Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal

Susana Gomes, Luis G. Dias, Leandro L. Moreira, Paula Rodrigues, Leticia Estevinho *

CIMO – Escola Superior Agrária, Instituto Politécnico de Braganca, Campus Santa Apolónia, Apartado 1172, 5301-855 Braganca, Portugal

ABSTRACT

The present study aimed to characterize five commercial honeys available in the Portuguese market in respect to their floral origins, physicochemical parameters and microbial safety and commercial quality assessment. Pollen profile, colour, moisture content, ash, acidity, electrical conductivity, pH, reducing sugars, apparent sucrose and HMF were the parameters analysed in each honey sample. Aerobic mesophiles, moulds and yeasts, fecal coliforms and sulphite-reducing clostridia were the microbial contaminants of interest studied. The antimicrobial effect against four fermentative yeasts was determined.

Concerning the physicochemical parameters, all honey samples were found to meet European Legislation (EC Directive 2001/110) for all parameters, except for HMF and apparent sucrose. Microbiologically, the commercial quality was considered good and all samples showed to be negative in respect to safety parameters. We also verified that the presence of honey differentially affected the growth of fermentative yeasts under study, depending on the type of yeast, but this growth was not significantly influenced by the type of honey used.

1. Introduction

Honey is a sweet and flavorful product which has been consumed as a high nutritive value food. It is essentially composed of a complex mixture of carbohydrates (of which fructose and glucose account for nearly 85–95%) and other minor substances, such as organic acids, amino acids, proteins, minerals, vitamins, and lipids (White, 1975).

The quality of honey is mainly determined by its sensorial, chemical, physical and microbiological characteristics. Honey physicochemical quality criteria are well specified by the EC Directive 2001/110 (EU, 2001). The major criteria of interest are moisture content, electrical conductivity, ash content, reducing and non-reducing sugars, free acidity, diastase activity and hydroxymethylfurfural (HMF) content.

On the other hand, EU legislation lacks specifications concerning microbial contamination and hygiene of the product. In fact, numerous studies have been reported on the physicochemical parameters of honeys from all over the world (Al-Khalifa and Al-Arify, 1999; Andrade et al., 1999; Azeredo et al., 2003; Terrab et al., 2002; Downey et al., 2005; Finola et al., 2007; Küçük et al., 2007; Al et al., 2009), but microbial contamination studies are rare and are essentially devoted to *Clostridium botulinum* (Snowdon and Cliver, 1995; Iurlina and Fritz, 2005; Nevas et al., 2002, 2005; Finola et al., 2007). Honey has several sources of microbial contamination. Primary sources include pollen, the digestive tracts of honey bees, dust, air, soil and nectar, and are somewhat difficult to eliminate. On the other hand, secondary sources, due to honey handlers and processing, are easier to control by the application of good manufacturing practices (Snowdon and Cliver, 1995). The major microbial contaminants include moulds and yeasts, as well the spores of *Bacillus* spp. and *Clostridium* spp. (Snowdon and Cliver, 1995), being their counts indicative of honeys' commercial quality and safety.

The antimicrobial activity of honeys has also been subject to extensive analysis. The interest is based mainly on the activity against pathogens and its use as a natural medicine (Al-Mamary et al., 2002; Küçük et al., 2007). To our knowledge, there are no studies on the antimicrobial activity of the various honeys towards fermentative yeasts, which can pose a commercial problem when high moisture content (>20%) is present.

The present study aimed to characterize five commercial honeys available in the Portuguese market in respect to floral nectar origin, physicochemical parameters, microbial safety and commercial quality evaluation. The pollens profile and the physicochemical parameters of each honey sample were obtained to differentiate them. Aerobic mesophiles, moulds and yeasts, fecal coliforms, sulphite-reducing clostridia and *Salmonella* were the microbial contaminants of interest. Furthermore, the antimicrobial effect against fermentative yeasts was also studied.

2. Materials and methods

2.1. Honey samples

Five commercial honeys of different floral sources (Table 1) and geographical origins where purchased from local market and left at room temperature until further analysis.

2.2. Pollen analysis

The samples were subjected to qualitative pollen analysis as *per* Erdtman's acetolysis method (Erdtman, 1986). The aim of that analysis was to confirm that the analyzed samples could be declared as heather monofloral honey. Briefly, pollen analyses are based on the extraction of pollen grains from 10 g of crude honey. The sample was dissolved in distilled water and the sediment was concentrated by repeated centrifuging 30' at 1500 rpm. About 10 mL of acetolysis mixture (9:1, $(C_2H_3O)_2O$, H_2SO_4) was added and the tubes were incubated in a water bath (100 °C for 3 min), stirred vigorously, then centrifuged and decanted. About 12 mL of water-free acetic acid was added, stirred thoroughly, centrifuged, and decanted. The precipitate was washed in about 12 mL of distilled water, centrifuged, and decanted. About 12 mL of 7% KOH was added, stirred thoroughly, centrifuged, and decanted. Finally, the pollen grains were stained with a solution of basic fuchsin and mixed with glycerin. The examination of the pollen slides were carried out with an optical microscope at 400× and 1000× in order to identify the pollen types. A minimum of 1000 pollen grains was counted per sample. To recognize the pollen types, the reference collection of the Escola Superior Agrária – Instituto Politécnico de Bragança and different pollen morphology guides were used. The following terms were used for pollen frequency classes: predominant pollen (P, more than 45% of pollen grains counted), secondary pollen (S, 16–45%) and important minor pollen (I, 3–15%). Additionally, the honey colours were classified according to the Pfund scale.

2.3. Physicochemical analyses

All physicochemical tests were performed in duplicate.

2.3.1. Moisture content

The determination of moisture (AOAC, 1990; Official Method 969.38) was ascertained by refractometry, using an Abbe refractometer (Digital refractometer Atago, Germany). All measurements were performed at 20 °C, after waiting for 6 min for equilibrium, and obtaining the corresponding% moisture (g/100 g honey) from the refractive index of the honey sample by consulting a standard table for the purpose.

2.3.2. Electrical conductivity

Electrical conductivity was determined by conductimetric assay (WTW Inolab conductivimeter), from a solution containing 10 g of honey in 75 mL of distilled water (Sancho et al., 1992).

2.3.3. Ash content

Total ash was estimated by conductimetry using the equation:

$$\text{Ash content}(\%) = 0.083 \times \text{conductivity} - 0.092 \ (\text{Sancho et al., 1992}).$$

Table 1
The most predominant pollen and colours in the five honeys analyzed.

Sample/Colour	Frequency class[a]	Pollen identification (frequency)
Honey 1	P	*Eucaliptus* sp. (70.7%)
Amber	S	*Pinus* sp. (18.8%)
	I	*Rubus* sp. (10.5%)
Honey 2	P	*Echium* sp. (69.4%)
Light amber	S	*Leotondon* sp. (15.4%)
	I	*Eucaliptus* sp. (8.7%); *Rubus* sp. (6.5%)
Honey 3	P	*Citrus* sp. (75.6%)
Extra light amber	I	*Lavandula* sp. (10.9%)
	I	*Echium* (13.5%)
Honey 4	P	*Eucaliptus* sp. (50.2%)
Amber	S	*Lavandula* sp. (23.0%); *Echium* sp. (16.8%)
	I	——————————
Honey 5	–	No pollen observed
Extra light amber		

[a] Frequency classes: P – predominant pollen (more than 45% of pollen grains counted); S – secondary pollen (16–45%); I – important minor pollen (3–15%).

2.3.4. pH

Honey pH was measured, with a combined pH glass electrode connected to pH-meter Basic 20, in a solution prepared with 10 g of honey in 75 mL of distilled water (NP 1309/1976).

2.3.5. Free acidity

Free acidity was determined by potentiometric titration (AOAC, 1990; Official Method 962.19). Honey samples were homogenized in a water bath and filtered through gauze, prior to analysis. Ten grams of honey were then dissolved in 75 mL of distilled water, and alcoholic solution of phenolphthalein added. The solution was titrated with 0.1 N NaOH. Acidity (milliequivalent of acid per kg of honey) was determined as 10 times the volume of NaOH used in titration.

2.3.6. Reducing sugars and apparent sucrose

Reducing sugars and apparent sucrose were determined by potentiometric titration using the Fehling's test (Lane and Eyon modified method).

2.3.7. Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural was determined by using the standard method AOAC (1990) Official Method 980.23. Five grams of honey were dissolved in 25 mL of distilled water, treated with a clarifying agent (0.5 mL of Carrez I and 0.5 mL of Carrez II solutions) and volume made up to 50 mL. The solution was filtered, and the first 10 mL discarded. The absorbance of the filtered solution was measured at 284 and 336 nm against an aliquot of the filtered solution treated with $NaHSO_3$. HMF was determined as:

$$\text{HMF}/100\ \text{g of honey} = (\text{Abs}_{284} - \text{Abs}_{336}) \times 14.97 \times (5/\text{g of sample}).$$

2.3.8. Diastase activity

Diastase activity (AOAC, 1990; Official Method 958.09) was determined using a buffered solution of soluble starch and honey incubated in a thermostatic bath at 40 °C. Thereafter, 1 mL aliquot of this mixture was removed at 5 min intervals and the absorption of the sample was followed at 660 nm in a Perkin Elmer Luminescence Spectrophotometer (Norwalk, USA).

The diastase value was calculated using the time taken for the absorbance to reach 0.235, and the results were expressed in Gothe degrees as the amount (mL) of 1% starch hydrolyzed by an enzyme in 1 g of honey in 1 h.

2.3.9. Water activity

Water activity of each sample was measured with a model Rotronic Hygroskop DT.

2.4. Microbial contamination

Ten grams of each honey sample were homogenized into 90 mL of peptone water solvent. Decimal dilutions were made into the same solvent. Aerobic mesophilic bacteria were counted onto standard plate count agar (PCA) and incubated at 30 °C for 48 h (NP-3788:2002). Moulds and yeasts counts followed the protocol of ISO 21527-2:2008. Microbial counts were expressed as colony-forming units per gram of honey (cfu/g).

For sulphite-reducing clostridia counting, aliquots of 10, 5, 1 and 0.1 mL of the initial suspension were added to an empty tube, thermally treated at 80 °C for 5 min and covered with SPS (sulphite–polymixin–sulfadiazine) agar media, tubes were incubated at 37 °C for 5 days. Then was analysed the fecal coliforms and *Salmonella* detection. Fecal coliforms were enumerated by the Most Probable Number technique defined in the protocol ISO 4831:2006. *Salmonella* detection followed the protocol of ISO 6579:2002(E).

All microbial tests were performed in triplicate.

2.5. Antimicrobial activity

Honey samples were pasteurized at 75 °C for 30 min prior to analysis. Yeast suspensions (approximately, 10^6 cfu/mL) were mixed in 0%, 10%, 25% and 50% of honey and made up to 25 mL with yeast broth (2% glucose, 1% peptone, and 0.5% yeast extract). Incubation was carried out for 24 h for *Saccharomyces cerevisiae ESA1* and for 48 h for *Zygosaccharomyces rouxii ESA 23, Zigosaccharomyces mellis ESA 35,* and *Zygosaccharomyces bailii ESA 11*, at 25 °C in a rotary shaker (Stuart Scientific SI50) at 150 rpm. The growth of yeast cultures was monitored by measuring optical density at 640 nm in a UV–Vis spectrophotometer (Varian Cary 50 Scan). Controls were carried out in the same conditions but in the absence of sample extract. Values for yeast growth rate were obtained by linear regression analysis in the exponential growth range in the graphs of optical density at 540 nm versus incubation time. The equation curve slope corresponded to the rate of microbial growth. Values of IC_{50} (honey concentration which inhibits 50% of microbial growth) were obtained.

All antimicrobial tests were performed in triplicate.

2.6. Statistical analysis

Each honey was analyzed in triplicate. Results are shown as mean values and standard deviation. In each parameter, the differences between honeys were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program.

3. Results and discussion

3.1. Pollinic analysis

Results of honey's pollen profile analysis permits to determinate its floral origin. The identified pollens and its frequency on the five analyzed honeys are presented on Table 1. The most relevant differences among the five honeys were the type and amount of the most predominant pollen present in them.

Four of the analyzed honeys are monofloral. In fact, according to Maia (1999), a honey is classified as monofloral if it contains pollen in quantities exceeding 45% on the remaining pollen identified. However, there are some exceptions depending on the type of pollen. For example, chestnut honey needs 90% of *Castanea* sp. pollen to be monofloral, while lavender honey only needs 15% of *Lavandula* sp. pollen.

By analyzing the data presented in Table 1, one can classify the honeys as: sample 1, a *Eucaliptus* sp. monofloral honey; sample 2, a *Echium* sp. monofloral honey; sample 3, a *Citrus* sp. monofloral honey; sample 4, a *Lavandula* sp. monofloral honey. In honey number 5, the amount of pollen was so low that it was not possible to identify the predominant pollen (possible honey pre-treatment with centrifugation).

3.2. Physicochemical analyses

Table 2 shows the results obtained for the physicochemical parameters analyzed in the five samples of honey. All samples were found to meet honeys quality European Legislation (EC Directive 2001/110) in all parameters except for HMF ($\leqslant$40 mg/kg) and apparent sucrose ($\leqslant$5%).

Visually, all honey samples showed no sign of fermentation or granulation before initiating the physicochemical analyses.

Honey moisture content depends on the environmental conditions and the manipulation from beekeepers at the harvest period, and it can vary from year to year (Acquarone et al., 2007). High moisture content could accelerate crystallisation in certain types of honey and increase its water activity to values where certain yeasts could grow. Moisture contents of honey samples ranged from 15.9 to 17.2, which are well below to the imposed limit of $\leqslant$20% (EU, 2001). There were no significant differences, using the Tukey test ($P < 0.05$), between humidity values obtained for the five honey samples. These results are indicative of good storage ability of these honeys, since high moisture content could lead to fermentation during storage. Electrical conductivity and free acidity values are also within the limits (lower than 0.8 mS/cm and 50 meq/kg, respectively). The free acidity of honey may be explained by taking into account the presence of organic acids in equilibrium with their corresponding lactones, or internal esters, and some inorganic ions, such as phosphate. High acidity can be indicative of fermentation of sugars into organic acids. None of the samples exceeded the limit allowed, which may be taken as indicative of freshness of all honey samples.

Water activity and pH, although not being legislated, meet the values reported by other authors for honeys of different sources (Azeredo et al., 2003; Terrab et al., 2002; Al-Khalifa and Al-Arify, 1999; Andrade et al., 1999) The low pH and a_w of honey inhibits the presence and growth of microorganisms. These parameters have great importance during the extraction and storage of honey, as they influence the texture, stability and shelf life of honey (Terrab et al., 2002).

None of the analyzed honey samples showed electrical conductivity values superior to 0.8 mS/cm (variation between 0.19 and 0.53 mS/cm), suggesting that all samples are from nectar honey, which is corroborated by the content of total ashes inferior to 0.6% (EU, 2001).

Honey colour depends on various factors, being their minerals content an important one. Light-coloured honeys usually have low ash contents, while dark-coloured honeys generally have higher ash contents (Al et al., 2009). In our case, the dark colour observed for most of the analyzed honeys corresponded to high ash contents, except for the honey sample number 4 (multifloral honey).

The HMF content is widely recognized as a parameter of honey samples freshness, because it is absent in fresh honeys and tends to increase during processing and/or aging of the product. Several factors influence the levels of HMF, such as temperature and time of heating, storage conditions, pH and floral source, thus it provides an indication of overheating and storage in poor conditions (Fallico et al., 2006). Two of the samples (samples 2 and 5) showed levels of HMF higher than the allowed limits of 80 mg/kg, which are indicative of temperature abuse during processing and/or bad storage practices.

Diastase is a natural enzyme of honey. Its level depends upon geographic and floral origins of the product, as well as on its freshness. As with HMF, diastase activity can be used as indicative of aging and temperature abuse, but with precaution, since its variability has been higher, confirmed in several honeys (Fallico et al., 2006). All honeys under analysis in the present study fall within imposed limits.

In respect to reducing sugars (fructose and glucose), EC Directive 2001/110 imposes reducing sugars $\geqslant$60 g/100 g, except for honeydew honey, which is $\geqslant$45 g/100 g. These samples do not only meet the standards but also correspond to the levels observed in other studies (Andrade et al., 1999; Rodríguez et al., 2004; Küçük et al., 2007). No significant differences were observed between

Table 2
Physicochemical parameters of honey samples (average ± standard deviation, $n = 3$)[*].

Parameters	Honey 1	Honey 2	Honey 3	Honey 4	Honey 5
Moisture (%)	17.2 ± 0.2a	16.8 ± 0.4a	15.9 ± 0.1a	17.03 ± 0.06a	17.2 ± 0.4a
Conductivity (mS/cm)	0.53 ± 0.01a	0.39 ± 0.01b	0.4 ± 0.1c	0.19 ± 0.01d	0.31 ± 0.03e
Ashes (%)	0.35 ± 0.02a	0.23 ± 0.01b	0.25 ± 0.01b	0.07 ± 0.01c	0.16 ± 0.02d
pH	4.3 ± 0.7a	3.9 ± 0.4a	4.2 ± 0.3a	4.0 ± 1.0a	3.7 ± 0.3a
HMF (mg/kg)	18.0 ± 3.0a	94.0 ± 8.0b	20.0 ± 1.0a	32.0 ± 7.0b	76.0 ± 8.0b
Free Acidity (meq Ac/kg)	27.0 ± 5.0a	25.0 ± 3.0b	32.0 ± 4.0c	16.0 ± 2.0d	25.0 ± 2.0b
Reducing sugars (%)	67.7 ± 0.5a	73.7 ± 0.4a	71.4 ± 0.6a	71.0 ± 0.4a	71.8 ± 0.3a
Apparent sucrose (%)	3.4 ± 0.5a	6.7 ± 0.2b	9.7 ± 0.2c	6.6 ± 0.3b	3.8 ± 0.2a
Water Activity	0.50 ± 0.01a	0.56 ± 0.01b	0.52 ± 0.01c	0.47 ± 0.01d	0.53 ± 0.01e
Diastase Activity (Gothe scale)	13.2 ± 0.5a	8.7 ± 0.2b	9.4 ± 0.2c	12.3 ± 0.3d	16.1 ± 0.2e

[*] The letters (a, b, c, d and e) represents which honeys are different by Tukey test with significance of $p = 0.05$.

reducing sugars values obtained for the five analyzed honey samples.

Non-reducing sugars (apparent sucrose) are set to be ⩽5 g/100 g for the majority of honeys, except for citrus and eucalyptus honeys, which have higher limits (⩽10 g/100 g), as well as lavender honeys (⩽15 g/100 g) (EC Directive 2001/110). Higher sucrose contents could be the result of an early harvest of honeys, i.e., the sucrose has not been converted to fructose and glucose (Azeredo et al., 2003). The honey sample number 3, does, in fact, meet the requirements for citrus honeys. The honey sample number 2 is the only sample that does not meet the legal requirements. The values obtained for honey samples 1, 4 and 5 are among the limits given by the European directive for this parameter. The sucrose determined for the *Lavandula* honey (6.59%) can be justified by its floral origin.

Table 3 shows the correlations between physicochemical parameters for the samples under study. As can be seen, we found a positive correlation between the electrical conductivity, acidity and ash content. This correlation has also been reported by. In fact, the honey electrical conductivity may be explained by taking into account the ash and acid content of honey, which reflects the presence of ions and organic acids; the higher their content, the higher the resulting conductivity. The positive correlation between diastase activity and the water content can be explained by the increase of the enzyme activity in water. The positive correlation verified between a_w values and HMF content can be explained by flaws during processing, in fact, HMF values can increase when storage conditions are not the most appropriated. Also in these conditions, we can verify an increase of a_w due to honey hygroscopic properties, therefore contributing to its deterioration (Silva et al., 2004).

3.3. Microbial contamination

Levels of microbial contamination of honey samples are presented in Table 4. Levels of quantification for the commercial quality parameters (aerobic mesophiles and moulds and yeasts) in the analyzed honey samples are generally lower than those reported by other authors. Iurlina and Fritz (2005) found higher levels of contamination for both aerobic mesophiles (average 244 cfu/g) and mould and yeasts (average 34 cfu/g) counts. In respect to sanitary quality (fecal coliforms) and safety (sulphite-reducing clostridia and *Salmonella*), all our samples were negative. In contrast, Iurlina and Fritz (2005) detected coliform contamination in one tested sample, and Finola et al. (2007) reported that 70% of 23 honey samples were contaminated with sulphite-reducing clostridia.

3.4. Antimicrobial activity

The concentration of honey required to reduce by 50% the specific microbial growth rate (IC 50%) was used as a parameter of the significant inhibitory effects induced by honey in the growth of several fermentative yeasts. Table 5 presents a comparison of five honey samples effect in four fermentation yeasts growth.

From this analysis we can verify that the yeasts growth, *Z. rouxii* ESA23 and *Z. mellis* ESA35 were not affected by the different honey concentrations tested. These results can be justified by the fact that these two yeasts have been isolated from honey by our work group (Carvalho et al., 2006). *S. cerevisiae* ESA1 and *Z. bailii* ESA11 showed a slight sensitivity to honey. In fact, although *S. cerevisiae* was isolated from the honey, it was, for all yeasts tested, the one that supported the lowest sugar concentrations (42.47%) (a_w optimal at

Table 3
Matrix of the correlation between analyzed physicochemical parameters for the different commercial honeys studied.

Parameters	H_2O	Ashes	Reducing sugars	Apparent sucrose	Diastase Activity	Conduct.	Free Acidity	a_w	HMF	pH
H_2O										
Ashes	−0.26									
Reduc. sugar	−0.25	−0.26								
Appar. sucrose	−0.42	−0.22	0.47							
Diastase act.	0.61	−0.16	−0.40	−0.48						
Conductivity	0.044	0.93	−0.40	−0.37	−0.004					
Acidity	−0.47	0.75	−0.18	0.056	−0.15	0.61				
a_w	−0.22	0.15	0.47	0.26	−0.23	0.065	0.36			
HMF	0.15	−0.51	0.53	0.12	−0.093	−0.48	−0.35	0.66		
pH	0.088	0.053	−0.40	0.37	−0.064	0.21	0.076	−0.26	−0.43	

Table 4
Microbial analyses of honey samples.

Honey Sample	Aerobic mesophiles (cfu/g)	Moulds and yeasts (cfu/g)	Fecal coliforms (MPN)	Sulphite-reducing clostridia (in 0.01 g)	*Salmonella* (in 25 g)
1	<10	<10	<1	Negative	Negative
2	<10	$1.3 \times 10^1 \pm 7.98$	<1	Negative	Negative
3	<10	<10	<1	Negative	Negative
4	$2 \times 10^1 \pm 0.35$	$2.2 \times 10^1 \pm 2.89$	<1	Negative	Negative
5	<10	$1.1 \times 10^1 \pm 5.78$	<1	Negative	Negative

Table 5
Honey's antimicrobial activity against selected microorganisms.

Microorganisms	IC 50 (%)				
	Honey 1	Honey 2	Honey 3	Honey 4	Honey 5
S. cerevisiae ESA1	43.27 ± 5.45	>50	42.71 ± 2.38	42.47 ± 1.99	43.27 ± 12.34
Z. rouxii ESA23	>50	>50	>50	>50	>50
Z. mellis ESA35	>50	>50	>50	>50	>50
Z. bailii ESA1307	48.39 ± 7.98	49.23 ± 3.67	>50	44.22	46.23 ± 8.98

0.89). *Z. bailii* ESA11 was isolated from wine, which could justify its susceptibility to honeys, probably because it is not adapted to the stress conditions found in this product, namely, low redox potential and high osmotic pressure.

It was also found that the growth of yeasts under study have not been influenced by the type of honey used, in spite of showing some differences between them, particularly in what concerns pH and acidity. These factors are pointed by many researchers as the primary factors responsible for antimicrobial activity. This fact can be explained by the presence, on honey, of other compounds with biological activity, such as, hydrogen peroxide, phenolic and volatile compounds (Pires et al., 2009).

Conflict of Interest

The authors declare that there are no conflicts of interest.

References

Acquarone, C., Buera, P., Elizalde, B., 2007. Pattern of pH and electrical conductivity upon honey dilution as a complementary tool for discriminating geographical origin of honeys. Food Chemistry 101, 695–703.

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.

Al-Khalifa, A.S., Al-Arify, I.A., 1999. Physicochemical characteristics and pollen spectrum of some Saudi honeys. Food Chemistry 67, 21–25.

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.

Andrade, P.B., Amaral, M.T., Isabel, P., Carvalho, J.C.M.F., Seabra, R.M., Cunha, A.P., 1999. Physicochemical attributes and pollen spectrum of Portuguese heather honeys. Food Chemistry 66, 503–510.

AOAC, 1990. Official Methods of Analysis, 15th ed. Association of Official Analytical Chemists, Inc., Arlington.

Azeredo, L.C., Azeredo, M.A.A., Souza, S.R., Dutra, V.M.L., 2003. Protein contents and physicochemical properties in honey samples of Apis mellifera of different floral origins. Food Chemistry 80, 249–254.

Carvalho, M.C., Rocha, A., Estevinho, L., Choupina, A.B., 2006. Identification of honey yeast species based on RELP analyses of the ITS region. Ciencia y Tecnologia Alimentaria 5 (1), 11–17.

Downey, G., Hussey, K., Kelly, J.D., Walshe, T.F., Martin, P.G., 2005. Preliminary contribution to the characterisation of artisanal honey produced on the island of Ireland by palynological and physico-chemical data. Food Chemistry 91, 347–354.

Erdtman, G., 1986. Pollen Morphology and Plant Taxonomy: An Introduction to Palynology. Brill Archive.

EU, 2001. Council Directive 2001/110 relating to honey. Official Journal of the European Communities.

Fallico, B., Arena, E., Verzera, A., Zappalà, M., 2006. The European Food Legislation and its impact on honey sector. Accreditation and Quality Assurance 11, 49–54.

Finola, M.S., Lasagno, M.C., Marioli, J.M., 2007. Microbiological and chemical characterization of honeys from central Argentina. Food Chemistry 100, 1649–1653.

ISO 21527-2:2008. Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Yeasts and Moulds – Part 2: Colony Count Technique in Products with Water Activity Less Than or Equal to 0.95. International Standards Organization, Switzerland.

ISO 4831:2006. Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of Coliforms – Most Probable Number Technique. International Standards Organization, Switzerland.

ISO 6579:2002(E). Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection of *Salmonella* spp. International Standards Organization, Switzerland.

Iurlina, M.O., Fritz, R., 2005. Characterization of microorganisms in Argentinean honeys from different sources. International Journal of Food Microbiology 105, 297–304.

Küçük, M., Kolaylı, S., Karaoglu, 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.

Maia, M., 1999. Contribuição para a caracterização do mel do Parque Arqueológico do Vale do Côa. Rel. final de Estágio, UTAD, 77.

Nevas, M., Hielm, S., Lindström, M., Horn, H., Koivulehto, K., Korkeala, H., 2002. High prevalence of *Clostridium botulinum* types A and B in honey samples detected by polymerase chain reaction. International Journal of Food Microbiology 72, 45–52.

Nevas, M., Lindström, M., Hautamäki, K., Puoskari, S., Korkeala, H., 2005. Prevalence and diversity of *Clostridium botulinum* types A, B, E and F in honey produced in the Nordic countries. International Journal of Food Microbiology 105, 145–151.

NP 1309:1976 (Ed. 1) – Mel – Determinação da acidez.

NP-3788:2002. Microbiologia Alimentar – Regras gerais para a contagem de microrganismos a 30 °C. Instituto Português da Qualidade, Portugal.

Pires, J., Estevinho, L., Feásc, X., Cantalapiedrad, J., Iglesias, A., 2009. Pollen spectrum and physicochemical attributes of heather (*Erica* sp.) honeys of the North of Portugal. Journal of the Science of Food and Agriculture 89, 1862–1870.

Rodríguez, G.O., Ferrer, B.S., Ferrer, A., Rodríguez, B., 2004. Characterization of honey produced in Venezuela. Food Chemistry 84, 499–502.

Sancho, M.T., Muniategui, S., Huidobro, J.F., Simal, J., 1992. Evaluating soluble and insoluble ash, alkalinity of soluble and insoluble ash and total alkalinity of ash in honey using electrical conductivity measurements at 20 °C. Apidologie 23, 291–297.

Silva, C., Queiroz, A., Figueiredo, R., 2004. Caracterização físico-química de méis produzidos no Estado de Piauí para diferentes floradas. Revista Brasileira de Engenharia Agrícola e Ambiental 6, 260–265.

Snowdon, J.A., Cliver, J.O., 1995. Microorganisms in honey. International Journal of Food Microbiology 31, 1–26.

Terrab, A., Diez, M.J., Heredia, F.J., 2002. Characterization of Moroccan unifloral honeys by their physicochemical characteristics. Food Chemistry 79, 373–379.

White, Jonathan W., 1975. Composition of honey. In: Crane, E. (Ed.), Honey, A Comprehensive Survey, vol. 5. Heinemann, London, UK, pp. 157–206.