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ORIGINAL RESEARCH ARTICLE

Preliminary characterization of a Moroccan honey with a predominance of *Bupleurum spinosum* pollen

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Honey with *Bupleurum spinosum* (zandaz) as a main pollen source has not been the subject of previous detailed study. Therefore, twelve Moroccan samples of this honey were subjected to melissopalynological, physicochemical and microbiological quality characterization, as well as antioxidant activity assessment. From a quality point of view, almost all samples were within the limits established by Codex Alimentarius, and/or the European legislation. All samples presented predominance of *B. spinosum* pollen (more than 48%). Relatively high levels of trehalose (1.3–4.0 g/100 g) and melezitose (1.5–2.8 g/100 g) were detected. Those sugars, not common in monofloral honeys, could be used as an important factor to discriminate zandaz honey. Flavonoid content correlated positively with the honey color, melanoidin and polyphenol content, and negatively with the IC_{50} values of scavenging ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) free radicals, while proline amount correlated negatively with IC_{50} values of nitric oxide scavenging activity and chelating power. This correlation supports the use of anti-oxidant activities as important variables for PCA (principal component analysis). Both components explained 70% from the given data, and showed certain homogeneity upon analyzed samples independent of the region, suggesting the importance of *B. spinosum* nectar in the resulting honey characteristics.

Caracterización preliminar de una miel marroquí con predominio del polen de *Bupleurum spinosum*

La miel con *Bupleurum spinosum* (pendejo) como principal fuente de polen no ha sido objeto de un estudio detallado previo. Por lo tanto, doce muestras marroquíes de esta miel fueron sometidas a una caracterización melisopalinológica, físico-química y microbiológica, así como a la evaluación de su actividad antioxidante. Desde el punto de vista de la calidad, casi todas las muestras estaban dentro de los límites establecidos por el Codex Alimentarius y/o la legislación europea. Todas las muestras presentaron predominio de polen de *B. spinosum* (más del 48%). Se detectaron niveles relativamente altos de trehalosa (1.3-4.0 g/100 g) y melecitosa (1.5-2.8 g/100 g). Esos azúcares, no comunes en mieles monoflorales, podrían ser utilizados como un factor importante para discriminar la miel de pendejo. El contenido de flavonoides se correlacionó positivamente con el color de la miel, el contenido de melanoidina y polifenol y negativamente con los valores de IC_{50} de la actividad de barrido de los radicales libres de ABTS (2,2'-azino-bis (3-etilbenzotiazolina-6-sulfónico), mientras que la cantidad de prolina se correlacionó negativamente con los valores de IC_{50} de la actividad de barrido de ON (óxido nítrico) y el poder quelante. Esta correlación apoya el uso de las actividades antioxidantes como variables importantes para el APC (análisis de componentes principales). Ambos componentes explican el 70% de los datos obtenidos y muestran cierta homogeneidad en las muestras analizadas independientemente de la región, lo que sugiere la importancia del néctar de *B. spinosum* en las características de la miel resultante.

Keywords: *Bupleurum spinosum*; Zandaz honey; melissopalynology; physicochemical analysis; antioxidant activity; botanic origin

Introduction

The floral origin determination of honey is very important in the field of apicultural research, due to its direct link with customer demand. For the purpose of consumer protection, the honey floral origin labeling has been required by the European Community legislation, i.e., Directive EEC/74/409 amended by the Proposal COM/95/0722 (1996).

Melissopalynolgical, sensory and physico-chemical analyzes together are needed for establishing the botanical denomination of a honey, because when considered individually, each one has its own limitations. Regarding melissopalynolgical analysis, whereas some honey types need high percentage of pollen to be considered as monofloral (90% for chestnut honey), for others only 15% is sufficient to declare their botanical origin

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(e.g., *Lavandula* spp.) (Gomes, Dias, Moreira, Rodrigues, & Estevinho, 2010).

Physico-chemical parameters are also important in the determination of the botanical origin of honey and are widely used in routine honey analysis. Persano Oddo et al. (2004) reported that color, electrical conductivity, specific rotation, diastase, acidity, fructose and glucose content have the most important value in discriminating honey origins. In addition, the physico-chemical analysis: sugars (de la Fuente, Ruiz-Matute, Valencia-Barrera, Sanz, & Martínez Castro, 2011); volatile compounds (Bouseta, Scheirman, & Collin, 1996); phenols (Escriche, Kadar, Juan-Borrás, & Domenech, 2014); and minerals (Anklam, 1998) amongst others, is largely influenced by honey variability (honey seasonal, geographical variability, among other factors) (Persano Oddo et al., 2004). The use of one cannot be sufficient in itself, and the discriminatory power only increases with the number of parameters used (Persano Oddo et al., 2004).

The whole image of the botanical origin can be established only, when the sensory correspondence is evaluated (Persano Oddo et al., 2004); because this tool can exclude some samples from being characterized as monofloral, based on the other parameters, if some botanic components altering the sensory characteristics are detected (Piana et al., 2004). Nevertheless, sensory evaluation is always linked to inherent subjective factors.

The main goal of the present work is to establish a first screening of melissopalynological and physicochemical characterization and, at same time some, to determine the *in vitro* antioxidant activity of 12 samples of a Moroccan honey with *Bupleurum spinosum* as the main pollen source. In Morocco, this honey is called zandaz, zentaz, aguerbaz or airbaz honey, referring to the berbere name of *B. spinosum* Gouan (Syn. *Bupleurum fruticescens* subsp. *spinosum* (Gouan) O. Bolòs & Vigo) (Figure 1(a)). This species can be found in the siliceous high peaks of eastern Anti-Atlas, generally above 2000 m a.s.l. (Peltier, 1983). Some authors also reported the presence of this species at high altitudes of the High Atlas (above approximately 2900 m a.s.l.) and in some mountains of the middle Atlas (Nassif & El Amiri, 2011) where the samples of this study were mainly collected. The flowering period occurs during August–September (Khabbch, Libiad, & Ennabili, 2014).

Material and methods

The twelve honey samples (n = 12) were directly purchased from beekeepers and centrifuged upon receipt at the laboratory. In Table 1 the sample codes, production years, places of collection and their most predominant pollen types are presented.

Chemicals, reagents and equipment

Culture media were purchased from Biokar, (Paris, France) or Oxoid (Basingstoke, Hampshire, UK); peptone water (Oxoid; Basingstoke, Hampshire, UK); NaOH, Na_2CO_3, sodium phosphate were purchased from Pronalab, Madalena, Portugal; HCl was purchased from Fisher Scientific UK Ltd.; Loughborough, UK; KCl was purchased from BHD Prolabo; Leuven, Belgium; acetic acid, ascorbic acid, Folin–Ciocalteu, fuchsin, KOH, methanol, 2-propanol were purchased from Merck KGaA; Darmstadt, Germany; 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ferrozine, $FeSO_4$, gallic acid, NBT, ninhydrine, PMS, proline were purchased from Acros Organics; New Jersey, USA; $AlCl_3$ and anhydride acetic were purchased from Panreac Química, Montcada i Reixac; Barcelona, Spain; catechin was purchased from Fluka. Biochemika. Sigma-Aldrich; Steinheim, Germany; glucose, fructose, sucrose, maltose, turanose, trehalose, melezitose, were purchased from Sigma Aldrich Chimie GmbH, Riedstr; Steinheim, Germany; H_2SO_4, sodium acetate, starch, potassium iodide, I_2, sodium nitroprusside (SNP), were purchased from Riedel-de-Haen; Germany; acetic acid was purchased from CHEM-Lab; Belgium; optic micro-

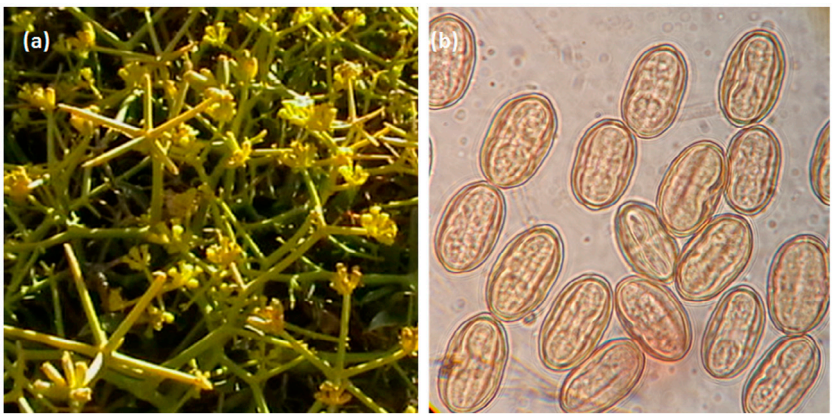

Figure 1. (a) Flowers of *B. spinosum* plant; (b) *B. spinosum* pollen under microscope (400×).

Table 1. Sample code, places of collection, year of production and the most predominant pollen of twelve *B. spinosum* honey samples from Morocco.

Sample code	*B. spinosum* (PP)	SP Species (%)	IMP	MP	Region name	Harvest year
S1	58.9	*Opuntia* spp. (17.7)	*Epilobium* spp. (13.8) + *Eragrostis* spp. (3.5) + *Thymus* spp. (3.0) + Others (3.0)		Ait Bazza	2011
S2	63.0	*Thymus* spp. (19.8)	*Opuntia* spp. (8.6) + *Phoenix* spp. (8.6)		Ait Bazza	2013
S3	62.9	*Opuntia* spp. (18.8)	*Olea* spp. (9.2) + *Thymus* spp. (4.3) + Others (4.9)		Ait Bazza	2013
S4	53.7		*Cactus* spp. (4.4) + *Epilobium* spp. (13.0) + *Eragrostis* spp. (5.9) + *Opuntia* spp. (11.2) + *Thymus* spp. (7.5) + Others (4.4)		Ait Bouilloul	2013
S5	83.0		*Olea* spp. (7.1) + *Opuntia* spp. (9.9)		Ait Bouilloul	2013
S6	62.4	*Thymus* spp. (21.3)	*Eragrostis* spp. (6.3) + *Phoenix* spp. (6.3) + Others (3.7)		Ait Ali	2013
S7	68.9		*Cardus* spp. (8.0) + *Eucalyptus* spp. (4.4) + *Olea* spp. (10.4) + *Thymus* spp. (8.0)		Ait ali	2012
S8	55.6	*Cardus* spp. (25.3)	*Phoenix* spp. (3.1) + *Thymus* spp. (11.4) + Others (4.7)		Ait bourais	2011
S9	48.8	*Cardus* spp. (24.5) + *Olea* spp. (17.4)	*Phoenix* spp. (6.5)	Others (2.8)	Boulemane	2011
S10	71.1	*Cardus* spp. (22.2)	*Acacia* spp. (3.9) + *Phoenix* spp. (3.9)		Bouiblane	2013
S11	65.0	*Cardus* spp. (30.8)	+ Others (4.2)		Bouiblane	2013
S12	56.8	*Cactus* spp. (24.2)	*Acacia* spp. (7.0) + *Phoenix* spp. (12.0)		Bouiblane	2013

Notes: PP – predominant pollen (>45%); SP – secondary pollen (16–45%); IMP – important minor pollen (3–15%); MP – minor pollen (<3%).

scope (Leitz Messtechnik GmbH; Wetzlar, Germany); HPLC: Dionex ICS3000 equipment; microplate reader (Tecan Infinite M200; Tecan, Austria); potentiometer combined with glass electrode (Thermo Electron Corporation, Orion 3 STAR; USA); analytical balance (Shimadzu, Aux 220; Philippines); electric furnace (Cassel; Portugal); conductivity meter (Thermo Electron Corporation, Orion 3 STAR; USA); Abbe Refractometer (HANNA, HI968601; Romania); HPLC (Hitachi, LaChrom Elite; Japan).

Melissopalynological analysis

The analysis of the honey samples' pollen qualitative and quantitative spectrum was performed according to the International Commission for Bee Botany (ICBB), as previously described in detail (Louveaux, Maurizio, & Vorwohl, 1978). Pollen identification and count were carried out using a light microscope (Leitz Messtechnik GmbH; Wetzlar, Germany) with 400× and 1000× objectives, the latter being used when greater detail was required for the morphological identification. For each honey sample, we counted and analyzed a minimum of 1000 pollen grains. Frequency classes were determined twice for each sample and designated as dominant pollen (>45% of a specific pollen type), secondary pollen (16–45%), important minor pollen (3–15%) and minor pollen (<3%).

Microbiological evaluation

The microbiological quality of the honey samples was examined by determining the counts of aerobic mesophilic bacteria, Enterobacteriaceae and sulfite-reducing *Clostridium* spp. The counts of aerobic mesophilic bacteria were done using the Plate Count Agar medium as described in the standard NP-4405:2002 (NP-4405:2002, 2002). The determination of Enterobacteriaceae and sulfite-reducing *Clostridium* spp. were performed according to the standards ISO 21528-2:2004 and ISO 15213:2003, respectively (ISO 21528-2:2004, 2004; ISO 15213:2003, 2003). Homogenates of 10 g of each honey sample with 90 ml of peptone water (Oxoid) were subject to decimal dilutions that were inoculated in appropriate medium. The viability counts were done in triplicate.

Physico-chemical analysis

All measurements described in this section were performed in triplicate, except the sugar content that was performed in duplicate. Results were then expressed as mean ± SD. The parameters pH, free acidity, lactonic acidity, total acidity, ash content, electrical conductivity, moisture, proline content, diastase activity and HMF content, were assessed following the Harmonized methods of the International Honey Commission (Bogdanov, 2002).

Honey color and melanoidins estimations

Color was determined by measuring the absorbance of aqueous solutions (10 g of honey in 20 ml of distilled water) at 635 nm (A_{635}) in a Shimadzu spectrophotometer (Naab, Tamame, & Caccavari, 2008). The mm Pfund values and honey color were obtained using the follow-

ing algorithm, mm Pfund = $-38.7 + 371.39 \times A_{635}$. Additionally, honey color was determined by spectrophotometry by calculating net absorbance ($A_{560} - A_{720}$). Melanoidin content was estimated based on the browning index (net absorbance = $A_{450} - A_{720}$) (Brudzynski & Miotto, 2011a). Spectrophotometric measurements were performed in a 1 cm quartz cell; results were expressed as absorbance units (AU).

Sugar content

The method used was in accordance with that reported by Anjos, Campos, Ruiz, and Antunes (2015).

Total content of polyphenols

The total polyphenol content was determined in honey solutions by the Folin–Ciocalteau method (Singleton & Rossi, 1965). To test sugar interference, a sugar solution was made taking into account the sugar profile of each sample and two further dilutions were prepared for each one. The prepared sugar solutions were in accordance with the sugar content in the 50% honey solution prepared for pholyenols' estimation. Experiments were made in triplicates, and the results were expressed as mean ± SD mg equivalent of gallic acid/100 g honey, after the elimination of sugar interference.

Total flavonoid content

The total flavonoid content (TFC) of different honey samples was determined using the aluminum chloride assay according to Samatha, Shyamsundarachary, Srinivas, and Swang (2012). To study a possible interference of sugars in this assay, the sugar solution made for polyphenol content estimation was also used. To avoid possible interaction of sample color with the reaction's reagents, the blank was constituted by water (substituting the other reaction's components) and the sample as reported by Sancho et al. (2016), and the resulting absorbance was subtracted from the whole reaction absorbance before TFC estimation. The TFC was expressed in mg of chatechin equivalents (CE) per 100 g of honey as the mean of three triplicate ± SD.

Antioxidant activity

All measurements described in this section were performed in triplicate, and the results were expressed as the mean ± SD.

Capacity for scavenging 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

The determination of ABTS radical scavenging capacity was carried out as reported previously (Miguel, Nunes, Dandlen, Cavaco, & Antunes, 2010). Briefly, the ABTS radical was generated by the reaction of ABTS aqueous solution (7 mM) with Potassium persulfate ($K_2S_2O_8$) (2.45 mM) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.7 at room temperature. The samples (25 µl) were added to 275 µl ABTS and the absorbance at 734 nm was read after 6 min. Several concentrations of samples were used and the percentage inhibition calculated by applying the formula: $[(A_0 - A_1/A_0) \times 100]$, plotted against sample concentration, and IC_{50}, or half maximal inhibitory concentration was determined. A_0 is the absorbance of the negative control (blank sample containing the same amount of water and ABTS solution); A_1 is the absorbance of the sample.

Nitric oxide scavenging capacity

The nitric oxide (NO) scavenging activity of samples was measured according to the method described by Ho, Tang, Lin, and Liew (2010). NO was generated from SNP and was measured by using the Griess reagent (*N*-(1-Naphthyl) ethylenediamine). In this method, 50 µl of serially diluted honey sample were added to 50 µl of 10 mM SNP ($Na_2[Fe(CN)_5NO]$) in phosphate buffer saline (PBS) into a 96-well plate, which was then incubated at room temperature for 90 min. Finally, an equal volume of Griess reagent was added to each well and the absorbance was read at 546 nm. Several concentrations of samples were made and the percentage inhibition calculated using the formula: $[1 - (A_{sample} - A_{sample\ blank})/(A_{control} - A_{control\ blank})] \times 100$, where $(A_{sample} - A_{sample\ blank})$ is the difference in the absorbance of a sample, with or without 10 mM SNP, and $(A_{control} - A_{control\ blank})$ is the difference in the absorbance of the PBS control, with or without 10 mM SNP. The inhibition percentage was plotted against sample concentration and IC_{50} was determined (concentration of sample able to scavenge 50% of NO free radical).

Chelating metal ions

The degree of chelating of ferrous ions by honey samples was evaluated according to Miguel et al. (2010). Briefly, samples were incubated with 0.05 ml of Iron(II) chloride, $FeCl_2{\cdot}4H_2O$ (2 mM). The addition of 0.2 ml of 5 mM ferrozine initiated the reaction, and after 10 min, the absorbance at 562 nm was measured. An untreated sample served as control. The percentage of chelating ability was determined according to the following formula: $[(A_0 - A_1)/A_0 \times 100]$, in which A_0 is the absorbance of the control and A_1 the absorbance of honey sample. The values of IC_{50} were determined as reported above for the previous assays.

Superoxide anion radical ($O_2^{\cdot-}$) scavenging activity (non-enzymatic method)

Measurements of $O_2^{\cdot-}$ scavenging activity of honey samples were based on Soares (1996) method. $O_2^{\cdot-}$ were

generated in a non-enzymatic phenazine methosulfatenicotinamide adenine dinucleotide (PMS-NADH) system based on the oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). The sample's IC_{50} was then determined in triplicate for each sample.

Statistical analysis

One-way ANOVA was performed to determine significant differences among samples with a Scheffe *post hoc* test (95% confidence level). The results were also subjected to a multivariate analysis (principal component analysis). All experimental data were analyzed using StatSoft Statistics (version 7).

Results

Melissopalynological analysis

The main question facing the analysis of pollen profile for monofloral honey determination is: at which level of a pollen species is necessary to reach for declaring the discriminating point between monofloral and multifloral honey. In all the analyzed samples of the present study, pollen grains of *B. spinosum* (Figure 1(b)) were predominant (Table 1), with percentages ranging from 48.8%, in the sample S9, to 83.0%, in the sample S5.

Regarding the previously mentioned limitations of melissopalynology, and the variability in the remaining pollens of each analyzed sample, the conclusion will not be final, and further detailed study will be needed. From the results of the present work it is possible to see that *Thymus* pollens could be detected in six samples, being the secondary pollen in two of them with percentages of 19.8% (S2) and 21.3% (S6), while *Cardus* spp. was secondary in S8 (25.3%), S9 (24.5%), S10 (22.2%), and S11 (30.8%). For the remaining samples the secondary pollen was *Olea* spp. (S9), *Cactus* spp. (S12), *Opuntia* spp. (S1 and S3) (Table 1). The occurrence of other pollens was also seen, namely: *Acacia* spp.; *Epilobium* spp.; *Eragrostis* spp.; *Eucalyptus* spp.; and *Phoenix* sp., although with percentages below 15% (important minor pollen).

Pollen qualitative analysis provides important data for honey characterization, regarding the occurrence of nectariferous plants, harvest season, honey extraction and filtration, the occurrence of fermentations, as well the geographical origin (Von Der Ohe, Persano Oddo, Piana, Morlot, & Martin, 2004). Keeping in mind the absence of any previous reference of this honey, the last indication is, in fact, one of the limitations of the present study to declare the "monoflorality" of this honey: honey production in different countries leads to the diffusion of different secondary plant nectars, and so changes the discriminatory percentage of the dominant pollen. This makes the 'ideal reference model' of monofloral type different from one analyst to another. This fact can be clearly seen in the variability of the same European monofloral honey, in terms of their pollen profile, from country to country (Persano Oddo et al., 2004).

More discussions about pollen profile will be addressed in the following sections of this work, as well as its possible influence on other honey parameters.

Microbiological evaluation

Microbial counts in organic honey samples are shown in Table 2. All honey samples had aerobic mesophilic bacteria, although the bacterial load was variable, with the highest level was recorded in sample S7. All samples were negative for Enterobacteriaceae and sulfite-reducing *Clostridium* spp. The counts of aerobic mesophilic bacteria in the samples S6, S7 and S12 suggest that the hygiene conditions of the honey production must be improved. The low numbers of aerobic mesophilic bacteria in the majority of the honey samples and the absence of Enterobacteriaceae and sulfite-reducing *Clostridium* spp. do, however, indicate the safe consumption of this honey.

Physico-chemical analyzes

Tables 2 and 3 present the results of the physico-chemical parameters analyzed in the twelve samples of honey. From a quality point of view, the analyzed samples were, in most cases, within European limits (EU Council, 2002) or the Codex Alimentarius (2001), and showed no alarming values. However a significant variability was observed.

In all the analyzed samples, pH values, with 3.82 ± 0.16 as a mean didn't show any outset value, to be in accordance with Malika, Mohamed, and Chakib (2005) and Bettar et al. (in press), who analyzed Moroccan honeys from different floral origin. The same observation made for lactonic acidity, free acidity, and the total acidity, for which the mean values were, respectively, 16.58 ± 1.40, 25.70 ± 6.46 and 42.28 ± 6.76 meq/kg. It has to be mentioned that (EU Council, 2002) fixed honey free acidity to values bellow 50 mEq/kg, which is important for avoiding undesirable fermentations.

Another determinant parameter in a possible fermentation process (Mendes, Proença, Ferreira, & Ferreira, 1998) is the moisture percentage, that ranged from 17.3% (S7) to 20.5% (S11) (Table 3), exceeding so 20%, or the maximum allowed by (Codex Alimentarius, 2001), and the EC directive (EU Council, 2002). Furthermore sample S4, S5 were also above the required limit.

According to the European Unit EU Council (2002), the values of electrical conductivity and ash must not exceed 800 μS/cm and 0.6%, respectively. Amongst the studied samples (Table 2), only S12 had an electrical conductivity higher than the maximum recommended value, which was coincident with the highest percentage of ash (0.37%). Indeed, the electrical conductivity depends on the ash and acid content, which, per se,

Table 2. Some physicochemical parameters and microbial analysis of twelve *B. spinosum* honey samples from Morocco.

Samples	AMB (CFU/g)*	pH	Ash (%)	Electrical conductivity (µS/cm)	Free acidity (meq/kg)	Lactonic acidity (meq/kg)	Total acidity (meq/kg)	Diastase activity (Shade units/g)
S1	<20	4.16 ± 0.01c	0.28 ± 0.01bc	635.67 ± 1.15f	14.15 ± 2.85a	16 ± 3.77ab	30.15 ± 0.96a	18.65 ± 0.38b
S2	<10	3.787 ± 0.04ab	0.19 ± 0.02a	443.33 ± 5.03b	27.85 ± 4.89bc	15.5 ± 1.5ab	43.35 ± 4.2bc	20.71 ± 1.3bc
S3	30 ± 0.0	3.62 ± 0.05a	0.16 ± 0.01a	405.33 ± 3.05a	29.7 ± 2.06bc	18.5 ± 3b	48.2 ± 1.04c	23.91 ± 1.2c
S4	<10	3.88 ± 0abc	0.18 ± 0.01a	453 ± 1b	28.25 ± 3.57bc	18 ± 3.77b	46.25 ± 7.09bc	30.3 ± 1.86d
S5	<10	3.61 ± 0.58a	0.2 ± 0.01ab	437.67 ± 2.08b	25.6 ± 0.22c	16.5 ± 1.73ab	42.1 ± 1.91bc	23.24 ± 1.01c
S6	425 ± 35.4	3.9 ± 0.05abc	0.19 ± 0.02a	495.67 ± 4.16cd	18.5 ± 1.06a	18.5 ± 1.49b	37 ± 2.12ab	17.53 ± 0.89b
S7	2.4×10^3 ± 183	3.9 ± 0.04abc	0.18 ± 0.03a	486 ± 4.35c	15.75 ± 1.13a	15 ± 2.29ab	30.75 ± 1.25a	11.33 ± 0.05a
S8	55.0 ± 7.1	3.79 ± 0.02ab	0.23 ± 0abc	573.67 ± 7.23e	28.75 ± 2.21bc	17.5 ± 0.86ab	46.25 ± 2.86bc	27.71 ± 1.07d
S9	50.0 ± 14.1	3.95 ± 0.01bc	0.3 ± 0cd	639 ± 8.28f	24.75 ± 0.12c	16.5 ± 0.7ab	41.25 ± 0.6ab	21.01 ± 0.27bc
S10	<10	3.67 ± 0.05ab	0.22 ± 0.03abc	508 ± 6.55d	28.55 ± 1.91bc	16 ± 2.29ab	44.55 ± 1.36bc	29 ± 0.27d
S11	<10	3.7 ± 0.01ab	0.18 ± 0.01a	442 ± 2.64b	30.55 ± 1.13bc	14 ± 1.5a	44.55 ± 0.91bc	28.57 ± 0.12d
S12	225 ± 63.6	3.84 ± 0.02ab	0.37 ± 0.01c	833 ± 7.54g	36 ± 1.98bc	17 ± 2.59ab	53 ± 0.74c	21.6 ± 0.39c
Means ± SD		3.82 ± 0.16	0.22 ± 0.06	529.4 ± 122.7	25.70 ± 6.46	16.58 ± 1.40	42.28 ± 6.76	22.80 ± 5.55
Min–Max		3.61–4.16	0.16–0.37	405.3–833.0	14.15–36.00	14.00–18.50	30.15–53.00	11.33–30.30

Note: AMB – Aerobic mesophilic bacteria.
*Data represent the mean ± standard deviation of duplicate replicates of each honey sample and are expressed as Colony Forming Units per gram (CFU/g).

Table 3. Some physicochemical parameters of twelve *B. spinosum* honey samples from Morocco.

Samples	Moisture (%)	HMF (mg/kg)	Proline (mg/kg)	Melanoidins	Polyphenols (mg GAE/100 g)	Flavonoids (mg QE/100 g)	Color Pfund scale (mm)
S1	19.53 ± 0.23cd	5 ± 0.25e	763.06 ± 16.43a	0.63 ± 0.01b	61.36 ± 10.85a	13.53 ± 1.52bc	42.37 ± 2.24a Light extra amber
S2	19.8 ± 0.12d	1.07 ± 0.07a	926.77 ± 18.14b	1.25 ± 0.02d	101.86 ± 11.78cd	20.87 ± 1.31c	72.48 ± 1.9a Light amber
S3	19.73 ± 0.23cd	10.9 ± 0.64e	970.43 ± 44.4b	0.93 ± 0.01c	85.03 ± 9.32b	20.65 ± 2.1c	63.46 ± 1.19a Light amber
S4	20.4 ± 0.12e	6.45 ± 0.21d	1007.49 ± 7.58bc	1.38 ± 0.01e	123.65 ± 3.58d	18.79 ± 2.59c	89.49 ± 0.81a Amber
S5	20.27 ± 0.11e	1.18 ± 0.06a	800.85 ± 40.78a	1.55 ± 0.015f	116.85 ± 7.75cd	20.3 ± 2.59c	116.91 ± 6.56b Dark amber
S6	19.07 ± 0.11c	5.35 ± 0.1cd	709.95 ± 36.85a	0.92 ± 0.01c	91.77 ± 5.86b	14.73 ± 0.93c	61.34 ± 1.43b Light amber
S7	17.27 ± 0.11a	15.97 ± 0.09f	717.8 ± 114.24a	0.56 ± 0.02a	59.32 ± 2.93a	5.11 ± 1.36ab	28.46 ± 2.28c Extra white
S8	17.93 ± 0.3ab	4.89 ± 0.09c	847.32 ± 22.3ab	0.61 ± 0.01b	61.08 ± 5.96a	9.86 ± 1.14c	32.21 ± 1.23cd White
S9	17.47 ± 0.09a	14.7 ± 0.63f	763.59 ± 7.18a	0.96 ± 0.01c	89.46 ± 2.19bc	4.87 ± 3.85a	44.24 ± 0.95d Light extra amber
S10	18.27 ± 0.11b	2.98 ± 0.11b	1005.35 ± 39.07bc	0.68 ± 0.02b	66.58 ± 7.15a	2.44 ± 1.21a	28.44 ± 0.18d White
S11	20.53 ± 0.11e	2.76 ± 0.08b	1202.53 ± 42d	0.97 ± 0.01c	99.04 ± 3.83bc	21.5 ± 2.77c	72.46 ± 0.73e Light amber
S12	19.2 ± 0cd	5.38 ± 0.3cd	1179.98 ± 18.68cd	0.67 ± 0.01b	72.72 ± 2.86a	1.74 ± 2.11a	34.46 ± 0.89f Light extra amber
Means ± SD	19.12 ± 1.14	6.97 ± 5.16	907.93 ± 169.70	0.93 ± 0.32	85.73 ± 22.01	12.86 ± 7.73	57.20 ± 27.49
Min–Max	17.27–20.53	1.07–15.97	709.95–1202.53	0.56–1.55	59.32–123.65	1.74–21.5	28.44–116.91

reflects the presence of ions and organic acids; the higher their content, the higher the resulting conductivity (Feás, Pires, Estevinho, Iglesias, & De Araujo, 2010).

It is well known that honey diastase activity is one of the indicators telling about storage conditions and/or processing, and it is used for honey freshness confirmation in routine analysis because of its sensitivity to heat or inadequate storage condition, (Anklam, 1998). Nevertheless, its level also depends on the geographic and floral origins of samples (Fallico, Arena, Verzera, & Zappalà, 2006). All honey samples studied in the present work had higher diastase activity than the minimum required (8.0 Shade units/g) by European legislation (EU Council, 2002). Nevertheless it is noteworthy to refer that a great variability was found among samples (11.3 in S7 to 30.3 Shade units/g in S4) (Table 2).

Hydroxymethylfurfural (HMF) is one of the most known Amadori compounds, formed during the thermal treatments of carbohydrate-containing foods as a result of Maillard reaction, becoming another honey freshness indicator. This compound is found only in trace amount in fresh honey, with values bellow 40 mg/kg, the maximal value allowed by Codex Alimentarius (2001) and EU Council (2002). Based on this parameter, all the analyzed samples showed freshness signs, with values ranging from 1.1 mg/kg in S2 to 16.0 mg/kg in S7, collected in 2013 and 2011, respectively (Table 3).

In the present work, proline evaluation was followed according to the IHC method, although other methods can be used, as previously reported by Truzzi, Annibaldi, Illuminati, Finale, and Scarponi (2014). All honey samples contained more than the minimum acceptable proline concentration, i.e., 200 mg/kg (Hermosín, Chicón, & Cabezudo, 2003), nevertheless its amounts varied greatly among samples (710.0 mg/kg in S6 and 1202.5 mg/kg in S11) (Table 3).

Honey color is an indicator of the presence of compounds, such as polyphenols, terpenes and carotenoids (Naab et al., 2008). In the analyzed honey samples a great variability of color, determined at A_{635}, was observed: from extra white (28.5 mm Pfund) in S7 sample to dark amber (116.9 mm Pfund) in S5 (Table 3). A strong correlation was observed between color and melanoidin content ($r = 0.948$; $p < 0.01$) (Figure 1), confirming their participation in the resulting honey color (Brudzynski & Maldonado-Alvarez, 2015), and explaining the variability seen amongst the analyzed samples (Table 3). Melanoidins, or Maillard reaction products, estimated as a difference between two wavelengths ($A_{560} - A_{720}$), are multi-component polymers consisting of protein–polyphenol–oligosaccharide complexes, which along with phenols are responsible for the antioxidant activity of honey (Brudzynski & Miotto, 2011; Moussa, Saad, & Noureddine, 2012).

As the Folin–Ciocalteau reagents can not only oxidize phenolic molecules, but also non-phenolic organic compounds, (Pękal & Pyrzynska, 2014), such as reducing sugars, sugar effect was studied. For this, the assay reaction was applied on sugar solutions for each sample based on its sugar profiling, and the values were subtracted from total polyphenol estimation, to obtain the results illustrated in Table 3. The effect of dilution on sugar interference was also evaluated, being the assay initiated with solutions with sugar content equivalent to that of 50% honey solution, and 12.5%. This concentration was also that used for the estimation of polyphenols in honey samples. The results showed a clear interference of sugars when they were in concentration of 50%, with values between 16.83 ± 0.67, for S10, and 23.67 ± 2.35 mg GA equivalent/100 g honey, for S1. In fact, polyphenol estimation was not possible in 50% honey solution, and a 1/4 dilution was used, so the same was done on sugar solutions. A clear decrease in sugar interference was observed with dilution and the values passed from 19.26 ± 2.43 mg GAE/100 g as the mean of all sugar solutions 50% to 7.28 ± 3.13 mg GAE/100 g in the 1/4 dilution.

Without sugar effect elimination, polyphenol content ranged from 66.15 mg GAE/100 g (S1) to a maximum of 130.04 mg GAE/100 g (S4) (data not shown). These values were significantly lower than that reported for other Moroccan honey types (Aazza, Lyoussi, Antunes, & Miguel, 2014). In addition and taking into account the sugar interference, the respective values were even lower because they ranged from 61.36 ± 10.85 mg GAE/100 g (S1) to 123.65 ± 3.58 mg GAE/100 g (S4).

Several methods are used for the quantification of total flavonoids, in spite of the interferences of some reagents with some flavonoid classes, being therefore a limitation of the method in unknown samples analysis (Mammen & Daniel, 2012; Pękal & Pyrzynska, 2014). The same authors proved that rutin, luteolin and catechins seem to be more accurate with the sodium nitrite ($NaNO_2$) used in some methods for flavonoid determination. For this reason, in the present work catechin was used as standard. As expected from the polyphenol content results, flavonoid content was also reduced compared to other Moroccan honeys, and the values ranged from 1.74 to 20.87 mg CE/100 g of honey. The effect of sugar on flavonoid estimation in honey samples was also studied and no interference was detected, independent of the dilution (data not shown).

Positive correlations were obtained between color, melanoidin, phenol and flavonoid contents (Figure 2). These results reveal that polyphenols and melanoidins play a role in honey color. Correlations between phenols, melanoidins and flavonoids may be attributed to the fact that all these compounds absorb light in the visible range, as previously reported by Aazza et al. (2014) for Moroccan honeys of diverse floral origin. The samples S2, S4, S5 and S11 from Ait Bazza, Ait Bouilloul and Bouiblane respectively, presented the highest values of phenols, melanoidins and flavonoids (Figure 2). Additionally the color and the electrical conductivity give an indication of the botanical origin of honey.

Sugar content

Fructose and glucose are the major monosaccharides in honey, and their proportion depends on nectar delivering plant (Anklam, 1998). Their sum in the analyzed honey samples ranged from 56.9% in S1 to 69.9% in S8, (Table 4) which were in accordance with those reported by other authors that analyzed honey from different origin (Anjos, Campos et al., 2015; Shin & Ustunol, 2005). The concentration of fructose ranged from 34.4 g/100 g in S1 sample to 42.3 g/100 g in S3 and could be found 5 homogenous groups with statistic differences from the 12 analyzed samples (Table 4). Sample S1 had also the lowest concentration of glucose (22.2 g/100 g), while S8 had the highest amount of this monosaccharide (31.0 g/100 g).

Practically all samples had sucrose lower that the quantification limit (<0.2 g/100) except S12 that presented 4.3 g/100 g of sucrose, but still lower than the limit of 5% allowed by the Codex Alimentarius (2001) for this sugar. Its structural isomer, turanose, as well as maltose were not detected also, except for sample S4 which had 1.8 g/100 g of maltose.

Trehalose was the most important disaccharide present in the zandaz honey samples, for which the occurrence with such relative high levels is, generally, not usual in honeys (Anjos, Campos et al., 2015) and could be a discriminant characteristic of zandaz honey.

Another relevant characteristic in this honey type was the occurrence of melezitose, which is absent in unifloral honeys with the exceptions of heather, chestnut and cardoon (Aazza, Lyoussi, Antunes, & Miguel, 2013; Mateo & Bosch-Reig, 1997), but is very common, even with higher levels, in honeydew (Bogdanov, Ruoff, & Persano-Oddo, 2004). In the present work, and independent of the region where the samples were collected, high levels of this trisaccharide were detected (1.5–2.8 g/100 g).

From a discriminatory point of view, and as expected from pollen spectrum, even with a predominance of *B. spinosum* pollen, the results of physico-chemical characterization showed variability. This makes the task to declare the monoflorality or not of the so called zandaz honey more complicated.

It is well established that the above studied parameters, when recorded at extreme low or high values, can be used as a discriminatory pattern to support the melissopalynological results. Low values of G/W ratio and high values of color, electric conductivity, enzymes, pH and F/G ratio, when associated with more than 90% of chestnut pollen, constitute a discriminatory characterization for this monofloral honey (Persano Oddo et al., 2004). In contrast, and in the same reference, the physico-chemical pattern of lavender unifloral honey is characterized by low values of electrical conductivity, slightly low F + G and high values of sucrose. Focusing on the means of some analyzed parameters, lead to the easy misclassification of the samples with other honey types. The electrical conductivity and color are similar to those of dandelion, and eucalyptus honeys (Persano Oddo et al., 2004).

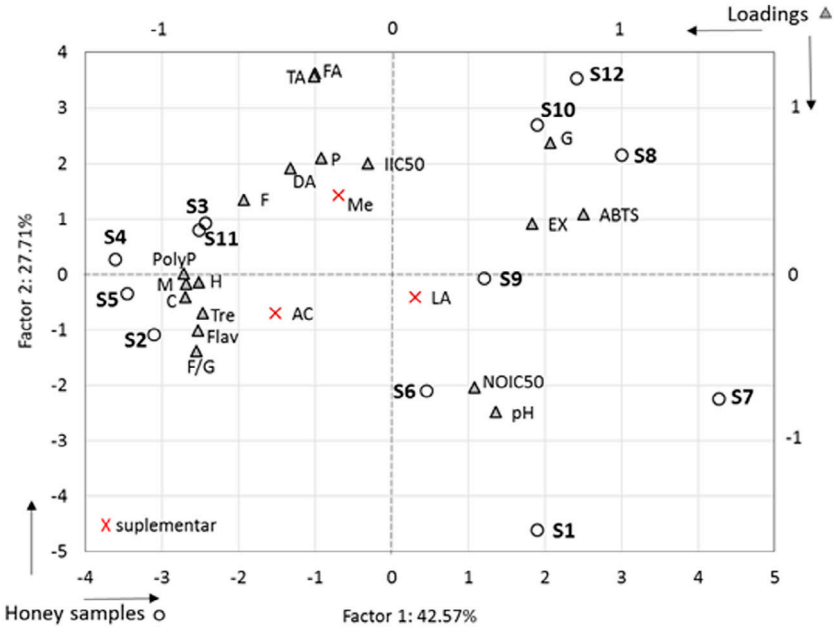

Figure 2. Principal component analysis with all measured parameter: projection of loadings and honey samples in the same system of vectors. Legend: FA – free acidity (mEq/kg); LA – lactonic acidity (mEq/kg); TA – total acidity (mEq/kg); EX – electrical conductivity mS/cm; AC – ash content %; H – water content %; C – color (Pfund); M – melanoidines; DA – diastase activity (shade number); P – proline mg/kg; PolyP – phenol (mg GAE/100 g); Flav – flavonoids mg QE/100 g; ABTS – ABTS IC_{50} (mg/ml); IIC50 – iron chelating power IC_{50} (mg/ml); NOIC50 – nitric oxide IC_{50} (mg/mL); F – fructose (g/100 g); G – glucose (g/100 g); Me – melezitose (g/100 g); Tre – trehalose (g/100 g); F/G – fructose/glucose ratio.

Table 4. Sugar content (g/100 g) of twelve *B. spinosum* honey samples from Morocco.

Samples	Fructose	Glucose	Sucrose	Maltose	Melezitose	Trehalose	Turanose	Fructose+ glucose	Fructose/ glucose
S1	34.7 ± 1.8a	22.2 ± 1.0a	<0.2	<0.2	2.8 ± 0.1b	2.1 ± 0.1ab	<0.2	56.9 ± 2.8cd	1.56 ± 0.01a
S2	40.0 ± 0.6d	23.4 ± 0.4b	<0.2	<0.2	1.7 ± 0.1a	4.7 ± 0.1b	<0.2	63.4 ± 1.0de	1.71 ± 0.00ab
S3	42.3 ± 0.2e	24.4 ± 0.1b	<0.2	<0.2	1.5 ± 0.1a	4.1 ± 0.1ab	<0.2	66.7 ± 0.4e	1.73 ± 0.00b
S4	39.8 ± 0.4d	24.6 ± 0.2b	<0.2	1.8 ± 0.1	2.2 ± 0.3ab	4.0 ± 0.1ab	<0.2	64.4 ± 0.6cd	1.62 ± 0.01ab
S5	38.7 ± 0.0cd	24.2 ± 0.0b	<0.2	<0.2	2.0 ± 0.1ab	2.2 ± 1.7ab	<0.2	62.9 ± 0.1cd	1.60 ± 0.00ab
S6	38.1 ± 0.3cd	26.8 ± 0.3d	<0.2	<0.2	1.7 ± 0.0a	3.1 ± 0.0ab	<0.2	64.9 ± 0.5b	1.42 ± 0.00b
S7	36.5 ± 0.8b	28.5 ± 0.2d	<0.2	<0.2	2.0 ± 0.1ab	1.6 ± 0.0a	<0.2	65.0 ± 1.0a	1.28 ± 0.02b
S8	38.9 ± 0.6cd	31.0 ± 0.3e	<0.2	<0.2	1.8 ± 0.2a	1.3 ± 0.0a	<0.2	69.9 ± 1.0a	1.25 ± 0.01b
S9	39.1 ± 0.5cd	27.0 ± 0.2d	<0.2	<0.2	2.0 ± 0.1ab	2.1 ± 0.0ab	<0.2	66.1 ± 0.4b	1.45 ± 0.01b
S10	36.5 ± 0.2b	28.7 ± 0.0d	<0.2	<0.2	1.9 ± 0.1a	2.0 ± 0.0ab	<0.2	65.2 ± 0.2a	1.27 ± 0.01b
S11	38.4 ± 1.8cd	25.4 ± 1.1c	<0.2	<0.2	1.9 ± 0.0a	4.0 ± 0.26ab	<0.2	63.8 ± 2.8bc	1.51 ± 0.01ab
S12	37.6 ± 1.9bc	28.9 ± 0.3d	4.3 ± 0.2	<0.2	2.0 ± 0.2a	1.1 ± 0.0a	<0.2	66.5 ± 2.1a	1.30 ± 0.05b
Mean ± SD	38.4 ± 2.0	26.3 ± 2.7			2.0 ± 0.3	2.7 ± 1.2		64.6 ± 3.1	1.48 ± 0.17
Min–max	34.7–42.3	22.2–31.0			1.5–2.8	1.1–4.7		56.9–69.9	1.25–1.74

Antioxidant activity

The antioxidant activities of the honey samples were measured using four different methods: three assays assessed the capacity for scavenging free radicals (ABTS, superoxide, and NO) and one assay to assess samples' capacity for chelating metal ions. The results are shown in Table 5. Sample S2 was the best for scavenging ABTS free radicals. The sample S4 was also a good scavenger of ABTS free radicals, along with S5. Samples S1, S2 and S6 were those honey samples that presented the best capacity for scavenging the superoxide anion radicals, whereas S9, S10 and S12 where those which had the best capacity for scavenging NO radicals. The capacity for chelating metal ions was higher in samples S1, S2 and S6 (Table 5).

The capacity for scavenging ABTS free radicals by samples, measuring the absorbance at 734 nm, changes proportionally with incubation time, nevertheless Sancho et al. (2016) observed that there were no differences between activity measuring the absorbance at 60 min and calculated activity at 60 min, by measuring absorbance at 6 min, independent on the type of honey and extract. The activity in the present work was followed by measuring the absorbance after 6 min of reaction.

The antioxidant activity of honey samples depends on the honey floral origin, because of the differences in the content of plant secondary metabolites such as polyphenolics, and enzyme activities (Alvarez-Suarez, Giampieri, & Battino, 2013; Sousa et al., 2016). However, the same monofloral honey may present diverse antioxidant activities as found in the present work, since the polyphenol content may vary. Such findings were already reported by Aazza et al. (2014) for the same monofloral honeys (thyme and jujube) from Morocco of different geographic origins. The results of the present work also show that the capacity for scavenging free radicals depends on the type of radicals used, whereby it is important to discriminate the type of radicals that honey samples are able to scavenge.

Flavonoid content correlated positively with the honey color, melanoidins and phenol content but negatively with the IC_{50} values of the scavenging capacity of ABTS free radicals (Figure 2). Proline amount positively correlated with the IC_{50} values of the capacity for chelating metal ions and scavenging superoxide free radicals. The negative correlation between HMF content and antioxidant activity (e.g., IC_{50} values for ABTS assay) was not as evident as observed for phenols and antioxidant activity. The weak correlation between those two factors was already reported (Gheldof, Wang, & Engeseth, 2002) for honey samples, being the breakdown products of HMF the most probable contributors for the antioxidant activity of honey samples.

The negative correlation between IC_{50} values of NO scavenging activity and proline content may reveal an important role of this amino acid on the capacity for scavenging that free radical. Antioxidant activity of some amino acids (histidine, taurine, glycine, alanine, proline) has been reported (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005; Wu, Shiau, Chen, & Chiou, 2003). Such property of proline may indicate some anti-inflammatory activity of these honeys since NO appears at higher concentrations during inflammatory reactions. Compounds with the capacity for scavenging NO can be therefore considered as possessing antioxidant and anti-inflammatory properties. Color was strongly correlated with the capacity of samples to scavenge ABTS free radicals (Figure 2). The same happened with the capacity for scavenging these free radicals and melanoidins as reported before. The correlation of color and antioxidant activity as well as between melanoidin and

Table 5. Antioxidant activity and capacity for scavenging free radicals of twelve *B. spinosum* honey samples from Morocco.

Samples	ABTS IC_{50} (mg/ml)	Superoxide IC_{50} (mg/ml)	Nitric oxide IC_{50} (mg/ml)	Iron chelating power IC_{50} (mg/ml)
S1	7.06 ± 0.26b	9.92 ± 0.05a	167.03 ± 16.78c	9.92 ± 0.05a
S2	4.94 ± 0.13a	7.97 ± 0.1a	121.25 ± 0.71ab	7.97 ± 0.1a
S3	7.40 ± 0.09b	86.8 ± 1.68c	135.39 ± 1.86b	86.80 ± 1.68c
S4	4.62 ± 0.08a	75.45 ± 6.23c	95.54 ± 0.99ab	75.45 ± 6.23c
S5	4.20 ± 0.03a	132.19 ± 2.94d	93.82 ± 15.67ab	132.19 ± 2.94d
S6	6.44 ± 0.15b	10.06 ± 0.26a	143.11 ± 0.34bc	10.06 ± 0.26a
S7	10.06 ± 0.86c	51.64 ± 2.75bc	189.60 ± 0.43c	51.64 ± 2.75bc
S8	12.18 ± 0.2d	49.74 ± 3.24bc	137.42 ± 5.22bc	49.74 ± 3.24bc
S9	6.50 ± 0.16b	31.88 ± 0.47ab	81.98 ± 3.44a	31.88 ± 0.47ab
S10	9.03 ± 0.3c	173.20 ± 33.84e	75.75 ± 1.9a	173.20 ± 33.84e
S11	6.36 ± 0.16b	80.94 ± 2c	110.12 ± 2.86ab	80.94 ± 2c
S12	9.56 ± 0.25c	101.97 ± 3.61d	69.60 ± 3.06a	101.97 ± 3.61d
Means ± SD	7.37 ± 2.42	51.31 ± 25.65	118.38 ± 37.45	67.65 ± 51.48
Min–Max	4.20–12.18	15.02–81.96	69.60–189.60	7.98–173.21

antioxidant activity were also previously reported by Aazza et al. (2014), in Moroccan honeys of different floral origins.

Variance explained by principal component analysis

A principal component analysis was performed with all analytical data of *B. spinosum* honey from the 12 sites (Figure 2) to establish the differences among samples. For the PCA, two replicates of the physicochemical parameters were used (minimum and maximum) since for the sugar content, only two duplicate values of each analysis were available. Three samples (variables) were projected as supplementary variable because they do not contribute significantly to variance explanation (ash content, lactonic Acidity, and melizetose) in the first two components.

Factor 1 could be identified as the phenol content component, includes a series of properties, which are significantly correlated amongst themselves: phenols content; melanoidins; flavonoids; color; F/G ratio as well trehalose, fructose and water content. ABTS IC_{50} and glucose content are also correlated with the previously mentioned group of properties, but negatively. The second component could be identified as the component related to the honey acidity. This factor correlated well iron chelating power IC_{50}, superoxide IC_{50} and nitric oxide IC_{50} with pH, diastase activity, proline, free acidity and total acidity.

The principal component analyzes explain 70.3% of the total variance observed (Figure 2). Moreover some samples are very similar, namely S10, S12, S8 and other group composed by the samples S2, S3, S4, S5, S11, the separation are not so large reason by which it was not possible to group the samples belonging to different geographical regions (Figure 2). The separation between the previously groups are given basically by the sugar content and total flavonoids and polyphenol content, that could be explained by the different composition in the secondary floral resources. Given this result it is possible conclude that the zandaz honey from the different regions under study possesses similar properties.

Discussion

While this is the first study reporting a preliminary investigation of some features of zandaz honey, characterized by the domination of *B. spinosum* pollen, and regarding the variability that can occur in the same type of honey, it was not easy to claim determinant conclusions. In all analyzed samples pollen count of *B. spinosum* exceeded 45%, suggesting the monofloraty of this honey. Other relevant feature that can be used for zandaz honey characterization is the occurrence of trehalose and melizetose, which is unusual in almost honey types.

The use of PCA allowed also seeing homogeneity in the analyzed samples, a reason that did not allow grouping them into geographical groups, suggesting that belonging, or not, to a given region, zandaz honey conserved similar properties. To support the conclusions of the present study about this honey, large size samples will be needed, and subjected to the same analyzed parameters, with others evolving, mainly, sensory investigation and composition profiling.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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