Antioxidant activity, color chromaticity coordinates, and chemical characterization of monofloral honeys from Morocco

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ABSTRACT

The bioprospecting of several monofloral Moroccan honeys was carried out. The antiradical activity expressed as mmol Trolox equivalents/kg of honey and evaluated by 2,2-diphenyl-1-picrylhydrazyl assay, ranged from 0.15 for euphorb honey to 1.08 for citrus honey. The antioxidant activity expressed as mmol Fe^{2+}/kg and evaluated by ferric ion reducing antioxidant power assay, ranged from 0.96 for euphorb honey to 4.74 for orange honey. The total phenol content was evaluated by colorimetric assay, while the color attributes were evaluated as transmittance data. Significant Pearson correlation factors were found between total polyphenol amount and antioxidant activity and between color attributes and antioxidant activity. Furthermore the chemical composition of volatile organic compounds was determined. The volatile organic compounds chemical composition of the studied honeys was mainly represented by terpene and benzene derivatives, Maillard reaction products, isoprenoids, and hydrocarbons. The volatiles fingerprint, as well as a targeted high-performance liquid chromatography analysis of the polar components, was used to tentatively confirm the declared botanical origin of the samples studied.

KEYWORDS

Floral marker; Biological activity; Polyphenols; Volatiles; Spurge honey

Introduction

As reported in Codex Alimentarius, honey is defined as:

> the natural sweet substance produced by Apis mellifera bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature. It is composed of a great number of substances but only a few main components, represented mainly by sugars and water. (ALINORM 01/25 APPENDIX II)

For this reason it is often defined as a supersaturated sugar solution in water. Alongside the main components are small quantities of organic acids, proteins, enzymes, amino acids, vitamins, volatile organic compounds (VOCs), polyphenols, and solid particles. Since most of these substances are derived directly from the plants, the chemical composition of honey is often related to the botanical, e.g.,[1] and geographical[2] origin of the honeybees collection. The color of honey varies from nearly colorless to dark brown and, in addition to the botanical origin, it depends from several parameters such as mineral content,[3] phenolic compounds,[4] and as a consequence of Maillard reactions or fructose caramelization. It can be fluid, viscous or partly to entirely crystallized.

Honey is an interesting food matrix and numerous studies have been carried out with the aim of elucidating its chemical composition as well as evaluating its biological activity. Honey has been

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recognized as antimicrobial,[5] anticancer,[6] antiviral,[7] gastroprotective,[8] hepatoprotective,[9] and hypoglycemic agent.[10] Great attention was given, and is currently still being given, to the antioxidant activity[11] which is usually attributed to the presence of phenolic compounds and flavonoids. Morocco, due to its pedoclimatic condition, is a suitable area for the honeybees activities. Even though Morocco has the largest honey production among the North Africa countries, mainly due to traditional beekeeping,[12] studies on Moroccan honeys are limited in number. Previous literature reports on Moroccan honeys are mainly focused on chemical characterization. The sugar components of Moroccan honey were studied by Terrab et al.[13] Several unifloral honeys were characterized by physicochemical data[12,14] and chromatic evaluation.[15–17] Furthermore the mineral composition and electrical conductivity of several unifloral Moroccan honeys was used by Terrab et al.[17] in the classification of the honey's botanical origin. More recently, the quality parameter and antioxidant activity study on several commercial Moroccan honeys was also carried out,[18,19] the studied honeys were within the limits established by European commis-sion, despite a high 5-hydroxymethyl-2-furfuraldehyde (HMF) content. Finally Bettar et al.[20] achieved the discrimination by means of multivariate analysis of minerals, physicochemical and colorimetric data, of two Euphorbia spp. honeys.

The antioxidant capacity of honey is usually linked to the phenolic components,[21] though recently some authors have demonstrated the capacity of the volatile fraction to act as an antioxidant.[22–24] Since the studies on antioxidant activity on Moroccan honeys are limited in number[18,19] as are investigations into their VOCs,[19] in the present study we report on the chemical characterization, focusing the analysis on the less studied antioxidant activity and the volatile fraction, of several monofloral Moroccan honeys. The volatile compounds were evaluated by solid phase micro extraction (SPME) followed by gas-chromatographic (GC) analysis, while antioxidant and antiradical activities were evaluated by ferric ion reducing antioxidant power (FRAP) and by 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assays. In addition to the antioxidant and antiradical activity, the total polyphenol content was also evaluated by the Folin-Ciocalteu reagent. Finally, the color chromatic attributes obtained by transmit-tance data were evaluated for all studied samples and selected marker compound analysis was carried out by means of targeted high-performance liquid chromatography (HPLC) technique. The honey metabolic profiles obtained with our analysis were furthermore compared with those of honeys from other countries already reported in literature.

Methods

Samples

A total of 10 samples of Moroccan honey types were analyzed. Samples were classified according to their brand names. Two multifloral honeys (MF1 and MF2) and eight monofloral honeys, namely Thyme (TY; Laminaceae spp.), two samples of Eucalyptus (EU1 and EU2; Eucalyptus spp.), two samples of Citrus (CI and OR; Citrus spp), Asparagus (AS; Asparagus acutifolius), Euphorb (EF; Euphorbia spp.), Carob tree (CT; Ceratonia siliqua L.) were obtained (2013–2014) from professional beekeepers in Morocco who declared the botanical origin. After acquisition, the honeys were stored at 4°C in the dark and analyzed within 3 months.

Chemical and reagents

MeOH, MeCN, and 85% H_3PO_4 were purchased from Merck (Darmstadt. Germany). The standards of p-hydroxybenzoic acid, methyl syringate, (2Z,4E)-abscisic acid, gallic acid, ferrous sulfate, 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, NaCl, Na_2CO_3, and ferric chloride were purchased from Sigma Aldrich (Milan, Italy). All the chemicals were of analytical grade. Ultrapure H_2O (18.0 MΩ•cm) was obtained with Milli-Q Advantage A10 system apparatus (Millipore, Milan, Italy).

Headspace solid-phase microextraction (HS-SPME)

A 100 μm PDMS/DVB/CAR (Polydimethylsiloxane/Divinylbenzene/Carboxen) coated fiber 50/30 Stableflex (Supelco, Sigma Aldrich, St. Louis, Mo., USA) was preconditioned prior to use at 270°C for 1 h in a Gerstel MPS bake-out station, according to the manufacturer's instructions. 5 g of homogenized honey, to which was added 5 mL of saturated aqueous NaCl solution, were placed in a 20 mL SPME vial, 75.5 × 22.5 mm, which was tightly closed with a septum and allowed to equilibrate for 15 min at 60°C. The preconditioned fiber was then exposed to the headspace. The extraction time was fixed at 40 min, based on a previous optimization.[19] All experiments were carried out under constant agitation at 250 rpm. After the extraction, the fiber was desorbed for 2 min into a Gerstel CIS6 PTV injector operating at 250°C in a splitless injection mode.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was carried out using an Agilent 7890 GC equipped with a Gerstel MPS autosampler, coupled with an Agilent 7000C MSD detector. The chromatographic separation was performed on a VF-Wax 60 m × 0.25 mm i.d., 0.5 μm film thickness column (Agilent), as well as on a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.17 μm). The following temperature program was used for the VF-Wax column: 40°C hold for 4 min, then increased to 150°C at a rate of 5.0°C/min, held for 3 min then increased to 240°C at a rate of 10°C/min, and finally held for 12 min. For the HP-5MS column the following temperature program was used: 60ºC hold for 3 min, then increased to 210ºC at a rate of 4ºC/min, then held at 210ºC for 15 min, then increased to 300ºC at a rate of 10ºC/min, and, finally, held at 300ºC for 15 min. Helium was used as the carrier gas at a constant flow of 1 mL/min for both columns. The data was analyzed using a MassHunter Workstation B.06.00 SP1, with identification of the individual components performed by comparison with the co-injected pure compounds and by matching the MS fragmentation patterns and retention indices with the built in libraries or literature data or commercial mass spectral libraries (NIST/EPA/NIH 2008; HP1607 purchased from Agilent Technologies).

A hydrocarbon mixture of n-alkanes (C7-C22) was analyzed separately under the same chromatographic conditions used on the HP-5MS and the VF-Wax capillary columns to calculate the retention indexes with the generalized equation by Van del Dool and Kartz,[25] $Ix = 100[(t_x - t_n) /(t_{n+1} - t_n) + n]$. 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.

CIE $L^*C_{ab}^*h_{ab}^\circ$ coordinates

The measurements of chromatic coordinates were performed with a ultraviolet-visible (UV-Vis) spectrophotometer and obtained data were processed using Cary Win UV Color Application V. 2.00 software.[26] 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).

Total polyphenol content, antiradical (DPPH), and antioxidant (FRAP) activities

The total content of polyphenolic compounds was measured spectrophotometrically with a modified Folin–Ciocalteu method.[27] Total polyphenol 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). The antiradical activity was evaluated with a spectrophotometric analysis using DPPH.[27] A calibration curve in the range 0.05–1.0 mmol/L was used for the Trolox, and data were reported as Trolox equivalent antioxidant capacity (TEAC, mmol/L). The total antioxidant activity was

performed with a modified FRAP assay.[27] Quantitative analysis was performed according to the external standard method ($FeSO_4$, 0.1–2 mmol/L), correlating the absorbance with the concentration and results were reported as mmol/L of Fe^{2+}. For the three assays, the honey samples were homogenized and diluted (1:5, w/v) with ultrapure water and all measurements were performed in triplicate.

HPLC-DAD analysis

The chromatographic analyses were performed using HPLC-diode array detector (DAD). A Varian system ProStar fitted with a pump module 230, an autosampler module 410 with a 10 µL loop and a Thermo Separation DAD Spectro System UV6000LP (Thermo Separation, San Jose, CA) were used and set at 280 nm.[28] The chromatographic separation was obtained on a Phenomenex Gemini C18 110 Å column (150 mm × 4.60 mm, 3 µm, 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 an Econofilter RC membrane (0,45 µm, 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 standards (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 HMF). Calibration curves were built with the method of external standard, correlating the area of the peaks versus the concentration. The correlation value for HMF was 0.9998 in the range of 0.1–20 mg/kg.

Statistical analysis

All experiments were repeated three times. All statistical analyses were performed comparing data with unpaired Student's t-test, when the data followed a normal distribution, using SigmaStat v 3.5 software. The distribution of the sample was evaluated by the Kolmogorov-Smirnov and Shapiro tests. The strength of association between variables was analyzed with the Pearson product moment correlation coefficient when the data were normally distributed. A $p \leq 0.05$ was considered statistically significant.

Results and discussion

The studied Moroccan honeys showed very different analytical parameters. Color characterization of the studied honeys was carried out by spectrophotometric transmittance method and CIE $L^*C_{ab}^*h_{ab}^\circ$ coordinates are reported in Table 1. With the exception of CI honey ($L^* = 77.6$) which could classified as light or slight-light honey, all other samples showed lower values of lightness ranging from $L^* = 47.1$ for OR honey to $L^* = 73.0$ for CT honey. Honey's chromatic attributes have been related, in addition to the Maillard reaction,[29] to several parameters such as minerals, electrical conductivity,[3] phenolic content, and antioxidant activity.[4] Tuberoso et al.[26] reported similar lightness values to our Eucalyptus and Citrus honeys, using spectrophotometric methods such as transmittance[19] or reflectance[20] data, for the determination of chromatic coordinates, making a further comparison with literature data non-trivial. In addition, some authors have determined the color attributes directly from honey[16] while some other authors determined the color by measuring the absorbance of aqueous honey solutions.[18]

The antioxidant capacity of the studied honeys was evaluated by two assays, namely DPPH and FRAP tests. Both assays show a variability among the monofloral Moroccan honeys and result are reported in Table 1. The total antiradical capacity (DPPH assay), expressed as mmol TEAC/kg of

Table 1. General characteristics, CIE L*C_{ab}*h_{ab}° chromatic coordinates, total phenols, antioxidant, and antiradical activities of the 10 honeys (mean ± SD).

Sample	AS	CI	CT	EF	EU1	EU2	MF1	MF2	OR	TY
Origin	Asparagus	Citrus	Carob tree	Euphorbie	Eucalyptus	Eucalyptus	Multiflower	Multiflower	Orange	Thyme
Year	2014	2014	2014	2013	2013	2014	2013	2014	2014	2014
L^{*a}	58.8 ± 0.0	77.6 ± 0.0	73.0 ± 0.0	72.2 ± 0.0	54.8 ±0.0	71.8 ± 0.0	54.3 ±0.03	65.6 ±0.0	47.1 ± 0.0	63.2 ± 0.0
$C_{ab}{}^{*b}$	77.4 ± 0.1	45.7 ± 0.0	52.4 ± 0.0	45.4 ± 0.0	72.7 ±0.0	46.8 ± 0.0	77.4 ±0.0	67.3 ±0.0	79.7 ± 0.1	71.5 ± 0.1
$h_{ab}{}^{\circ c}$	77.0 ± 0.0	87.6 ± 0.0	85.2 ± 0.1	85.1 ± 0.0	76.6 ±0.0	84.9 ± 0.0	74.6 ±0.0	80.4 ±0.0	70.2 ± 0.1	78.7 ± 0.0
TP[d] mg GAE/kg	423.8 ± 2.9	214.0 ± 22.0	290.3 ± 8.3	214.6 ± 27.0	495.7 ±47.2	237.7 ± 22.7	477.0 ±36.4	286.4 ±25.4	477.0 ± 26.6	299.1 ± 10.3
FRAP[e] mmol Fe^{2+}/kg	2.89 ± 0.07	1.40 ± 0.09	2.68 ± 0.15	0.96 ± 0.13	2.91 ±0.09	1.65 ± 0.17	2.71 ±0.10	1.69 ±0.09	4.74 ± 0.32	2.23 ± 0.13
DPPH[f] mmol Trolox/kg	0.86 ± 0.06	0.31 ± 0.01	0.73 ± 0.04	0.15 ± 0.08	0.89 ±0.24	0.31 ± 0.07	0.86 ±0.28	0.4 ±0.05	1.08 ± 0.09	0.58 ± 0.01

[a]lightness.
[b]chroma.
[c]hue, deg.
[d]Total phenols (TP) values are expressed as gallic acid equivalent (GAE).
[e]FRAP values expressed as millimolar concentration of Fe^{2+} obtained from a dilution of $FeSO_4$ having an equivalent antioxidant capacity as the honey solution.
[f]<DPPH values expressed as millimolar concentration of TEAC obtained from a dilution of Trolox having an equivalent antiradical capacity as the honey solution.

honey, ranged from 1.08 for OR honey to 0.15 for the EF honey sample. The total antioxidant activity, in terms of capacity of components present in the samples able to reduce the Fe^{3+}/Fe^{2+} couple, confirm the tendency found by DPPH tests: the greater value of antioxidant activity found by FRAP assay was an OR honey sample (4.74 mmol Fe^{2+}/kg), whereas the FRAP assay carried out on EF showed a lower value (0.96 mmol Fe^{2+}/kg). By plotting FRAP data against DPPH assay a positive correlation factor ($p < 0.001$) was found, supporting the result of Thaipong et al.[30] The total phenolic compounds, evaluated by colorimetric Folin–Ciocalteu assay and expressed as mg GAE/kg of honey, ranged from 477.0 for MF1 and OR honey to 213.0 for EU2 honey sample (Table 1). Honey polyphenols are considered to be mainly responsible for the antioxidant activity, indeed positive correlation factors have been found among total phenol and FRAP values ($p < 0.01$), and total phenol and DPPH values ($p < 0.01$). Aazza et al.[18] in a recent study on commercial Moroccan honeys, reported values of total phenol that are slightly greater than our results here; conversely our OR honey samples total phenol values are greater than those reported by Aazza et al.[18]

For all our studied Moroccan honey samples, both the DPPH and the FRAP values were generally lower than literature data.[31,32] However, as reported in the literature,[31,32] the reducing power and radical-scavenging ability show a general variability according the botanical source of nectar. Significant correlation factors (negative) were also found by plotting the lightness values L* against DPPH ($p < 0.01$), L* against FRAP ($p < 0.01$) and L* versus total phenols ($p < 0.001$). HS-SPME/GC-MS revealed the presence of 82 different components (Table 2) some of which were detected in all honey samples. Overall the volatile compounds in honey that are responsible for its biological activity are classified into three principal categories: terpenes, isoprenoids and benzene derivatives,[33] all of which are well represented in the volatiles of the Moroccan samples. Monoterpenes are well known to have antimicrobial properties as well as to be able to preserve beneficial microorganisms like lactic acid bacteria.[34] By contrast norisoprenoids are usually derived from carotenoid derivatives and, in addi-tion to the important impact on the sensory characteristics of honey, their presence has been linked to the antiradical activity. Compounds like benzene acetaldehyde, benzaldehyde or phenyl ethyl alcohol have shown antibacterial activity.[33]

The major compounds in chemical composition of AS honey volatiles were phenyl ethyl alcohol, furfural and benzeneacetaldehyde. Previous literature reports on AS honey, focused on the identification of volatile compounds, identified exo-bornyl acetate as a major component.[35] Even though the target of that study was the detection of aromatic hydrocarbons as possible environmental contamination, the authors also reported the presence of several components that we also detected in our asparagus honey sample, such as benzeneacetaldehyde, nonanal, decanal and dodecanal.

The two samples of Citrus honeys had significant differences: OR volatiles chemical composition is dominated by HMF while the CI honey has benzeneacetaldehyde as a major component of the volatile fraction. Linalool derivatives are considered chemical markers for Citrus honey.[36,37] The VOCs analysis supports the botanical origin of our OR honey samples since it shows the presence of both linalool oxide derivatives as well as the three isomers of lilac aldehydes, which are considered to be linalool derivatives.[36] Beside the linalool derivatives, methyl anthranilate has also been suggested as a citrus honey chemical marker;[38] in any event, the use of additional markers is recommended since the methyl anthranilate concentration could be lower than the limit of detection of commonly used analytical techniques.[39] The HS-SPME/GC-MS analysis applied to CT honey shows some similarities with literature data. In addition to hotrienol, Boi et al.[40] detected as major components in the volatile fraction of CT honey cis- and trans-linalool oxide which we detected among the main compounds of our Moroccan CT sample.

The chemical profile of the eucalyptus (EU1 and EU2) honey's VOCs show the presence of several main compounds such as benzaldehyde, p-cymene, benzeneacetaldehyde, linalool oxide, nonanal, 2-phenyletha-nol, and nonanoic acid, all of which have already been detected in eucalyptus honeys[1,36] from other countries. Among these compounds p-cymene and nonanoic acid were classified as floral markers for eucalyptus honey.[33] Among the 18 components detected in EF honey, nonanal was the major compound, followed by n-octane. To the best of our knowledge, no previous studies on VOCs composition have been

Table 2. Compounds identified by GC-MS analysis of the honeys.

Compounds	AS	CI	CT	EF	EU1	EU2	MF1	MF2	OR	TY	RI_{HP5}	RI_{VF-VAX}	ID
Dimethyl disulfide#						0.9			1.4		<700	<700	MS, RI
Dichloromethane	5.2				0.4	1.0	1.2			0.3	<700	755.6	STD, MS, RI
Hexane	0.4					0.9				0.2	<700	<700	STD, MS, RI
pentanal#			0.4			1.2		2.9			<700	<700	MS, RI
Heptane	2.0		0.4							0.4	700.0	700.0	STD, MS, RI
Disulfide dimethyl#				0.7		1.0			0.2		733.4	nd	MS, RI
Amyl alcohol#			0.6	0.4	0.4			1.1			739.0	1336.0	MS, RI
Isoamyl alcohol#	1.7		0.7	0.7	3.9		2.2	1.5			739.8	1210.0	MS, RI
Toluene	0.4		7.7	2.6	0.4	3.4	2.8	2.4		0.3	777.3	1054.0	STD, MS, RI
1-octene	3.0		0.5	0.2					0.3		790.0	840.0	STD, MS, RI
Octane	5.2		21.6	12.7	0.3	3.8		9.2	3.1	0.7	802.0	799.3	STD, MS, RI
furfural#	8.3	3.8	1.1	2.6	14.7	2.1	12.3	1.3	5.7	5.7	830.5	1488.3	MS, RI
Nonane	0.6		2.4	1.0				1.1		0.5	896.0	898.6	STD, MS, RI
2-acetylfuran#	0.5				0.4				1.2		911.0	1534.8	MS, RI
Trycyclene										0.4	917.0	1031.0	STD, MS, RI
α–thujene#										0.4	922.5	1038.0	MS, RI
α–pinene	0.8									3.1	927.1	1036.0	STD, MS, RI
Camphene										0.9	942.0	1074.0	STD, MS, RI
Thuja–2,4(10)–diene#										0.4	947.6	1133.5	MS, RI
Benzaldehyde	3.1	1.9	0.6	0.8	2.7	1.7	2.8	2.0	3.9	1.5	952.5	1563.1	STD, MS, RI
Furfural 5 methyl#	0.4				0.4	0.3	1.3			0.3	958.8	1602.9	MS, RI
β–pinene									0.2	0.3	968.9	1126.0	STD, MS, RI
Verbenene#										Overlap	985.0	1138.4	MS, RI
Linalool oxide dehydro trans#								0.8		Overlap	987.0	1220.5	MS, RI
Octanal#	0.3								0.6		1001.0	1302.9	MS, RI
Carene d2										0.4	1002.0	1157.2	STD, MS, RI
Linalool oxide dehydro cis#		1.8						1.0			1003.0	nd	MS, RI
P–cymene	0.3	0.0	1.0	1.0	0.4	0.3		0.4	0.2	0.8	1019.6	1291.0	STD, MS, RI
Limonene			0.4	0.2		0.5	1.7	0.5		1.2	1021.4	1210.1	STD, MS, RI
Isopulegol#	0.9										1022.4	nd	MS, RI
Benzeneacetaldehyde#	6.4	12.5		2.4	22.4	0.8	14.4	0.3	0.4	0.6	1034.8	1680.8	MS, RI
γ–terpinene										2.1	1050.5	1259.3	STD, MS, RI
Linalool oxide furanoid cis#	4.4	8.5	12.3	1.2	1.3	0.8	1.7	30.4	0.5	0.8	1070.0	1457.0	MS, RI
Terpinolene#										Overlap	1085.0	1310.5	MS, RI
Para cimenene#	0.5		0.4		0.3					Overlap	1085.0	1459.7	MS, RI
Linalool oxide furanoid trans#		3.2				1.3		6.2			1089.0	1487.0	MS, RI
Linalool			2.5		0.1			1.0		3.1	1096.0	1551.8	STD, MS, RI
Nonanal#	4.0		11.5	28.2	0.8	3.7	1.9	5.0		1.5	1100.3	1408.6	MS, RI
Hotrienol#		8.0									1106.0	nd	MS, RI
Rose oxide cis#										0.3	1107.0	1369.1	MS, RI
Phenyl ethyl alcohol#	8.3	2.0			4.4	1.7	1.8		0.6	1.0	1108.0	1943.5	MS, RI
Isophorone (4-keto) #									1.4		1141.0	1738.1	MS, RI
Ilac aldehyde*#		2.8				0.9					1144.8	nd	MS, RI
Lilac aldeihyde*#		2.8			1.9						1153.2	nd	MS, RI
Borneol										18.7	1160.0	1728.8	STD, MS, RI
Ethyl benzoate#					1.7		1.7				1165.5	1698.0	MS, RI
Linalool oxide cis Pyranoid#			0.4					0.4			1167.0	1756/1774	MS, RI
Nonanol#	0.8		0.6	1.8	0.4				0.7		1168.0	1664.6	MS, RI
Lilac aldehyde*#					4.5						1171.0	nd	MS, RI
Linalool oxide trans pyranoid trans#		4.5	0.6					0.6			1173.0	1756/1774	MS, RI
Terpinen-4-ol										0.7	1172.0	1616.4	STD, MS, RI
P–cymen-8-ol#									0.8		1187.0	1878.0	MS, RI
Methyl salicilate			0.5	0.2							1191.3	1822.2	STD, MS, RI
α–terpineol					0.2					4.9	1194.0	1716.3	MS, RI
Ethyl octanoate#	2.5				0.9						1200.0	1441.6	MS, RI
Decanal#	1.2		0.6	2.0	0.2	0.4			1.3	0.9	1206.4	1516.6	MS, RI
Pulegone#	0.4									0.6	1228.2	1694.0	MS, RI
HMF	0.4		2.0	7.8	18.0	12.5	9.0	4.7	16.8	1.8	1229.0	>2300	STD, MS, RI
Carvacrol methyl ether#										4.3	1233.3	1626.5	MS, RI

(Continued)

Table 2. (Continued).

Compounds	AS	CI	CT	EF	EU1	EU2	MF1	MF2	OR	TY	RI_{HP5}	RI_{VF-VAX}	ID
2–phenyl ethyl acetate#	2.9				0.9					0.6	1250.0	1847.1	MS, RI
Nonanoic acid		1.4			1.5	2.1			6.9	0.1	1270.0	2174.0	STD, MS, RI
Bornyl acetate#										0.8	1275.0	1609.4	MS, RI
Thymol		2.7			0.5					12.4	1292.0	2202.0	STD, MS, RI
Carvacrol										0.8	1301.0	2237.5	STD, MS, RI
Decanoic acid#									4.0		1367.0	2279.0	MS, RI
β–bourbonene#										0.5	1373.1	nd	MS, RI
Ethyl decanoate#	3.3				0.9					0.9	1392.0	1648.4	MS, RI
E caryophyllene										0.3	1406.7	1637.0	STD, MS, RI
Dodecanal#	0.7				0.4						1408.0	1728.0	MS, RI
δ cadinene#										0.4	1510.5	nd	MS, RI
Thymohydro quinone#										0.4	1554.2	nd	MS, RI
Total identified	68.7	55.7	68.8	66.5	85.3	41.3	54.7	72.8	50.3	76.3			

Compounds are listed according to crescent retention times in HP5 column.
ID: identification method, RI: retention index, nd: not detected, STD: pure standard coinjecton.
*Not identified isomer.
#Tentatively identified.
Results are expressed as TIC area relative percentage.

carried out on euphorbia honey so that any comparison with literature data is impossible. Although a greater number of samples are needed to achieve a classification of any floral marker, our data suggests that nonanal and the correspondent alcohol, nonanol seem to be floral markers for this honey since they look to be, in comparison with other samples, characteristic of EF honey.

The TY honey was the richest sample in VOCs since its volatiles chemical composition show the presence of 43 molecules, mainly represented by terpenoid compounds (28/43). Although ethyl phenyl acetate has been suggested as chemical marker for TY honey,[1] our results show the presence of this compound in AS, TY, and in EU1 samples. Alessandrakis et al.[41] proposed phenylacetaldehyde as a chemical marker of TY honey, along with other compounds. This compound was found also in our TY honey sample, but also in almost all examined samples. On the other hand the high concentration of thymol derivatives has been linked to an adulteration in thyme honey by thyme essential oil.[42]

The polar compounds identified in the Moroccan honey samples by targeted HPLC analysis are reported in Table 3. Several compounds belonging to phenolic derivatives (4-hydroxybenzoic acid, methyl syringate and methyl benzaldehyde), sugar derivatives (kojic acid and 5-hydroxymethylfurfural, HMF), amino acids (tyrosine and phenylalanine), and isoprenoids ([E/E]-abscisic acid ABA) were also detected, along with lumichrome and caffeine. The aromatic amino acids detected in the samples were phenylalanine and tyrosine and were present in very varying amounts: tyrosine ranged from 4.8 ± 0.2 to 350.0 ± 12.9 mg/kg in TY and AS honeys, respectively; the highest amount of phenylalanine was found in MF2 honeys (152.8 ± 6.3 mg/kg) while TY showed the lower amount (4.8 ± 0.2 mg/kg). The detected phenolic compounds are not useful for confirming unifloral origins because 4-hydroxybenzoic acid and methyl benzaldehyde are very common compounds in honey and methyl syringate can be marker for specific honeys, but only at higher concentrations than those detected in the analyses Moroccan honeys. HMF is the compound typically evaluated as a marker of heating treatments or inadequate storage and a maximum value of 40 mg/kg is normally established by the international laws. The 10 samples generally showed a low amount of HMF, except OR, CI and MF2 samples where it ranged between 18.7 ± 1.2 and 15.8 ± 1.3 mg/kg. The high HMF amounts in OR sample, also detected by GC analysis, explains the unusual $L^*C_{ab}^*h_{ab}^\circ$ values that describe a darker honey for this typology of honey. Kojic acid was reported in Manuka and Coffea spp. honeys.[43] Similar amounts of aromatic amino acids were found in other unifloral honeys.[44] Among the other compounds, the presence of caffeine in sample CI confirmed its botanical origin because this alkaloid has been reported to be a possible chemical marker for Citrus spp. honeys, alongside Coffea spp. honeys.[43,45]

Table 3. Targeted polar compound dosed by HPLC-DAD (mg/kg, mean ± SD).

Sample	AS	CI	CT	EF	EU1	EU2	MF1	MF2	OR	TY
(E/E)-ABA	nd	nd	nd	nd	2.3 ± 0.1	nd	tr	nd	nd	nd
(Z/E)-ABA	nd	nd	nd	nd	2.8 ± 0.5	nd	2.0 ± 0.1	nd	nd	nd
4-hydroxy benzoic Acid	tr	3.2 ± 0.2	nd	tr	11.6 ± 0.2	nd	4.1 ± 0.1	nd	nd	tr
Caffeine	nd	2.6 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd
HMF	8.6 ± 1.0	16.0 ± 2.5	4.3 ± 0.3	2.9 ± 0.3	4.5 ± 0.5	9.9 ± 0.3	11.6 ± 1.4	15.8 ±1.3	18.7 ± 1.2	14.0 ± 1.0
Kojic acid	nd	3.3 ± 0.2	2.1 ± 0.2	nd	nd	nd	nd	nd	tr	3.0 ± 0.3
Kynurenic acid	3.6 ± 0.1	nd	nd	nd	3.0 ± 0.3	nd	nd	nd	nd	nd
Lumichrome	nd	nd	nd	nd	tr	nd	4.2 ± 0.1	nd	nd	nd
Methyl benzaldehyde	tr	nd	tr	0.3 ± 0.1	0.5 ± 0.1	tr	0.4 ± 0.0	nd	nd	tr
Methyl syringate	nd	nd	nd	nd	tr	nd	3.4 ± 0.2	nd	3.7 ± 0.4	1.3 ± 0.2
Phenylalanine	112.9 ± 4.6	116.9 ± 7.5	64.4 ± 2.1	26.8 ± 2.0	45.5 ± 1.9	169.1 ± 9.9	33.2 ± 2.5	152.8 ± 6.3	7.2 ± 0.4	4.8 ± 0.2
Tyrosine	350.0 ± 12.9	313.9 ± 33.3	149.1 ± 13.1	57.0 ± 4.0	262.7 ± 35.8	330.3 ± 15.8	4.2 ± 0.2	254.0 ±13.5	7.6 ± 0.9	294.2 ± 5.5

Conclusion

The antioxidant capacity of the ten Moroccan honeys was determined by two different in vitro assays, namely the FRAP and DPPH tests. A comparison of the antioxidant values with those of honey samples from the same floral origin, but from other countries, was carried out. Overall the Moroccan samples showed a slightly lower antioxidant capacity than the literature data from other conuntries. Our study confirms that polyphenols are mainly responsible for the antioxidant capacity, with significant ($P \leq 0.05$) correlation factors found by plotting DPPH values, as well as FRAP values, versus total polyphenols. In addition significant correlation factors (negative) were found between lightness (L*) and both DPPH and FRAP values. In addition to their important contributions to the sensory characteristics and the potential biomedical activities, the honey volatiles are a powerful tool for the determination of floral origin. The fingerprints of honey volatiles that we recorded, together with the targeted HPLC analysis on polar compounds, supports the declared botanical origin of the samples. In addition, the VOCs analysis suggested a possible contamination of thyme honey with thyme essential oil.

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