SUPPLEMENTARY MATERIAL

Volatiles, Color Characteristics and Other Physico-chemical Parameters of Commercial Moroccan Honey

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Abstract

Seven commercial Moroccan honeys were considered for chemical characterization. Volatile fraction, total polyphenols content, and antioxidant and antiradical activities were evaluated by employing different analytical methodologies. Several physical parameters such as refractive index, pH, water content, solids content, and colour were measured. Volatile fraction revealed an abundant presence of *cis*- and *trans*-linalool oxide in the seven studied samples. The presence of high levels of compounds related to the Maillard reaction, like furfural and hydroxymethylfurfural, could be the result of thermal treatments used to liquefy commercial honeys or of long storage times. The CIE $L^*a^*b^*C_{ab}^*h_{ab}^\circ$ chromatic coordinates confirmed the advanced stage of the Maillard reaction, showing L^* values lower than the common values found for honey of similar typologies.

Keywords: furfural; hydroxymethylfurfural; chromatic coordinates; HS-SPME;

1. Experimental

1.1. Samples

A total of seven commercial samples of Moroccan honey types were analyzed. Samples were classified according to their brand names. Five multifloral honeys, namely bitter, black, forest, herbal, mountain and two monofloral honeys, namely acacia (*Robinia pseudoacacia*) and eucalyptus (*Eucalyptus* spp.), were obtained (2013) from local market in Casablanca (Morocco). After acquisition, the honeys were stored at 4 °C in the dark and analysed within 3 months.

1.2. Chemical and reagents

MeOH, MeCN, and 85% H₃PO₄ were purchased from Merck (Darmstadt, Germany). The standards of *p*-hydroxybenzoic acid, methyl syringate, (2Z,4E)-abscisic acid, gallic acid, ferrous sulfate, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), Folin-Ciocalteu reagent, Na₂CO₃ and ferric chloride were purchased from Sigma Aldrich (Milan, Italy). All the chemicals were of analytical grade. Ultrapure H₂O (18,0 MΩ) was obtained with Milli-Q Advantage A10 system apparatus (Millipore, Milan, Italy).

13. Headspace Solid-Phase Microextraction (HS-SPME)

A 100 μm PDMS/DVB/CAR (Polydimethylsiloxane/Divinylbenzene/Carboxen) coated fiber (Supelco, Sigma Aldrich, St. Louis, Mo., USA) was conditioned prior to use according manufacturer instruction. The conditioned fiber was injected through the septum, and suspended in the headspace of a 40 mL glass vial containing 5 g of homogenized honey, to which was added 5 mL of saturated aqueous NaCl solution.

The fiber was exposed for extracting volatiles for 36 min at 60 °C then the temperature was decreased to 40° for 18 min and then the temperature was decreased in ice bat to 0°C for 6 min, according to the method reported by Bianchin and Coworkers (2014). The fiber was then retracted and removed from the vial, and placed immediately into the injector of the GC. Thermal desorption was performed into the injector at a temperature of 250 °C for 5 min in splitless injection mode.

1.4. GC-FID analysis

The GC analyses were carried out using an Agilent 4890N (Palo Alto, USA). The instrument was equipped with a FID detector and a HP5 capillary column (30 m x 0.25 mm, film thickness 0.17 μm). The column temperature was kept at 50 °C for 2 min (isothermal), then programmed to 200 °C at a rate of 4 °C/min and kept constant at 200 °C for 10 minutes (isothermal), then programmed to 270 °C at a rate of 10 °C/min. Nitrogen was used as carrier gas at a flow rate of 1.2 mL/min in splitless mode. The relative proportions percentages of the HS constituents (HS-SPME semi-quantitative analysis) were obtained by FID peak area normalization.

1.5. GC-MS analysis

The GC-MS analysis was carried out using a Hewlett Packard 5890 GC (Palo Alto, USA) equipped with a Hewlett Packard 5971 (Palo Alto, USA) MS system operating in the EI mode at 70 eV, using the column and operating conditions analogous to those reported in the GC-FID section.

Identification of the individual components was performed by comparison with the co-injected pure compounds or by matching the MS fragmentation patterns and retention indices with the built in libraries or literature data and commercial mass spectral libraries (NIST/EPA/NIH 1999; HP1607 purchased from Agilent Technologies).

1.6. Retention index

A hydrocarbon mixture of *n*-alkanes (C5-C22) was analysed separately under the same chromatographic conditions using the HP-5 capillary column to calculate the retention indexes with the generalized equation by Van del Dool and Kartz (1963). $I_x = 100[(t_x - t_n)/(t_{n+1} - t_n) + n]$. Where *t* is the retention time, *x* is the analyte, *n* is the number of carbons of alkane that elutes before analyte and *n*+1 is the number of carbons of alkane that elutes after analyte.

1.7. Refractive index, water content, solids content and pH

Refractive index was measured using a refractometer ATAGO EX-5000. The sample was homogenized prior to the analysis by manual slow stirring with a spatula during 3 minutes. (Gazzetta Ufficiale 2003) The measurements have been performed at 20 °C in duplicate.

Water content was calculated from the refractive index value measured by applying the equation of Wedmore: (Sesta and Lusco 2008; Wedmore 1955) $WWed(\%) = [-0.2681 - \log(RI - 1)] / 0.002243$ where RI is the refraction index measured.

Solids content

The Brix degree represents the concentration of the water soluble solids in the sample considered. It indicates the concentration of sugars, being calibrated on a water solution containing 100g of sugar (NIST circular 1942).

pH

10 g of honey sample were dissolved in 75 mL of distilled water and the pH was measured with glass electrode pH-meter (BioBlock pH-metre Microprocessor 99621) at room temperature.

1.8. CIE $L^*a^*b^*$ C_{ab} h_{ab} coordinates

The measurements of chromatic coordinates were performed with a UV/VIS spectrophotometer and obtained data were processed using Cary Win UV Color Application V. 2.00 software (Tuberoso et al. 2014). Transmittances in a wavelength interval between 380 and 780 nm were measured using D65 illuminant with a 10° observation angle. Prior to the analysis, the honeys were completely liquefied in ultrasonic bath ($t < 40$ °C, max 60 min) and the analysis was performed on transparent samples, without any dilution, in a 10 mm polystyrene cuvettes (Kartell 01937).

1.9. Total polyphenol content

The total content of phenolic compounds was measured spectrophotometrically with a modified Folin-Ciocalteu method (Tuberoso et al. 2009). The honey samples were homogenized and diluted (1:5, w/v) with ultrapure water. 100 mL of the honey solution was mixed with 0.5 mL of Folin-Ciocalteu reagent. After 5 min, 3 mL of 100 g/L solution of Na₂CO₃ (w/v) was added. The mixture was shaken and brought up to a volume of 10 mL with ultra pure water. After 90 min of incubation at room temperature, the absorbance was read against blank at 725 nm in a 10 mm polystyrene cuvettes (Kartell 01937) by a spectrophotometer Varian Cary 50 Scan (Varian, Milano, IT). Total phenol content, expressed as milligrams of gallic acid equivalent (GAE) per kilogram, was calculated using a calibration curve prepared with fresh gallic acid standard solution (10-200 mg/L) that was analysed in the same mode as the honey samples. All measurements were performed in triplicate.

1.10. Antiradical activity (DPPH)

A spectrophotometric analysis of antiradical activity was performed using DPPH assay (Tuberoso et al. 2009). 50 µL of the honey solution (1:5, w/v), diluted in ultrapure water, were dissolved in 2 mL of 0.04 mmol/L DPPH in methanol. After 60 min of incubation at room temperature in a dark place, the absorbance readings were carried out with a spectrophotometer at 517 nm using disposable polystyrene cuvettes. A calibration curve was prepared with Trolox solution (0.05-1.0 mmol/L). The data were expressed as Trolox equivalent antioxidant capacity per kilogram of the honey (TEAC, mmol/kg). All measurements were performed in triplicate.

1.11. Antioxidant activity (FRAP)

The ferric reducing antioxidant assay (FRAP) is based on the reduction of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe(III)-TPTZ] to the ferrous complex at low pH, followed by spectrophotometric analysis. The analyses were performed as in previous research (Tuberoso et al. 2009). Briefly, the reagent was prepared by mixing 10 mmol/L TPTZ reagent with 20 mmol/L ferric chloride in acetate buffer (pH 3.6). The quantitative analyses were performed by the external standard method using ferrous sulphate (0.1 -2 mmol/L) as the standard and correlating the absorbance (593 nm) with the concentration. Twenty µL of the honey diluted with H₂O (1 : 5, w/v) were dissolved in 2 mL of ferric complex. The results were calculated and expressed as mmol of Fe²⁺ per kilogram of the honey. The value of absorbance was read in disposable polystyrene cuvettes using a spectrophotometer. All measurements were performed in triplicate.

1.12. HPLC-DAD analysis

The chromatographic analyses were performed using HPLC-DAD. A Varian system ProStar fitted with a pump module 230, an autosampler module 410 with a 10 μ L loop and a Thermo Separation diode array detector Spectro System UV6000LP (Thermo Separation, San Jose, CA) were used and set at 280 nm (Tuberoso et al. 2011). The chromatographic separation was obtained on a Phenomenex Gemini C18 110 Å column (150 mm x 4.60 mm, 3 μ m, Chemtek Analitica, Anzola Emilia, BO, IT) using 0.2 M phosphoric acid (solvent A), and acetonitrile (solvent B) at a constant flow rate of 1.0 mL/min. The gradient (v/v) was generated keeping an isocratic condition for 5 min then decreasing from 90% of solvent A to 65% in 15 min; to 10% in 20 min and again keeping an isocratic condition A/B (10%:90% v/v) for 10 min. Before each injection, the system was stabilized for 10 min with initial ratio A/B (90:10, v/v).

The honey samples were diluted with ultrapure water (1:5, p/v) then filtered through Econofilter RC membrane (0,45 μ m, Ø 25 mm, Agilent Technologies, Milano, IT). The chromatogram and the spectra were elaborated by ChromQuest V. 4.0. (ThermoQuest, Rodano, Milano, IT). The qualitative identification of compounds was achieved by comparison of retention time and UV spectra with those of pure commercial standard (4-hydroxy benzoic acid, kynurenic acid, 4-hydroxy phenyl acetic acid, riboflavin, phenyl lactic acid, p-coumaric acid, benzoic acid, lumichrome, methyl syringate, (E/E)-abscisic acid, (Z/E)- abscisic acid, salicylic acid, methyl benzaldehyde and 5-hydroxymethylfurfural, HMF). Calibration curves were built with the method of external standard, correlating the area of the peaks vs. the concentration. The correlation value for HMF was 0.9998 in the range of 0.1–20 mg/kg.

Table S1. HMF, Refractive index, solids content, density, pH and water content for different honey samples at 20 C°.

Honey Sample	HMF (mg/kg) mean $\pm$ sd	Refractive Index	Solids content (°Bx)	pH	Water content (g/100g)
Acacia	25.63 $\pm$ 1.47	1.49217	80.4	4.33	17.7
Bitter	34.09 $\pm$ 1.85	1.49071	79.9	4.46	18.2
Black	34.98 $\pm$ 3.26	1.49136	80.2	4.51	18.1
Eucalyptus	23.97 $\pm$ 1.61	1.49028	79.7	4.40	18.5
Forest	24.27 $\pm$ 1.44	1.49259	80.6	4.69	17.6
Herbal	30.59 $\pm$ 2.11	1.49300	80.7	4.23	17.4
Mountain	18.87 $\pm$ 0.95	1.49129	80.0	4.44	18.1

Table S2. CIE L*a*b*C_{ab}* h_{ab}*coordinates (result are expressed as mean $\pm$ standard deviation).

Honey Sample	L*		a*		b*		C _{ab} *		h _{ab} *	
	mean	$\pm$ sd	mean	$\pm$ sd	mean	$\pm$ sd	mean	$\pm$ sd	mean	$\pm$ sd
Acacia	49.09	0.02	25.54	0.04	75.79	0.01	79.97	0.05	71.38	0.03
Bitter	25.76	0.03	31.14	0.05	44.30	0.04	54.15	0.02	54.90	0.02
Black	9.24	0.05	27.96	0.01	16.14	0.02	32.29	0.05	30.00	0.02
Eucalyptus	41.77	0.03	27.65	0.03	68.76	0.04	74.11	0.02	68.10	0.01
Forest	21.32	0.04	28.47	0.05	36.79	0.03	46.51	0.04	52.27	0.04
Herbal	44.39	0.01	27.91	0.02	72.25	0.03	77.45	0.04	68.88	0.04
Mountain	56.74	0.04	23.66	0.02	80.54	0.03	83.94	0.03	73.63	0.02

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