Journal of ApiProduct and ApiMedical Science 1 (3): 72-81 (2009)
DOI 10.3896/IBRA.4.01.3.03

ORIGINAL RESEARCH ARTICLE

Expanded parameters to assess the quality of honey from Venezuelan bees *(Apis mellifera)*

Patricia Vit[1*], Antonio Rodríguez-Malaver[2], David W Roubik[3], Enrique Moreno[3], Bruno Almeida Souza[4], María Teresa Sancho[5], Miguel Fernández-Muiño[5], Daniela Almeida-Anacleto[6], Luis Carlos Marchini[6], Florimar Gil[7], Carolina González[7], Greana Aguilera[7] and Beatriz Nieves[7]

[1] Apiterapia y Bioactividad, Departamento Ciencia de los Alimentos, Facultad de Farmacia y Bioanálisis, Universidad de Los Andes, Mérida, Venezuela.

[2] Laboratorio de Bioquímica Adaptativa, Departamento de Bioquímica, Facultad de Medicina, Universidad de Los Andes, Mérida, Venezuela.

[3] Smithsonian Tropical Research Institute, Ancón, Balboa, Republic of Panamá.

[4] Núcleo de Pesquisas com Abelhas, Embrapa Meio-Norte, Teresina, Piauí, Brasil.

[5] Área de Nutrición y Bromatología, Departamento de Biotecnología y Ciencia de los Alimentos , Facultad de Ciencias, Universidad de Burgos, Plaza Misael Bañuelos García, E-09001 Burgos, Castilla y León, Spain.

[6] Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, São Paulo, Brasil.

[7] Laboratorio Roberto Gabaldón, Departamento de Microbiología y Parasitología, Facultad de Farmacia y Bioanálisis, Universidad de Los Andes, Mérida, Venezuela.

Received 22 October 2008, accepted subject to revision 26 March 2009, accepted for publication 18 May 2009.

*Corresponding author: Email: vit@ula.ve

Summary

Hive samples from seven Venezuelan states were studied to determine the quality of honeys from the naturalized tropical honey bee *Apis mellifera*, submitted for a national honey competition. The physicochemical composition varied as follows: antibacterial activity as minimal inhibitory concentration for each of *S. aureus* and *E. coli* was 25.0-50.0 g/100 mL, antioxidant activity was 34.90-203.21 µmoles Trolox equivalents/100 g, ash was 0.03-0.13 g/100 g, diastase activity was 3.00-47.81 DN, flavonoids was 2.32-14.41 mg EQ/100 g, free acidity was 24.40-54.55 meq/kg, HMF was 17.70-631.73 mg/kg, moisture content was 17.2-20.2 g/100 g and nitrogen was 28.68-107.29 mg/100 g. Non aromatic organic acids, such as D-gluconic acid, was 13.5-69.3 g/kg, citric acid was 8.0-135.4 mg/kg, and malic acid was 11.2-60.9 mg/kg. Polyphenols were 38.15-182.10 mg EGA/100g, reducing sugars were 62.05-77.57 g/100 g, sucrose was 0.93-13.86 g/100 g, and vitamin C was 12.86-37.05 mg/100 g. Botanical origins of the nine honeys, determined by pollen analysis, indicate that these honeys often were derived from non-forest, non-native and weedy species. The results are a first step to better characterisation of honeys, and some of the parameters were determined for the first time in Venezuelan *A. mellifera* honey. They can be used for research, educational purposes, and to better understand market values, natural occurrence and chemistry of tropical honey harvested from *Apis mellifera.*

Keywords: antioxidant activity, antibacterial activity, *Apis mellifera*, honey competition, melissopalynological analysis, physicochemical analysis, organic acids, quality control.

Introduction

National exhibitions featuring honey offer a venue for information exchange between beekeepers, food analysts, consumers and researchers, and may also help to maintain honey quality standards. In Venezuela, three previous honey contests have taken place (1986 -1988). Beekeepers sent their honeys to compete, and a fourth competition that was held in 2005 is analysed in depth here. In local markets honey is valued not only as a food, but also as medicine. Venezuelan honeys from Zulia state are rich in potassium and phosphates (Sulbarán *et al.*, 2004). Previous works on physicochemical composition of Venezuelan commercial honeys (Vit *et al.*, 1994) and comb honey from one state concluded that certain quality factors were not met (acidity, ash, diastase activity, hydroxymethylfurfural, sugars) (Piccirillo *et al.*, 1998; Ojeda de Rodríguez *et al.,* 2004).

The Codex Alimentarius Commission has produced international quality standards for the honeys of *Apis mellifera* (1969, 1987, 1990, 2001), which are useful references. Venezuelan standards were provided by the Venezuelan Commission of Industrial Standards (Spanish: *Comisión Venezolana de Normas Industriales*) in 1984 (COVENIN 1984 a, b). Government organizations currently include apiculture networks in a few states. The Venezuelan Federation of Beekeepers is attempting to answer the educational needs of professional beekeepers and the present work emphasizes the quality of honey in a national competition. This effort led to the formation of an international multidisciplinary team for honey analysis. In addition to the physicochemical parameters now included in honey quality control (ash, diastase activity, free acidity, HMF, moisture content, reducing sugars and sucrose), some new parameters were included here: antibacterial and antioxidant activities, flavonoid, nitrogen, non aromatic organic acids, polyphenol, and vitamin C contents, as well as pollen identification. These more comprehensive factors of considerable practical and commercial interest will improve biological quality criteria in honey from tropical *A. melllifera.*

Materials and methods

Honey competition

A honey competition is minimally based upon an HMF (hydroxymethylfurfural) and moisture content for physicochemical analysis. Sucrose content, an indicator of incomplete ripening, is optional. However, among the scant total of nine samples gathered for this edition, intensive analyses were carried out, including antioxidant and antibacterial activities, ash, diastase activity, flavonoids, free acidity, nitrogen, non aromatic organic acids, polyphenols, reducing sugars, sucrose and vitamin C, in addition to pollen analysis, the latter utilizing acetolyzed honey samples (Roubik and Moreno Patiño, 1991) to estimate percentages or frequency classes (Louveaux *et al.*, 1978).

Honey samples

Nine honey samples were received from several locations in Venezuela, as indicated in Table 1.

Routine parameters

Routine parameters were measured in duplicates.

Ash

A honey sample of 2.5 ± 0.1 g was burned in a crucible with a drop of olive oil, and incinerated in muffle furnace, to obtain g ash/100 g honey (COVENIN, 1984a).

Diastase activity

Diastase activity was measured using a buffered solution of soluble starch and honey, which was incubated in glass tubes in a bath with controlled temperature. The time required to reach a specified reaction endpoint (absorbance < 0.235 nm) was determined spectrophotometrically (Metrolab 1700 UV/VIS Spectrophotometer). The diastase activity, as Gothe's degrees, is expressed as mL of 1% starch hydrolyzed by the enzyme in one gram in one hour, called the diastase number (DN) (CAC, 1990).

Free Acidity

A honey sample of 2.5 ± 0.1 g was diluted with distilled water and titrated with a 0.1 N NaOH solution (COVENIN, 1984a).

Hydroxymethylfurfural (HMF)

The HMF was determined after the method of White (AOAC, 1990) in a spectrophotometer (Metrolab 1700 UV/VIS Spectrophotometer). Results were expressed in mg HMF/kg honey.

Table 1. Locations for geographical origin of honey samples.

Honey	Geographical origin of honey	
	Location	**State**
1	Altamira de Cáceres	Barinas
2	Altamira de Cáceres	Barinas
3	Vía Guanare-Barrancas	Barinas
4	Tinaquillo	Cojedes
5	El Tigre	Anzoátegui
6	Caicara del Orinoco	Bolívar
7	Bobare	Lara
8	San Diego de Los Altos	Miranda
9	Boconó	Trujillo

Moisture content

The refractometric index of honey was measured at room temperature IR_{Ta} and modified to IR_{25}, then converted into moisture content using the Chataway Table (COVENIN, 1984a).

Reducing sugars and apparent sucrose

The Lane-Eynon hot titration method was used to measure the content of reducing sugars and apparent sucrose (COVENIN, 1984a).

Non-routine parameters

Additional parameters were measured in triplicates except duplicates for antibacterial activity, organic acids and nitrogen. A control was made with 40 g fructose, 30 g glucose, 8 g maltose and 2 g sucrose up to 100 mL with distilled water, autoclaved at 121°C for 15 minutes (Taormina *et al.*, 2001).

Antibacterial activity

The minimal inhibitory concentration (MIC) method according to the Clinical and Laboratory Standards Institute (CLSI, 2008) was used with *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 chosen as Gram positive and Gram negative strains. Results are expressed as g honey/100 mL culture media dilution. The two bacterial strains were chosen because *S. aureus* is associated with multiresistance phenomena and intrahospital infections, and *E. coli* may cause enteric human infections.

Antioxidant activity

Trolox equivalent antioxidant capacity (TEAC) was measured by decolouration of the $ABTS^{+\bullet}$ radical cation. To prepare the ABTS reagent, 7 mM ABTS and 4.9 mM ammonium persulfate were mixed 1:1, covered with foil for 16 h, and diluted up to an absorbance of 0.7 ± 0.2 at 734 nm, which was approximately 40 µL of reagent + 960 µL 20% ethanol. For the TEAC measurement 10 µL of the diluted honey (0.1 g of honey plus 1 mL of 20% v/v ethanol) were added to approximately 40 µL of ABTS reagent + 960 µL ethanol (previously fixed), and shaken vigorously. Variations of absorbance were recorded at 734 nm at 0 and 6 min. A calibration curve with 0.000 -0.625-1.250-2.500 µmoles Trolox was used to measure the percentage of decolouration, to estimate mM Trolox equivalents TE/100 g (Re *et al.*, 1999).

Flavonoids

Total flavonoid contents in the sample were determined by the method of Woisky and Salatino (1998), with minor modifications. To 0.1 mL of the honey sample solution (10% w/v), 0.5 mL of 20 mg $AlCl_3$/mL ethanol 95% (v/v) solution was added. After 20 min at 37°C, the absorbance was measured at 420 nm. Total flavonoids are calculated as mg quercetin equivalents QE/100 g honey from a calibration curve.

Non-aromatic organic acids

Organic acids (total D-gluconic acid, citric acid and L-malic acid) were measured by enzymatic methods previously described (Mato *et al.*, 1997, 1998a, 1998b).

Nitrogen

A standard micro Kjeldahl digestion of 200 ± 10 mg honey (AOAC, 1990) was followed to measure nitrogen content. Nitrogen is reported instead of protein because it is also an indicator of non protein nitrogen added to honey (Vit, 1987).

Polyphenols

Total polyphenol contents were determined after the Folin-Ciocalteu colorimetric method (Singleton *et al.*, 1999). Sample solution (0.1 mL) was mixed with 0.5 mL of the Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, USA) and 0.4 mL of 7.5% (w/v) Na_2CO_3, and the absorbance was measured at 765 nm after 10 min at 37°C. Total polyphenol contents were expressed as mg gallic acid equivalents GAE/100 g honey.

Vitamin C

The 2,4-dinitrophenylhidrazine reagent was used to measure vitamin C content. A protein-free supernatant was obtained by treatment with metaphosphoric acid. Ascorbic acid was oxidized by copper ions to dihydroascorbic acid and diketogulonic acid. These acids reacted with 2,4-dinitrophenylhidrazine in the presence of sulphuric acid, yielding phenylhydrazones that absorbed at 520 nm (Pesce and Kaplan, 1990).

Melissopalynological analysis

The original sample consisted of 25 mL honey, diluted with the same quantity of distilled water, before treatment by acetolysis. To obtain comparative data on relative pollen abundance among samples, an internal standard of spores, *Lycopodium clavatum*, was added, a quantity of 10,700 spores for each honey sample (Stockmarr, 1971). Pollen preparation was carried out by acetolysis, followed by transect counts and identifications of an average of 360 pollen grains on microscope slides prepared from each honey sample. The honeys were identified from a Neotropical pollen guide prepared from the lowlands of central Panama (Roubik and Moreno Patiño, 1991) and supplemented with additional references and published pollen floras.

Statistical analysis

A standard statistical package SPSS 12.0 was used to calculate mean ± SD for all variables and Pearson correlations. All variables were measured in duplicates except triplicates for TEAC, flavonoids, polyphenols, MIC and vitamin C.

Results

Routine parameters

Data on routine parameters of honey composition of the nine honey samples are shown in Table 2. The minimum and maximum values varied as follows: Ash (0.03-0.13) g/100 g honey; diastase activity (3.00-47.81) DN, free acidity (24.40-54.55) meq/kg honey, HMF (17.70-631.73) mg/kg honey, moisture content (17.2-20.2) g/100 g honey, reducing sugars (62.05-77.57) g/100 g honey, sucrose (0.93-13.86) g/100 g honey. From the additional composition and quality factors included in the annex of the Codex Alimentarius Commission (CAC, 2001), diastase activity, free acidity and HMF content were analyzed for the honey exhibit and competition. These values are suggested for voluntary application but not for official use, although they are still present in the official regulations of Venezuela (COVENIN 1984b). Values not within standards of ash, diastase activity, free acidity, HMF, moisture, reducing sugars and sucrose, are in bold.

Non-routine parameters

Expanded data measured to honey, namely non-routine parameters, are also shown in Table 2. The minimum and maximum values varied as follows: Antibacterial activity as minimal inhibitory concentration (MIC) for *S. aureus* (25.0-50.0% w/v) and for *E. coli* (25.0-50% w/v), antioxidant activity (34.90-203.21) µmol TE/100 g, flavonoids (2.32-14.41 mg EQ/100 g), nitrogen (28.68-107.29) mg/100 g honey, non aromatic organic acids such as D-gluconic acid (13.5-69.3) g/kg, citric acid (8.0-135.4) mg/kg, and malic acid (11.2 -60.9) mg/kg, polyphenols (38.15-182.10 mg EGA/100 g), and vitamin C (12.86-37.05) mg/100g honey. Antibacterial and antioxidant activities, flavonoids, nitrogen, non aromatic organic acids, polyphenols, and vitamin C are not included in the standards for honey, therefore there is no reference for comparison of their values.

Melissopalynological analysis

Honey samples contained pollens from 23 to 73 plant species listed in Table 3. The three most common species in each honey were often from plants that are not strictly a source of honey – they are nectarless. Their pollen nonetheless contributes to honey quality. The top three species of each honey sample were uniformly of non-forest species, indicating open and deforested habitats, and even included mango (*Mangifera indica*), a fruit tree imported from India. Fourteen plant species comprised the top three pollen types. They varied between 25.16 to 66.78% in a sample, and summed to account for 54.22 to 80.10% of the total pollen grains counted. Some taxa were present only in one honey (*M. indica*, Asteraceae type 2, *Roystonea* sp., *Alchornea* sp., *Hyptis* sp., *Mimosa casta*, *Eugenia* sp., Poaceae), whereas others were present in two of nine honeys (*Doliocarpus* sp., *Psidium* sp., *Piper* sp.), three in all nine (*Cecropia*, a pioneer native tree which grows in newly cleared areas), and in five of the nine honeys (Asteraceae type 1, *Mimosa pudica*). However, the significance of the presence of these pollen types has different implications to explain honey components, being either from nectariferous and/or polliniferous plants. For instance, the common *Mimosa pudica* pollen is from flowers that have no nectar, as is *Cecropia*, *Piper* and the grasses (Poaceae).

The analytical results of this event were published in a booklet (Almeida *et al.*, 2005) and discussed in a workshop held at Universidad Central de Venezuela in the city of Caracas under the auspices of the *Convención Anual de AsoVAC* (in Spanish, Venezuelan Association of Science), on the 23rd of November, 2005. During the final stage of the honey contest held at *Asociación de Profesores de la Universidad de Los Andes* APULA (in Spanish Association of Professors of Universidad de Los Andes), in the city of Mérida, on the 29th of November, 2005. In both meetings, beekeepers, contest participants and the public received information on quality factors of honey physicochemical composition, compared to *A. mellifera* standards. Honey was displayed in trays for a comparative sensory evaluation,

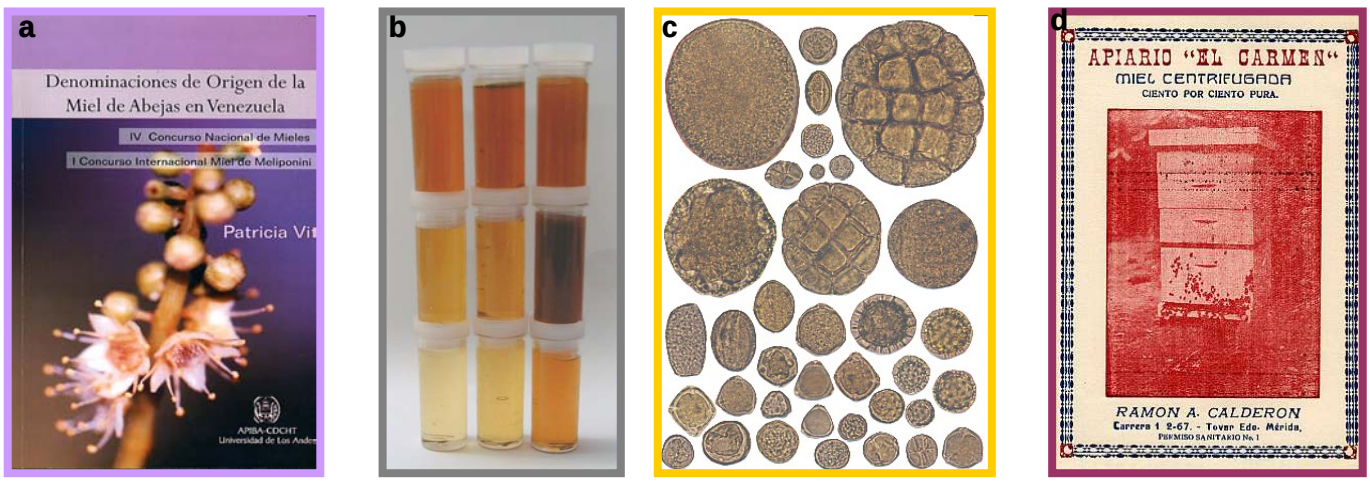

Fig. 1. Information on Venezuelan honey: a) Booklet cover. b) Honey colours. c) Pollen images. d) Label with the first Venezuelan sanitary permit for honey.

Table 2. Mean composition of nine honey samples.

Honey components	Honey samples									Mean of n=9	Overall SD
	1	2	3	4	5	6	7	8	9		
Routine parameters											
Ash (g/100 g)	0.03	0.03	0.07	0.13	0.03	0.08	0.07	0.03	0.09	0.06	0.04
	(0.00)	(0.00)	(0.01)	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.01)		
Diastase activity (DN)	47.81	21.90	17.90	14.00	16.55	**3.00**	**3.50**	**3.50**	17.00	16.13	13.86
	(0.21)	(2.55)	(1.84)	(2.69)	(1.63)	(0.28)	(0.71)	(0.14)	(1.56)		
Moisture (g/100 g)	17.2	17.3	19.4	18.2	17.4	18.4	**20.2**	18.2	18.4	18.3	1.0
	(0.0)	(0.1)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)		
Free acidity (meq/kg)	24.40	33.90	47.35	31.95	27.35	38.15	27.80	30.85	**54.55**	35.14	9.97
	(1.84)	(1.98)	(0.35)	(0.07)	(2.33)	(0.21)	(1.41)	(1.90)	(0.35)		
HMF(mg/kg)	17.70	26.90	**101.80**	83.23	**181.01**	**631.73**	**168.90**	**150.70**	**102.40**	162.71	184.94
	(1.70)	(3.68)	(1.98)	(0.28)	(12.16)	(60.10)	(8.63)	(12.02)	(3.25)		
Reducing sugars (g/100 g)	71.76	71.41	75.05	73.03	77.57	72.70	75.20	72.29	**62.05**	72.34	4.34
	(0.27)	(0.27)	(0.30)	(0.00)	(0.96)	(0.28)	(0.30)	(0.28)	(0.41)		
Sucrose (g/100 g)	0.93	1.35	1.25	1.98	1.53	**13.86**	1.67	3.52	0.99	3.01	4.14
	(0.26)	(0.27)	(0.00)	(0.00)	(0.91)	(0.66)	(0.59)	(0.04)	(0.59)		
Non-routine parameters											
TEAC (moles TE/100 g)	34.90	35.66	93.43	109.66	50.40	203.21	129.81	54.01	104.41	90.61	54.61
	(28.05)	(2.23)	(38.53)	(75.98)	(17.24)	(25.55)	(14.17)	(27.88)	(23.67)		
Flavonoids (mg EQ./100 g)	2.84	4.51	6.21	3.73	2.32	14.41	5.70	5.32	6.84	5.76	3.58
	(1.05)	(1.40)	(1.46)	(1.49)	(1.09)	(3.62)	(1.56)	(1.71)	(1.78)		
Antibacterial activity[1]											
(MIC g/100 mL) *E coli*	50.0	25.0	50.0	50.0	50.0	50.0	50.0	50.0	25.0	44.44	11.02
	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)		
S aureus	50.0	25.0	25.0	25.0	50.0	50.0	50.0	50.0	25.0	38.89	13.18
	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)		
Nitrogen (mg N/100 g)	70.50	56.93	69.93	28.68	107.29	62.66	42.12	55.03	70.82	62.66	21.91
	(0.75)	(0.06)	(1.16)	(0.50)	(0.42)	(0.31)	(0.07)	(0.55)	(0.20)		
Non-aromatic organic acids											
D-gluconic acid (g/kg)	13.5	55.6	69.3	36.7	62.0	63.6	59.9	44.5	17.6	47.0	20.4
	(0.28)	(0.00)	(0.14)	(0.42)	(0.14)	(0.14)	(0.14)	(0.42)	(0.42)		
Citric acid (mg/kg)	14.9	28.6	41.2	29.4	8.0	26.8	23.4	12.7	135.4	35.6	38.8
	(0.14)	(2.12)	(1.41)	(0.00)	(0.42)	(0.28)	(0.28)	(0.56)	(0.14)		
Malic acid (mg/kg)	19.7	11.2	17.6	60.9	18.4	38.2	26.3	12.2	50.7	28.4	17.7
	(0.71)	(0.71)	(0.14)	(0.28)	(0.57)	(0.42)	(0.71)	(0.71)	(0.43)		
Polyphenols	71.85	38.15	67.04	55.28	52.00	164.08	125.22	182.10	85.74	93.50	51.62
(mg EGA./100 g)	(4.33)	(2.60)	(1.15)	(5.97)	(3.77)	(20.01)	(16.99)	(9.63)	(3.21)		
Vitamin C	27.91	17.45	16.36	17.56	12.86	37.05	20.26	24.53	24.41	22.04	7.35
(mg/100 g)	(23.81)	(4.11)	(2.42)	(0.17)	(0.23)	(9.90)	(8.71)	(5.54)	(13.03)		

Means and (SD) values are presented. Values in bold do not meet the *A. mellifera* international honey standards (CAC, 2001), reducing sugars and sucrose are compared to local standards (COVENIN, 1984b). Minimum and maximum values for each parameter are underlined except for MIC.

[1] MIC (minimum inhibitory concentration) values for the control honey were 50 g/100 mL against *E. coli* and *S. aureus*.

Table 3. Percentages of the three main pollen types of the honey samples.

Locality	1 Barinas, Altamira de Cáceres	2 Barinas, Altamira de Cáceres	3 Barinas, Guanare-Barrancas	4 Cojedes, Tinaquillo	5 Anzoategui, El Tigre	6 Bolivar, Caicara	7 Lara, Bobare	8 Miranda, San Diego	9 Trujillo, Bocono
TAXA	**PERCENTAGE**								
ANACARDIACEAE. *Mangifera indica*									4.28
ASTERACEAE. Type 1			6.92	10.81	32.57	7.46	22.09		
ASTERACEAE. Type 2			6.40						
ARECACEAE. *Roystonea* sp.		16.67							
CECROPIACEAE. *Cecropia* sp.	16.56	14.62						18.96	
DILLENIACEAE. *Doliocarpus* sp.				16.22	18.86				
EUPHORBIACEAE. *Alchornea* sp.	25.16								
LAMIACEAE. *Hyptis* sp.					21.71				
FABACEAE. *Mimosa casta*									59.47
FABACEAE. *Mimosa pudica*			66.78	38.74		10.77	14.06	13.77	
MYRTACEAE. *Eugenia* sp.	13.06								
MYRTACEAE. *Psidium* sp.						50.00	18.07		
PIPERACEAE. *Piper* sp.		26.90						35.84	
POACEAE. Not determined.									9.57
TOTAL 3 major pollen types	54.78	58.19	80.10	65.77	73.14	68.23	54.22	68.57	73.32
PERCENTAGE major/3 major pollen types	45.9	46.2	83.4	58.90	44.5	73.28	40.74	52.27	81.1
TOTAL NUMBER OF TAXA	31	23	34	44	26	73	60	30	47

Maximum percentage of pollen taxa in each honey are underlined .

and tasted to choose the favourite honey. All beekeepers received institutional plates as recognition for their work. Instead of a final prize for a competition, participants received the compositional data of their honey in the booklet shared with an international honey contest of stingless bee honey. Few images illustrate educational information (Figure 1).

Discussion

Almost 80% of the honey entries showed high HMF (>80 mg HMF/ kg honey) an indicator of inadequate heating and/or aging. This parameter is generally used to disqualify participating honeys. However, with only nine honeys from all Venezuela, it is more relevant to discuss possible problems with good practice of manufacture than failed standards or techniques. Honey harvest procedures affect the product before it reaches the consumer. Besides high HMF, diastase activity was lower than the suggested standard in three honeys. Generally, 33% of the honeys did not fulfill three quality indicators, one of them including free acidity higher than 50 meq/kg. Sucrose was higher than 5% only for one honey, as well as water content which was higher than 20% also for one honey. In contrast with the previous screening of 500 Venezuelan commercial honeys (Vit *et al.*, 1994), ash content was not higher than the allowed 0.5 g/100 g, in any of the entries.

The antioxidant capacity ranged from 34.90 to 203.21 µmoles Trolox equivalents/100 g, being 17.39 in the control honey. Using three proposed TEAC categories for honey of *Apis mellifera*: low (0-100), medium (101-200) and high (200-300) (Vit *et al.*, 2008), five of the current honeys would be considered low in TEAC, three with medium TEAC and only one with high TEAC. TEAC may become an indicator of medicinal power related with the action of honey in reducing the damage caused by reactive oxygen (Schramm *et al.*, 2003).

Measurement of individual acids in honey is important because they are probably responsible for the antimicrobial action of honey (Bogdanov, 1997). From the seventeen organic acids of honey (Crane, 1990), non-aromatic organic acids can also be used as predictors of fermentation, antioxidant activity and as botanical/ geographical markers (Mato *et al.*, 2003).

The content of the three non aromatic organic acids in Venezuelan honeys varied from 13.5 to 69.3 g D-gluconic acid/kg (average 47.0), 8.0 to 135.4 mg citric acid /kg (average 35.6), and 11.2 to 60.9 mg L-malic acid/kg (average 28.4). In Spanish honeys the following amounts of those acids were found: 3.7-14 g/kg, 71-351 mg/kg and 21-539 mg/kg (Mato *et al.*, 2006), showing lower quantities of D-gluconic acid, but higher concentrations citric and L-malic acids. Also compared to reported averages of 7-11 g/kg in honeys from Spain (Mato *et al.*, 1997; Nozal *et al.*, 1998; Mato, 2004), Italy (Cherchi *et al.*, 1994; Casella and Gatta, 2001), Hungary, USA and Germany (Horvath and Molnár-Perl, 1998), the concentration of D-gluconic acid in Venezuelan honey was four times higher. Gluconic acid has flavor enhancing properties.

The average value of 35.6 mg citric acid/100 g honey was similar to values reported for honeys of multifloral origin and unifloral *Citrus* (Horvath and Molnár-Perl, 1998), *Rosmarinus* (Nozal *et al.*, 1998), and *Eucalyptus* (Mato, 2004) honeys, but was considerably lower than mean values reported for multifloral, *Lavandula*, *Erica* (Nozal *et al.*, 1998), *Lavandula* (Horvath and Molnár-Perl, 1998), multifloral, *Castanea* and *Trifolium* (Mato, 2004) honeys. On the other hand, the average of 28.4 mg L-malic acid/100 g was similar to *Citrus*, multifloral (Horvath and Molnár-Perl, 1998), and *Eucalyptus* honeys (Mato, 2004), but resulted higher than the values reported for *Trifolium* honeys (Horvath and Molnár-Perl, 1998; Mato, 2004). Finally, 28.4 mg L-malic acid/100g resulted lower than the values reported for multifloral (Cherchi *et al.*,1994; Nozal *et al.*,1998; Mato, 2004), *Lavandula* (Horvath and Molnár-Perl, 1998) and *Castanea* honeys (Mato, 2004).

A recent study was demonstrated the bee enzyme system producing acids in honey (Cocker, 2006), to confirm the seminal proposal that citric and malic acid are present in all floral honeys (Nelson and Mottern, 1931), as well as in pomegranate juice where both are principle organic acids (Tezcan *et al.*, 2009). The acidity of honey is mainly attributed to malic acid, although it is not the major organic acid of honey (Root and Root, 2005). As found here, free acidity was highly correlated to malic acid (r^2 =0.846 $p < 0.01$).

The flavonoid content varried between 2.32 and 14.41 mg EQ/100 g, and the polyphenols varied between 38.15 and 182.10 mg EGA/100g, similar to values from the literature (Meda *et al.*, 2005; Vit *et al.*, 2008; Marghitas *et al.*, 2009), with a narrower range.

The nitrogen content varied between 28.68-107.29 mg/100 g, corresponding to a total protein content of 180 to 670 mg/ 100 g, similar to values found in European honeys (Bogdanov, 1981), and genuine Venezuelan commercial honeys (Vit, 1987).

The honey of *A. mellifera* has reportedly a low concentration of vitamin C <5 mg/100g (White, 1975), 2.5 mg vitamin C/100 g honey are found in the literature (Bogdanov *et al.*, 2008). Here we report slightly higher values from 12.86 to 37.05.

Bioactive properties of honey, such as the antibacterial and the antioxidant activities, and flavonoid content, are interesting additions to traditional routine quality standards. The antioxidant capacity TEAC ranged from 34.90 to 203.21 µmoles TE/100 g honey. In this work, the antioxidant activity of nine honeys did not increase with free acidity, but it did in a previous work with stingless bee honeys ($r = 0.97$, $p < 0.01$) (Vit *et al.*, 2006), therefore more investigation is needed to explain this difference. The antioxidant activity correlated significantly to the ash ($r^2 = 0.70$, $p < 0.05$), HMF ($r^2 = 0.81$, $p < 0.01$), flavonoid (r^2 =0.85, $p < 0.01$), and sucrose ($r^2 = 0.73$, $p < 0.05$) contents but failed to correlate significantly to

any other parameter in Table 2. A positive correlation between the antioxidant effects and the concentration of Maillard products and HMF has been found before (Turkmen *et al.*, 2006). The fact that the antioxidant capacity of Venezuelan honeys correlated significantly with the flavonoid content but did not to the polyphenol content confirms a controversy found in literature. In one study TEAC correlated significantly with both the flavonoid and the polyphenol contents (Marghitas *et al.*, 2009). However, in another study the antioxidant activity correlated significantly only with the polyphenols, but not to the flavonoid content (Meda *et al.*, 2005). In the present work as well as in the mentioned work with stingless bee honey, a lack of correlation between ascorbic acid and TEAC was found. Summarising the above findings, the total antioxidant activity of honey could be better explained by interactions of a wide range of compounds, including phenolics, peptides, organic acids, enzymes, and Maillard reaction products (Gheldof *et al.*, 2002).

The antibacterial activity as minimal inhibitory concentration for *S. aureus*, the Gram positive bacteria, varied between 25-50%. The sugar control inhibited bacterial growth also at 50%. This means that 5 out of 9 honeys had no significant antibacterial activity. *Staph* MIC concentrations between 1.5 to 50% have been found in the literature (Molan, 2001). On the other hand, *E. coli*, the Gram negative bacteria was sensitive to only two honeys with a MIC of 25.0 g/100 mL, seven honeys with 50.0 g/100 mL, similar to the control honey made with a pasteurized mixture of sugars and distilled water. MIC values for *E. coli* found in the literature varied between 7 and 50% (Willix *et al.*, 1992; Cabrera *et al.*, 2003; Basson and Grobler, 2008; Fangio *et al.*, 2007).

The highest anti-*Staph* activity was found in honey samples 2, 3, 4 and 9. One of these honeys (number 9) also had the highest citric acid content (135.4 mg/kg honey) and was the most resistant against *E. coli*. However another honey (number 2) was also the most effective against *E. coli* and *S. aureus*, but had only moderate citric acid content (28.6 mg/kg honey). These results are similar to previous reports with honeys from Argentina and South Africa (Cabrera *et al.*, 2003; Fangio *et al.*, 2007; Basson and Grobler, 2008). The relatively high MIC values found in the most recent studies cited here suggest that is better to consume undiluted or slightly diluted honey to receive the antibacterial benefits of this product of the hive.

The honey sample with highest TEAC and flavonoid content (number 6), showed 50% pollen counts of *Psidium* sp., and was the unique unifloral honey in the study. Honey made with other Myrtaceae from New Zealand, like *Lepstospermum scoparium*, called manuka honey, is known for the strong non-peroxide antimicrobial activity (Allen *et al.*, 1991; Al Somai *et al.*, 1994; Molan, 2005). The honey with *Psidium*, a fruit crop called guava, came from apiaries where bees used more plant species than found in the other honeys. However, this honey sample was not well processed because an HMF>600 would indicate overheating or long storage at high temperature. Also, such a high sucrose content has not been reported in genuine honey before.

In addition to the established analysis supported by the Codex Alimentarius Commission honey standards (CAC, 2001), the antibacterial and the antioxidant activities could be considered biological activity indicators. TEAC may become an indicator of medicinal power related to the action of honey in reducing the damage caused by reactive oxygen species (Schramm *et al.*, 2003). The non-aromatic organic acids, flavonoids, nitrogen, polyphenols and vitamin C, also contribute to this list. The fact that antioxidant activity of honey increases with HMF, may call for regulated standards. New questions on heating, ageing and other factors causing a high content of HMF in honey, also are significant as health factors, not only for marketing purposes. The pollen analysis establishes indicators for apiary habitat, quality and origin. Therefore, expanded analytical techniques are needed to unravel the ancient matrix of honey, besides the routine interpretations associated with its quality and current norms. A recent outcome of this innovative approach to honey is appreciated in the study of Guerrini *et al.* (2009) with a comprehensive pharmaceutical background.

Although only nine honey samples were analyzed in this work, many different honey quality parameters were measured. These were later discussed with beekeepers, suggesting that besides the routine analysis supported by the Codex Alimentarius Commission (CAC, 2001), and Comisión Venezolana de Normas Industriales (COVENIN, 1984b), honey standards, the antibacterial and the antioxidant activities could be considered to be included in honey labels, as well as the content of organic acids, flavonoids, polyphenols and vitamin C. The quality of honey is a team work beyond botanical and bee species, interpreted by men.

Acknowledgement

The authors wish to thank CDCHT-ULA for the financial support provided for the honey contest with the project SULA-FA-06-05-03. To beekeepers and participants in the honey contest Agustín Leal, Istvan Locsi, Juan Carlos Schwartzenberg, Giovanna Olivier, Renato Guida, José Gregorio Rodríguez and Mario Mutone, who sent their honeys for this event. Also to editorial improvement of the manuscript. To the memory of Prof. Werner Jaffé, founder member of AsoVAC, for his unconditional ability to transform academic research into social problem solutions.

References

AL SOMAI, N; COLEY, K E; *MOLAN,* P C; HANCOCK, B M (1994) Susceptibility of *Helicobacter pylori* to the *antibacterial* activity of *manuka honey*. *Journal of the Royal Society of Medicine* 87(1): 9-12.

ALMEIDA, D; MARCHINI, LC; RODRÍGUEZ-MALAVER, A; SOUZA, BA; VIT P (2005) IV Concurso Nacional de mieles. In Vit, P (ed) (2005) *Denominación de Origen de la Miel de Abejas en Venezuela*. APIBA-CDCHT, Universidad de Los Andes; Mérida, Venezuela. 15-37 pp.

ALLEN, K L; MOLAN, P C; REID, G M (1991) A survey of the antibacterial activity of some New Zealand honeys. *Journal of Pharmacy and Pharmacology* 43(12): 817-822.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (AOAC) (1990) *Official Methods of Analysis.* 15th., supl 2. 1990.

BASSON, N; GROBLER, S (2008) Antimicrobial activity of two South African honeys produced from indigenous *Leucospermun cordifolium* and *Erica* species on selected micro-organisms. *BMC Complementary Alternative Medicine* 8(41): 1-4.

BOGDANOV, S (1981) Bestimmung von Honigprotein mit Coomassie Brillantblau G 250. *Mitteilungen aus dem Gebiete der Lebensmitteluntersuchung und Hygiene* 72: 411-417.

BOGDANOV, S (1997) Nature and origin of the antibacterial substances in honey. *Lebensmittel Wissenschaft und Technologie* 30 (7): 748-753.

BOGDANOV, S; JURENDIC, T; SIEBER, R; GALLMANN, P (2008) Honey for Nutrition and Health: A Review. *Journal of the American College of Nutrition* 27: 677-689.

CABRERA, L; OJEDA DE RODRÍGUEZ, G; CÉSPEDES, E; COLINA, A (2003) Actividad antibacteriana de miel de abejas multiflorales (*Apis mellifera scutellata*) de cuatro zonas apícolas del estado Zulia Venezuela. *Revista Científica* 13(3): 205-211.

CASELLA, I G; GATTA, M (2001) Determination of electroactive organic acids by anion-exchange chromatography using a copper modified electrode. *Journal of Chromatography*. A 912: 223-233.

CHERCHI, A, SPANEDDA, L, TUBEROSO, C, CABRAS, P (1994) Solid-phase extraction and high-performance liquid chromatographic determination of organic acids in honey. *Journal of Chromatography* A 669(1-2): 59-64.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) (2008) *Performance standards for antimicrobial susceptibility testing.* Fifteenth Informational Supplement; M100-S18, 25(1). CLSI; Wayne, PA; USA.

COCKER, L (2006) The enzymic production of acid in honey. Journal of the Science of Food and Agriculture 2(9):411-414.

CODEX ALIMENTARIUS COMMISSION (CAC) (1969) CAC/RS 12-1969. Norma Regional Europea para Miel. Programa Conjunto FAO/OMS; Roma, Italia. 29 pp.

CODEX ALIMENTARIUS COMMISSION (CAC) (1987) CODEX STAN 12-1981. Revised Codex Standard for Honey. CAC; Rome, Italy. 17 pp.

CODEX ALIMENTARIUS COMMISSION (CAC) (1990) Official methods of analysis. Rome, v.3, supl.2.

CODEX ALIMENTARIUS COMMISSION (CAC) (2001) CODEX STAN 12-1981. Revised Codex Standard for Honey. CAC; Rome, Italy. 7 pp.

COMISIÓN VENEZOLANA DE NORMAS INDUSTRIALES (COVENIN) (1984a) Miel de Abejas. Métodos de Ensayo. COVENIN 2136-84. Fondonorma; Caracas, Venezuela 32 p.

COMISIÓN VENEZOLANA DE NORMAS INDUSTRIALES (COVENIN) (1984b) Miel de Abejas. COVENIN 2191-84. Fondonorma; Caracas, Venezuela. 5 p.

CRANE E (1990) Bees and Beekeeping. Science, Practice and World Resources. Heinemann; Oxford, UK. 614 pp.

FANGIO M, LURLINA M, FRITZ R (2007) Actividad antimicrobiana de mieles del sudeste de la provincia de Buenos Aires frente a *Escherichia coli*. Revista Argentina de Microbiología 39 (2): 1-6.

GHELDOF, N; WANG, X H; ENGESETH, N J (2002) Identification and quantification of antioxidant components of honey from various floral sources. *Journal of Agriculture and Food Chemistry* 50: 5870-5877.

GUERRINI, A; BRUNI, R; MAIETTI, S; POLI, F; ROSSI, D; PAGANETTO, G; MUZZOLI, M; SCALVENZI, L; SACCHETTI, G (2009) Ecuadorian stingless bee (Meliponinae) honey: A chemical and functional profile of an ancient health product. *Food Chemistry* 114: 1413-1420.

HORVATH, K; MOLNÁR-PERL, I (1998) Simultaneus GC-MS quantitation of o-phosphoric, aliphatic and aromatic carboxylic acids, proline, hydroxymethylfurfural and sugars and their TMS derivatives in honeys. *Chromatographia* 48(1-2): 120-126.

LOUVEAUX, J; MAURIZIO, A; VORWOHOL, G (1978) Methods of melissopalynology. *Bee World* 59(4): 139-157.

MARGHITAS, L; DEZMIREAN, 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 (4): 863-867.

MATO, I (2004) Estudio de los ácidos orgánicos no aromáticos en la miel. Tesis Doctoral. Universidad de Santiago de Compostela. España.

MATO, I; HUIDOBRO, J F; SÁNCHEZ, M P; MUNIATEGUI, S; FERNÁNDEZ-MUIÑO, M A; SANCHO, M T (1997) Enzymatic determination of total D-gluconic Acid in honey. *Journal of Agricultural and Food Chemistry* 45: 3550-3553.

MATO, I; HUIDOBRO, J F; SÁNCHEZ, M P; MUNIATEGUI, S; FERNÁNDEZ-MUIÑO, M A; SANCHO, M T (1998a) Enzymatic determination of total citric acid in honey by using polyvinylpyrrolidona clarification. *Journal of Agricultural and Food Chemistry* 46(1): 141-144.

MATO, I; HUIDOBRO, J F; SÁNCHEZ, M P; MUNIATEGUI, S; FERNÁNDEZ-MUIÑO, M A; SANCHO, M T (1998b) Enzymatic determination of L-malic acid in honey. *Food Chemistry* 62(4): 503-508.

MATO, I; HUIDOBRO, J F; SIMAL-LOZANO, J; SANCHO, M T (2003) Significance of nonaromatic organic acids in honey. *Journal of Food Protection* 66(12):2371-6.

MATO, I; HUIDOBRO, J F; SIMAL-LOZANO, J; SANCHO, M T (2006) Rapid determination of nonaromatic organic acids in honey by capillary zone electrophoresis with direct ultraviolet detection. *Journal of Agricultural and Food Chemistry* 54 (5): 1541-1550.

MEDA, A; LAMIEN, C E; ROMITO, M; MILLOGO, J; NACOULMA, O G (2005) Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry* 91 (3): 571-577.

MOLAN, P C (2001) Honey as a topical antibacterial agent for treatment of infected wounds. Report 1-13. http://www.worldwidewounds.com/2001/november/Molan/honey-as-topical-agent.html

MOLAN, P C (2005) Manuka honey as a medicine. *Public Service Review* (5): 52-54 www.bio.waikato.ac.nz/pdfs/honeyresearch/bioactives.pdf

NELSON, E K; MOTTERN, H H (1931) Some organic acids in honey. *Industrial and engineering chemistry* 23(3):335-336.

NOZAL, M J del; BERNAL, J L; MARINERO, P; DIEGO, J C; FRECHILLA, J I; HIGES, M; LLORENTE, J (1998) High performance liquid chromatographic determination of organic acids in honey from different botanical origin. *Journal of Liquid Chromatography and Related Technologies* 21(20): 3197-3214.

OJEDA DE RODRÍGUEZ, G; SULBARÁN DE FERRER, B; FERRER, A; RODRÍGUEZ, B (2004) Characterization of honey produced in Venezuela. *Food Chemistry* 84: 499-522.

PESCE, A J; KAPLAN, L A (1990) *Química clínica. Métodos*. Médica Panamericana; Buenos Aires, Argentina. 590 pp.

PICCIRILLO, G A; ROGRÍGUEZ , B; OJEDA DE RODRÍGUEZ, G (1998) Estudio de algunos parámetros físicoquímicos en mieles cosechadas durante la época seca de ocho zonas apícolas del Estado Zulia, Venezuela. *Revista de la Facultad de Agronomía (LUZ)* 15: 486-497.

RE, R; PELLEGRINI, N; PROTEGGENTE, A; PANNALA, A; YANG, M; RICE-EVANS, C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 26(9/10): 1231-1237.

ROUBIK, D W; MORENO PATIÑO, J E (1991) *Pollen and spores of Barro Colorado island*. Monographs in Systematics Botany; Missouri Botanical Garden, Sant Louis, Montana, USA. 270 pp.

ROOT, A I; ROOT, E R (2005) *The ABC and XYZ of Bee Culture*. Kessinger Publishing; Whitefish, MT. 740 pp.

SCHRAMM, D D; KARIM, M; SCHRADER, H R; HOLT, R R; CARDETTI, M; KEEN, C L (2003) Honey with high levels of antioxidants can provide protection to healthy human subjects. *Journal of Agricultural and Food Chemistry* 51: 1732-1735.

SINGLETON, V L; ORTHOFER, R; LAMUELA-RAVENTOS, R M (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin Ciocalteu reagent. *Methods in Enzymology* 299: 152-178.

SULBARÁN DE FERRER, B; OJEDA DE RODRÍGUEZ, G; PEÑA, J; MARTÍNEZ, J; MORÁN, M (2004) Mineral content of the honey produced in Zulia state, Venezuela. *Archivos Latinoamericanos de Nutrición* 54(3): 346-348.

STOCKMARR, J (1971) Tablets with spores used in absolute pollen analysis. *Pollen et Spores* 13(4): 615-621.

TAORMINA, P; NIEMIRA, B; BEAUCHAT, L (2001) Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. *International Journal of Food Microbiology* 69: 217-225

TEZCAN, F ; GÜLTEKIN-ÖZGÜVEN, M ; DIKEN, T ; ÖZÇELIK, B; ERIM, FB (2009) Antioxidant activity and total phenolic, organic acid and sugar content in commercial pomegranate juices. *Food Chemistry* 115(3) :873-877.

TURKMEN, N; SARI, F; POYRAZOGLU, E S; VELIOGLU, Y S (2006) Effects of prolonged heating on antioxidant activity and colour of honey. *Food Chemistry* 95 (4): 653-657.

VIT, P (1987) Utilidad de la determinación del contenido de nitrógeno en el control de calidad de mieles venezolanas. *Acta Científica Venezolana* 38(4):511-512.

VIT, P; GONZÁLEZ, I; LÓPEZ-PALACIOS, S (1994) Clasificación de mieles comerciales venezolanas. *Archivos Latinoamericanos de Nutrición* 44(1): 47-56.

VIT, P; RODRÍGUEZ-MALAVER, A; ALMEIDA, D; SOUZA, BA; MARCHINI, LC; FERNÁNDEZ DÍAZ, C; TRICIO, A E; VILLAS-BÔAS, J K; HEARD, T A (2006) A scientific event to promote knowledge regarding honey from stingless bees: 1. Physical-chemical composition. *Magistra* 18(4):270-276.

VIT, P; GUTIÉRREZ, M G; TITĚRA, D; BEDNÁŘ, M; RODRÍGUEZ-MALAVER, A J (2008) Mieles checas categorizadas según su actividad antioxidante. *Acta Bioquímica Clínica Latinoamericana* 42(2): 237-244.

WHITE, J W (1975) Composition of honey. In Crane, E. (ed). *Honey, a comprehensive survey*. Heinemann; London, UK; pp. 157-206.

WILLIX, D J; MOLAN, P C; HARFOOT, C G (1992) A comparison of the sensitivity of wound-infecting species of bacteria to the antibacterial activity of manuka honey and other honey. *Journal of Applied Bacteriology* 73 (5): 388-394.

WOISKY, R; SALATINO, A (1998) Analysis of propolis: some parameters and procedures for chemical quality control. *Journal of Apicultural Research* 37: 99-105.