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Microbiological and chemical characterization of honeys from central Argentina

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Abstract

The characterization of some honey samples from southern Córdoba (Argentina) was carried out on the basis of their microbiological (*Clostridium*, fungi and yeasts), physical (colour) and chemical (carbohydrates, HMF, water and free acidity) analysis. The results showed that honeys produced in this region are of good quality. HMF content and free acidity values were mostly low, indicating honey freshness. Most of the samples contained less than 20% water. Glucose and fructose accounted for more than 60% of the weight. The amount of yeast and fungi found in the honey samples was less than 1×10^2 CFU/g. Low quantities of vegetative cells and spores of *Clostridia* were found in some honey samples. A standardization and a rationalization of beekeeping techniques throughout southern Córdoba may further improve honey quality, and ensure it over the years.

Keywords: Honey; Chemical analysis; Microbiology; *Clostridium*; Fungi; Yeast

1. Introduction

Honey is a natural food, mainly composed of a complex mixture of carbohydrates and other minor substances, such as organic acids, amino acids, proteins, minerals, vitamins, and lipids (White, 1975 chap. 5). In almost all honey types, fructose predominates, glucose being the second main sugar. These two account for nearly 85–95% of the honey carbohydrates. More complex sugars made up of two or more molecules of glucose and fructose constitute the remaining carbohydrates, except for a trace of polysaccharide. Honey also contains volatile substances which are responsible for the characteristic flavour.

Argentinian honeys are recognized throughout the world because of their quality. Their chemical and microbiological characteristics and their attractive flavour have positioned Argentinian honeys at the top of the world preferences. In 2002, Argentina became the world's second honey manufacturer and the first honey exporter. However, there is little scientific research published on Argentina's honey on its physicochemical and microbiological quality.

The quality of honey is mainly determined by its sensorial, chemical, physical and microbiological characteristics. Internationally, honey quality criteria are specified in Regulatory Standards, compiled in a Codex Alimentarius standard which at present is under revision (Bogdanov, 1999). Regionally, honey commercialized in the MERCOSUR area is required to meet the Technical Documentation regulations.

The Codex Alimentarius Standard for honey quality includes several chemical and physical parameters, comprising moisture content, mineral content, acidity, hydroxymethylfurfural (HMF) content, diastase activity, apparent sugar content, and water insoluble solids content. These analyses help the food analyst to determine the "chemical" quality of the honeys analyzed. Moreover, Devillers,

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Morlot, Pham-Delègue, and Doré (2004) suggest that they may be used in association with multivariate analyses to assign floral origin. These authors reported 100% good predictions by analyses of conductivity, pH, free acidity and percentages of fructose, glucose and raffinose as variables for the principal component analysis.

On the other hand, honey has two sources of contamination with microorganisms: primary sources include pollen, the digestive tracts of honey bees, dust, air, soil and nectar; secondary sources are those arising from honey manipulation by people, they include air, food handlers, cross-contamination, equipment and buildings. Primary sources of honey contamination are very difficult to control. Conversely, secondary sources of honey contamination can be controlled by good manufacturing practices.

The microbes of concern in honey are fungi, yeasts and spore-forming bacteria. Fungi and yeasts are responsible for honey fermentation when the moisture content is high (i.e., above 21%). *Penicillium* and *Mucor* are microorganisms usually found in honey. Moreover, the presence of strains of *Bettsya alvei*, *Acosphaera apis* and *Acosphaera major* may be indicative of bad bee-hive management practices. On the other hand, strains of *Saccharomyces*, *Schizosaccharomyces* and *Torula* predominate among yeasts (Migdal, Owczarczyk, Kedzia, Holderna-Kedzia, & Madajczyk, 2000). Bacterial spores, particularly those of the *Bacillus* and *Clostridium* genus, are regularly found in honey. Sulfite-reducing *Clostridium* is an indicator organism, whose presence in honey provides evidence of contamination or pollution (Collins, Lyne, & Grange, 1999). Spores of *C. botulinum* may be found in honey, usually at low levels (Monetto et al., 1999). The presence of spores of *Clostridium* is specially dangerous for infants and small children (Centorbi et al., 1999). Infant botulism is mainly caused by the consumption of honey contaminated with *C. botulinum*.

These facts clearly indicate the need to count on a detailed characterization of Argentinian honeys. Consequently, the aim of the present work was to analyze the chemical and microbiological quality of some honey samples produced in southern Córdoba (Argentina) to find out whether they meet national and international compositional standards of honey specifications. Thus, colour, acidity, moisture content, hydroxymethylfurfural content, sugar content, fungi and yeasts, and sulfite-reducing *Clostridia* were analyzed in 23 honey samples from the south of the Córdoba province in Argentina.

2. Materials and methods

2.1. Honey samples

Twenty three samples were obtained from local producers. The botanical origin of the honey samples was unknown, although most of the honeys produced in southern Córdoba (Argentina) are multifloral. All the samples were less than 3 months old, as indicated by the producers.

2.2. Physicochemical characteristics

2.2.1. Colour

The colour of honey was measured with a honey colour meter (IPTEA, Argentina). Approximately 5 g of the sample was loaded into the measuring tube and the colour compared with colour standards. The results are expressed in the Pfund scale (mm Pfund).

2.2.2. Acidity

Ten grammes of homogenized honey was weighed in a glass beaker, 75 ml of water was then added, and this solution was titrated with carbonate-free 0.10 NaOH until the pH reached 8.5. An Orion 720A (USA) pH-meter, provided with a Ross Combination Electrode Orion 8102 SC, with a precision of ± 0.002 pH units, was used for pH measurements.

2.2.3. Ash content

Ash percentage was measured by ashing in a Muffle furnace at 550 °C for 6 h.

2.2.4. Moisture content

The determination of moisture was performed by refractometry, using an Atago (Japan) model 1T Abbe refractometer. All measurements were performed at 25 °C.

2.2.5. Hydroxymethylfurfural content

The Winkler method was used to determine the HMF content of honey samples. Five grammes of each of the samples were treated with a clarifying agent (Carrez), the volume was completed to 50 ml and the solution was filtered. The absorbance of the filtered solution was measured at 284 and 336 nm against an aliquot treated with $NaHSO_3$.

2.2.6. Carbohydrate analysis by HPLC

A Gilson (France) model 307 solvent delivery module was used for the flow experiments. Samples were injected with a Rheodyne 7125 (USA) injection valve with a 20 μl loop. The separation of the carbohydrates was achieved with a Hamilton RCX-10 250×4.6 mm column (USA), packed with 10 μm particles, and coupled with a guard column, packed with the same stationary phase. A homemade potentiostat was used as an amperometric detector. The electrochemical signal was fed to a PC-compatible computer, equipped with Peak Simple II data processing software (SRI, USA). The graphic output was obtained with a conventional printer.

2.2.7. Absorbance of honey

Honey samples were loaded into a 1 cm path-length cuvette, and the absorbance at 660 nm was measured with a Hewlett–Packard (USA) model 8453 UV–vis spectrophotometer, controlled by a Hewlett–Packard (USA) Kayak XA computer.

2.3. Microbiological parameters

2.3.1. Yeast and fungi counting method

Ten grammes of honey taken from the surface of the container was diluted in 90 ml of phosphate buffer, pH 5.3, containing 0.1 g of agar (10^{-1} dilution). The same procedure was performed with honey from the bulk of the container. A series of dilutions (10^{-2} and 10^{-3}) were then obtained from these solutions. One millilitre of each of these dilutions (10^{-1}, 10^{-2} and 10^{-3}) was then mixed in Petri dishes with 12 ml of culture medium (pH 3.5) containing yeast extracts, glucose, minerals and chloramphenicol (10 mg/ml). Finally, they were incubated at 25 °C for 5 days. The experiments were carried out in duplicate.

2.3.2. Expression of results

Average number of colonies, multiplied by the dilution factor, was considered for the counting of yeast and fungi colonies. Results were expressed as colony forming units (CFU) of yeast or fungi per gramme of honey.

2.3.3. Isolation of vegetative cells of sulfite-reducing Clostridia

Anaerobic bacteria were isolated on SPS agar (containing per litre: peptone 15 g; yeast extract, 10 g; sodium sulfite, 0.5 g; ferric citrate, 0.5 g; Polymyxin B sulfate, 0.01 g; sulfadiazine sodium salt, 0.12 g, and agar, 13.5 g). Black colonies indicated the presence of these microorganisms.

The methodology proposed by Fernández et al. (1999) for the isolation of vegetative cells of sulfite-reducing *Clostridia* was followed: 20 g of honey was suspended in 150 ml of 1‰ peptone in water, homogenized, and centrifuged at 8500 g and 4 °C during 60 min. The sediment was re-suspended in 7 ml of 1‰ peptone in water. Then, a series of dilutions (1:10) were cultured in Miller–Pricket tubes containing SPS medium. The tubes were sealed with Vas-Par and incubated at 37 °C for 48 h. The whole experiment was carried out in duplicate.

2.3.4. Isolation of spores of sulfite-reducing Clostridia

The above-mentioned dilutions were heated to 80 °C for 20 min and quickly cooled in water to obtain the spores of sulfite-reducing *Clostridia*, and cultured in Miller–Pricket tubes containing SPS medium. The tubes were sealed with Vas-Par and incubated at 45 °C during 48 h. The whole experiment was carried out in duplicate.

2.3.5. Expression of results

The average number of colonies, multiplied by the dilution factor, was considered for the counting of vegetative forms and spores. Results were expressed as colony forming units (CFU) of sulfite-reducing *Clostridia* per gramme of honey.

3. Results and discussion

3.1. Physical and chemical analysis

Table 1 shows the means, standard deviations and ranges of the various physicochemical parameters analyzed: free acidity, ash, glucose, fructose, water and HMF content.

The colours of the honey samples analyzed were: water white (27%), extra white (30%), white (27%), extra light amber (13%), and amber (3%).

Honey colour depends on various factors, their mineral content being an important one. Light-coloured honeys usually have low ash contents, below 0.1 g%, while dark-coloured honeys generally have higher ash contents (Gómez Pajuelo, 1995). In our case, the light colour observed for most of the analyzed honeys corresponded with their low ash contents. Also, nectar honeys are usually light-coloured, and this was the informed botanical origin of the honey samples provided by the beekeepers.

The free acidity values of the honeys analyzed ranged from 11.9 to 29.4 meq/kg (mean value ± standard deviation = 20.6 ± 5.6 meq/kg).

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. None of the samples exceeded the limit allowed by national (40 meq/kg) and international regulations (50 meq/kg), indicating the absence of unwanted fermentations. Moreover, the mean value of 20.6 meq/kg may be taken as indicative of honey freshness.

The ash content of the honeys ranged from 0.02 to 0.18 g% (mean value ± standard deviation = 0.063 ± 0.036 g%).

The ash content of the analyzed honeys was low, characteristic of floral honeys, and consistent with the information provided by the producers. The high dispersion observed in the honey's ash content may indicate that the harvest processes and/or the beekeeping techniques used

Table 1
Physicochemical characteristics of 23 honey samples

	Free acidity (meq/kg)	Ash (%)	Water content (%)	Glucose (%)	Fructose (%)	HMF (mg/kg)
Minimum	11.9	0.015	16	24.0	33.0	1.1
Maximum	29.4	0.179	23.4	39.7	48.4	44.8
Mean	20.6	0.063	18.4	31.7	41.1	14.8
SD	5.6	0.036	1.6	4.6	4.8	11.1

SD: standard deviation.

by the producers are non-uniform. However, it has also been proposed that the ash content of honey depends on the material collected by the bees during the foraging on the flora (Ojeda De Rodríguez, Sulbarán De Ferrer, Ferrer, & Rodríguez, 2004).

An inverse relationship could be observed between the free acidity value of honey and its ash content (Fig. 1). Free acidity slightly decreased with increasing ash content. This dependence might be rationalized by considering that a higher mineral content of honey corresponds to a higher salinized fraction of the acids present.

Percent moisture in the analyzed honeys ranged from 16 to 23.4 (mean value ± standard deviation = 18.4 ± 1.6%).

The water content of honey depends on various factors, for example the harvesting season, the degree of maturity reached in the hive and climatic factors. The maximum amount of water contained by honey is regulated for safety against fermentation. Eleven samples (47.8%) contained less than 18% water, the maximum amount allowed by local regulations. Twenty one samples (91.3%) contained less than 20% water, the maximum amount allowed by international regulations. The mean water content value coincided with honeys harvested at the beginning of the summer. The high variation observed in water content between the samples may be due to the different bee-hive handling practices applied by beekeepers. This high variation might be reduced by homogenization of the bee-hive handling practices.

The content of hydroxymethylfurfural in honeys ranged from 1.1 to 44.8 mg/kg (mean value ± standard deviation = 14.8 ± 11.1 mg/kg).

The HMF content is indicative of honey freshness (Terrab, Diez, & Heredia, 2002), and from this point of view the majority of the analyzed samples are fresh, in agreement with the information provided by the producers. Only one honey sample had HMF over the local regulations limit of 40 mg/kg. However, the amount of HMF contained in this sample does not represent a sanitary risk. Moreover, some authors have proposed increasing the allowed HMF maximum limit to 60 mg/kg (Bogdanov, 1999).

Honey is mainly composed of the monosaccharides glucose and fructose. Our results show a mean glucose content of 31.7 g% and a mean fructose content of 41.1 g%.

It has been proposed that the proportion of fructose to glucose depends largely on the nectar source (Anklam, 1998). It has been reported that the average ratio of fructose to glucose contents is approximately 1.2 (Ojeda De Rodríguez et al., 2004). In our study, this ratio was approximately 1.3, which may have an effect on honey flavour given that fructose is sweeter than glucose.

The absorbance of honey at 660 nm was measured twice a week during a twenty week period. Honey samples were kept at ambient temperature (approximately 25 °C) during measurements. Typical results obtained in this experiment are shown in Fig. 2 for representative honey samples.

Turbidity of honey increases with granulation, and an intensity increase in the absorbance at 660 nm is considered a valid measure of determining the granulation extent (Lupano, 1997). Considering that glucose is less water soluble than fructose, honey's glucose content is the main cause for honey granulation. It has been proposed (Ojeda De Rodríguez et al., 2004) that high fructose to glucose ratios may influence the speed of honey granulation. Thus, honeys with high fructose to glucose ratios may remain liquid for longer periods. However, it has also been proposed that the ratio, glucose/water, is a better indicator for predicting honey crystallization (Manikis & Thrasivoulou, 2001). The latter authors have proposed that glucose/water ratios below 1.7 are indicative of slowly crystallizing honeys. In our case, 21 honey samples had glucose/water ratios below 1.7, and 20 honey samples showed a slow crystallization rate. Thus, this index allowed us to predict the slow crystallization rate of honey in 95% of the cases.

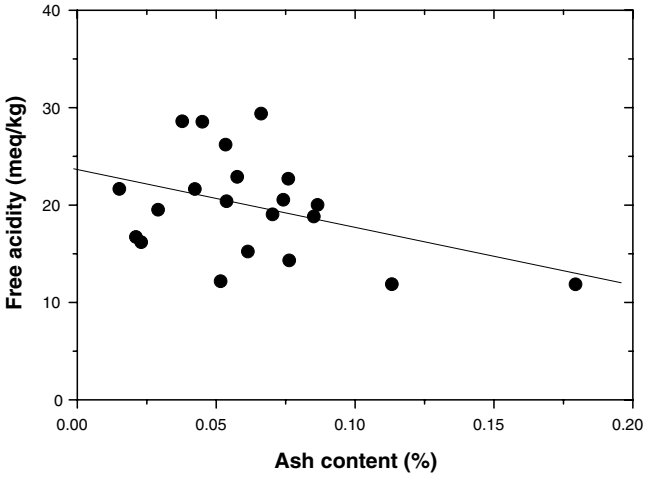

Fig. 1. Linear regression between free acidity and ash content in 23 honey samples from southern Córdoba (Argentina).

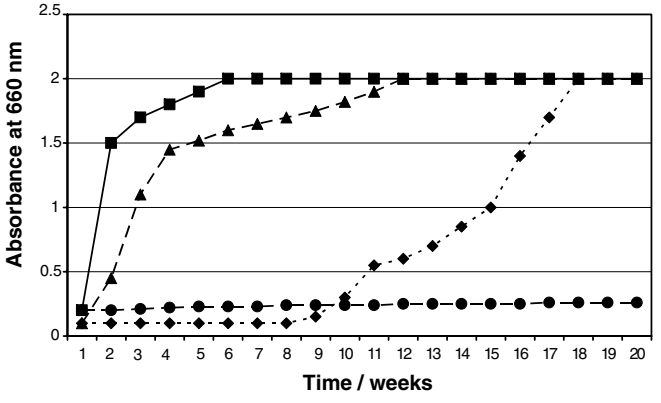

Fig. 2. Absorbance at 660 nm of honey stored at 25 °C, as a function of storage time. ■ and ▲: samples with high crystallization rate (samples15 and 22); ♦: sample with medium crystallization rate (sample 5), and ●: sample with low crystallization rate (sample 8).

3.2. Microbiological analysis

The counting of yeast and fungi showed that all the analyzed honeys had less than 10 CFU/g. This value is well below the maximum limit value ruled by MERCOSUR (1×10^2 CFU/g).

The low free acidity value previously observed in the chemical analysis is concurrent with the low count of yeast and fungi. From the microbiological point of view, the low count of yeast and fungi is indicative of an appropriate management of apiaries. From this point of view, it can be said that the quality of the analyzed honeys is good, which facilitates its national and international commercialization.

The count of sulfite-reducing *Clostridia* showed that approximately 70% of the samples (16 out of 23) contained this microorganism. Approximately 56% of the samples (13 out of 23) contained vegetative cells, and approximately 39% of the samples (9 out of 23) contained the spores. Only 6 out of 23 samples (approximately 26%) showed a vegetative cell count above the MERCOSUR stipulated value of 1×10^2 CFU/g, and only 4 out of 23 samples (approximately 17%) showed a spore count above the MERCOSUR stipulated value of 1×10^2 CFU/g.

Soil is the main source of *Clostridium*, although dust, equipment, buildings and the environment can also contain this microorganism. Thus, the isolation of sulfite-reducing *Clostridia* in honey is important because it may indicate contamination or pollution. Especially, the isolation of sulfite reducing *Clostridia* may indicate the presence of spores of *C. botulinum* which are the main cause of infant botulism (Monetto et al., 1999). To reduce the possibility of honey contamination with *C. botulinum* spores, the manufacturing chain and the maturity of the product at harvest must be controlled.

From our results, the presence of sulfite-reducing *Clostridia* in approximately 70% of the samples, shows the need to perform microbiological analyses in honey to guarantee its highest quality from a human safety point of view. It may be noted that a dynamic relationship between the producer and the quality control laboratory is essential for improving the quality of the final product.

4. Conclusions

The physicochemical and microbiological analytical results of honeys, produced in southern Córdoba (Argentina), indicate a good level of quality. Both the HMF content and the free acidity value were mostly low, indicating honey freshness. Also, most of the samples contained less than 20% water, the maximum amount allowed by international regulations. Among carbohydrates, glucose and fructose accounted for more than 60% of the weight. The ratio between fructose and glucose percentages was above 1.3, which influences honey flavour and may have an effect on its crystallization tendency. The analyzed honeys contained yeast and fungi much below 1×10^2 CFU/g, the maximum amount allowed by MERCOSUR regulations. Vegetative cells and spores of *Clostridia* were found in some honey samples, indicating the need to improve cleanness and the handling practices of honey. It may be advisable to standardize and rationalize the beekeeping techniques throughout southern Córdoba to further contribute to improved honey quality.

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