006		2009		2006		2009	
		Sample	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
AREA B	Ameixal	9	0,114	0,001	0,120	0,040	0,253	0,001	0,184	0,003	4,03	0,01	4,09	0,03
		6	0,060	0,001	0,036	0,001	0,140	0,002	0,107	0,001	3,45	0,02	3,63	0,01
		31	0,050	0,000	0,066	0,016	0,234	0,001	0,119	0,000	3,49	0,04	3,69	0,01
	Cachopo	12	0,050	0,002	0,069	0,002	0,129	0,003	0,114	0,000	3,77	0,01	3,79	0,03
		13	0,070	0,005	0,067	0,003	0,144	0,001	0,114	0,000	3,35	0,01	3,59	0,03
		4	0,050	0,001	0,062	0,006	0,198	0,001	0,131	0,001	3,60	0,02	3,79	0,02
		3	0,050	0,000	0,061	0,003	0,196	0,001	0,138	0,001	3,50	0,00	3,76	0,00
		2	0,048	0,000	0,017	0,006	0,199	0,001	0,353	0,001	3,57	0,02	3,81	0,00
		1	0,050	0,000	0,050	0,007	0,162	0,001	0,122	0,000	3,64	0,01	3,73	0,02
		26	0,050	0,000	0,118	0,002	0,440	0,003	0,224	0,001	3,55	0,00	3,78	0,01
		11	0,050	0,002	0,069	0,002	0,180	0,002	0,134	0,001	3,57	0,01	3,65	0,02
		7	0,080	0,003	0,087	0,005	0,193	0,000	0,149	0,001	4,28	0,02	3,84	0,02
		27	0,070	0,000	0,129	0,007	0,400	0,001	0,216	0,001	3,55	0,03	3,86	0,00
		21	0,060	0,002	0,085	0,001	0,345	0,001	0,157	0,000	3,46	0,01	3,74	0,03
		8	0,060	0,003	0,065	0,010	0,177	0,002	0,128	0,000	3,75	0,02	3,74	0,01
	Martinlongo	22	0,070	0,000	0,114	0,000	0,487	0,001	0,210	0,001	3,53	0,05	3,73	0,00
		29	0,070	0,000	0,049	0,001	0,435	0,001	0,127	0,001	3,37	0,10	3,72	0,01
		20	0,070	0,000	0,078	0,002	0,233	0,000	0,153	0,002	3,27	0,24	3,76	0,01
		10	0,050	0,005	0,065	0,006	0,158	0,001	0,123	0,001	3,79	0,01	3,79	0,01
		Average	**0,06**	**0,02**	**0,07**	**0,03**	**0,248**	**0,114**	**0,158**	**0,059**	**3,61**	**0,24**	**3,76**	**0,10**
AREA D	Sta. Catarina	16	0,180	0,000	0,168	0,003	0,537	0,000	0,296	0,001	3,39	0,00	4,10	0,02
		32	0,050	0,000	0,055	0,013	0,528	0,001	0,118	0,001	3,41	0,00	3,64	0,00
		33	0,050	0,000	0,066	0,003	0,560	0,001	0,146	0,001	3,27	0,03	3,63	0,01
		28	0,050	0,006	0,090	0,000	0,242	0,001	0,174	0,000	3,35	0,01	3,60	0,00
		18	0,070	0,000	0,182	0,004	0,628	0,021	0,272	0,000	3,84	0,04	4,27	0,03
		30	0,070	0,000	0,077	0,015	0,234	0,001	0,148	0,000	3,49	0,04	3,72	0,00
		19	0,050	0,000	0,216	0,007	0,245	0,000	0,319	0,001	3,99	0,01	4,22	0,02
		17	0,080	0,000	0,080	0,003	0,528	0,001	0,174	0,001	3,84	0,04	3,67	0,02
		25	0,070	0,000	0,130	0,008	0,350	0,001	0,234	0,002	3,43	0,02	3,72	0,01
	Tavira	15	0,010	0,002	0,079	0,000	0,537	0,001	0,171	0,000	3,82	0,00	3,65	0,02
		24	0,060	0,000	0,146	0,004	0,526	0,001	0,262	0,001	3,41	0,03	3,68	0,01
		23	0,100	0,000	0,172	0,007	0,477	0,001	0,257	0,002	3,88	0,02	4,14	0,01
		Average	**0,07**	**0,04**	**0,12**	**0,05**	**0,449**	**0,141**	**0,214**	**0,067**	**3,59**	**0,26**	**3,84**	**0,26**

**2006 results unpublished by Figueira et al.*

Table 9.3.1 (Continued).

			Total acidity [meq/kg]				MC [%]				TSS [º Brix]			
			2006		2009		2006		2009		2006		2009	
		Sample	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
AREA B	Ameixal	9	19,34	0,81	22,05	0,07	14,20	0,00	14,85	0,01	84,00	0,00	85,15	0,01
		6	19,95	0,01	19,33	0,02	16,20	0,26	16,24	0,04	81,85	0,26	83,76	0,04
		31	15,80	0,28	20,22	0,06	15,90	0,00	15,29	0,20	82,00	0,00	84,71	0,20
		12	13,41	0,28	16,59	0,63	16,36	0,48	16,58	0,92	81,83	0,58	83,42	0,92
		13	15,00	0,28	19,60	0,64	16,20	0,00	15,82	0,01	82,17	0,29	84,18	0,01
		4	20,00	0,04	19,83	0,77	17,01	0,02	15,01	0,07	82,00	0,00	84,99	0,07
		3	18,90	0,36	19,66	0,67	15,40	0,00	15,75	0,28	82,90	0,00	84,25	0,28
	Cachopo	2	18,99	0,36	26,82	0,67	15,80	0,00	16,65	0,13	82,50	0,00	83,45	0,13
		1	19,58	0,48	18,07	0,54	16,08	0,00	15,90	0,10	82,00	0,00	84,10	0,10
		26	17,00	0,18	32,56	1,28	16,50	0,00	15,42	0,04	82,00	0,00	84,58	0,04
		11	22,77	0,26	23,92	0,68	17,27	0,02	16,82	0,05	81,00	0,00	83,18	0,05
		7	19,45	1,16	21,37	0,46	15,05	0,39	15,32	0,00	82,93	0,26	84,89	0,00
		27	15,80	0,02	22,72	0,84	16,50	0,00	15,10	0,23	82,00	0,00	84,90	0,23
		21	17,00	0,28	23,75	0,40	15,80	0,00	15,82	0,11	82,50	0,39	84,18	0,11
		8	19,13	1,38	21,68	0,77	14,80	0,00	15,57	0,04	82,50	0,00	84,43	0,04
	Martinlongo	22	17,00	0,28	26,92	0,74	16,60	0,00	16,02	0,47	81,50	0,39	84,00	0,47
		29	17,58	0,21	19,83	0,71	16,00	0,00	15,28	0,09	81,50	0,00	84,72	0,09
		20	16,00	0,28	24,81	0,73	16,20	0,00	15,79	0,20	82,00	0,00	84,21	0,20
		10	17,04	0,98	18,01	1,36	15,51	0,18	15,26	0,07	82,53	0,30	84,74	0,07
		Average	**17,88**	**2,21**	**21,99**	**3,85**	**15,97**	**0,75**	**15,71**	**0,56**	**82,20**	**0,65**	**84,31**	**0,57**
AREA D	Sta. Catarina	16	15,50	0,28	31,20	1,64	17,00	0,00	16,29	0,29	81,00	0,39	83,71	0,29
		32	17,10	0,32	21,27	0,15	16,60	0,00	15,67	0,09	81,00	0,12	84,33	0,09
		33	15,94	0,13	27,81	0,75	16,60	0,00	16,63	0,20	81,00	0,46	83,14	0,20
		28	15,80	0,01	39,56	0,80	16,50	0,00	15,29	0,30	82,00	0,00	84,71	0,30
		18	17,00	0,19	25,26	1,66	15,80	0,00	15,26	0,05	82,00	0,00	84,74	0,05
		30	16,50	0,21	24,59	0,72	15,90	0,00	16,07	0,44	82,00	0,00	83,93	0,44
		19	17,00	0,28	28,72	0,76	15,80	0,00	15,65	0,41	82,50	0,00	84,35	0,41
		17	16,00	0,28	28,77	2,26	16,60	0,00	15,73	0,11	81,67	0,39	84,27	0,11
		25	15,80	0,32	34,63	0,12	16,50	0,00	15,13	0,38	82,00	0,00	84,87	0,38
	Tavira	15	16,50	0,28	30,16	0,02	16,20	0,00	16,05	0,04	81,33	0,14	83,95	0,04
		24	16,15	0,25	33,93	0,80	16,60	0,00	15,32	1,08	81,67	0,29	84,68	1,08
		23	17,00	0,20	26,25	1,83	16,50	0,00	15,74	0,50	82,00	0,00	84,26	0,50
		Average	**16,36**	**0,57**	**29,34**	**5,00**	**16,38**	**0,38**	**15,74**	**0,46**	**81,68**	**0,49**	**84,25**	**0,50**

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Table 9.3.1 (Continued).

Honeys samples	Moisture [%] 2006 Mean	SD	TSS [º Brix] 2006 Mean	SD	Moisture [%] 2009 Mean	SD	TSS [º Brix] 2009 Mean	SD
1	16,08	0,00	82,00	0,00	15,90	0,10	84,10	0,10
2	15,80	0,00	82,50	0,00	16,65	0,13	83,45	0,13
3	15,40	0,00	82,90	0,00	15,75	0,28	84,25	0,28
4	17,01	0,02	82,00	0,00	15,01	0,07	84,99	0,07
6	16,20	0,26	81,85	0,26	16,24	0,04	83,76	0,04
7	15,05	0,39	82,93	0,26	15,32	0,00	84,89	0,00
8	14,80	0,00	82,50	0,00	15,57	0,04	84,43	0,04
9	14,20	0,00	84,00	0,00	14,85	0,01	85,15	0,01
10	15,51	0,18	82,53	0,30	15,26	0,07	84,74	0,07
11	17,27	0,02	81,00	0,00	16,82	0,05	83,18	0,05
12	16,36	0,48	81,83	0,58	16,58	0,92	83,42	0,92
13	16,20	0,00	82,17	0,29	15,82	0,01	84,18	0,01
15	16,20	0,00	81,33	0,14	16,05	0,04	83,95	0,04
16	17,00	0,00	81,00	0,39	16,29	0,29	83,71	0,29
17	16,60	0,00	81,67	0,39	15,73	0,11	84,27	0,11
18	15,80	0,00	82,00	0,00	15,26	0,05	84,74	0,05
19	15,80	0,00	82,50	0,00	15,65	0,41	84,35	0,41
20	16,20	0,00	82,00	0,00	15,79	0,20	84,21	0,20
21	15,80	0,00	82,50	0,39	15,82	0,11	84,18	0,11
22	16,60	0,00	81,50	0,39	16,02	0,47	84,00	0,47
23	16,50	0,00	82,00	0,00	15,74	0,50	84,26	0,50
24	16,60	0,00	81,67	0,29	15,32	1,08	84,68	1,08
25	16,50	0,00	82,00	0,00	15,13	0,38	84,87	0,38
26	16,50	0,00	82,00	0,00	15,42	0,04	84,58	0,04
27	16,50	0,00	82,00	0,00	15,10	0,23	84,90	0,23
28	16,50	0,00	82,00	0,00	15,29	0,30	84,71	0,30
29	16,00	0,00	81,50	0,00	15,28	0,09	84,72	0,09
30	15,90	0,00	82,00	0,00	16,07	0,44	83,93	0,44
31	15,90	0,00	82,00	0,00	15,29	0,20	84,71	0,20
32	16,60	0,00	81,00	0,12	15,67	0,09	84,33	0,09
33	16,60	0,00	81,00	0,46	16,63	0,20	83,14	0,20

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Table 9.3.1 (Continued).

			Proline content [mg/kg]				Water activity				DA [Gothe]			
			2006		2009		2006		2009		2006		2009	
		Sample	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
AREA B	Ameixal	9	202,3	27,6	446,4	4,2	0,490	0,003	0,487	0,001	16,16	0,00	7,60	1,08
		6	139,8	7,5	288,0	8,9	0,530	0,001	0,508	0,002	23,01	0,00	8,11	1,97
		31	190,3	14,6	502,1	0,0	0,540	0,002	0,486	0,006	26,57	0,00	7,32	0,77
	Cachopo	12	102,6	7,8	326,5	10,2	0,550	0,020	0,540	0,002	9,64	0,00	7,03	0,96
		13	115,3	8,7	326,5	0,0	0,520	0,007	0,503	0,001	23,01	0,00	8,19	0,07
		4	44,5	0,9	417,9	93,0	0,510	0,001	0,503	0,001	11,17	0,00	5,31	0,48
		3	43,5	28,5	484,3	14,2	0,510	0,000	0,521	0,001	13,20	0,00	8,15	0,19
		2	115,8	12,6	676,2	34,0	0,520	0,000	0,527	0,003	39,96	0,01	9,62	0,11
		1	76,5	12,9	370,5	15,5	0,520	0,000	0,510	0,001	18,99	0,00	12,71	0,63
	Martinlongo	26	243,3	24,8	846,3	3,5	0,520	0,006	0,509	0,004	17,36	0,00	9,15	0,49
		11	145,8	7,2	380,0	10,2	0,540	0,000	0,546	0,002	10,04	0,00	6,97	0,63
		7	169,8	18,9	420,7	3,5	0,520	0,001	0,513	0,002	21,32	0,00	7,58	0,74
		27	189,1	33,1	450,8	1,0	0,520	0,003	0,498	0,003	5,60	0,00	3,55	0,46
		21	182,1	11,8	559,6	1,9	0,530	0,002	0,519	0,003	14,69	0,00	7,71	0,25
		8	85,5	5,4	437,4	17,7	0,520	0,003	0,512	0,001	16,63	0,00	6,42	0,20
		22	196,3	33,5	412,9	1,0	0,560	0,001	0,504	0,004	26,57	0,00	10,38	0,05
		29	94	1,1	515,0	1,9	0,520	0,004	0,503	0,002	12,14	0,00	8,67	0,18
		20	141,3	20,3	381,7	3,3	0,530	0,001	0,526	0,001	15,66	0,00	7,62	0,23
		10	140,3	4,8	252,3	1,9	0,510	0,010	0,509	0,003	16,16	0,00	6,66	0,30
		Average	**137,8**	**55,5**	**447,11**	**138,1**	**0,52**	**0,02**	**0,51**	**0,02**	**17,78**	**7,84**	**7,83**	**1,92**
AREA D	Sta. Catarina	16	317,6	29,1	680,6	2,6	0,540	0,002	0,533	0,001	14,18	0,00	8,86	0,05
		32	200,3	16	706,3	1,7	0,540	0,001	0,515	0,002	16,57	0,00	9,04	0,14
		33	180,3	15,7	788,8	1,0	0,540	0,001	0,536	0,001	16,67	0,00	9,51	0,24
		28	190,1	40,5	401,8	0,0	0,570	0,002	0,493	0,001	4,92	0,00	2,64	0,03
		18	169,6	2,3	463,1	69,1	0,530	0,003	0,516	0,003	9,82	0,00	6,72	0,11
		30	148,8	10	488,8	1,7	0,540	0,002	0,507	0,005	15,32	0,00	8,12	0,29
	Tavira	19	260,6	49	557,4	0,0	0,530	0,003	0,514	0,001	13,90	0,00	8,23	0,00
		17	190,3	15,6	561,3	5,9	0,530	0,001	0,517	0,003	16,18	0,00	10,88	0,57
		25	204,3	9,7	624,9	1,0	0,550	0,004	0,505	0,003	15,20	0,00	9,63	1,38
		15	181,3	4,7	438,0	1,9	0,540	0,001	0,514	0,001	11,85	0,00	8,20	0,20
		24	269,6	10,4	914,9	1,9	0,530	0,003	0,516	0,002	25,87	0,00	12,16	0,30
		23	233,1	37,3	594,2	1,7	0,530	0,006	0,509	0,003	12,25	0,00	6,26	0,27
		Average	**212,2**	**48,8**	**601,7**	**151,7**	**0,54**	**0,01**	**0,51**	**0,01**	**14,39**	**4,94**	**8,35**	**2,42**

**2006 results unpublished by Figueira et al.*

Table 9.3.1 (Continued).

			HMF content [mg/kg]				2006				COLOR		2009					
			2006		2009		***L***		***a***		***b***		***L***		***a***		***b***	
		Sample	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Δ E
AREA B	Ameixal	9	1,32	0,02	107,38	2,10	33,08	0,42	-1,17	0,12	7,82	0,29	30,00	0,29	-0,68	0,09	7,15	0,31	3,19
		6	0,99	0,00	87,35	5,76	31,26	0,42	-1,30	0,06	5,22	0,30	32,33	0,17	-1,06	0,10	6,76	0,56	1,89
		31	11,21	0,00	131,64	0,00	28,22	2,33	-1,13	0,09	5,28	0,79	30,17	0,21	-0,85	0,20	5,84	0,73	2,05
	Cachopo	12	2,21	0,00	106,93	4,65	33,76	1,69	-1,03	0,08	3,51	0,03	31,04	0,24	-0,98	0,13	7,13	0,25	4,53
		13	0,02	0,00	109,16	3,49	34,46	0,47	-1,03	0,08	3,51	0,03	31,16	0,45	-0,93	0,13	6,48	0,31	4,44
		4	11,51	0,00	99,02	0,82	32,00	0,00	-1,00	0,05	7,00	0,21	31,35	0,49	-0,62	0,14	7,03	0,42	0,75
		3	12,53	0,00	101,24	0,60	32,21	0,32	-0,95	0,05	7,39	0,44	33,61	0,74	-0,64	0,20	6,18	1,22	1,87
		2	8,23	0,00	106,20	2,46	31,09	0,09	-0,99	0,07	6,57	0,21	29,72	0,10	0,54	0,04	7,79	0,16	2,39
		1	2,71	0,00	179,51	2,97	33,64	0,24	-1,32	0,20	7,49	0,16	31,67	0,39	-1,00	0,07	7,99	0,62	2,06
	Martinlongo	26	11,80	0,00	127,52	1,62	31,34	0,67	-0,97	0,09	3,37	0,70	30,54	0,31	-0,07	0,14	8,07	0,56	4,85
		11	2,04	0,02	135,19	0,64	31,47	0,25	-1,12	0,02	7,26	0,53	31,92	0,23	-1,23	0,16	7,42	0,30	0,49
		7	2,36	0,00	103,00	0,20	31,72	0,24	-0,91	0,08	7,28	0,32	30,80	0,43	-0,75	0,18	8,00	0,56	1,18
		27	5,92	0,00	76,57	5,30	29,44	0,66	-0,97	0,09	3,40	0,80	31,27	1,51	-0,94	0,09	8,66	0,29	5,57
		21	11,12	0,00	100,03	5,93	29,22	1,67	-0,95	0,09	4,57	0,79	32,28	0,40	-0,99	0,10	7,44	0,45	4,19
		8	1,14	0,32	77,87	0,87	33,62	0,42	-1,22	0,08	5,79	0,32	30,26	0,11	-1,05	0,12	6,13	0,16	3,38
		22	11,21	0,00	130,20	2,09	28,22	0,00	-0,98	0,09	3,37	0,88	31,77	0,06	0,45	0,07	8,01	0,51	6,01
		29	12,90	0,00	104,44	3,33	25,98	0,89	-0,79	0,00	5,42	0,54	32,17	0,61	-1,22	0,12	8,24	0,67	6,81
		20	14,59	0,00	113,40	1,10	32,22	0,67	-0,96	0,00	4,37	0,79	33,86	0,48	-1,06	0,11	8,42	0,18	4,37
		10	1,32	0,02	74,21	1,32	32,74	0,30	-1,18	0,13	7,26	0,33	30,46	0,44	-0,96	0,11	6,69	0,42	2,36
		Average	**6,59**	**5,21**	**108,99**	**24,71**	**31,35**	**2,23**	**-1,05**	**0,14**	**5,57**	**1,66**	**31,39**	**1,14**	**-0,74**	**0,51**	**7,34**	**0,83**	**1,79**
AREA D	Sta. Catarina	16	0,01	0,00	70,47	5,01	30,44	0,45	-0,89	0,09	8,23	0,79	30,42	0,39	0,06	0,14	8,44	0,61	0,96
		32	12,21	0,00	126,36	0,00	28,22	0,47	-1,17	0,12	5,48	0,63	30,90	0,24	-1,08	0,12	6,78	0,34	2,98
		33	14,21	0,00	138,64	4,31	34,12	0,67	-0,92	0,00	4,37	0,57	30,28	0,14	-0,17	0,19	8,46	0,25	5,66
		28	28,29	0,00	331,71	10,44	25,22	0,67	-0,96	0,09	3,43	0,00	25,07	0,17	2,30	0,24	2,28	0,30	3,46
		18	5,70	0,00	77,04	0,88	33,12	0,67	-1,17	0,01	4,37	0,44	31,15	0,34	-0,90	0,15	7,54	0,17	3,74
		30	11,43	0,00	16,77	0,00	29,87	0,01	-1,17	0,10	4,38	0,79	30,14	1,26	-0,74	0,11	7,38	1,28	3,04
		19	8,16	0,00	71,42	7,50	34,22	1,00	-0,96	0,09	4,87	0,79	30,43	0,26	1,40	0,18	9,34	0,42	6,32
		17	n.d.		61,96	0,65	33,22	1,67	-1,16	0,09	5,37	0,79	31,26	0,29	-0,83	0,11	7,99	0,31	3,29
	Tavira	25	17,87	0,00	103,84	8,20	23,95	0,44	-0,98	0,00	3,37	0,79	29,98	0,22	0,91	0,14	8,75	0,22	8,30
		15	n.d.		67,82	3,84	31,74	0,47	-1,24	0,07	5,60	0,04	32,27	0,34	-0,07	0,11	4,68	0,52	1,58
		24	26,98	0,00	75,04	10,03	28,20	1,00	-0,96	0,09	5,37	0,00	32,14	0,36	-0,64	0,14	7,98	0,36	4,74
		23	9,80	0,00	65,49	0,65	32,21	1,00	-0,97	0,09	4,47	0,00	30,34	0,23	0,56	0,19	8,39	0,20	4,61
		Average	**13,47**	**8,89**	**100,55**	**79,39**	**30,38**	**3,40**	**-1,05**	**0,12**	**4,94**	**1,27**	**30,36**	**1,83**	**0,07**	**1,04**	**7,33**	**1,99**	**2,64**

9.2 Annexes to Chapter 4

Table 9.4.1 Pollen frequency (%) of strawberry tree honey samples

Pollen type	**Mean (%)**	**SD**
Acacia spp.	**0,34**	**0,02**
Anarrhinum bellidifolium	**0,17**	**0,01**
Anthemis arvensis	**0,17**	**0,01**
Apiaceae	**0,17**	**0,01**
Arbutus unedo	**31,96**	**3,50**
Calendula arvensis	**1,53**	**0,08**
Calluna vulgaris	**0,17**	**0,01**
Campanula spp.	**0,51**	**0,03**
Centaurea calcitrapa	**0,17**	**0,01**
Ceratonia siliqua	**5,37**	**0,28**
Cistaceae	**20,45**	**1,06**
Citrus spp.	**3,92**	**0,20**
Crataegus monogyna	**0,68**	**0,04**
Crepis capillaris	**0,17**	**0,01**
Cytisus scoparius	**5,11**	**0,26**
Echium plantagineum	**4,52**	**0,23**
Erica spp.	**0,51**	**0,03**
Eucalyptus spp.	**0,17**	**0,01**
Frangula alnus	**0,17**	**0,01**
Lavandula stoechas	**15,05**	**0,64**
Myrtus spp.	**1,36**	**0,07**
Olea europea	**1,87**	**0,10**
Pistacia terebinthus	**1,98**	**0,10**
Plantago spp.	**0,17**	**0,01**
Raphanus spp./ *Sinapsis* spp.	**0,51**	**0,03**
Reseda luteola	**3,92**	**0,20**
Rhamnus spp.	**0,34**	**0,02**
Rosmarinus officinalis	**0,85**	**0,04**
Rubus spp.	**0,51**	**0,03**
Senecio vulgaris	**0,17**	**0,01**
Trifolium arvense	**0,17**	**0,01**

Table 9.4.2 Color expressed in Pfund scale (from Naab et al., 2008)

Honey color	Pfund scale (mm)
Water white	< 8
Extra white	9–17
White	18–34
Extra light amber	35–50
Light amber	51–85
Amber	86–114
Dark amber	> 114

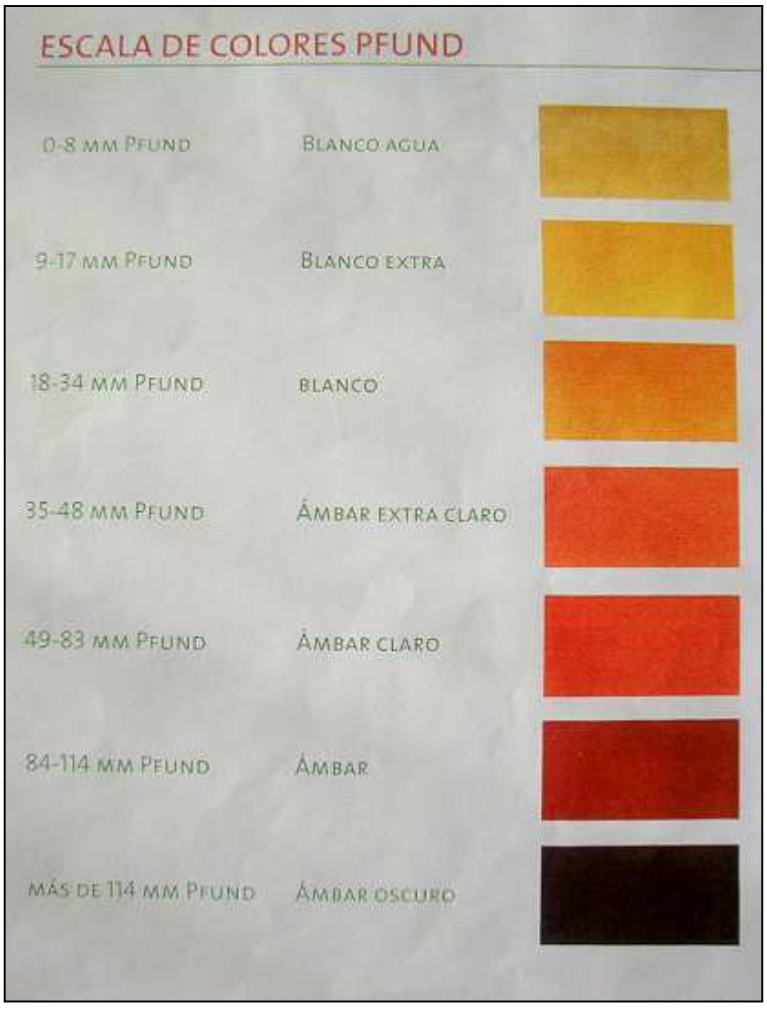

Figure 9.4.1 Color table according Pfund scale (from Pajuelo, 2004).

Table 9.4.3 Data of flow behaviour of strawberry tree determined by viscometer.

Temperature (ºC)	RPM	% torque	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)
20	0	0,3	0	0,05	0
	0,3	16,5	33000	2,8	0,08
	0,5	26,7	32160	4,5	0,14
	0,6	31,7	31700	5,3	0,17
	1	50,3	30180	8,5	0,28
	1,5	75,5	30200	12,7	0,42
	2	98,7	29640	16,6	0,56
25	0	-0,7	0	0	0
	0,3	8,9	18000	1,5	0,08
	0,6	16,7	16700	2,8	0,17
	1	27,4	16440	4,6	0,28
	1,5	40,3	16160	6,8	0,42
	2	52,6	15780	8,8	0,56
	3	77	15380	12,9	0,84
30	0	-	-	-	-
	0,3	4,1	8200	0,69	0,08
	0,6	8,5	8500	1,4	0,17
	1	14,5	8700	2,4	0,28
	2	29,5	8850	4,9	0,56
	2,5	36,7	8808	6,2	0,7
	3	44,6	8900	7,5	0,84
	4	59	8865	9,9	1,12
	5	72,3	8864	12,2	1,4
	6	84,7	8460	14,2	1,68
35	0	-0,4	0	0	0
	0,5	2,3	2760	0,39	0,14
	1	5,8	3480	0,97	0,28
	2	11,8	3570	2	0,56
	2,5	14,8	3528	2,5	0,7
	3	18,2	3640	3,1	0,84
	4	24,4	3660	4,1	1,12
	5	29,9	3588	5	1,4
	6	35,2	3500	5,9	1,68
	10	57,6	3456	9,7	2,8
	12	68,8	3435	11,5	3,36

Table 9.4.2 (continued).

Temperature (ºC)	RPM	% torque	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)
40	0	-0,1	0	0	0
	0,3	1	2000	0,17	0,08
	1,5	3,7	1480	0,62	0,42
	3	7,8	1540	1,3	0,84
	5	12,8	1536	2,2	1,4
	10	24,5	1470	4,1	2,8
	20	48,2	1446	8,1	5,6
	30	71,9	1440	12,1	8,4

9.3 Annexes to Chapter 5

Table 9.5.1 Results of pollen spectrum of commercial honey samples.

	Commercial honeys samples			
Pollen types	**L-1 (%)**	**G-1 (%)**	**R-1 (%)**	**B-1 (%)**
Anacardiaceae	0,00	0,00	0,00	0,00
Pistacia terebinthus	5,15	6,86	0,00	0,00
Apiaceae	0,00	0,48	0,23	0,52
Aquifoliaceae	0,00	0,00	0,00	0,00
Ilex aquifolium	0,00	0,00	0,00	0,00
Asteraceae	0,00	0,00	0,00	0,00
Anthemis arvensis	0,04	0,32	0,00	0,52
Calendula arvensis	0,08	0,16	0,23	1,57
Carthamus lanatus	0,00	0,00	0,00	0,00
Centaurea calcitrapa	0,00	0,00	0,00	0,00
Crepis capillaris	0,00	1,27	0,45	1,04
Senecio vulgaris	0,51	1,06	0,86	2,09
Helianthus annuus	0,00	24,60	0,00	0,00
Betulaceae	0,00	0,00	0,00	0,00
Alnus glutinosa	0,00	0,00	0,00	0,00
Boraginaceae	0,00	0,00	0,00	0,00
Borago oficinallis	0,00	0,16	0,00	0,00
Echium plantagineum	11,43	14,10	8,05	6,27
Lithodora fruticosa	0,00	0,00	0,00	0,00
Brassicaceae	0,00	0,00	0,00	0,00
Raphanus spp. / Sinapsis spp.	1,73	0,90	0,00	0,00
Caesalpiniaceae	0,00	0,00	0,00	0,00
Ceratonia siliqua	0,00	0,16	0,00	7,83
Campanulaceae	0,00	0,00	0,00	0,00
Campanula spp.	0,00	1,11	2,90	0,00
Caryophyllaceae	0,00	0,00	0,00	0,00
Silene spp.	0,00	0,00	0,00	0,00
Chenopodiaceae	0,00	0,00	0,00	0,00
Chenopodium album	0,08	0,53	0,00	0,00
Cistaceae	10,92	16,16	30,54	10,44
Clusiaceae	0,00	0,00	0,00	0,00
Hypericum perforatum	0,20	0,00	0,00	0,00
Crassulaceae	0,00	0,00	0,00	0,00
Sedum spp.	0,00	0,00	0,00	0,00
Ericaceae	0,00	0,00	0,00	0,00
Arbutus unedo	0,16	0,00	0,00	20,37
Calluna vulgaris	0,00	0,00	0,04	0,00

Table 9.5.1 (Continued).

	Commercial honeys samples			
Pollen types	**L-1 (%)**	**G-1 (%)**	**R-1 (%)**	**B-1 (%)**
Erica spp.	1,10	0,00	0,00	5,22
Euphorbiaceae	0,00	0,00	0,00	0,00
Fabaceae	0,00	0,00	0,00	0,00
Anthyllis lotoides	0,00	0,00	0,00	0,00
Cytisus scoparius	6,84	0,21	5,53	0,52
Medicago sativa	0,00	0,00	0,00	0,00
Lotus creticus	0,00	0,69	0,00	0,00
Ononis repens	0,00	0,00	0,00	0,00
Ornithopus compressus	0,00	0,00	0,00	0,00
Psoralea butiminosa	0,00	0,00	0,00	0,00
Trifolium arvense	0,00	0,16	0,45	1,04
Trifolium repens	0,00	0,00	0,00	0,00
Fagaceae	0,00	0,00	0,00	0,00
Castanea sativa	0,00	0,00	0,00	0,00
Quercus spp.	4,01	5,07	1,28	0,00
Fumariaceae	0,00	0,00	0,00	0,00
Fumaria officinalis	0,00	0,00	0,00	0,00
Geraniaceae	0,00	0,00	0,00	0,00
Geranium molle	0,00	0,00	0,00	0,00
Graminea	0,24	0,69	0,00	0,00
Lamiaceae	0,00	0,00	0,00	0,00
Lavandula stoechas	2,24	5,44	54,18	13,05
Marrubium spp.	0,00	0,00	0,00	0,00
Thymus spp.	0,00	0,00	0,00	0,00
Rosmarinus officinalis	1,14	3,01	0,00	3,13
Liliaceae	0,00	0,00	0,00	0,00
Aspargus spp.	0,00	0,00	0,00	0,00
Asphodelus albus	0,08	0,00	0,00	0,00
Muscaris comosum	0,00	0,00	0,00	0,00
Malvaceae	0,00	0,00	0,00	0,00
Mimosaceae	0,16	0,00	0,08	0,00
Acacia spp.	0,98	0,00	1,39	0,00
Moraceae	0,00	0,00	0,00	0,00
Morus alba	0,00	0,00	0,00	0,00
Myrtaceae	0,00	0,00	0,08	0,00
Eucalyptus spp.	1,45	2,96	1,39	0,00
Myrtus spp.	0,00	0,00	1,69	0,00
Oleaceae	0,00	0,00	0,00	0,00

Table 9.5.1 (Continued).

	Commercial honeys samples			
Pollen types	**L-1 (%)**	**G-1 (%)**	**R-1 (%)**	**B-1 (%)**
Fraxinus angustifolia	0,00	0,00	0,00	0,00
Olea europea	4,75	5,28	1,47	5,74
Onagraceae	0,00	0,00	0,00	0,00
Oxalidaceae	0,00	0,00	0,00	0,00
Oxalis corniculata	0,63	0,05	0,00	0,00
Papaveraceae	0,00	0,00	0,00	0,00
Papaver rhoeas	0,00	0,00	0,00	0,00
Plantaginaceae	0,00	0,00	0,00	0,00
Plantago spp.	0,39	0,21	0,00	0,00
Pinaceae *(Pinus spp.)*	0,00	0,00	0,00	0,00
Polygonaceae	0,00	0,00	0,00	0,00
Rumex spp.	0,43	0,00	0,08	0,00
Rafflesiaceae	0,00	0,00	0,00	0,00
Cytinus hypocistis	0,00	0,00	0,00	0,00
Resedaceae	0,00	0,11	0,00	0,00
Reseda luteola	4,44	0,69	0,23	9,14
Reseda alba	0,00	0,00	0,00	0,00
Rhamnaceae	0,00	0,00	0,00	0,00
Frangula alnus	0,59	0,21	0,00	0,00
Rhamnus spp.	0,16	0,00	1,32	0,00
Rosaceae	0,00	0,00	0,00	0,00
Crataegus monogyna	2,04	0,53	0,71	2,61
Prunus spp.	0,55	0,00	0,00	0,00
Rubus spp.	7,03	3,48	5,57	0,00
Sanguisorba minor	0,00	0,00	0,00	0,00
Rutaceae	0,00	0,00	0,00	0,00
Citrus spp.	30,22	2,85	0,15	7,83
Ruta spp.	0,00	0,00	0,00	0,00
Salicaceae	0,00	0,00	0,00	0,00
Salix spp.	0,00	0,00	0,00	0,00
Scrophulariaceae	0,00	0,00	0,00	0,00
Anarrhinum bellidifolium	0,04	0,53	0,08	1,04
Scrophularia canina	0,00	0,00	0,00	0,00
Thymelaeaceae	0,00	0,00	0,00	0,00
Thymelaea villosa	0,00	0,00	0,00	0,00
Tiliaceae	0,00	0,00	0,00	0,00
Tilia spp.	0,20	0,00	0,00	0,00
Violaceae	0,00	0,00	0,00	0,00

Table 9.5.1 (Continued).

	Commercial honeys samples			
Pollen types	**L-1 (%)**	**G-1 (%)**	**R-1 (%)**	**B-1 (%)**
Viola spp.	0,00	0,00	0,00	0,00
Others (crystal, ash, bees parts, spores, etc)	0,00	0,00	0,00	0,00
Honeydew elements	0,00	0,00	0,00	0,00
Total pollen types	0,00	0,00	0,00	0,00
Total pollen grains	100,00	100,00	100,00	100,00

Table 9.5.2 Data for linear regression ash content (%) and electrical conductivity (EC; mS/cm).

Honey Sample	**Ash content (%)**	**EC (mS/cm)**
B-1	0,304	0,475
	0,295	0,486
	0,300	0,483
G-1	0,145	0,330
	0,154	0,337
	0,149	0,334
L-1	0,108	0,217
	0,107	0,216
	0,108	0,217
R-1	0,043	0,112
	0,055	0,113
	0,049	0,113
MJ	0,631	0,636
	0,465	0,628
	0,640	0,645

Table 9.5.3 Data of flow behaviour of commercial honey (B-1, G-1 and L-1) determined by viscometer.

	Honey B-1					Honey G-1					Honey L-1				
T (ºC)	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**
20	0	-1,8	0			0	2,8	0	0,47	0	0				
	0,3	13,4	26800	2,3	0,08	0,3	27,5	55000	4,6	0,08	0,3	17,6	35200	3	0,08
	0,5	20,4	24480	3,4	0,14	0,5	44,3	53160	7,4	0,14	0,5	28,1	33720	4,7	0,14
	0,6	24	24000	4	0,17	0,6	52,8	52800	8,9	0,17	0,6	33	33100	5,6	0,17
	1	39,9	23940	6,7	0,28	1	87,7	52560	14,7	0,28	1	54,5	32700	9,2	0,28
	1,5	60,5	24200	10,2	0,42						1,5	82,1	32840	13,8	0,42
	2	79,3	23790	13,3	0,56										
	2,5	98,7	23680	16,6	0,7										
T (ºC)	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**
25	0	0,8	0	0,13	0	0					0	-1	0	0	0
	0,3	6,4	0	1,1	0,08	0,3	12,6	25200	2,1	0,08	0,3	7	14000	1,2	0,08
	0,6	11,8	11800	2	0,17	0,5	19,3	23160	3,2	0,14	0,5	10,9	13080	1,8	0,14
	1	19,7	11820	3,3	0,28	0,6	21,7	21700	3,7	0,17	0,6	12,9	12900	2,2	0,17
	2	38,4	11520	6,5	0,56	1	36	21480	6	0,28	1	21,4	12840	3,6	0,28
	2,5	48,7	11680	8,2	0,7	1,5	53,2	21320	9	0,42	1,5	32	12800	5,4	0,42
	3	59,2	11840	10	0,84	2	70,8	21240	11,9	0,56	2	42,6	12780	7,2	0,56
	4	78,6	11790	13,2	1,12	2,5	89,4	21450	15	0,7	2,5	52,9	12720	8,9	0,7
	5	96,9	11610	16,3	1,4						3	64,7	12940	10,9	0,84
T (ºC)	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**
30	0	-0,7	0	0	0	0	3,2	0	0,54	0	0	0,2	0	0,02	0
	0,3	3,2	6600	0,55	0,08	0,3	5,4	10800	0,92	0,08	0,3	3,8	7600	0,64	0,08
	0,6	5,6	5600	0,94	0,17	0,5	8,1	9720	1,4	0,14	0,5	4,7	5640	0,79	0,14
	1	9,6	5760	1,6	0,28	0,6	9,9	9900	1,7	0,17	1	10,1	6120	1,7	0,28
	2	19,6	5880	3,3	0,56	1	17,2	10320	2,9	0,28	2	22,2	6660	3,8	0,56

Table 9.5.3 (Continued).

T (ºC)	RPM	Torque %	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)	RPM	Torque %	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)	RPM	Torque %	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)
	2,5	26,6	6384	4,5	0,7	1,5	27,2	10880	4,6	0,42	2,5	28,8	6912	4,8	0,7
	3	33,7	6740	5,7	0,84	2	35,4	10620	6	0,56	3	35,8	7160	6	0,84
	4	45,5	6825	7,6	1,12	3	57,4	10500	9,7	0,84	4	48,2	7230	8,1	1,12
	5	56,6	6792	9,5	1,4	4	77,2	11590	13	1,12	5	59,7	7164	10	1,4
	6	67,3	6740	11,3	1,68	5	96,2	11540	16,2	1,4	6	71,6	7160	12,1	1,68
T (ºC)	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**
35	0	-0,2	0	0	0	0	5,2	0	0,81	0	0	1,8	0		
	0,5	2,6	3120	0,44	0,14	0,3	4,8	9600	0,79	0,08	0,5	3	3600	0,5	0,14
	1	5,5	3300	0,92	0,28	0,5	4,9	5880	0,82	0,14	1	6,1	3660	1	0,28
	2	11,2	3360	1,9	0,56	1	9,5	5700	1,6	0,28	1,5	9,6	3840	1,6	0,42
	4	22,5	3375	3,8	1,12	2	19,8	5940	3,4	0,56	2	12,7	3810	2,2	0,56
	5	27,6	3324	4,6	1,4	3	32,1	6420	5,4	0,84	3	20	3980	3,4	0,84
	6	32,9	3280	5,5	1,68	4	43,2	6495	7,3	1,12	4	27,2	4080	4,6	1,12
	10	53,4	3204	9	2,8	5	53,6	6432	9	1,4	5	33,6	4032	5,6	1,4
	12	64,1	3205	10,8	3,36	6	64,4	6430	10,8	1,68	6	40,3	4030	6,8	1,68
											10	66,1	3972	11,1	2,8
											12	79,9	3995	13,4	3,36
T (ºC)	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**	**RPM**	**Torque %**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**
40	0	0,7	0	0,12	0	0	0,4	0	0,07	0	0	2,4	0	0,34	0
	0,6	1,5	1500	0,25	0,17	0,3	1,3	2600	0,24	0,08	0,6	2,4	2400	0,4	0,17
	1	2,7	1680	0,47	0,28	0,5	2,4	2880	0,4	0,14	1	3,9	2340	0,67	0,28
	2	5,6	1680	0,94	0,56	1	5,4	0	0,91	0,28	2	7,8	2340	1,3	0,56
	4	12,2	1830	2,1	1,12	2	11,5	3450	1,9	0,56	3	12,4	2480	2,1	0,84
	6	17,6	1780	3	1,68	3	17,9	3560	3	0,84	4	16,7	2520	2,8	1,12
	10	28,3	1704	4,8	2,8	4	24,3	3645	4,1	1,12	5	20,4	2460	3,4	1,4

Table 9.5.3 (Continued).

T (ºC)	RPM	Torque %	η (mPa s)	Shear stress (N/m2)	Shear rate (1/s)	RPM	Torque %	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)	RPM	Torque %	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)
	20	55,8	1677	9,4	5,6	5	29,9	3588	5	1,4	6	24,8	2470	4,1	1,68
	30	83,3	1668	14	8,4	6	36	3600	6,1	1,68	10	40,3	2418	6,8	2,8
						10	59,3	3564	10	2,8	20	79,9	2400	13,5	5,6
						12	72,1	3605	12,1	3,36					

Table 9.5.4 Data of flow behaviour of commercial honey (R-1) determined by viscometer.

Honey R-1					
T (ºC)	RPM	Torque (%)	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)
	0	0	0	0	0
20	0,3	29,7	59400	0,08	5
	0,5	48	57600	0,14	8,1
	0,6	57,7	57700	0,17	9,7
	1	96	57600	0,28	16,1
T (ºC)	**RPM**	**Torque (%)**	**η (mPa s)**	**Shear stress (N/m2)**	**Shear rate (1/s)**
25	0	6,3	0	0	1,1
	0,3	13,5	27000	0,08	2,3
	0,5	21,5	25800	0,14	3,6
	1	43,8	26280	0,28	7,4
	1,5	67,2	26880	0,42	11,3
	2	88,6	26610	0,56	14,9
T (ºC)	**RPM**	**Torque (%)**	**η (mPa s)**	**Shear stress (N/m2)**	**Shear rate (1/s)**
30	0	5,8	0	0	0,97
	0,3	6,7	13200	0,08	1,1
	0,5	10,8	13080	0,14	1,8
	1	22,6	13560	0,28	3,8

Table 9.5.4 (Continued).

T (ºC)	RPM	Torque (%)	η (mPa s)	Shear stress (N/m^2)	Shear rate (1/s)
	1,5	34,9	14000	0,42	5,9
	2	46,3	13920	0,56	7,8
	2,5	60,3	14470	0,7	10,1
	3	74	14800	0,84	12,5
	4	99,4	14910	1,12	16,7
T (ºC)	**RPM**	**Torque (%)**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**
35	0	0,6	0	0	0,1
	0,3	3,8	7600	0,08	0,64
	0,5	5,4	6480	0,14	0,91
	1	11,6	7020	0,28	2
	1,5	18,6	7440	0,42	3,1
	3	38,9	7800	0,84	6,5
	4	52,2	7830	1,12	8,8
	5	64,5	7740	1,4	10,8
	6	77,6	7760	1,68	13
T (ºC)	**RPM**	**Torque (%)**	**η (mPa s)**	**Shear stress (N/m^2)**	**Shear rate (1/s)**
40	0	0	0	0	0
	0,3	2,4	4800	0,08	0,4
	0,5	3	3600	0,14	0,5
	1	6,1	3660	0,28	1
	2	13,1	3960	0,56	2,2
	3	21,9	4380	0,84	3,7
	4	29,1	4365	1,12	4,9
	5	35,7	4284	1,4	6
	6	42,7	4270	1,68	7,2
	10	70,8	4242	2,8	11,9
	12	85,2	4265	3,36	14,3

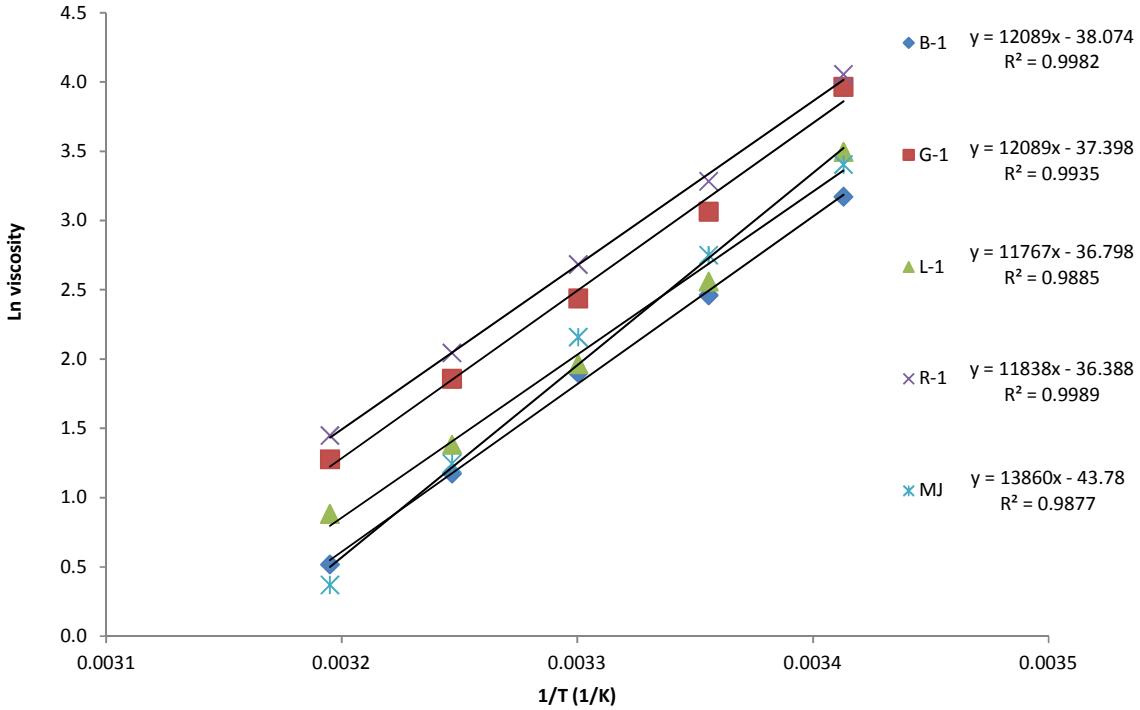

Figure 9.5.1 Arrhenius model for all commercial honey (B-1, G-1, L-1 and R-1) and artisanal honey (MJ).

Table 9.5.5 Results for antioxidant capacity, radical scavenging DPPH and total phenol content for all honey samples analyzed.

Honeys	Antioxidant (mg QEAC/100g of honey)	Antioxidant (mg AEAC/100g of honey)	DPPH (%)	Phenol content (GAE/100g of honey)
B-1	11,58	21,62	39,11	92,24
	11,47	21,43	39,58	91,13
	11,47	21,43	38,41	92,13
G-1	3,06	6,05	27,87	39,64
	3,11	6,14	26,23	39,33
	3,00	5,95	18,03	39,64
L-1	1,57	3,25	14,29	27,33
	1,53	3,17	18,97	27,33
	1,24	2,65	17,56	27,44
R-1	1,84	3,76	16,86	22,18
	1,80	3,68	17,10	21,96
	1,84	3,76	15,93	22,18
MJ	9,73	18,17	42,39	95,60
	9,78	18,25	40,28	96,10
	9,64	18,01	42,15	91,70

Table 9.5.6 Results for sensory analysis to five attributes to honey samples analyzed.

Sample	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor
Honey B-1	1	3	4	4	4	4	1	3	5	2	4	3
	2	4	5	3	4	2	2	5	5	3	5	2
	3	3	3	4	4	4	3	3	4	4	4	3
	4	5	3	3	5	3	4	5	3	2	4	3
	5	3	4	4	5	5	5	3	4	4	4	5
	6	2	4	4	4	5	6	5	4	2	4	2
	7	5	3	3	3	4	7	5	5	3	2	3
	8	5	1	4	5	5	8	5	3	3	4	4
	9	5	3	3	3	4	9	5	4	4	2	4
	10	5	4	2	5	4	10	5	5	4	4	4
	11	4	5	2	5	3	11	5	5	4	4	3
	12	2	2	2	5	4	12	3	2	2	4	4
	13	5	3	2	5	4	13	5	2	3	5	4
	14	2	2	4	4	3	14	2	2	4	4	3
	15	5	3	3	2	5	15	2	3	3	4	4
	Average	3,9	3,3	3,1	4,2	3,9	Average	4,1	3,7	3,1	3,9	3,4
	SD	1,2	1,1	0,8	0,9	0,9	SD	1,2	1,2	0,8	0,8	0,8
Sample	**Panellist**	**Color**	**Aroma**	**Viscosity**	**Crystal presence**	**Flavor**	**Panellist**	**Color**	**Aroma**	**Viscosity**	**Crystal presence**	**Flavor**
Honey G-1	1	2	5	4	1	5	1	2	5	5	2	5
	2	2	4	4	3	5	2	2	3	4	2	5
	3	2	4	4	1	5	3	2	4	4	2	5
	4	1	2	5	1	4	4	2	5	5	1	2
	5	1	3	5	4	5	5	2	3	4	3	5
	6	1	2	5	1	4	6	2	2	5	1	4
	7	1	4	5	2	4	7	1	4	5	1	4
	8	2	2	5	2	4	8	3	3	5	1	4
	9	2	2	5	2	3	9	2	1	5	2	5
	10	2	1	4	1	4	10	2	5	5	1	4
	11	2	4	4	2	4	11	2	3	4	1	5
	12	1	5	5	2	3	12	2	2	5	1	5
	13	2	5	4	2	5	13	2	3	2	1	5
	14	1	2	4	3	4	14	2	2	4	2	3

Table 9.5.6 (Continued).

Sample	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor
Honey G-1	15	2	3	4	2	5	15	1	4	5	1	5
	Average	1,6	3,2	4,5	1,9	4,3	Average	1,9	3,3	4,5	1,5	4,4
	SD	0,5	1,3	0,5	0,9	0,7	SD	0,5	1,2	0,8	0,6	0,9

Sample	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor
Honey L-1	1	1	3	3	2	4	1	1	3	4	2	4
	2	1	2	3	4	5	2	1	4	3	2	5
	3	1	2	3	2	5	3	1	2	4	2	4
	4	1	1	3	2	2	4	1	4	4	2	3
	5	1	1	4	5	5	5	1	3	4	4	5
	6	1	2	5	1	5	6	1	5	4	1	3
	7	1	1	4	2	5	7	1	1	4	2	5
	8	1	1	5	2	2	8	1	1	4	2	3
	9	1	2	5	3	2	9	1	2	4	4	3
	10	1	2	5	1	4	10	1	3	4	2	5
	11	1	2	3	3	4	11	1	4	3	3	3
	12	1	1	3	3	3	12	1	1	5	1	4
	13	1	5	1	3	4	13	1	4	1	3	4
	14	1	2	4	3	4	14	1	2	3	4	4
	15	1	2	4	3	4	15	1	3	4	2	4
	Average	1,0	1,9	3,7	2,6	3,9	Average	1,0	2,8	3,7	2,4	3,9
	SD	0,0	1,0	1,1	1,1	1,1	SD	0,0	1,3	0,9	1,0	0,8

Sample	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor
Honey R-1	1	1	2	4	5	5	1	1	3	5	5	5
	2	1	1	5	5	5	2	1	1	5	5	5
	3	1	2	5	5	4	3	1	2	4	5	4
	4	1	4	5	5	4	4	1	5	4	5	5
	5	1	4	5	5	5	5	1	1	5	5	5
	6	1	1	5	5	4	6	1	1	4	5	5
	7	1	2	5	5	4	7	1	3	4	5	5
	8	2	1	5	5	5	8	2	2	5	5	5
	9	1	2	5	5	4	9	1	2	5	3	4
	10	1	2	3	5	4	10	1	3	3	5	4

Table 9.5.6 (Continued).

Sample	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor
Honey R-1	11	1	4	5	5	5	11	1	2	5	5	4
	12	1	1	4	5	5	12	1	1	1	5	5
	13	1	3	2	5	4	13	1	2	4	5	4
	14	1	2	4	5	4	14	1	2	4	5	4
	15	1	1	4	5	4	15	1	3	4	5	5
	Average	1,1	2,1	4,4	5,0	4,4	Average	1,1	2,2	4,1	4,9	4,6
	SD	0,3	1,1	0,9	0,0	0,5	SD	0,3	1,1	1,1	0,5	0,5

Sample	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor	Panellist	Color	Aroma	Viscosity	Crystal presence	Flavor
Honey MJ	1	1	5	4	2	1	1	2	4	3	4	1
	2	2	5	4	5	1	2	4	5	3	4	1
	3	1	2	4	4	1	3	2	4	3	4	1
	4	1	3	5	3	1	4	1	3	3	4	1
	5	1	3	4	3	3	5	2	3	3	5	3
	6	1	5	5	3	1	6	1	3	4	3	1
	7	1	5	4	3	1	7	1	5	4	3	1
	8	1	3	4	4	1	8	2	3	3	4	1
	9	1	1	4	4	2	9	1	2	4	3	1
	10	1	3	2	3	1	10	1	5	2	4	1
	11	1	4	3	4	1	11	2	4	2	4	1
	12	1	3	4	5	1	12	1	1	2	5	1
	13	2	2	5	4	1	13	2	1	3	5	1
	14	1	2	4	4	2	14	1	2	3	5	2
	15	2	4	2	3	2	15	1	4	3	4	1
	Average	1,2	3,3	3,9	3,6	1,3	Average	1,6	3,3	3,0	4,1	1,2
	SD	0,4	1,3	0,9	0,8	0,6	SD	0,8	1,3	0,7	0,7	0,6

Table 9.5.7. Results of sensory analysis to preferences/acceptability to honey samples.

Panellist	Honey B-1	Honey G-1	Honey L-1	Honey R-1	Honey MJ
1	3	7	8	9	1
2	5	7	7	9	4
3	8	6	8	7	1
4	2	7	2	9	2
5	6	7	2	8	4
6	6	2	3	8	1
7	4	7	6	8	2
8	9	6	1	9	1
9	8	3	3	8	3
10	4	4	5	9	1
11	6	8	5	6	2
12	5	7	6	9	1
13	8	4	7	7	3
14	4	6	7	8	4
15	4	7	5	7	3
Average	5,5	5,9	5,0	8,1	2,2
SD	2,1	1,8	2,3	1,0	1,2

Panellist	Honey B-1	Honey G-1	Honey L-1	Honey R-1	Honey MJ
1	3	6	7	9	1
2	6	8	9	8	4
3	6	6	5	7	2
4	7	3	3	9	4
5	7	7	6	2	4
6	3	6	4	8	1
7	3	6	7	8	2
8	7	8	5	9	1
9	8	8	6	6	3
10	7	4	4	9	2
11	7	9	5	5	2
12	7	6	5	9	1
13	7	4	6	7	3
14	4	6	7	8	4
15	4	7	4	6	3
Average	5,7	6,3	5,5	7,3	2,5
SD	1,8	1,7	1,6	2,0	1,2

9.4 Annexes to Chapter 6

Table 9.6.1 Data to electronic tongue with four electrodes (Al, Pt, Au and ITO) for all honey samples analyzed.

Honey Sample Girassol 2012													
		Electrode Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	1380000	118,66	14,52	2371000	455,34	55,70	1656000	273,13	33,41	2360000	250,96	30,70
25	1,40	1225000	145,54	16,57	1842000	503,02	57,27	1341000	305,05	34,73	1976000	306,2	34,86
30	1,48	1105000	170,67	18,39	1484000	540	58,18	1108000	333,27	35,91	1680000	352,48	37,98
40	1,60	916280	220,5	21,91	1045000	594,9	59,10	812050	374,89	37,24	1273000	426,5	42,37
50	1,70	778130	259,71	24,33	788170	629,77	59,00	620200	409,12	38,33	1008000	481	45,06
60	1,78	661450	291,1	26,06	622310	660,72	59,14	501200	432,8	38,74	827080	524,4	46,94
80	1,90	511300	351,7	29,41	423660	700,3	58,57	347600	465,4	38,92	590400	587,8	49,16
100	2,00	406310	396,23	31,53	313990	728,63	57,98	261050	489,7	38,97	450400	634,7	50,51
120	2,08	333150	432	33,07	245350	749,55	57,38	207300	507,69	38,86	358200	670,6	51,33
150	2,18	258990	437,2	31,98	181190	772,6	56,51	155490	527,99	38,62	268100	710,3	51,95
200	2,30	182780	522,31	36,13	122400	798,8	55,25	107600	551,23	38,13	182300	756,3	52,31
250	2,40	138030	556,7	36,95	90860	817	54,23	81370	567,6	37,67	134500	787,8	52,29
300	2,48	109000	581,8	37,38	71330	830,14	53,34	64900	580,4	37,29	104400	809,3	52,00
400	2,60	74920	617,8	37,79	4918	850,3	52,01	45880	599,77	36,68	70220	840,5	51,41
500	2,70	55880	642,66	37,90	37160	864,9	51,00	35280	613,16	36,16	51640	862,2	50,84
600	2,78	43980	661,56	37,90	29680	876,5	50,21	28560	625,95	35,86	40230	878,3	50,32
800	2,90	30100	688,71	37,76	20900	894,63	49,05	20470	644,33	35,32	27200	902,4	49,47
1000	3,00	22420	708,9	37,61	15960	908,06	48,17	15770	658,86	34,95	20070	919,4	48,78
1200	3,08	17620	722,9	37,36	12770	919,7	47,54	12710	670,28	34,65	15630	932,5	48,20
1500	3,18	13110	739,3	37,05	9690	932,72	46,74	9720	683,75	34,26	11480	947,2	47,46
2000	3,30	8980	758,5	36,57	6750	948,54	45,73	6840	700,9	33,79	7670	964,2	46,49

Table 9.6.1 (Continued).

Honey Sample Girassol 2014													
	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	1476000	132,49	16,21	2500000	516,46	63,18	1691000	292,29	35,76	2401000	295,89	36,08
25	1,40	1312000	156,32	17,80	1924000	567,3	64,59	1350000	326,78	37,20	1962000	352,37	39,99
30	1,48	1181000	187,5	20,20	1548000	605,98	65,29	1106000	354,5	38,20	1634000	398,3	42,78
40	1,60	975400	238,27	23,67	1078000	662,9	65,86	801910	397,2	39,46	1202000	466,72	46,22
50	1,70	819020	286,18	26,81	814300	700,46	65,62	615500	426,5	39,95	920550	515,63	48,15
60	1,78	695130	323,03	28,91	642730	730	65,34	485230	450,9	40,36	739200	554,96	49,52
80	1,90	526600	381,74	31,92	438090	774	64,73	340390	486,2	40,66	504490	605,28	50,46
100	2,00	413340	429,86	34,21	325620	803,2	63,92	255400	509,78	40,57	375180	642,26	50,95
120	2,08	335230	464,2	35,53	254700	825,2	63,17	201980	527,53	40,38	292250	669,01	51,05
150	2,18	254400	506,7	37,06	188380	849,42	62,12	151300	547,2	40,02	214500	698,13	50,90
200	2,30	178400	552,5	38,21	128360	877,2	60,67	104900	570,43	39,45	143100	730,03	50,34
250	2,40	133760	584,89	38,82	95820	897,6	59,58	79550	586,9	38,95	104500	751,17	49,70
300	2,48	105800	607,83	39,05	75820	912,7	58,64	63580	599,99	38,55	80890	766,6	49,10
400	2,60	72800	642,62	39,31	52810	934,84	57,18	45060	620	37,92	54470	788,5	48,08
500	2,70	54380	665,53	39,25	40190	951,56	56,11	34690	635,8	37,49	40240	804,2	47,27
600	2,78	42920	684,7	39,23	32250	965,04	55,29	28030	647,7	37,11	31510	816,82	46,65
800	2,90	29610	712,5	39,06	22810	986,1	54,06	19980	666,05	36,51	21440	835,65	45,67
1000	3,00	22200	730,3	38,74	17410	1002,1	53,16	15310	680,79	36,12	15860	847,59	44,82
1200	3,08	17500	745,15	38,51	13920	1014,5	52,44	12280	691,18	35,73	12390	857,68	44,19
1500	3,18	13140	761,3	38,15	10540	1029,3	51,58	9360	704,94	35,32	9130	869,14	43,42
2000	3,30	9140	781,3	37,67	7350	1046,7	50,47	6590	720,48	34,74	6130	882,19	42,40

Table 9.6.1 (Continued).

Honey Sample Girassol G-1													
	Electrode	Al			Electrode Pt			Electrode			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	1427333,33	123,37	15,09	2490333,33	491,96	60,18	1709000,00	284,54	34,81	2399000,00	274,63	33,56
25	1,40	1265666,67	150,37	17,12	1928666,67	542,02	61,71	1373666,67	318,85	36,30	1985666,67	330,86	37,63
30	1,48	1142000,00	177,76	19,15	1554000,00	580,63	62,56	1133333,33	347,37	37,43	1672333,33	377,34	40,61
40	1,60	947963,33	228,03	22,65	1090666,67	637,31	63,31	825680,00	390,69	38,81	1249333,33	449,55	44,61
50	1,70	803043,33	272,75	25,55	824096,67	675,25	63,26	634460,00	422,97	39,62	974130,00	501,55	46,93
60	1,78	685180,00	306,33	27,42	651123,33	705,62	63,16	506173,33	447,13	40,02	790623,33	543,22	48,57
80	1,90	524536,67	366,85	30,68	444310,00	748,64	62,61	353396,67	482,32	40,34	554486,67	602,31	50,32
100	2,00	415273,33	413,92	32,94	329940,00	778,26	61,93	265510,00	506,75	40,33	417710,00	644,87	51,26
120	2,08	339060,00	449,91	34,44	258206,67	800,09	61,24	210250,00	524,95	40,18	328876,67	676,27	51,71
150	2,18	260896,67	480,62	35,15	190823,33	824,39	60,29	157566,67	545,22	39,88	243686,67	711,44	51,98
200	2,30	183430,00	541,00	37,42	129383,33	852,10	58,94	109010,00	568,96	39,35	164063,33	750,63	51,87
250	2,40	137873,33	575,12	38,17	96150,00	868,06	57,61	82406,67	585,55	38,86	120296,67	776,86	51,51
300	2,48	108906,67	599,36	38,51	75700,00	885,71	56,91	65750,00	598,57	38,46	93196,67	795,64	51,07
400	2,60	74796,67	635,00	38,84	37612,67	907,09	55,48	46486,67	618,34	37,82	62590,00	822,23	50,24
500	2,70	55793,33	658,79	38,85	39643,33	922,71	54,41	35700,00	633,11	37,33	46053,33	841,11	49,55
600	2,78	43950,00	678,15	38,85	31696,67	935,11	53,57	28843,33	644,90	36,95	35923,33	855,00	48,93
800	2,90	30156,67	705,64	38,68	22333,33	955,23	52,37	20603,33	663,83	36,39	24316,67	876,12	47,98
1000	3,00	22490,00	724,70	38,45	17016,67	969,62	51,44	15813,33	678,37	35,99	17950,00	890,62	47,20
1200	3,08	17713,33	738,75	38,18	13596,67	981,53	50,73	12718,33	689,29	35,63	13983,33	902,06	46,58
1500	3,18	13230,00	754,95	37,83	10296,67	995,34	49,88	9703,33	702,80	35,22	10276,67	915,01	45,81
2000	3,30	9130,00	774,50	37,34	7170,00	1011,75	48,78	6830,00	718,92	34,66	6880,00	929,93	44,79

Table 9.6.1 (Continued).

Honey Sample Laranjeira Bravura													
	Electrode	**Al**			**Electrode Pt**			**Electrode Au**			**Electrode ITO**		
Freq	**Log freq**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**
20	1,30	897790	101,35	12,40	1193000	324,35	39,68	879780	195,22	1,18	1350000	214,38	26,23
25	1,40	763530	120,4	13,71	902870	346,58	39,46	678110	212,94	1,22	1070000	244,02	27,78
30	1,48	660900	137,04	14,77	715340	363,71	39,19	543020	225,27	1,24	880270	268,92	28,98
40	1,60	516800	166,25	16,52	486220	389,21	38,67	387980	245,65	1,29	632010	303,96	30,20
50	1,70	414940	192,09	17,99	368020	407,11	38,14	283880	254,65	1,30	478000	331,99	31,10
60	1,78	335600	209,2	18,73	288670	417,05	37,33	226590	268,55	1,36	382400	350,02	31,33
80	1,90	243290	236,59	19,79	196180	435,34	36,41	155990	280,8	1,42	261260	376,04	31,45
100	2,00	186210	255,8	20,36	145610	447,92	35,65	116540	290,5	1,47	193670	394,8	31,42
120	2,08	148750	270,17	20,68	114150	457,21	35,00	92340	297,87	1,52	150090	408,69	31,28
150	2,18	111950	286,65	20,97	85050	467,49	34,19	69850	306,21	1,57	111100	423,57	30,98
200	2,30	77260	305,19	21,11	58620	479,81	33,19	49100	316,22	1,65	74660	440,48	30,47
250	2,40	57760	317,92	21,10	44300	488,6	32,43	37660	323,7	1,72	55020	452,39	30,03
300	2,48	45530	327,23	21,03	35400	496,11	31,88	30400	329,63	1,77	42890	461,37	29,64
400	2,60	31360	341,61	20,90	24960	507,2	31,02	21820	339,32	1,86	29100	474,17	29,00
500	2,70	23500	351,64	20,74	19090	515,95	30,43	16860	346,68	1,93	21540	483,83	28,53
600	2,78	18580	358,26	20,52	15330	523,11	29,97	13630	352,85	2,00	16880	491,3	28,15
800	2,90	12820	370,92	20,34	10780	533,52	29,25	9700	361,82	2,09	11410	501,7	27,51
1000	3,00	9670	377,78	20,04	8180	541,28	28,72	7440	368,65	2,17	8400	509,09	27,01
1200	3,08	7710	383,93	19,84	6520	547,2	28,28	5970	373,89	2,24	6520	514,7	26,60
1500	3,18	5860	391,02	19,59	4940	554,3	27,78	4560	380,12	2,32	4790	520,1	26,06
2000	3,30	4141	400,12	19,29	3430	562,7	27,13	3220	387,91	2,42	3210	528,4	25,48

Table 9.6.1 (Continued).

Honey Sample Laranjeira Brito

	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	**Log freq**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**
20	1,30	1136000	129	15,78	1280000	359,47	43,97	1014000	220,59	26,98	1406000	243,99	29,85
25	1,40	974700	155,69	17,73	966800	382,85	43,59	783050	240	27,32	1098000	274,85	31,29
30	1,48	845850	176,73	19,04	764630	400,46	43,15	682830	255,02	27,48	888570	298,42	32,15
40	1,60	662310	215,49	21,41	524170	425,75	42,30	442220	277,66	27,58	623460	333,21	33,10
50	1,70	533340	245,36	22,98	391460	442,69	41,47	328070	292,7	27,42	466720	356,89	33,43
60	1,78	442270	269,58	24,13	305430	456,4	40,85	260090	302,6	27,08	366300	374,8	33,55
80	1,90	317090	305,76	25,57	206250	474,71	39,70	180080	318,53	26,64	246520	399,06	33,37
100	2,00	245620	330,37	26,29	152550	487,25	38,77	134830	328,98	26,18	180450	415,39	33,06
120	2,08	195680	349,57	26,76	119620	496,57	38,01	106880	336,9	25,79	139670	426,98	32,68
150	2,18	147630	370,69	27,11	89140	506,95	37,08	80760	346,6	25,35	102100	439,52	32,15
200	2,30	102470	395,45	27,35	61650	519,3	35,92	57070	357,47	24,73	68510	454,84	31,46
250	2,40	76300	412,18	27,36	46710	528,6	35,08	43770	365,63	24,27	50530	463,8	30,78
300	2,48	60210	425	27,31	37410	535,96	34,44	35420	372,59	23,94	39520	471,36	30,28
400	2,60	41460	443,75	27,14	26490	547,5	33,49	25450	383,87	23,48	26940	482,73	29,53
500	2,70	30990	456,45	26,92	20260	556,28	32,80	19650	391,96	23,11	20040	491,2	28,97
600	2,78	24380	466,47	26,72	16260	563,61	32,29	15880	399,8	22,90	15710	497,71	28,51
800	2,90	16800	480,72	26,35	11400	573,19	31,42	11270	409,67	22,46	10650	507,32	27,81
1000	3,00	12590	490,24	26,01	8620	582,2	30,89	8600	417,78	22,16	7840	514	27,27
1200	3,08	9980	497,9	25,74	6850	588,26	30,41	6880	423,2	21,87	6100	519,2	26,84
1500	3,18	7550	506,73	25,39	5160	595,06	29,82	5230	430,15	21,55	4470	524,85	26,30
2000	3,30	5330	517,88	24,97	3570	603,7	29,11	3690	438,7	21,15	2990	531,56	25,63

Table 9.6.1 (Continued).

Honey Sample Laranjeira Brito													
	Electrode	**Al**			**Electrode Pt**			**Electrode Au**			**Electrode ITO**		
Freq	**Log freq**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**
20	1,30	1105966,67	123,62	15,12	1183666,7	333,8	122,5	852073,33	197,93	24,21	1347666,67	228,57	27,96
25	1,40	943313,33	149,27	16,99	895843,3	355,4	121,4	658443,33	214,30	24,40	1061250,00	258,11	29,39
30	1,48	815933,33	170,34	18,35	709816,7	371,7	120,1	547343,33	226,93	24,45	864086,67	281,03	30,28
40	1,60	630643,33	207,67	20,63	488580,0	395,4	117,9	372073,33	244,80	24,32	611296,67	315,42	31,34
50	1,70	509900,00	235,92	22,10	364344,0	411,8	115,7	281490,00	257,35	24,11	465950,00	339,36	31,79
60	1,78	417983,33	259,49	23,23	285836,7	424,0	113,9	221256,67	267,53	23,95	367540,00	356,88	31,94
80	1,90	300916,67	292,69	24,48	193540,0	441,4	110,7	153643,33	281,22	23,52	249990,00	382,09	31,95
100	2,00	231703,33	316,71	25,20	143586,7	453,6	108,3	115776,67	290,59	23,12	184573,33	399,19	31,77
120	2,08	184486,67	335,01	25,64	112730,0	462,1	106,1	89506,67	297,74	22,79	143726,67	411,54	31,50
150	2,18	139006,67	354,77	25,95	84213,3	472,2	103,6	70103,33	306,24	22,40	105840,00	425,07	31,09
200	2,30	96610,00	378,31	26,17	58386,7	484,0	100,4	49766,67	316,54	21,89	71133,33	440,93	30,50
250	2,40	72433,33	394,44	26,18	44310,0	493,2	98,2	38376,67	323,86	21,49	52883,33	451,33	29,96
300	2,48	57340,00	406,41	26,11	35460,0	499,9	96,4	31156,67	330,28	21,22	41446,67	459,22	29,51
400	2,60	39566,67	424,82	25,98	25123,3	511,0	93,8	22513,33	340,29	20,81	28323,33	471,83	28,86
500	2,70	29640,00	437,44	25,80	19213,3	519,3	91,9	17476,67	347,92	20,52	21096,67	480,81	28,35
600	2,78	23343,33	447,41	25,63	15406,7	526,3	90,4	14166,67	354,57	20,31	16550,00	487,82	27,95
800	2,90	16056,67	461,28	25,29	10803,3	536,1	88,2	10113,33	363,93	19,95	11216,67	498,06	27,30
1000	3,00	11993,33	470,87	24,98	8156,7	544,0	86,6	7750,00	371,19	19,69	8256,67	505,32	26,81
1200	3,08	9473,33	478,61	24,74	6463,3	549,8	85,3	6220,00	374,99	19,38	6410,00	510,79	26,40
1500	3,18	7130,00	486,98	24,40	4856,7	556,1	83,6	4746,67	382,98	19,19	4690,00	516,85	25,90
2000	3,30	4970,00	497,26	23,97	3340,0	564,4	81,6	3350,00	390,92	18,85	3120,00	523,87	25,26

Table 9.6.1 (Continued).

Honey Sample Medronho Brito													
	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	1782000	130,1	15,92	2929000	572,5	70,03	2158000	313,99	38,41	2778000	319,9	39,13
25	1,40	1615000	167,23	19,04	2268000	632	71,95	1750000	360	40,99	2297000	384,69	43,80
30	1,48	1474000	202,6	21,83	1826000	677,38	72,99	1455000	398,2	42,90	1936000	438,09	47,20
40	1,60	1250000	264,8	26,31	1282000	743,8	73,89	1059000	456,3	45,33	1441000	521,55	51,81
50	1,70	1073000	324,4	30,39	970790	789,12	73,92	820300	497,5	46,60	1126000	538,78	50,47
60	1,78	928450	372,4	33,33	770000	825	73,84	660270	530,8	47,51	913900	630,4	56,42
80	1,90	721360	455,25	38,07	530780	875,56	73,22	461700	577,7	48,31	643900	701	58,62
100	2,00	580100	519,67	41,35	395900	911,3	72,52	348000	610,4	48,57	484700	750,4	59,71
120	2,08	477500	570,7	43,69	311060	937,7	71,78	275500	634,5	48,57	381500	787,3	60,27
150	2,18	370800	631,3	46,17	231250	967,3	70,75	206700	661,9	48,41	282600	827,7	60,54
200	2,30	263500	702,09	48,56	157800	1001,8	69,29	14300	693,1	47,94	190200	873,1	60,39
250	2,40	200200	750,7	49,83	117710	1025,4	68,06	108200	715,2	47,47	139500	903,1	59,94
300	2,48	159300	787,3	50,58	92980	1042,9	67,01	86020	732,3	47,05	108300	924,8	59,42
400	2,60	110230	840,7	51,42	64550	1070,3	65,46	60600	757,8	46,35	72790	955,5	58,44
500	2,70	82710	878,7	51,82	48930	1090,2	64,29	46430	777,4	45,84	53600	977,5	57,64
600	2,78	65220	906,6	51,94	39170	1105,4	63,33	37450	792,3	45,39	41950	993,7	56,93
800	2,90	44830	946,5	51,89	27600	1130,8	61,99	26670	816,9	44,78	28470	1018,2	55,82
1000	3,00	33520	974,1	51,68	21990	1149,9	61,00	20450	835,2	44,31	21070	1036	54,96
1200	3,08	26350	997,6	51,56	167500	1163,6	60,14	16420	849,5	43,91	16420	1049,4	54,24
1500	3,18	19670	1021,2	51,17	12630	1181	59,18	12500	866,4	43,42	12070	1065,1	53,37
2000	3,30	13510	1050,3	50,64	8730	1201,4	57,92	8760	886,8	42,76	8070	1083,2	52,23

Table 9.6.1 (Continued).

Honey Sample Medronho MJ													
	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	1782333,33	90,41	11,06	5946000,00	609,14	74,52	3272000,00	279,07	34,14	3925000,00	238,90	29,22
25	1,40	1682533,33	116,78	13,30	5021666,67	750,49	85,44	2874000,00	349,86	39,83	3578666,67	321,35	36,59
30	1,48	1591666,67	143,51	15,46	4288333,33	870,82	93,83	2542666,67	415,69	44,79	3260000,00	401,15	43,22
40	1,60	1445333,33	193,94	19,27	3233666,67	1063,16	105,62	2030000,00	530,54	52,71	2717666,67	549,24	54,56
50	1,70	1326333,33	244,78	22,93	2538333,33	1204,47	112,83	1663666,67	624,46	58,50	2294000,00	678,33	63,54
60	1,78	1227666,67	294,16	26,33	2056000,00	1314,07	117,62	1386333,33	700,44	62,69	1962333,33	787,83	70,52
80	1,90	1066810,00	390,70	32,67	1451666,67	1470,50	122,98	1013933,33	818,53	68,45	1485333,33	961,41	80,40
100	2,00	939196,67	483,85	38,50	1098333,33	1579,83	125,72	782680,00	902,84	71,85	1172333,33	1092,50	86,94
120	2,08	836196,67	564,30	43,20	871253,33	1661,93	127,22	626513,33	966,31	73,97	955883,33	1194,23	91,41
150	2,18	710940,00	678,89	49,65	652906,67	1754,10	128,29	473356,67	1037,70	75,90	735276,67	1312,27	95,98
200	2,30	559530,00	837,95	57,96	447233,33	1861,77	128,77	326236,67	1119,23	77,41	515363,33	1451,63	100,40
250	2,40	453210,00	965,13	64,06	332160,00	1935,27	128,45	243573,33	1174,60	77,96	386610,00	1548,60	102,78
300	2,48	375796,67	1069,21	68,70	259973,33	1990,50	127,89	191666,67	1215,40	78,09	303656,67	1620,63	104,13
400	2,60	272980,00	1225,50	74,96	176596,67	2068,50	126,52	131556,67	1273,33	77,88	205513,33	1721,40	105,29
500	2,70	209563,33	1337,13	78,85	131020,00	2122,20	125,14	98726,67	1313,97	77,48	150926,67	1789,27	105,51
600	2,78	167510,00	1421,50	81,44	102933,33	2162,17	123,87	78426,67	1344,80	77,04	117130,00	1838,07	105,30
800	2,90	116553,33	1541,23	84,49	70823,33	2220,60	121,74	55023,33	1389,93	76,20	78446,67	1906,73	104,53
1000	3,00	87433,33	1623,10	86,11	53353,33	2262,83	120,05	42086,67	1424,43	75,57	57590,00	1953,80	103,65
1200	3,08	68990,00	1685,60	87,12	42483,33	2296,43	118,70	33966,67	1452,53	75,08	31136,67	1986,47	102,68
1500	3,18	51563,33	1755,33	87,96	32233,33	2336,10	117,06	26153,33	1486,33	74,48	32976,67	2029,63	101,71
2000	3,30	35340,00	1837,53	88,59	22596,67	2387,17	115,09	18680,00	1529,67	73,75	20223,33	2088,57	100,70

Table 9.6.1 (Continued).

Honey Sample Medronho Pb-2009													
	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	1765000	87,55	10,71	5076000	580,67	71,04	3100000	244,34	29,89	3551000	239,2	29,26
25	1,40	1659000	114,17	13,00	4192000	701,65	79,88	2742000	313,71	35,72	3192000	316,74	36,06
30	1,48	1567000	141,64	15,26	3520000	801,07	86,31	2435000	378,5	40,78	2873000	390,53	42,08
40	1,60	1406000	196,82	19,55	2598000	953	94,68	1953000	491,7	48,85	2348000	523,04	51,96
50	1,70	1273000	251,55	23,57	2015900	1060	99,30	1588000	583,9	54,70	1953000	633,33	59,33
60	1,78	1168000	305,15	27,31	1621000	1144	102,40	1323000	658,3	58,92	1650000	725,23	64,91
80	1,90	986300	403,36	33,73	1140000	1262	105,54	966480	770,57	64,44	1238000	867,2	72,53
100	2,00	847850	491,77	39,13	862600	1346	107,11	743230	850,7	67,70	971240	973,9	77,50
120	2,08	738040	570,17	43,65	684590	1409	107,86	594420	910,71	69,71	788850	1057	80,91
150	2,18	611350	670,9	49,07	513900	1482	108,39	448800	977,74	71,51	605010	1153	84,33
200	2,30	465700	804,4	55,64	352900	1566	108,32	309980	1054	72,90	422880	1266	87,57
250	2,40	369400	906,18	60,15	262610	1624	107,79	231950	1106	73,41	316520	1345	89,27
300	2,48	302200	985,92	63,35	206120	1668	107,17	182960	1144,7	73,55	248400	1404	90,21
400	2,60	216590	1103	67,47	140550	1731	105,88	126230	1200,3	73,42	167840	1486	90,89
500	2,70	165500	1187	70,00	104670	1774	104,61	95080	1239	73,06	123210	1541	90,87
600	2,78	132300	1249	71,55	82460	1807	103,52	75730	1269	72,70	95590	1582	90,63
800	2,90	92500	1340	73,46	56920	1856	101,75	53310	1315	72,09	64040	1639	89,86
1000	3,00	69940	1404	74,49	42860	1892	100,38	40780	1350	71,62	46970	1678	89,02
1200	3,08	55640	1453	75,10	34030	1920	99,24	32820	1377	71,18	36460	1708	88,28
1500	3,18	42020	1509	75,62	25640	1954	97,92	25140	1411	70,71	26720	1742	87,29
2000	3,30	29260	1576	75,99	17730	1995	96,19	17760	1452	70,01	17840	1781	85,87

Table 9.6.1 (Continued).

Honey Sample Medronho Pb-2010													
	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	1916000	89,15	10,91	5815000	576,17	70,48	3285000	264,82	32,40	3912000	242,79	29,70
25	1,40	1811000	115,87	13,19	4913000	716,28	81,55	2895000	337,89	38,47	3555000	326,78	37,20
30	1,48	1720000	143,9	15,51	4191000	825,66	88,96	2656000	406,78	43,83	3229000	408,33	44,00
40	1,60	1558000	202,22	20,09	3151000	1024	101,73	2039000	527,43	52,40	2675000	555,9	55,23
50	1,70	1428000	258,9	24,25	2475000	1163	108,95	1648000	621,65	58,24	2252000	681,9	63,88
60	1,78	1311000	317,12	28,38	2007000	1267	113,41	1369000	700,45	62,70	1917000	789,01	70,62
80	1,90	1124000	427,49	35,75	1420000	1419,9	118,75	989660	815,77	68,22	1447000	958,12	80,13
100	2,00	976390	526,8	41,92	1078800	1527	121,52	755940	896,8	71,37	1140000	1085	86,34
120	2,08	856070	618,14	47,32	858170	1608	123,09	601260	956,94	73,25	929570	1184	90,63
150	2,18	715330	736,25	53,85	645350	1700	124,34	450730	1023	74,82	714840	1299	95,01
200	2,30	549830	894,08	61,84	444170	1808	125,06	308480	1099	76,02	501000	1435	99,26
250	2,40	438740	1016	67,44	330760	1884	125,05	229660	1150	76,33	375630	1530	101,55
300	2,48	359640	1112	71,45	259550	1939	124,58	179700	1187	76,27	294860	1601	102,87
400	2,60	258570	1254	76,70	176760	2019	123,50	122700	1241	75,91	199210	1699	103,92
500	2,70	197660	1355	79,90	131270	2075	122,36	91980	1279	75,42	146120	1765	104,08
600	2,78	157570	1430	81,92	103100	2116	121,22	72760	1308	74,93	113240	1813	103,87
800	2,90	109730	1539	84,37	70740	2177	119,35	50660	1349	73,96	75710	1881	103,12
1000	3,00	82750	1613	85,57	53140	2221	117,83	38470	1381	73,27	55400	1927	102,23
1200	3,08	65540	1671	86,37	42080	2256	116,61	30790	1405	72,62	42950	1961	101,36
1500	3,18	49290	1737	87,04	31640	2297	115,11	23420	1436	71,96	31440	2001	100,27
2000	3,30	34090	1814	87,46	21860	2347	113,16	16480	1473	71,02	20960	2047,7	98,73

Table 9.6.1 (Continued).

Honey Sample Rosmaninho Brito-2010

	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	506490	98,01	11,99	433590	176,3	21,57	317680	118,6	14,51	642760	151,73	18,56
25	1,40	390770	109,266	12,44	321170	183,69	20,91	248320	123,14	14,02	490710	164,82	18,76
30	1,48	313170	117,2	12,63	250810	188,24	20,28	194800	127,6	13,75	390100	175,36	18,89
40	1,60	220300	127,38	12,65	168130	196,18	19,49	138480	132,83	13,20	260740	190	18,88
50	1,70	156130	137,75	12,90	125560	202,82	19,00	110470	138,4	12,96	195780	196	18,36
60	1,78	125890	141,81	12,69	93650	205,2	18,37	80440	140,6	12,58	155800	207,2	18,55
80	1,90	86780	150,15	12,56	64340	210,61	17,61	57290	143,99	12,04	101600	215,32	18,01
100	2,00	64010	155,6	12,38	48160	214,12	17,04	43340	147,52	11,74	74340	221,37	17,62
120	2,08	50300	159,74	12,23	38070	216,95	16,61	34920	150,16	11,49	57500	226,01	17,30
150	2,18	37370	164,38	12,02	28850	220,27	16,11	26790	153,53	11,23	42190	231,1	16,90
200	2,30	25680	169,86	11,75	20310	224,57	15,53	19250	157,82	10,92	28410	237,2	16,41
250	2,40	19140	174,97	11,61	15440	227,91	15,13	14920	161,03	10,69	20830	241,29	16,01
300	2,48	15070	176,73	11,35	12460	230,55	14,81	12020	163,72	10,52	16290	244,78	15,73
400	2,60	10380	181,24	11,09	8770	234,59	14,35	8540	167,8	10,26	10950	249,63	15,27
500	2,70	7810	184,48	10,88	6660	237,65	14,01	6540	170,68	10,06	8020	253,09	14,92
600	2,78	6200	186,84	10,70	5310	240,05	13,75	5240	173,05	9,91	6210	255,6	14,64
800	2,90	4350	190,56	10,45	3700	243,6	13,35	3690	176,59	9,68	4130	259,27	14,21
1000	3,00	3310	193,16	10,25	2780	246,2	13,06	2810	179,15	9,50	2990	261,91	13,89
1200	3,08	2650	195,29	10,09	2190	248,2	12,83	2240	181,19	9,37	2300	263,84	13,64
1500	3,18	2010	198,02	9,92	1630	250,5	12,55	1680	183,65	9,20	1650	265,92	13,33
2000	3,30	1400	201,27	9,70	1090	253,2	12,21	1150	186,55	8,99	1075	268,26	12,93

Table 9.6.1 (Continued).

Honey Sample Rosmaninho R-1													
	Electrode	Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	Log freq	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log	Cp (pF)	G (uS)	G/W log
20	1,30	565240,00	105,34	12,89	450446,67	193,91	23,72	360910,00	129,37	15,83	600510,00	161,04	19,70
25	1,40	440703,33	118,33	13,47	333833,33	201,42	22,93	275200,00	135,15	15,39	451143,33	172,67	19,66
30	1,48	357923,33	127,17	13,70	260726,67	206,64	22,26	217740,00	139,91	15,07	354410,00	181,50	19,56
40	1,60	253623,33	140,41	13,95	175536,67	214,39	21,30	151640,00	146,40	14,54	238510,00	193,56	19,23
50	1,70	186096,67	150,99	14,14	131703,33	220,39	20,65	117273,33	151,41	14,18	175863,33	200,69	18,80
60	1,78	150236,67	156,92	14,05	100616,67	223,99	20,05	91030,00	154,45	13,82	136553,33	207,39	18,56
80	1,90	103310,00	167,05	13,97	69470,00	229,41	19,19	64020,00	159,46	13,34	90803,33	215,27	18,00
100	2,00	76796,67	173,84	13,83	52263,33	233,39	18,57	48886,67	163,44	13,01	66673,33	220,58	17,55
120	2,08	60266,67	178,90	13,69	41623,33	236,58	18,11	39470,00	166,54	12,75	51883,33	224,67	17,20
150	2,18	45013,33	184,71	13,51	31696,67	240,40	17,58	30383,33	170,35	12,46	38340,00	229,29	16,77
200	2,30	30916,67	191,49	13,24	22380,00	245,23	16,96	21803,33	175,30	12,13	26020,00	234,89	16,25
250	2,40	23020,00	196,74	13,06	17043,33	248,92	16,52	16806,67	178,93	11,88	19226,67	238,87	15,85
300	2,48	18113,33	199,77	12,84	13673,33	251,91	16,19	13540,00	181,99	11,69	15046,67	242,08	15,55
400	2,60	12453,33	205,21	12,55	9576,67	256,40	15,68	9570,00	186,52	11,41	10150,00	246,66	15,09
500	2,70	9356,67	210,03	12,39	7233,33	259,62	15,31	7303,33	189,73	11,19	7453,33	249,86	14,73
600	2,78	7430,00	211,91	12,14	5750,00	262,14	15,02	5846,67	192,26	11,01	5780,00	252,26	14,45
800	2,90	5216,67	216,14	11,85	3990,00	265,95	14,58	4126,67	196,12	10,75	3860,00	255,71	14,02
1000	3,00	3970,00	219,53	11,65	2993,33	268,71	14,26	3146,67	198,97	10,56	2810,00	258,16	13,70
1200	3,08	3183,33	222,10	11,48	2356,67	270,85	14,00	2511,67	201,27	10,40	2166,67	260,02	13,44
1500	3,18	2424,00	225,33	11,29	1746,67	273,33	13,70	1893,33	203,91	10,22	1563,33	262,03	13,13
2000	3,30	1694,33	229,08	11,04	1170,00	276,17	13,32	1296,67	207,17	9,99	1019,37	273,34	13,18

Table 9.6.1 (Continued).

Honey Sample Rosmaninho Serra Mel

		Electrode Al			Electrode Pt			Electrode Au			Electrode ITO		
Freq	**Log freq**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**	**Cp (pF)**	**G (uS)**	**G/W log**
20	1,30	642400	120,32	14,72	488400	217,2	26,57	410380	147,71	18,07	613820	177,85	21,76
25	1,40	501500	134,8	15,35	361300	225,14	25,63	309200	154,46	17,59	455600	189,39	21,56
30	1,48	408110	145,1	15,63	281900	231	24,89	245680	159,68	17,20	354580	197,75	21,31
40	1,60	288400	160,7	15,96	191000	239,2	23,76	169550	166,81	16,57	236550	209,3	20,79
50	1,70	215240	171	16,02	141400	245,05	22,96	129050	172,3	16,14	170350	216,59	20,29
60	1,78	171420	179,89	16,10	109300	249,01	22,29	101060	176,19	15,77	132700	221,54	19,83
80	1,90	117980	190,7	15,95	75300	254,91	21,32	71290	182,02	15,22	88260	229,18	19,17
100	2,00	87700	198,27	15,78	56650	259,16	20,62	54440	186,35	14,83	64640	234,19	18,64
120	2,08	68860	203,9	15,61	45190	262,57	20,10	43930	189,8	14,53	50320	238,04	18,22
150	2,18	51380	210,48	15,39	34410	266,71	19,51	33780	194,06	14,19	37210	242,39	17,73
200	2,30	35300	218,06	15,08	24300	271,9	18,81	24180	199,4	13,79	25310	247,78	17,14
250	2,40	26330	223,57	14,84	18530	275,7	18,30	18590	203,3	13,49	18770	251,72	16,71
300	2,48	20760	227,24	14,60	14820	279	17,93	14990	206,79	13,29	14680	254,79	16,37
400	2,60	14340	233,68	14,29	10370	283,8	17,36	10590	211,71	12,95	9920	259,3	15,86
500	2,70	10820	237,86	14,03	7820	287,2	16,94	8100	215,04	12,68	7300	262,39	15,47
600	2,78	8650	241,2	13,82	6220	289,8	16,60	6490	217,87	12,48	5670	264,77	15,17
800	2,90	6130	246,04	13,49	4320	293,9	16,11	4610	222,1	12,18	3800	268,2	14,70
1000	3,00	4710	250,02	13,26	3240	296,8	15,75	3530	225,3	11,95	2770	270,6	14,36
1200	3,08	3810	253,3	13,09	2550	299,2	15,46	2820	227,9	11,78	2140	272,5	14,08
1500	3,18	2932	257,08	12,88	1890	301,8	15,12	2140	230,7	11,56	1550	274,5	13,76
2000	3,30	2073	261,75	12,62	1270	304,89	14,70	1470	234,47	11,30	1010	276,9	13,35

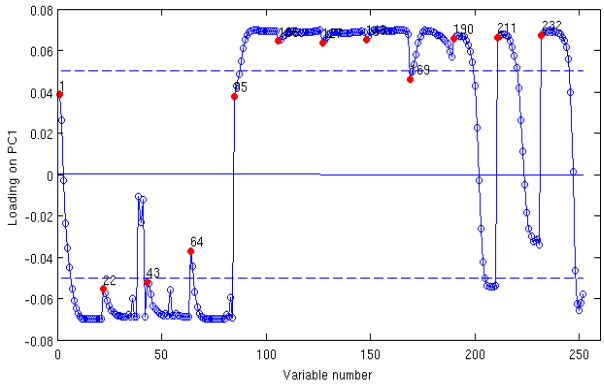

Figure 9.6.1 PC1 loadings plot for the model based on the derivative preprocessing (obtained by R. Guerra).

Table 9.6.2 Data experimental to absoption of water using Vis-NIR spectroscopy from Hale and Querry (1974) and Palmer and Williams (1974) for wavelentgth range (400-1040 nm).

Data from Hale and Querry (1973)		**Data from Palmer and Williams (1974)**	
Wavelenght (nm)	**Absorption (cm^{-1})**	**Wavelenght (nm)**	**Absorption (cm^{-1})**
400	0.0006	690	0.005
425	0.0004	702	0.007
450	0.0003	714	0.010
475	0.0002	725	0.013
500	0.0003	735	0.024
525	0.0003	746	0.028
550	0.0005	752	0.028
575	0.0008	758	0.027
600	0.0023	769	0.025
625	0.0028	781	0.023
650	0.0032	794	0.021
675	0.0042	806	0.020
700	0.0060	813	0.019
725	0.0159	820	0.020
750	0.0260	833	0.031
775	0.0240	847	0.039
800	0.0200	862	0.043
810	0.0199	877	0.051
820	0.0239	893	0.061
825	0.0280	909	0.075
830	0.0291	926	0.119
840	0.0347	935	0.158

Table 9.6.2 (Continued).

Data from Hale and Querry (1973)		Data from Palmer and Williams (1974)	
Wavelenght (nm)	**Absorption (cm^{-1})**	**Wavelenght (nm)**	**Absorption (cm^{-1})**
850	0.0430	943	0.214
860	0.0468	952	0.322
870	0.0520	962	0.471
875	0.0560	973	0.514
880	0.0560	980	0.502
890	0.0604	990	0.469
900	0.0680	1000	0.416
910	0.0729	1010	0.351
920	0.1093	1020	0.285
925	0.1440	1031	0.231
930	0.1730	1042	0.190
940	0.2674		
950	0.3900		
960	0.4200		
970	0.4500		
975	0.4500		
980	0.4300		
990	0.4100		
1000	0.3600		
1020	0.2700		
1040	0.1600		