We are IntechOpen,
the world's leading publisher of
Open Access books
Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS
BOOK CITATION INDEX
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Physicochemical Characterization of Maltese Honey

Everaldo Attard and Adrian Bugeja Douglas

Additional information is available at the end of the chapter

Abstract

The Maltese Islands are renowned for the production of genuine honey from different floral sources depending on the season and the location of the apiary. Honey samples were collected directly from local beekeepers over a period of 4 years. Each sample was coded and the details provided by the beekeepers were recorded. A total of 259 samples were collected. The distribution of the apiaries was also considered for the three honey seasons: spring, summer and autumn. All samples were tested for the parameters according to the EU Directive on Honey (2001/110/EC) and the Harmonised Methods of the International Honey Commission (2009). The samples were analysed for consistency (by appearance), moisture content and Brix (by refractometry), colour index, diastase, proline and hydroxymethylfurfural (by spectrophotometry), pH and electrical conductivity (by pH/conductivity meters), salinity (chloride meter), free acidity (by titrimetry), polyphenols (by the Folin-Ciocalteu test), sugar content (high performance liquid chromatography), antioxidant activity (by DPPH and FRAP) and antimicrobial activity. The Maltese honey can be classified into three seasons with distinctive physicochemical characteristics. Honey originating from particular season showed significantly different values for specific parameters. Typically, high sucrose content is found in spring honey and a high conductivity in autumn honey.

Keywords: Maltese honey, physicochemical, seasons, sugars, polyphenols

1. Introduction

1.1. Maltese Islands and local honey history

The production of high-quality Maltese honey has been renowned since ancient times. The Ancient Greeks and Romans used to call the island ελίτη (Melite) meaning "honey-sweet". Under the Arab rule, the name "Melite" was changed to "Malta".

1.2. Historical aspect

In ancient history, honey from Malta was considered as a delicacy and was also exported from the island. Research suggests that it was the Phoenicians who introduced the domestication of beekeeping in Malta. The Phoenicians brought the knowledge of apiaries and earthenware jars construction. In fact some Punic apiaries cut out in the rock still remain today (**Figure 1**). During the Roman rule beekeeping continued in the Maltese Islands, as observed by the presence of Roman beehives. Honey was very important to the Roman Empire and documents were discovered where people who stole honey were brought to justice. The Maltese honey was renowned for its spicy and blended taste [1].

In the Maltese Islands, there are a number of sites and places that have names originating from the apicultural industry e.g. "Wied il-Għasel" which means Valley of Honey and "Imġiebaħ" which means Apiaries. These names from the apicultural industry and the many ancient remains found around the island prove the basis of the bee population and honey production on the Maltese Islands.

The production of honey in ancient times was much less than today's production. In fact, there was only one harvest season, the wild thyme honey season, which was on the 26th of July (religious Feast of St. Anne) [1]. The traditional techniques used in bee-honey production started to change in the 1950s as the first movable frame hives and tools were introduced. The hives and tools needed were generally imported from Britain. The hives imported were copied and then produced locally. Till today, the British Standard hive is usually used in Malta. The biggest drop in the use of jars was in the early 1990s when a Varroa mite infestation resulted in the elimination of about two-thirds of the entire bee colonies on the islands [2].

1.3. The Maltese bee

The Maltese Islands have their own endemic bee type, called *Apis melliferaruttneri* [3]. This endemic sub-species of honeybee is known to have inhabited the Maltese Islands for

Figure 1. An ancient apiary in Malta, dating from the Punic era. The jars used to be placed in the wall holes. The beekeepers used to enter from the small door to add extensions to the back of the jars.

centuries. It is a sub-species of the Western honeybee or European honeybee (*Apis mellifera*), but is different from other Mediterranean bees. Up to recent times, it was the sole honeybee species in the Maltese Islands.

The Maltese bee is slightly smaller in size, dark in colour with apparently no yellow bands, and is resistant to certain diseases, but is vicious and highly active. Comparing the Maltese bee to the North African bee and the Sicilian bee, it results that it has shorter legs and wings. The wings of the Maltese bee are also much narrower. It has also a very wide abdomen. The hair on its abdomen is also very long. After centuries of local colonization, the Maltese bee has adapted well to the Maltese Islands climate and environment [3].

However, *Apis melliferaruttneri* is a very productive bee as it can work on windy days and also during very hot weather. During the hot summer of Malta, when temperatures can increase to 40°C, the bee tends to work early in the morning and in the afternoon till sunset while taking a short break during mid-day. From local beekeepers' observations, it cleans the hive very well and removes any foreign material promptly. Nevertheless the drawback is its aggressiveness.

1.4. Local honey production

The local honey is produced from different floral sources depending on the season and the location of the apiaries. In Malta, beekeepers usually harvest three times during the year; the first harvest takes place in spring, the second harvest in summer and the third harvest in autumn.

The first type of honey that is harvested in the Maltese Islands is the spring multi-flora honey. This type of honey is produced from the nectar of several types of flowers that are present in spring time. Typical plants include red clovers (*Hedysarum coronarium*), bore thistle (*Galactites tomentosa*) and starflower (*Borago officinalis*). This type of honey is collected during May and has the tendency to solidify in a few months [1].

The second type is the summer wild thyme honey. Wild thyme (*Thymus capitatus*) is a shrub that starts flowering by late May in Malta. The nectar of wild thyme produces honey which has a very delicious and spicy taste. In the summer season, honey usually starts in the last week of May and ends between the end of June and the first week of July. This honey has been very famous since ancient times and is a type of honey that is sought by both locals and foreigners.

The last season of Maltese honey production is the autumn season. This honey season usually starts from the month of August and ends in November. By the end of August the bees start collecting nectar from the flowers of Eucalyptus (*Eucalyptus melliodora*) and later in October, nectar from the carob trees (*Ceratonia siliqua*). Some beekeepers harvest the eucalyptus honey and then afterwards harvest the carob honey. Other beekeepers harvest only once in late autumn to produce a multi-floral honey made from eucalyptus flowers, carob flowers and some other flowers that the bees might find in the beginning of the season. Local tradition states that honey produced from carob is good for sore throats and for people who smoke.

The historical unique attributes that have been assigned to the Maltese honey must be due to the vast range of wild flowers within the Maltese Islands. There are about 1000 species of

mostly wild flowers identified in Malta such as wild thyme, white clover, bitumen clover, white mustard and borage [4]. The carob, citrus and stone fruit trees also help to give the honey a more special taste.

1.5. Aims of the study

This research was primarily characterised by two main aims:

(a) To determine the quality of Maltese honey. The main attributes were studied in accordance with the Food Safety Act Honey Regulations [5], the Council Directive 2001/110/EC [6] and the Harmonised Methods of the International Honey Commission [7]. Thus, local honey was valued by colour designation, sugar content, moisture content, water insolubility content, electrical conductivity, free acid, diastase activity, pollen types and HMF (hydroxymethylfurfural) content. Additional analyses included antioxidant activity (by DPPH and FRAP) and antimicrobial activity.

(b) To determine any particular characteristics for honey collected from the three honey seasons. Geographical map of the foraging areas around Malta and Gozo was one of the outputs.

2. Materials and methods

2.1. Honey samples

Honey samples were collected from Malta, Gozo and Comino between 2011 and 2014. All samples were obtained directly from the beekeepers. Each honey sample was assigned a code that was used throughout the experimental trail. Information on the physical status (colour, consistency, etc.) of the honey samples, and location data were recorded for future reference. The seasonal distribution on a yearly basis is illustrated in **Table 1**. No winter samples were collected as the foraging area usually lacks plants in flower during this season. Beekeepers were provided with their honey analysis for their information.

2.2. Physical appearance: colour and consistency

The colour of the honey samples was determined by the absorbance measurement at 560 nm using the UV-Vis spectrophotometer (WPA Lightwave II). Values exceeding 2.5 A were

	Spring	Summer	Autumn
2011	21	34	22
2012	42	34	12
2013	9	5	10
2014	34	27	9
	106	100	53

Table 1. The collection of honey samples by season and by year.

diluted and then the absorbance value was multiplied by the dilution factor to obtain the final absorbance. The viscosity of the honey sample was determined on the physical appearance of the honey.

2.3. Determination of brix and moisture content in honey

Before any measurements the refractometer was calibrated with distilled water. Approximately 0.3 ml of sample was placed on the prism. For each honey sample, three consecutive readings with independent honey were recorded for brix and moisture content.

2.4. Determination of pH and free acidity of honey

A honey sample of 10 g was accurately weighed in a 250 ml beaker on an electronic balance. The honey sample was then dissolved in 75 ml of carbon dioxide-free water (freshly produced de-ionised water) in a 250 ml beaker. The pH was recorded (Orion Star A215 Thermo Scientific). For the measurement of free acidity, the above solution was titrated with 0.1 M NaOH solution to pH 8.30 using the automatic titrator (Kern. Model: ABT-120-5DM).

2.5. Determination of electrical conductivity

A solution containing 20% of honey dry matter in 100 ml distilled water [7] was prepared for each sample. The 20% dry matter was determined from the moisture content reading. The amount of honey, equivalent to 20.0 g anhydrous honey, was dissolved in 70 ml distilled water, and made to a 100 ml volume. The conductivity was determined using a pH/conductivity meter (Orion Star A215 Thermo Scientific) in μS/cm) in triplicates.

2.6. The determination of HMF after White

The method according to White [8] was followed. Briefly, 5 g of honey were weighed accurately and dissolved in 25 ml of distilled water. 0.5 ml of Carrez solution I and 0.5 ml of Carrez solution II were added, and the solution made to a volume of 50 ml with water. Following filtration and dilution, the absorbance of the samples was read at 284 and 336 nm in 10 mm quartz cells within 1 hour. The HMF content in mg/kg was then obtained.

2.7. The determination of diastase activity

The Megazyme test kit (Megazyme Ireland, lot number 30602) was used for this determination. Briefly, 2 g of honey sample was dissolved in 40 ml of 100 mM sodium maleate buffer (pH 5.6) and topped to 50 ml. The Amylazyme tablet was added and following an incubation period of 10 min at 40°C, 10 ml of Trizma base (2% w/v) solution were added. The absorbance of the solution was read at 590 nm. The α-amylase activity of a sample (as Schade per gram of honey) was determined by use of the associated regression equation.

2.8. The determination of proline

The method outlined in Ref. [7] was followed. Briefly, 5 g of honey were made to the 100 ml volume with distilled water. 0.5 ml of the sample solution in one tube, 0.5 ml of water (blank test)

into a second tube and 0.5 ml of proline standard solution into a third tube were pipetted. 1 ml of formic acid and 1 ml of ninhydrin solution were added to each tube. After 15 min shaking, the tubes were incubated at 70°C for 10 min. 5ml of the 2-propanol-water-solution were added to each tube and the absorbance read at 510 nm after 45 min. The proline content in mg/kg honey was calculated.

2.9. Determination of sugar content

The respective standards and honey samples were prepared as 1% solution prior to analysis. Each standard and sample (2 μl) was injected in triplicates in a Dionex Thermo Fisher Ultra High Performance Liquid Chromatography with a charged aerosol detector, equipped with an amino column from Supelco (250 × 4.6 mm, 5 μm particles). Mobile phase consisted of a water/acetonitrile mixture (volume ratio 75/25), with a flow rate of 1.5 mL/min. Detection was performed at a Data Collection Rate of 20 Hz, filtered at 5 s, peak width was 0.02 mm and oven temperature set to 35°C.

2.10. Polyphenolic content

The total phenolic content (TP) was determined using a Folin Ciocalteu test [9]. 100 μl of Folin-Ciocalteu reagent and 80 μl of sodium carbonate (1 M) were added to 10 μl of each honey stock solution (triplicates) and incubated for 20 min at room temperature. The absorbance was read at 630 nm. Tannic acid was used as a standard for the test.

2.11. Total flavonoid content

The total flavonoid (TF) content was determined by a spectrophotometric method [10, 11]. Honey samples were prepared as 50% (w/v) solutions. 25 μl of honey solutions and 100 μl of 0.15% $NaNO_2$ solution (Fisher Scientific, UK) were allowed to mix for 6 min. 100 μl of 4% NaOH solution and 25 μl of distilled water were added for a final volume of 250 μl. After 15 min, the absorbance was read at 510 nm. Rutin (Sigma-Aldrich, USA) was used as a standard for the quantification of the total flavonoid content (0–500 mg/mL; r^2= 0.9962) [12].

2.12. Radical scavenging activity: the DPPH assay

The DPPH assay (2,2-diphenyl-1-picrylhydrazyl, Sigma Aldrich, USA) was carried out according to Moien et al. [10]. Honey samples were prepared as 12.5% (w/v) solutions. 200 μl of a 100 μM solution of DPPH radical in methanol were added to 20 μl of honey solution, and incubated for 30 min in the dark at room temperature. The radical inhibition was measured at 490 nm against a blank containing DPPH and methanol. Ascorbic acid (BDH, UK) was used as a standard (10–100 μg/mL). The AAE-DPPH and ascorbic acid equivalence (mg AEAC/100 g honey) (r^2= 0.9928) were calculated [12].

2.13. Reducing power: the FRAP assay

The FRAP (Ferric Reducing Antioxidant Power) activity of honey samples was determined according to Oyaizu [13]. 250 μl of honey solutions (6.25–50%) or 250 μl of distilled water

(blank) were mixed with 250 µl of 0.2 M phosphate buffer (pH 6.6) and 250 µl of 1% potassium ferricyanide. Following an incubation period of 20 min at 50°C, mixtures were immediately cooled in an ice bath for 30 s. 250 µl of 10% trichloroacetic acid were added and centrifuged at 3000 rpm for 10 min. 500 µl of the upper layer were mixed with 500 µl of distilled water and 100 µl of 0.1% ferric chloride. The absorbance was read at 700 nm against a blank [14]. The EC_{50} was used to define the specific reducing capability (mg AEAC/100 g honey) using ascorbic acid (10–100 µg/ml; r^2= 0.9981) as a positive control.

2.14. Antimicrobial activity

Maltese honey was tested against *Escherichia coli* ATCC® 25922, *Staphylococcus aureus* ATCC® 259213 and *Pseudomonas aeruginosa* ATCC® 27853. The medium of choice for broth dilution testing was cation-adjusted MHB (CAMHB). The activity was compared against artificial honey so as to show whether the activity is merely the result of the high osmotic potential of honey. This was prepared by dissolving 81 g D-fructose, 67 g D-glucose, 15 g maltose and 3 g sucrose in 34 ml filter sterilised distilled water [15]. A broth macro-dilution assay was used to analyse the honey and control samples [16]. 1 ml of each honey sample and artificial honey (0.0625–1.0 g/ml) and 1 ml of inoculum suspension (5×10^5 CFU/ml) were incubated for 20 hours at 35°C. The minimum inhibitory concentration (in g/ml) was then obtained.

2.15. Statistical analysis

Statistical significance was set at $p < 0.05$. One-way ANOVA with Bonferroni post-hoc test was conducted on all the parameters studied to compare between the seasons. These were performed with GraphPad Prism ver.5.0 for Windows (San Diego, CA, USA). The parameters were then analysed with multivariate analysis of all the honey samples. The correlation matrix was calculated, giving the correlation coefficients between each pair of variables present. To identify variability and to reduce the dimensions of the dataset, principal component analysis (PCA) was performed, using the XLSTAT Version 2011.5.01 software (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. Honey sampling

According to the population density map (**Figure 2**), the Western and Northern Districts are the least populated on the island of Malta. This reflects the highest vegetation density in these two districts when compared to the others. The Northern district shows a high sample percentage during the summer season (37%) as compared to the autumn and spring seasons (17 and 21%, respectively).

3.2. Physical appearance: colour and consistency

The results obtained for the colour and consistency of honey are illustrated in **Table 2**, and **Figure 3** and **Figure 4**, respectively. The darkest colour was observed for the autumn honey.
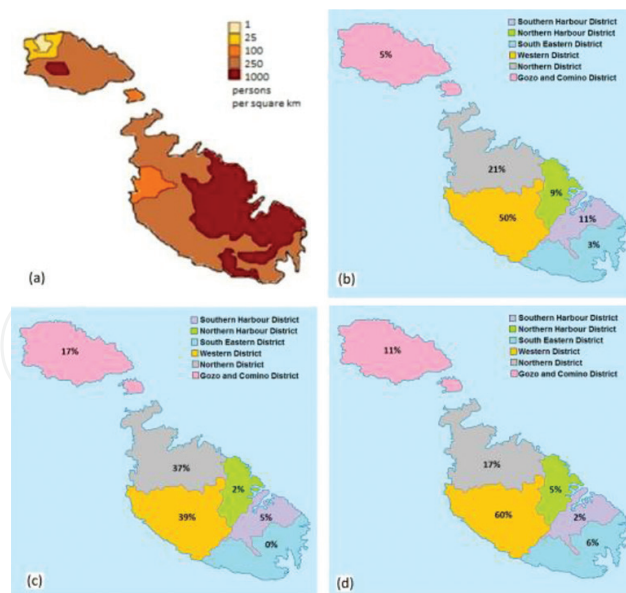
Figure 2. Spatial maps for (a) population density (adopted with permission from [17]) (b) spring, (c) summer and (d) autumn honey sample collection.

	Summer	**Autumn**	**Spring**
Colour (560 nm)	1.844 ± 0.242	3.909 ± 0.207[***]	2.143 ± 0.299
Consistency	2.563 ± 0.190	2.227 ± 0.254	1.750 ± 0.083[**]

[**]$p < 0.01$.
[***]$p < 0.001$.

Table 2. Mean absorbance values at 560 nm and mean consistency values for honey samples from the three seasons.
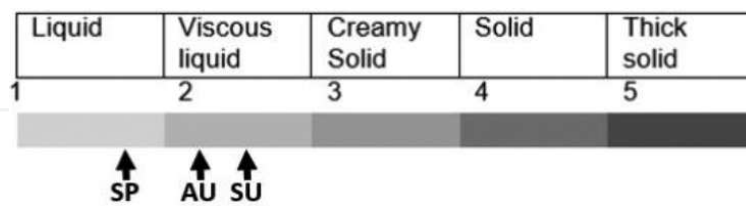

Figure 3. The colour scale for honey samples from the three honey seasons. SU = summer, SP = spring, AU = autumn.

Figure 4. The consistency scale for honey samples from the three honey seasons. SU = summer, SP = spring, AU = autumn.

The autumn honey is characterised by carob and eucalyptus sources. This honey is so distinctive, compared to other honey types ($p < 0.001$), that it is sometimes confused with carob syrup. Spring honey has a more liquid consistency than honey from the other two seasons ($p < 0.01$). As the nectar type determines seasonality, this has no direct impact on the consistency of honey and therefore some other factor might influence this parameter. This can only be determined through the investigation of other physicochemical characteristics.

3.3. Determination of brix and moisture content in honey

The brix and moisture contents of honey are illustrated in **Table 3**. Although in general there are minimal differences between the brix values for each particular season with year, seasonal statistical analysis reveals a significantly lower brix values for the autumn honey samples as compared to the other two seasons, that is, less than 79.90% (autumn) as opposed to more than 80.33% (spring and summer). The moisture content is practically opposite to the brix value, in which case autumn honey moisture content is significantly higher ($p < 0.001$) than that of the other two seasons. That is, more than 18.52% (autumn) and opposed to less than 18.46% (spring and summer). The main reason for this difference may be due to the abundance of water during the beginning of autumn, which is considered as the rainy season. Although during winter it is likely that 'winter honey' is produced, this is removed before the spring season starts, as this honey is mainly made from syrup. This is mandatory as syrup honey is considered as adulterated honey. The use of syrup during the winter months is only allowed so as to maintain the bee colony alive and healthy, considering that during winter very few plant species flower.

	2011		2012		2013		2014	
	Brix (%)	Moisture (%)	Brix (%)	Moisture (%)	Brix (%)	Moisture (%)	Brix (%)	Moisture (%)
Spring	80.88 ± 0.33	17.68 ± 0.33	81.02 ± 0.15	17.29 ± 0.16	80.33 ± 0.37	17.91 ± 0.43	80.40 ± 0.23	17.81 ± 0.22
Summer	80.26 ± 0.20	18.46 ± 0.16	80.63 ± 0.22	17.72 ± 0.24	81.20 ± 0.38	16.88 ± 0.33	80.76 ± 0.16	17.49 ± 0.16
Autumn[***]	79.20 ± 0.27	19.20 ± 0.24	79.90 ± 0.36	18.52 ± 0.42	76.69 ± 0.67	20.69 ± 0.88	79.15 ± 0.68	19.23 ± 0.71

[***]$p > 0.001$.

Table 3. Mean percentage brix and moisture values for honey samples from the three seasons between 2011 and 2014.

3.4. Determination of pH and free acidity of honey

The acidic nature of honey is important for several reasons. The most important reason is that the low pH inhibits the presence and growth of microorganisms. Other aspects of food technology, permit the honey to be blended with other food products, due to its low pH. The acidic nature also contributed to the flavour of honey particularly in monofloral honeys [18]. **Table 4** shows the mean pH and mean acidity values for honey samples from the three seasons. It was observed that there is slight yearly variation between mean pH values for the separate seasons. However, seasonal statistics reveal significant differences between the three season, the pH being the highest for the autumn honey (pH > 3.95), followed by summer honey (pH < 3.95) and finally spring (pH < 3.84). On the other hand, the total acidity was not statistically different for the three seasons, meaning that the organic

	2011		2012		2013		2014	
	pH	Acidity	pH	Acidity	pH	Acidity	pH	Acidity
Spring[**]	3.77 ± 0.03	32.55 ± 1.39	3.75 ± 0.02	34.12 ± 1.38	3.73 ± 0.08	41.13 ± 5.39	3.84 ± 0.03	33.29 ± 1.16
Summer	3.84 ± 0.03	34.74 ± 1.16	3.87 ± 0.06	45.52 ± 2.82	3.73 ± 0.11	40.04 ± 4.12	3.95 ± 0.02	29.71 ± 1.53
Autumn[***]	4.01 ± 0.04	29.48 ± 2.77	4.04 ± 0.07	43.53 ± 4.73	3.95 ± 0.03	40.85 ± 2.76	3.98 ± 0.09	30.68 ± 3.02

[**]$p < 0.01$.
[***]$p > 0.001$ for pH values.

Table 4. Mean pH and mean acidity (mM/kg) values for honey samples from the three seasons.

acid content did not seem to differ of these three seasons. pH mirrors the moisture content of the seasonal honeys. This may reflect the mobility of more free protons (H^+) with a higher moisture content.

3.5. Determination of electrical conductivity

The electrical conductivity of honey is measured at 20°C using a 20% solution of honey on dry weight basis. Conductivity is measured in mS/cm or μS/cm, reflecting the presence of ionizable substances, such as minerals [19], typically not exceeding 800 μS/cm. **Table 5** shows the mean conductivity values for honey samples from the three seasons throughout the project period. It was observed that there is slight yearly variation between mean pH values for the separate seasons. However, seasonal variations were significant. Autumn honey has the highest and a significantly different conductivity of all three seasons ($EC_{autumn} > 963.6$ μS/cm compared to the other two seasons (<752.5 and <767.1 μS/cm for summer and spring, respectively). The high salt content for autumn honeys may occur due to the arid summer conditions that result in the salting out of minerals during this period (summer). When precipitation commences in autumn, the high salt content is dissolved leading to a higher uptake in plants, and the accumulation of salt in the nectar. The salt accumulation on autumn plants following a dry period was observed in other studies under local conditions [20].

3.6. The determination of HMF after White

5-Hydroxymethylfurfural (5-HMF) is an aldehyde, which can be used as an indicator of honey quality deterioration. 5-HMF forms through the Maillard reaction, a complex series of reactions between amino acids and reducing sugars (hexoses). The International Honey Commission [7] recommends three methods for the determination of HMF. The method described by White [8] involves the measurement of UV absorbance of clarified aqueous honey solutions with and without bisulphite. An HPLC method is also described in the IHC harmonized methods [7].

The Codex Alimentarius [21] established that processed or blended honey should not contain HMF levels higher than 80 mg/kg. The European Union [6] adopted the same upper limit for honey coming from Countries or Regions with tropical temperatures. In most cases, an upper limit of 40 mg/kg is applicable in EU member states.

Table 5 shows the mean HMF values for honey samples from the three seasons throughout the project period. HMF was exceptionally higher in autumn samples as opposed to summer and

	2011	2012	2013	2014
	Mean conductivity (µS/cm) values			
Spring	669.00 ± 54.11	569.30 ± 40.19	590.80 ± 85.45	767.10 ± 24.27
Summer	739.40 ± 73.97	685.50 ± 68.13	708.00 ± 108.60	752.50 ± 26.35
Autumn***	1895.00 ± 143.60	1296.00 ± 146.70	963.60 ± 87.18	1028.00 ± 190.10
	Mean HMF (mg/kg) values			
Spring	8.57 ± 2.02	27.60 ± 6.02	12.56 ± 2.74	10.85 ± 2.40
Summer	16.91 ± 5.80	36.15 ± 6.13	18.60 ± 2.94	25.11 ± 1.76
Autumn*	27.50 ± 7.71	59.50 ± 17.79	15.80 ± 3.01	16.83 ± 1.14
	Mean diastase (Schade units) values			
Spring	4.84 ± 0.66	9.10 ± 0.79	11.35 ± 0.49	11.55 ± 1.00
Summer	5.77 ± 0.41	10.89 ± 0.94	2.98 ± 1.06	8.62 ± 0.65
Autumn**	5.27 ± 0.62	7.87 ± 1.78	6.54 ± 0.51	8.70 ± 0.33
	Mean proline (g/kg) values			
Spring	0.28 ± 0.06	0.66 ± 0.13	0.67 ± 0.02	0.57 ± 0.06
Summer	0.24 ± 0.02	0.71 ± 0.05	0.16 ± 0.04	0.57 ± 0.05
Autumn	0.32 ± 0.03	0.78 ± 0.08	0.25 ± 0.02	0.69 ± 0.09

*$p < 0.05$;
**$p < 0.01$.
***$p < 0.001$.

Table 5. The mean conductivity (µS/cm), mean HMF (mg/kg), mean diastase (Schade units) and mean proline (g/kg) values for honey samples from the three seasons.

spring honeys ($p < 0.05$). It was observed that 2012 honeys from all three seasons exhibited higher HMF content with respect to other years. It was expected that summer honey may contain more HMF. However, with a higher brix level and lower water content, the HMF production is favoured. Honey samples turn darker (browner) in colour due to the accumulation of HMF.

3.7. The determination of diastase activity

Diastase, also referred to as any α-, β- or γ-amylase, can break down carbohydrates. Hence, diastase is the enzyme that converts the long chain starch to dextrins and sugars. This enzyme is produced by the bees and introduced into honey by the bees themselves. Diastase is used an indication of adulteration as honey that is harvested from hives which are feed sucrose to produce high volumes will have a diastase content which is low.

The α-amylase (alternative names: 1,4-α-D-glucan glucanohydrolase; glycogenase) is a calcium metalloenzyme, completely unable to function in the absence of calcium. As opposed to HMF, diastase activity decreases with time. However, this is another quality parameter where the degradation of honey enzymes indicates a decline in the functionality of the honey as a food supplement and also as a medicine.

Table 5 shows the mean diastase values for honey samples from the three seasons throughout the project period. Diastase was exceptionally lower in autumn samples (<8.70 Schade units) as opposed to spring honeys (>9.10 Schade units, $p < 0.01$). However, for 2011, the spring diastase level was low compared to the other years. The summer samples showed a varied diastase level, with the lowest values obtained during 2013 (2.98 Schade units) and highest values obtained during 2012 (10.89 Schade units).

Possible heating of honey to skim waxes should be avoided. Unfortunately this is a common local practice amongst beekeepers as the Maltese consumer prefers liquefied honey. It seems that enzymatic activity is more sensitive to heat than HMF and perhaps diastase activity may be considered as a more significant indicator of quality than HMF. However, diastase degradation seems to have less implications on human health than HMF accumulation.

3.8. The determination of proline

Honey is very low in protein. As a matter of fact it contains less than 1%. The protein portion is mainly made up of several amino acids. A chemical marker that represents proteins, i.e. amino acids, is proline. Proline is not an amino acid as there is no free amine group in its structure. Although proline content is not considered as one of the main indicators of honey quality, legal issues can be resolved by taking into consideration this parameter. A honey that contains less than 180 mg of proline per kilo of honey is an altered honey [7].

Table 5 shows that all mean proline values for honey from the three seasons (>240 mg/kg) were well above the 180 mg of proline per kilo of honey standard, except for the summer 2013 honeys (160 mg/kg). The years 2012 and 2014, showed a very high content of proline throughout the three seasons. Compared to the other two seasons, autumn 2012 and 2014 showed exceptionally high proline content. Combining all the 4 years for the three seasons, the highest proline content was observed for spring. However, the difference in proline contents was not significant with the other two seasons.

3.9. Determination of sugar content

Honey is made up of a matrix of sugars. Although the brix content provides a good indication of the content of sugars, the individual sugars are not identified by this method. Honey sugars are formed by the action of several honey bee enzymes on the floral nectar. The result is a complex mixture composed of 70% of monosaccharides and 10–15% disaccharides. Honey is also used as a sweetener in hundreds of products manufactured [22]. The oligosaccharide content of honey contributes to its prebiotic properties, promoting the growth of Bifidobacteria and Lactobacilli [23]. Many scientists attempted to characterize the sugars in many honey types [24–26]. Different techniques such as HPLC [27, 28] or GC-MS [29] were used. These methods have been standardized by the "International Honey Commission" [7]. The HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection) is one of the most used techniques for the analysis oligosaccharides [30].

In some studies [31, 32], the disaccharide content was used to characterize the type and origin of the honey. Moreover, maltose, turanose and nigerose were useful for differentiating Brazilian honey in several other geographic areas [33]. The sugar profiles are also used to

	Fru	Glu	Fru+Glu	Suc	Mal	Mel
Spring	44.07 ± 0.27	40.56 ± 0.29	84.62***	11.69 ± 0.50	3.58 ± 0.19	0.00 ± 0.00
Summer	47.19 ± 1.06	39.89 ± 0.66	87.08**	8.45 ± 0.73	4.13 ± 0.11	0.00 ± 0.00
Autumn	48.64 ± 0.82	41.90 ± 0.71	90.54	5.41 ± 0.51***	3.66 ± 0.27	0.38 ± 0.38

Fru, fructose; Glu, glucose; Suc, sucrose; Mal, maltose; Mel, melezitose
**$p < 0.01$.
***$p < 0.001$.

Table 6. Mean percentage sugar values for honey samples from the three seasons throughout the project period.

differentiate honeydew honey from flowers. Indeed, the honeydew presents lower concentrations of glucose and fructose and higher oligosaccharides including melezitose or erlose [34]. It is worth noting that the concentrations of fructose and glucose are used to classify floral honey [7]. Two disaccharides of importance are sucrose and maltose. According to Council Directive [6], the content should not exceed 5% of the total sugars.

Table 6 provides the mean percentage sugar values for honey samples from the three seasons throughout the 4-year period. **Figure 5** shows a typical chromatogram for the Maltese honey samples. The most abundant sugar is fructose followed by glucose. The disaccharides, sucrose and maltose, are found in lower concentrations. Maltose is more consistent than sucrose. Melezitose is only present in autumn honey. The content of glucose and fructose for the three seasons, as shown in **Table 6**, is well above the content stated in Ref. [5], which is a minimum of 60 g/100 g. For the three honey types the amount exceeded 84 g/100 g.

Fructose is significantly high in autumn honey (48.64%) and least in spring honey (44.07%). Glucose is relatively similar in all seasonal honey types ranging from 39.89% for summer honey to 41.90% for autumn honey. Sucrose varies significantly between the three seasons; in spring honey being the highest (11.69%) and in autumn honey being the lowest (5.41%). This goes in accordance with the brix results, which partially concluded the possible feeding of a sucrose syrup to bees during winter, when flora is scarce, and the possible incorporation of this 'syrup honey' within the spring honey. Therefore, beekeepers should be advised that the honey produced from the artificial syrup should be discarded prior to the commencement of honey production in spring. Melezitose (**Table 6**) was only present in autumn honey. However, not all autumn honey samples contained this saccharide. This sugar is typical of honeydew honey. Honeydew is a sugar-rich sticky liquid,
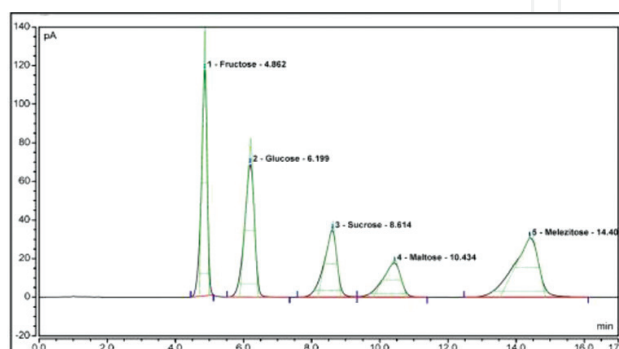

Figure 5. A typical chromatogram for honey samples.

produced mainly by aphids and some scale insects as they feed on plant sap. According to Mifsud et al. [35], aphids are present on carob and citrus during autumn, particularly *Aphis* (Aphis) *gossypii* on carob, *Aphis* (Aphis) *craccivora* and *Toxopteraaurantii* on citrus. This may explain the presence of honeydew honey within the floral honey during autumn. Honeydew honey may be produced during summer and autumn [36]. However, due to limitations in tree numbers, this cannot be produced on a large scale in Malta. Therefore, a possible indicator of pure autumn honey may be melezitose, although this needs to be further investigated.

3.10. Polyphenolic content

The mean total polyphenolic content in mg TAE/100 g honey for spring (56.943 ± 7.027) was significantly lower ($p < 0.05$) than for the other two seasons (AU: 79.692 ± 8.000 and SU: 69.598 ± 3.208 mg TAE/100 g honey) (**Table 7**). It was observed that the darker the honey colour, the higher was the total polyphenolic content. This was the case with autumn honey samples [12].

3.11. Total flavonoid content

In spite of the higher flavonoid content for some autumn honey samples (212.86 and 197.57 mg RE/100 g), there was no statistical difference in content for honey samples from the three seasons (**Table 7**). The mean flavonoid content for the three seasons was 31.154 ± 17.729, 37.651 ± 8.460, and 31.420 ± 11.373 mg RE/100 g honey, respectively. As compared to similar studies, the Maltese honey samples contained superior flavonoid content to that observed in other similar studies with quantities ranging between 1.35 and 9.78 mg RE/100 g honey [37, 38].

3.12. Radical scavenging activity: the DPPH assay

The mean DPPH inhibition in mg AEAC/100 g honey for autumn, spring and summer was 9.300 ± 1.292, 5.805 ± 0.610 and 5.238 ± 0.657, respectively (**Table 7**). The autumn honey samples had a superior DPPH inhibitory activity with respect to the other two seasons ($p < 0.01$). This may be due to the presence of carob nectar in autumn honey which contains high amounts of polyphenols and tannins, as noted in Ref. [39].

	TPC	TF	DPPH	Red Pow
Spring	$56.943 \pm 7.027^*$	37.651 ± 8.460	5.805 ± 0.610	16.600 ± 1.979
Summer	69.598 ± 3.208	31.420 ± 11.373	5.238 ± 0.657	14.250 ± 0.035
Autumn	79.692 ± 8.000	31.154 ± 17.729	$9.300 \pm 1.292^{**}$	12.67 ± 1.093

*p < 0.05.
**p < 0.01.

Table 7. Total polyphenolic (mg TAE/100 g honey), total flavonoid (mg RE/100 g honey), DPPH (AAE-DPPH mg AEAC/100 g honey) and reducing power (mg AEAC/100 g honey) values for honey samples from the three seasons.

3.13. Reducing power

The Maltese honey samples had antiradical activity values between 3.33 and 15.62 mg AEAC/100 g honey (**Table 7**). The mean reducing power in mg AEAC/100 g honey for the

autumn, spring and summer seasons were 12.67 ± 1.093, 16.600 ± 1.979 and 14.250 ± 0.035 mg AEAC/100 g, respectively. The reducing power values were similar to those obtained by Savatović et al. [38], i.e. 1.43–7.82 mg AEAC/100 g honey but lower than those obtained by Meda et al. [40], i.e. 10.20–37.87 mg AEAC/100 g honey. In the study by Savatović et al. [38], it was pointed out that monofloral honeys provide a higher reducing power than multifloral honeys. This was also observed for the Maltese honey with the monofloral autumn and summer samples showing higher activity (7.54 and 6.96 mg AEAC/100 g honey) than the multifloral spring samples (5.98 mg AEAC/100 g honey).

3.14. Antimicrobial activity

Maltese honey exhibited MIC values ranging between 0.067 and 0.205 g/ml (**Table 8**). In spite of the statistical insignificance, the spring honey samples showed the best MIC values compared to the other two seasons. The honey samples were compared against artificial honey as highlighted earlier. Only spring samples against *S. aureus* and *P. aeruginosa* showed a significantly lower MIC than the artificial honey ($p < 0.001$ and $p < 0.05$, respectively) [41]. *S. aureus* strains are known to be involved in acquired and nosocomial infections, while *P. aeruginosa* may cause diabetic ulcers, wound infections and urinary tract infections [42]. Therefore, Maltese honey may be potentially useful for the topical treatment of microbial infections particularly associated with wounds and ulcers.

	Spring	Summer	Autumn	Artificial honey
E. coli	0.165 ± 0.0255	0.172 ± 0.0392	0.203 ± 0.0468	0.250 ± 0.0000
S. aureus	0.067 ± 0.0162[***]	0.125 ± 0.0255	0.157 ± 0.0182	0.250 ± 0.0000
P. aeruginosa	0.110 ± 0.0155[*]	0.157 ± 0.0403	0.172 ± 0.0202	0.250 ± 0.0000

[*]$p < 0.05$.
[***]$p < 0.001$.

Table 8. Total minimum inhibitory concentrations (g/ml) for honey samples from the three seasons.

3.15. PCA analysis of physicochemical parameters and sugar content

It was observed from the scree plot that the first three components accounted for 50.31% of the total variance. However, the parameters studied fall within different components. The scores plot (**Figure 6**) shows the physicochemical parameters of honey samples in the space of the two new variables, F1 and F2. The parameters plot shows that brix and moisture are inversely related, while acidity, diastase and proline are particularly inversely related to HMF and pH.

Moving along F1, it was observed that the honey samples were distributed by those with low moisture and brix contents on the left and those with the highest moisture and brix contents on the right. From left to right, the samples moved from summer to spring to autumn.

To determine any possible clustering for the seasonal honey, the sugar content was used subjected to principal component analysis. It was observed from the scree plot that the first two components accounted for 71.843% of the total variance. The parameters studied fell within the first two components. The scores plot (**Figure 7**) shows the sugar content of honey samples in the space of the two new variables, F1 and F2. The parameters were grouped as factor 1 for the most common sugars in honey (fructose, glucose and sucrose). This analysis shows that fructose and glucose are inversely related to sucrose.

Moving along F1, it was observed that the honey samples were distributed by those with a high fructose and glucose and low sucrose on the left and those with the lower fructose and
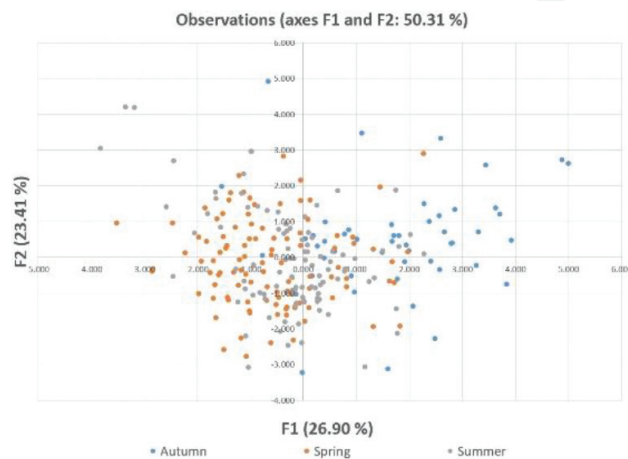
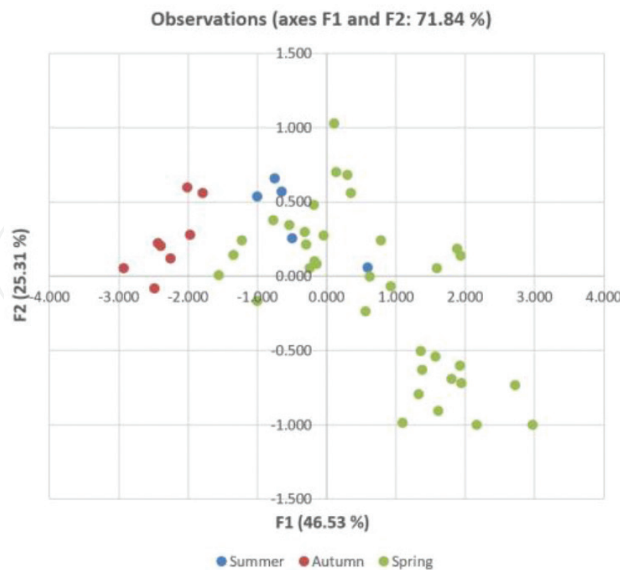**Figure 6.** Score plot of seasonal honey analysed by PCA (physicochemical).

Figure 7. Score plot of seasonal honey analysed by PCA (sugars).

glucose and higher sucrose on the right. From left to right, the samples moved from autumn to summer to spring. Autumn honey samples were particularly distinctive from the other two seasonal honey types. This may be due to the fact that autumn honey samples contained melezitose as opposed to the other two types.

3.16. Concluding remarks on Maltese honey

The physicochemical characterisation of Maltese honey was conducted over a period of 4 years, in order to determine any typical similarities and differences that may be attributed to the different seasonal characteristics. The main characteristics are meteorological conditions that typify the season and the seasonal floral diversity. Data is not shown for the latter parameter, as the floral distribution is beyond the scope of this present study. The typical characteristics of seasonal Maltese honey are as follows.

Spring honey is typically reddish yellow in colour with a liquid consistency. It may be classified as a multifloral honey, featuring nectar and pollens from a vast number of plant that flower during spring. Summer honey is usually yellowish coloured with a viscous consistency. This typically features thyme due to the translocation of hives to areas (North of Malta) rich in thyme during early summer. Autumn honey is dark (reddish-brown) in colour and may contain honeydew due to the tree-related nectars. Therefore, it may contain Melezitose as a minor sugar. It usually has a higher conductivity in relation to the other seasonal honeys, but HMF tends to be high too. This typically contains carob and eucalyptus nectar and pollens. **Table 9** illustrates the typical physicochemical parameters for the three seasonal honey types. In conclusion, **Figure 8** shows a radial plot for the three seasonal honey-types.

Test	Range*	Spring honey	Summer honey	Autumn honey
Moisture (%)	<20	17.29-17.91	16.88-18.46	18.52-20.69
Brix (%)	≈80	80.33-81.02	80.26-81.20	76.69-79.90
Conductivity (μS/cm)	<800	569-767	685-752	963-1895
pH	3-5.5	3.7-3.8	3.7-3.9	3.9-4.0
Acidity (mM/kg)	8.7-46.8	32.5-41.1	29.7-45.5	29.5-43.5
HMF (mg/kg)	<40	9-28	17-36	16-60
Proline (mg/kg)	>180	280-670	160-710	250-780
Diastase activity (Schade units)	>8	5-12	3-11	7-9
Fructose and glucose (%)	>60	≈85	≈87	≈91
Sucrose (%)	<5	≈12	≈8	≈5

*According to Ref. [6]

Table 9. Typical ranges for physicochemical parameter for spring honey.
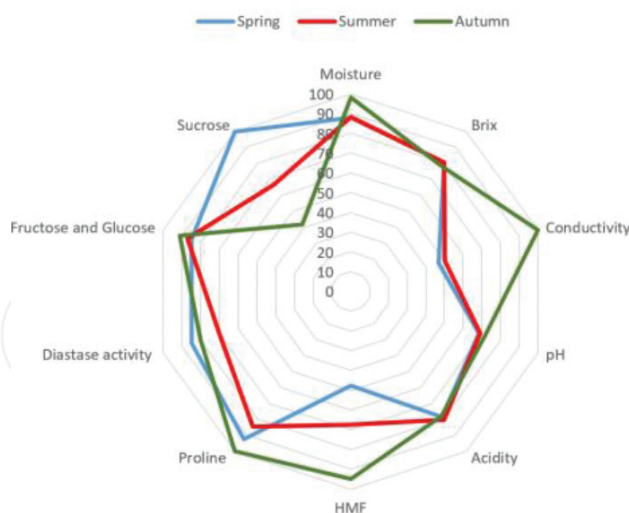
Figure 8. A radial plot for seasonal honey.

Acknowledgements

Author details

Everaldo Attard* and Adrian Bugeja Douglas

*Address all correspondence to: everaldo.attard@um.edu.mt

Institute of Earth Systems, University of Malta, Msida, Malta

References

[1] Bugeja-Douglas A. Maltese Islands and local honey history. Aricilik Arastirma Dergisi (Beekeeping Research Journal). 2011; 3: 36–38.

[2] Duca E. Bees Dream of gold. Think. 2014; 8: 20–26.

[3] Sheppard WS, Arias MC, Grech A, Meixner MD. Apis mellifera ruttneri, a new honey bee subspecies from Malta. Apidologie. 1997; 28: 287–294. hal-00891462

[4] Attard E. Progress report on medicinal and aromatic plants in Malta, 2002–2004. In: Report of a Working Group on Medicinal and Aromatic Plants, Second Meeting; 16–18 December 2004, Strumica, Macedonia FYR; 2009. pp. 154–157.

[5] Food Safety Act (CAP. 449). Honey regulations L.N. 213 of 2004. The Malta Government Gazette. 2004; 17574: 03733–03746.

[6] Council Directive. Council directive of 20 December relating to honey 2001/110/EC. Official Journal of the European Communities. 2001; 10: 47–52.

[7] Bogdanov S, Martin P, Lullmann C. Harmonised Methods of the International Honey Commission. Swiss Bee Research Centre, FAM, Liebefeld. 2009; pp. 1–63.

[8] White JW Jr. Spectrophotometric method for hydroxymethylfurfural in honey. J Assoc Off Anal Chem. 1979; 3: 509–514.

[9] Attard E. A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. Cent Eur J Biol. 2013; 8: 48–53.

[10] Moien S, Farzami B, Khaghani S, Moein MR, Larijani B. Antioxidant properties and protective effect on cell cytotoxicity of Salvia mirzayani. Pharm Biol. 2007; 45: 1–6.

[11] Moein S, Moein MR. Relationship between antioxidant properties and phenolics in Zhumeria majdae. J Med Plants Res. 2010; 4: 517–521.

[12] Meinen N, Camilleri L, Attard E. Antioxidant properties of Maltese Honey. J Apic Res. 2014; 58: 65–74. DOI: 10.2478/jas-2014-0004

[13] Oyaizu M. Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr. 1986; 44: 307–315.

[14] Mohamed TK, Issoufou A, Zhou H Antioxidant activity of fractionated foxtail millet protein hydrolysate. Int Food Res J. 2012; 19: 207–213.

[15] Cooper RA, Molan PC, Harding KG. The sensitivity to honey of Gram-positive cocci of clinical significance. J Appl Microbiol. 2002; 93: 857–863.

[16] CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.

[17] Formosa S, Magri V, Neuschmid J, Schrenk M Sharing integrated spatial and thematic data: the CRISOLA case for Malta and the European project Plan4all process. Future Internet. 2011; 3: 344–361.

[18] National Honey Board. Food Technology/Product Research Program. pH and Acids in Honey. [Internet]. 2006. Available from http://www.honey.com/images/downloads/ph-acidsinhoney.tif [Accessed: 2016-09-04]

[19] Lobreau-Callen D, Marie-Claude C. Les miels, Techniques de l Ingénieur (Honeys, Practical Techniques). Traité Agroalimentaire (Agri-Food Law), 2001. p. 20

[20] Attard E, Zammit ML, Vella E. The exogenous and endogenous parameters affecting the Cypress tree: the Maltese case. In: CYPFIRE Project–P.O.MED 2G-MED09-070 VII MEETING; 9–11 April 2013; Corte, Corse.

[21] Codex Alimentarius. Draft revised standard for honey at step 8 of the Codex procedure. Alinorm 01/25. 2000.

[22] Cavia MM, Fernandez-Muino MA, Gomez-Alonso E, Montes-Pérez MJ, Huidobro JF, Sancho MT. Evolution of fructose and glucose in honey over one year: Influence of induced granulation. Food Chem. 2002; 78: 157–161.

[23] Sanz ML, Polemis N, Morales V, Corzo N, Drakoularakou A, Gibson GR. In vitro investigation into the potential prebiotic activity of honey oligosaccharides. J Agric Food Chem. 2005; 53: 2914–2921.

[24] Esti M, Panfili G, Marconi E, Trivisonno MC. Valorization of the honeys from the Molise region through physico-chemical, organoleptic and nutritional assessment. Food Chem. 1997:58: 125–128.

[25] Goodall I, Dennis MJ, Parker I, Sharman M. Contribution of high performance liquid chromatographic analysis of carbohydrates to authenticity testing honey. J Chromatogr A. 1995; 706: 353–359.

[26] Sanz ML, Sanz J, Martinez-Castro I. Gas chromatographic–mass spectrometric method for the qualitative and quantitative determination of disaccharides and trisaccharides in honey. J Chromatogr A. 2004; 1059: 143–1483.

[27] Fantoni E, Ball S, Lloyd L, Mapp K. Honey compositional analysis by HPLC. Application Note. Food Testing and Agriculture. Agilent Technologies. 2012; SI-01920:1–3.

[28] Ouchemoukh S, Schweitzer P, Bey MB, Djoudad-Kadji H, Louaileche H. HPLC sugar profiles of Algerian honeys. Food Chem. 2010; 121: 561–568.

[29] Lazaridou A, Biliaderis CG, Bacandritsos N, Sabatini AG. Composition thermal and rheological behaviour of selected Greek honeys. J Food Eng. 2004; 64: 9–21.

[30] Morales V, Corzoa N, Sanz ML. HPAEC-PAD oligosaccharide analysis to detect adulterations of honey with sugar syrups. Food Chem. 2008; 107: 922–928.

[31] Mateo R, Bosch-Reig F. Sugar profiles of Spanish unifloral honeys. Food Chem. 1997; 60: 33–41.

[32] Mateo R, Bosch-Reig F. Classification of Spanish unifloral honeys by discriminant analysis of electrical conductivity, color, water content, sugars, and pH. J Agric Food Chem. 1998; 46: 393–400.

[33] Da Costa Leite JM, Trugo LC, Costa LSM, Quinteiro LMC, Barth OM, Dutra VML. Determination of oligosaccharides in Brazilian honeys of different botanical origin. Food Chem. 2000; 70: 93–98.

[34] Weston RJ, Brocklebank LK. The oligosaccharide composition of some New Zealand honeys. Food Chem. 1999; 64: 33–37.

[35] Mifsud D, Mangion M, Azzopardi E, Espadaler X, Cuesta Segura D, Watson GW, Perez Hidalgo N. Aphids associated with shrubs, herbaceous plants and crops in the Maltese Archipelago (Hemiptera, Aphidoidea). Bull Entomol Soc Malta. 2011; 4: 5–53.

[36] Stanway P. The Miracle of Lemons: Practical Tips for Health, Home and Beauty. Watkins Media Limited; London; 2012.

[37] Kaškoniene V, Maruška A, Kornyšova O, Charczun N, Ligor M, Buszewski B. Quantitative and qualitative determination of phenolic compounds in honey. Chem Tech. 2009; 52: 74–80.

[38] Savatovic SM, Dimitrijevic DJ, Dilas SM, Canadanovic-Brunet JM, Cetkovic GS, Tumbas VT, Štajner DI. Antioxidant activity of three different Serbian floral honeys. Acta Periodica Technol. 2011; 4242: 145–155.

[39] Avallone, R, Plessi M, Baraldi M, Monzani A. Determination of chemical composition of carob (Ceratonia siliqua): protein, fat, carbohydrates, and tannins. J Food Comp Anal. 1997; 10: 166–172.

[40] Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem. 2005; 91: 571–577.

[41] Muscat C, Bugeja-Douglas A, Sauret C, Attard E. Physicochemical, polyphenol, anti-oxidant and antibacterial properties of Maltese honey. In: The International Symposium on Bee Products, 3rd edition, Annual meeting of the International Honey Commission (IHC); 28th September to 3rd October 2014; Opatija Croatia; 2014:BP.13.

[42] Miorin PL, Levy Junior NC, Custodio AR, Bretz WA, Marcucci MC. Antibacterial activity of honey and propolis from Apis mellifera and Tetragonisca angustula against Staphylococcus aureus. J Appl Microbial. 2003; 95: 913–920.