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Zantaz honey "monoflorality": Chemometric applied to the routinely assessed parameters

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ABSTRACT

The comparison of Zantaz honey samples harvested in Morocco in two different years was performed through chemometric analysis of routinely assessed parameters. The objective was to study how the pollen profile of this newly reported honey shapes its physicochemical characteristics as well as to determine its monoflorality threshold, which has not yet been defined. The predominance of *B. spinosum* pollen was confirmed in the majority of samples reaching 45%, generally requested for monoflorality declaration. The principal component analysis (PCA) was used for clustering and variables correlations. Pollen qualitative and quantitative differences could discriminate the samples belonging to both seasons when combined with the sugar analysis (59.44%) better than the combination with physicochemical parameters (pH, acidity, ash content, electrical conductivity and color) (60.62%). Positive correlation between the presence of *B. spinosum* pollen and melanoidins, color, fructose, moisture, trehalose, melezitose, iron, manganese and calcium could be seen. Integrating all the parameters except the pollen data allowed distinguishing two groups with significant differences ($P < 0.05$) in *B. spinosum* representability (58 ± 11.24% against 40 ± 15.98%). This may suggest that 40% is the monoflorality threshold of the Moroccan Zantaz honey, although a confirmation with sensorial analysis is required.

1. Introduction

Chemometrics application to food chemistry parameters is attracting more attention from the scientific community due to its power on the elaboration of conclusions about the traceability (Bertacchini et al., 2013) and the classification/clustering problems (Anjos et al., 2015a; Silva, Gauche, Gonzaga, Costa, & Fett, 2016). These techniques were previously applied on the analysis of several food products, among which coffee (Briandet, Kemsley, & Wilson, 1996) and amino acids content in honey (Zhao et al., 2018). Honey does not make an exception; indeed the chemometric tools were not only used as a discriminatory tool between honeys with different geographical (Estevinho, Chambó, Pereira, Carvalho, Alencar, 2016; Karabagias et al., 2017a, 2017b) or botanical origins (Corbella & Cozzolino, 2006; Devillers, Morlot, Pham-Delègue, & Doré, 2004; Terrab, Díez, & Heridia, 2002) but also as an effective way to detect honeys' adulteration (Wang et al., 2014). Several tools can be employed depending on the study aim, implicating the filtration of the parameters. This parameters' filtration helps choosing the adequate parameters/variables carrying the adequate information as well as the elimination of the "noising" parameters (Bevilacqua, Bucci, Magrì, Magrì, & Nescatelli, 2013).

Honey monoflorality determination can be done using the combination of pollen spectrum analysis, physicochemical parameters and sensorial qualities (Bogdanov, Ruoff, & Oddo, 2004). This last step is highly expensive, requiring panels of assessors which go through controlled experimental protocols. The results need to be statistically reproducible to be considered (Piana et al., 2004). In fact, a predictive relationship has been established between physicochemical characteristics and sensory properties (Pestorić et al., 2015), although this should

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be interpreted cautiously (Estevinho, Chambó, Pereira, De Carvalho, & De Alencar Arnaut De Toledo, 2016). This relationship can be highlighted by the fact that both sensorial and physicochemical properties are determined by the nectar of the visited melliferous plants. As there is no way to evaluate the nectar delivered by each plant, researchers used to estimate it by using the pollen profiling, or mellissopalynology. The monoflorality threshold, considering a pollen species, is then the percentage above which honey samples becomes governed by the physicochemical and sensorial properties of the corresponding nectar.

Generally, a honey sample is considered to be monofloral, or governed by the physicochemical and sensorial properties of the main nectar, when the pollen of its main originating melliferous plant exceed 45% (Louveaux, Maurizio, & Vorwohl, 1978). Two exceptions to this rule can be mentioned: lavender honey, for which the presence of 15% of lavender pollen is enough to be labelled as monofloral (Estevinho et al., 2016), and chestnut honey which is labelled only when presenting more than 90% of chestnut pollen (Louveaux et al., 1978). Those exceptions emphasize the importance of establishing a specific threshold for a given honey type, especially when it is newly introduced to the scientific community, which is the case of the Moroccan Zanataz honey. A holistic view needs to be given not only about its characterizing parameters, but on how the pollen profiling shapes them. In other words, at which level the main pollen representability needs to achieve to consider a Zantaz honey sample as monofloral.

In fact, establishing monoflorality threshold for a given honey is not a trivial task. The variability of the contribution of the participating melliferous plants belonging to two different seasons, or geographical regions, is highly influential in shaping the final characteristics of honey samples (Estevinho et al., 2016). The present contribution aimed a second characterization of a new set of the Moroccan Zantaz honey samples known by the prevalence of *Bupleurum spinosum* pollen exceeding 45%, as a sequence of the previously published study (Elamine et al., 2018). Furthermore, the data of both studies will be integrated using the appropriate multivariate analysis to assess the effect of seasonal variability on the identified pollen species, and how the pollen spectrum correlate the acquired physicochemical characteristics of Zantaz honey samples. In addition, PCA will be applied using all analysed parameters. The pollen profile of the output clusters will be statistically compared in an attempt to determine how the homogeneity of the samples forming each cluster is determined by the presence of *B. spinosum* pollen.

2. Materials and methods

2.1. Sample preparation

Honey samples (n = 18) were purchased from beekeepers, centrifuged and kept under ambient temperature until their use. To strengthen the conclusions of the multivariate analysis section of the present work, the data of the samples (N = 12) published in the previous study (Elamine et al., 2018) was also used.

2.2. Pollen analysis

The qualitative and quantitative analysis of pollen spectrum was accomplished following the International Commission for Bee Botany (ICBB) method, as described previously (Louveaux et al., 1978). An optic microscope (Leitz Messtechnik GmbH, Wetzlar, Germany) with 400× and 1000× objectives, was used for pollen identification and count. One thousand pollen grains were counted for each sample, and the frequent classes were determined twice. A pollen species was considered dominant, when it represents more than 45% from the pollen spectrum, secondary with percentage between 16% and 45%, important minor pollen from 3 to 15% and minor pollen when it is less than 3%.

2.3. Acidity, pH, conductivity, moisture, color, proline, diastase activity

Free acidity, lactonic acidity, total acidity, pH, ash content, electrical conductivity, moisture, diastase activity, were assessed following the Harmonized Methods of the International Honey Commission (Bogdanov, 1997, pp. 1–62).

2.4. Colour and melanoidins

Colour was determined by measuring the absorbance of aqueous honey solutions (10 g in 20 mL H_2O) at 635 nm (A_{635}) in a Shimadzu spectrophotometer (Naab, Tamame, & Caccavari, 2008). The mm Pfund values and honey colour were obtained using the following algorithm, mm Pfund = $-38.7 + 371.39 \times A_{635}$. Additionally, honey colour 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, 2011). Spectrophotometric measurements were performed in a 1 cm quartz cell;

2.5. Sugars

The sugar contents were analysed by High Performance Liquid Chromatography (HPLC) with refraction index (IR) detector according the methodology proposed by Bogdanov (1997, pp. 1–62). The HPLC analysis was performed on analytical stainless-steel column, Purospher® STAR – NH2 with 4 mm in diameter, 250 mm length and with 5 mm particle size, using a flow rate of 1.3 mL/min; mobile phase: acetonitrile: water (80:20, v/v); column and detector temperature: 30 °C; and sample volume injection of 10 ml.

The different sugars (fructose, glucose, sucrose, melezitose, turanose, maltose, xilose, rhamnose, arabinose, melibiose, trehalose and erlose) were identified and quantified by comparison of the retention times and the peak areas of the honey sugars with those of calibration curves of standard sugars. The values expressed in g/100 g of honey. Retention time (RT), limit of quantification (LQ) of the analysed sugars are illustrated in Table 1.

2.6. Minerals

Sample preparation is initiated by calcination of 5 g honey at 550 °C. After cooling, 5 mL of 0.1 M nitric acid was added to the ash and the mixture was stirred on a heating plate to almost complete dryness. Then, 10 mL of the same nitric acid solution was added, and the mixture was brought up to a final volume of 25 mL with distilled water. Afterwards, Ca, Mg, Mn, Zn, Cu, and Fe were measured by flame atomic absorption, while Na, K by emission spectrometry (air-acetylene) using a novAA 350 (Analytik Jena, Germany). After the determination of mineral concentrations in the prepared solutions, their values were extrapolated to be expressed as mg/kg honey.

Table 1
Retention time, limit of quantification of the analysed sugars.

Analyte	N	RT (min)	LQ (g/100 g)
Ramnose	3	3.83	0.17
Xylose	1	4.24	0.87
Arabinose	6	4.96	0.13
Fructose	31	5.46	0.77
Glucose	31	6.42	1.61
Sucrose	7	9.45	0.23
Turanose	31	10.45	0.06
Maltose	31	11.55	0.66
Melibiose	31	12.69	0.12
Trehalose	31	15.27	0.16
Melezitose	13	18.32	0.08
Erlose	6	22.60	0.16

Table 2
Pollen profiling of the studied honey samples.

Sample code	Predominant pollen (> 45%)	Secondary pollen (16–45%)	important minor pollen (3–15%)	Minor pollen (< 3%)
S1	*Bupleurum spinosum* (Apiaceae) (60.9)		*Artocarpus altilis* (Moraceae) (10.47), *Eragrostis pilosa* (Poaceae) (12.8), *Populus* sp (Salicaceae) (10.15), *Prunus* sp (Rosaceae) (3.08)	*Celtis* sp (2.6)
S2	*Bupleurum spinosum* (Apiaceae) (50.75)		*Artocarpus altilis* (Moraceae) (11.34), *Celtis* sp (6.95), *Eragrostis pilosa* (Poaceae) (11.9), *Populus* sp (Salicaceae) (8.05), *Prunus* sp (Rosaceae) (3.96), *Quercus* sp (Fagaceae) (5.175)	*Pinus* sp (Pinaceae) (1.875)
S3	*Bupleurum spinosum* (Apiaceae) (49.84)	*Artocarpus altilis* (Moraceae) (18.335), *Eragrostis pilosa* (Poaceae) (19.94)	*Populus* sp (Salicaceae) (11.885)	
S4	*Bupleurum spinosum* (Apiaceae) (79.795)		*Artocarpus altilis* (Moraceae) (4.165), *Eragrostis pilosa* (Poaceae) (5.935), *Populus* sp (Salicaceae) (8.21)	*Mimosa* sp (Fabaceae) (1.895), *Quercus* sp (Fagaceae) (2)
S5	*Bupleurum spinosum* (Apiaceae) (76.77)	*Populus* sp (Salicaceae) (19.4)		*Artocarpus altilis* (Moraceae) (2.715), *Eucalyptus* sp (Myrtaceae) (1.115)
S6		*Bupleurum spinosum* (Apiaceae) (37.18), *Populus* sp (Salicaceae) (20.575), *Salvia* sp (Lamiaceae) (17.42)	*Cytisus* sp (Fabaceae) (8.8), *Eragrostis pilosa* (Poaceae) (5.795), *Euphorbia* sp (Euphorbiaceae) (5.85)	*Artocarpus altilis* (Moraceae) (1.915), *Quercus* sp (Fagaceae) (2.475)
S7		*Bupleurum spinosum* (Apiaceae) (30.025), *Populus* sp (Salicaceae) (21.65)	*Eragrostis pilosa* (Poaceae) (3.015), *Eucalyptus* sp (Myrtaceae) (8.92), *Euphorbia* sp (Euphorbiaceae) (14.695), *Phytolacca americana* (Phytolaccaceae) (4.065), *Raphanus* sp (Brassicaceae) (6.585)	*Celtis* sp (0.875), *Citrus* sp (Rutaceae) (0.875), *Cytisus* sp (Fabaceae) (2.785), *Lactuca* sp (Asteraceae) (0.625), *Phoenix* sp (Arecaceae) (0.875), *Quercus* sp (Fagaceae) (2.295), *Salvia* sp (Lamiaceae) (2.715)
S8	*Bupleurum spinosum* (Apiaceae) (64.565)	*Cytisus* sp (Fabaceae) (22.95)	*Eucalyptus* sp (Myrtaceae) (7.675)	*Phytolacca americana* (Phytolaccaceae) (1.15), *Populus* sp (Salicaceae) (2.18), *Salvia* sp (Lamiaceae) (1.48)
S9	*Bupleurum spinosum* (Apiaceae) (55.02)	*Cytisus* sp (Fabaceae) (27.185)	*Eucalyptus* sp (Myrtaceae) (12.39)	*Artocarpus altilis* (Moraceae) (1.97), *Citrus* sp (Rutaceae) (2.08), *Populus* sp (Salicaceae) (1.355)
S10	*Bupleurum spinosum* (Apiaceae) (52.895)	*Cytisus* sp (Fabaceae) (36.04)	*Artocarpus altilis* (Moraceae) (5.6), *Populus* sp (Salicaceae) (5.465)	
S11	*Bupleurum spinosum* (Apiaceae) (51.05)	*Cytisus* sp (Fabaceae) (38.28)	*Populus* sp (Salicaceae) (7.775)	*Artocarpus altilis* (Moraceae) (0.995), *Eragrostis pilosa* (Poaceae) (1.9)
S12	*Bupleurum spinosum* (Apiaceae) (47.81)	*Cytisus* sp (Fabaceae) (29.82)	*Eragrostis pilosa* (Poaceae) (4.05), *Populus* sp (Salicaceae) (4.4)	*Artocarpus altilis* (Moraceae) (2.355), *Salvia* sp (Lamiaceae) (1.155)
S13	*Bupleurum spinosum* (Apiaceae) (45.945)	*Cytisus* sp (Fabaceae) (23.76)	*Eragrostis pilosa* (Poaceae) (11.915), *Populus* sp (Salicaceae) (12.26), *Salvia* sp (Lamiaceae) (6.105)	
S14	*Cytisus* sp (Fabaceae) (45.775)	*Bupleurum spinosum* (Apiaceae) (18.97), *Populus* sp (Salicaceae) (15.065)	*Eragrostis pilosa* (Poaceae) (3.33), *Eucalyptus* sp (Myrtaceae) (10.005), *Raphanus* sp (Brassicaceae) (4.125)	*Salvia* sp (Lamiaceae) (2.73)
S15	*Bupleurum spinosum* (Apiaceae) (56.995)	*Artocarpus altilis* (Moraceae) (18.1)	*Eragrostis pilosa* (Poaceae) (13.84), *Populus* sp (Salicaceae) (11.065)	
S16	*Bupleurum spinosum* (Apiaceae) (49.295)		*Artocarpus altilis* (Moraceae) (11.665), *Cytisus* sp (Fabaceae) (9.86), *Eragrostis pilosa* (Poaceae) (6.375), *Euphorbia* sp (Euphorbiaceae) (7.435), *Populus* sp (Salicaceae) (6.375), *Raphanus* sp (Brassicaceae) (4.125)	*Eucalyptus* sp (Myrtaceae) (2.52), *Phytolacca americana* (Phytolaccaceae) (2.35)
S17		*Artocarpus altilis* (Moraceae) (28.545), *Bupleurum spinosum* (Apiaceae) (22.02), *Eragrostis pilosa* (Poaceae) (26.335), *Populus* sp (Salicaceae) (19.995)		*Euphorbia* sp (Euphorbiaceae) (1.93), *Salvia* sp (Lamiaceae) (2.14)
S18	*Bupleurum spinosum* (Apiaceae) (68.6)		*Pinus* sp (Pinaceae) (3.55), *Populus* sp (Salicaceae) (7.4), *Prunus* sp (Rosaceae) (10.9), *Quercus* sp (Fagaceae) (4.9)	*Artocarpus altilis* (Moraceae) (2.45), *Erica* sp (Ericaceae) (1.1), *Eucalyptus* sp (Myrtaceae) (1.1)

2.7. Statistical tools

To estimate the differences amongst the analysed samples regarding each of the assessed parameters, ANOVA, followed by a post hoc Tukey test was used and the means were considered to be significantly different when $p < 0.05$.

The PCA was used, as a powerful multivariate analysis for scales reduction, to integrate the information provided by several parameters in few principal components (PC). Prior to each PCA running, the data was normalized by subtracting the mean value and dividing by the standard deviation of each of the given values. To run the PCA a MultBiplot64 was used on MATLAB R2017a.

3. Results and discussion

3.1. Preliminary descriptive characterization

Firstly, a descriptive approach was adopted for a preliminary characterization of the 18 honey samples. Table 2 shows their pollen analysis spectrum. *Bupleurum spinosum* pollen was the predominant species, exceeding 45%, except for S6, S7, S14 and S17, presenting values of 37.18%, 30.02%, 18.97% and 28.54%, respectively. Regardless being secondary pollen (16–45%) in the four samples, *Bupleurum spinosum* was the most abundant except for S14, where *Cytisus* sp., was the predominant species (45.77%), and S17 where it was third after *Artocarpus altilis* (28.54%) and *Eragrostis pilosa* (26.33%). Besides being predominant pollen in sample S14, the *Cytisus* sp. was the secondary pollen in 6 samples with values between 22.95% and 38.28% in S8 and S11, respectively. Other secondary pollen species were: *Eragrostis pilosa* (S3), *Populus* sp. (S5), and *Artocarpus altilis* (S15 and S17).

An overview of the pollen analysis applied previously to the samples of the same botanical origin (Elamine et al., 2018), revealed more contribution of *B. spinosum* pollen presenting an average of 62.50 ± 8.66% compared to the samples of the current work (51.02 ± 16.11%). Besides this quantitative difference, some pollen species were only identified in one of the two groups, making a qualitative difference between them. Namely, *Thymus* sp., *Opuntia* sp., *Phoenix* sp., *Olea* sp., *Epilobium* sp., *Cactus* sp. and *Acacia* sp. were identified only in the samples of the previous study, while the current work was specifically characterized by the presence of *Populus* sp., *Prunus* sp., *Quercus* sp., *Raphanus* sp., *Salvia* sp., *Euphorbia* sp., *Lactuca* sp., *Mimosa* sp., *Artocarpus altilis, Phytolacca* sp., *Pinus* sp., *Erica* sp., *Citrus* sp., *Celtis* sp. and *Cytisus* sp. The effect of the mentioned differences on the assessed parameters will be discussed with details in the multivariate analysis section.

The studied physicochemical parameters are summarized in Table 3. The pH values ranged from 3.74 to 4.39 with an average value of 4.03 ± 0.21 considering the 18 samples. The same average can be seen in the previously analysed samples (previous season). The lactonic acidity varied also amongst the analysed samples between 8.00 ± 1.49 mEq/kg and 16 ± 0.86 mEq/kg. The average values of the free and lactonic acidity (20.28 ± 6.36 mEq/kg - 12.11 ± 1.82 mEq/kg) were slightly lower than those observed in the previous work (25.70 ± 6.46 mEq/kg and 16.58 ± 1.40 mEq/kg).

The ash content and the electrical conductivity were evaluated. A correlation between the two parameters is reported in addition to their correlation with the mineral composition of honey samples (Aazza, Lyoussi, Antunes, & Miguel, 2013). The correlation can be seen by the fact that samples presenting low ash content had also low electrical conductivity, and *vice versa*. The lowest ash content percentage was 0.11 ± 0.02%, while the highest ash value was 0.32 ± 0.03%. For the electrical conductivity, the minimum and the maximum values, were 351.66 ± 0.57 µs/cm and 900.33 ± 3.05 µs/cm, respectively. Both assessed parameters can be used, not only to predict the richness on mineral elements, and so its nutritional value (Ribeiro et al., 2014) but also for honey quality control purpose. According to the international legislation the ash content and the electrical conductivity are not allowed to be more than 800 µS/cm and 0.6%, respectively in honey (European Union Directive (EU) (2014). Considering this, all analysed Zantaz honey samples were within the established values, except for one sample that had a value of 900.33 ± 3.05 µs/cm for electrical conductivity. Furthermore, the comparison with the samples used in previous studies of our team showed no significant differences and the average value of the samples maintained almost the same: 0.20 ± 0.05% and 507.63 ± 144.95 µs/cm in comparison to the previous season 0.22 ± 0.06% and 529.4 ± 122.70 µs/cm.

Table 3
Physicochemical characterization and sugar profile of the analysed honey samples.

Assessed parameter (Unit)	Means	SD	Min	Max
Ash content (%)	0.19	0.05	0.11	0.32
Water content (%)	19.59	0.81	18.40	21.13
Electrical conductivity µs/cm	507.63	144.95	351.66	900.33
pH	4.02	0.21	3.73	4.38
Free acidity (mEq/kg)	20.28	6.36	8.40	32.80
Lactonic Acidity (mEq/kg)	12.11	1.82	8.00	16.00
Total Acidity (mEq/kg)	32.39	7.37	18.40	43.30
Diastase (Shad number)	21.81	5.25	12.38	31.89
Melanoidins	0.94	0.30	0.40	1.45
Colour (mmPfund)	56.62	20.77	22.24	96.42
Fructose (g/100 g)	37.43	2.42	33.52	41.83
Glucose (g/100 g)	24.44	2.73	19.61	30.88
Fructose/Glucose Ratio	1.52	0.22	1.12	2.13
Melebiose (g/100 g)	2.66	0.96	0.62	4.31
Turanose (g/100 g)	1.71	0.28	1.08	2.05
Maltose (g/100 g)	1.56	0.63	0.34	3.03
Arabinose (g/100 g)	1.44	0.67	0.33	2.44
Trehalose (g/100 g)	0.88	0.39	0.41	2.03
Xylose (g/100 g)	0.31	0.04	0.25	0.42
Melezitose (g/100 g)	0.51	0.01	0.50	0.51
Sucrose (g/100 g)	–	–	–	–
Rhamnose (g/100 g)	–	–	–	–
Erlose (g/100 g)	–	–	–	–

Abbreviations: SD = standard deviation; Min = Minimal; Max = maximal.

The diastase activity of the samples analysed in the present work was very similar to that observed on the previous harvest, and the average values were 22.80 ± 5.55 Shade units/g and 22.21 ± 5.30 Shade units/g, successively. Although, an important variability could be seen amongst the analysed samples with a minimum activity of 12.38 ± 0.13 Shade units/g, and a maximum of 31.89 ± 0.73 Shade units/g, being all above the minimum value (8.0 Shade units/g) required for honey freshness confirmation by the European council (European Union Directive (EU), 2014).

The ripeness is another important step in the quality control of honey. Usually, the fast parameter used is the moisture content (Terrab, Díez, & Heredia, 2002), which may be a factor favouring the growth and development of undesirable germs. Therefore, a maximum value was fixed to be 20% (European Union Directive (EU), 2014). In the present work eight of the analysed samples had moisture values between 20% and 21% (data not shown), and one sample presented a value of 21.13 ± 0.23% being the maximum observed. The remaining samples did not exceed the 20%, while the minimum value was 18.4 ± 0.20%.

Honey colour and melanoidins content are also important parameters in the characterization of honey samples. A well-established relationship is reported between the colour, bioactive compounds, and mineral content (Silva et al., 2016). Both parameters ranged in the analysed samples between 22.24 ± 10.52 mm Pfund and 0.40 ± 0.02 AU as minimum values and maximum values of 96.42 ± 6.99 mm Pfund and 1.45 ± 0.03 for colour and melanoidins respectively.

The results of the sugars profiling are summarized in Table 3. In all samples the fructose prevailed, and the average of the ratio fructose/glucose was 1.56 ± 0.25 which is a usual value for honey (Anjos,

Table 4
Mineral content of honey samples.

	[K] mg/kg	[Ca] mg/kg	[Na] mg/kg	[Mg] mg/kg	[Fe] mg/kg	[Cu] mg/kg	[Mn] mg/kg	[Zn] mg/kg
S1	566.30 ± 3.18^{f}	189.95 ± 1.21^{b}	44.63 ± 0.41^{k}	32.14 ± 0.45^{def}	15.50 ± 0.06^{b}	0.92 ± 0.002^{j}	0.96 ± 0.001^{d}	0.41 ± 0.008^{k}
S2	679.34 ± 3.40^{e}	170.73 ± 7.62^{cde}	77.69 ± 0.56^{c}	52.80 ± 1.02^{a}	18.22 ± 0.21^{a}	1.67 ± 0.005^{d}	1.12 ± 0.015^{b}	0.86 ± 0.011^{f}
S3	702.39 ± 6.87^{e}	221.12 ± 17.72^{a}	39.82 ± 0.30^{l}	34.69 ± 0.62^{cd}	9.41 ± 0.13^{g}	1.62 ± 0.015^{de}	1.16 ± 0.003^{a}	0.69 ± 0.015^{i}
S4	492.35 ± 2.04^{g}	124.15 ± 1.41^{ij}	38.39 ± 0.05^{m}	26.98 ± 0.98^{gh}	11.93 ± 0.19^{e}	0.89 ± 0.022^{j}	0.75 ± 0.014^{i}	0.36 ± 0.003^{l}
S5	448.50 ± 2.03^{gh}	138.78 ± 3.22^{hi}	64.13 ± 0.25^{e}	31.89 ± 0.55^{def}	11.55 ± 0.17^{ef}	1.02 ± 0.007^{i}	0.69 ± 0.007^{j}	0.26 ± 0.008^{n}
S6	594.02 ± 1.15^{f}	104.15 ± 2.43^{kl}	64.97 ± 0.12^{e}	20.94 ± 0.57^{i}	6.78 ± 0.054^{j}	1.06 ± 0.002^{i}	0.46 ± 0.005^{m}	0.44 ± 0.004^{k}
S7	558.61 ± 1.45^{f}	103.72 ± 3.67^{kl}	58.04 ± 0.04^{h}	27.78 ± 0.61^{gh}	6.72 ± 0.10^{j}	1.39 ± 0.013^{f}	0.51 ± 0.001^{l}	1.29 ± 0.016^{c}
S8	755.55 ± 0.95^{d}	124.41 ± 2.52^{ij}	90.90 ± 0.26^{b}	24.25 ± 0.09^{hi}	7.61 ± 0.01^{i}	1.07 ± 0.008^{i}	0.67 ± 0.007^{j}	1.72 ± 0.002^{b}
S9	1177.47 ± 18.29^{a}	173.79 ± 4.71^{cd}	59.59 ± 0.26^{g}	50.22 ± 1.006^{a}	15.61 ± 0.08^{b}	1.95 ± 0.019^{b}	0.87 ± 0.008^{f}	0.81 ± 0.006^{g}
S10	429.15 ± 2.31^{h}	153.43 ± 1.88^{fgh}	51.23 ± 0.48^{i}	24.35 ± 1.89^{hi}	14.01 ± 0.12^{d}	1.061 ± 0.003^{i}	0.51 ± 0.004^{l}	0.42 ± 0.004^{k}
S11	584.34 ± 0.94^{f}	169.82 ± 0.98^{cde}	49.33 ± 0.19^{j}	29.92 ± 0.92^{efg}	18.23 ± 0.15^{a}	1.80 ± 0.023^{c}	0.88 ± 0.012^{f}	0.88 ± 0.014^{f}
S12	468.73 ± 3.27^{gh}	180.08 ± 2.14^{bc}	50.02 ± 0.20^{j}	24.21 ± 0.09^{hi}	15.59 ± 0.15^{b}	1.57 ± 0.009^{e}	0.84 ± 0.005^{g}	0.32 ± 0.014^{m}
S13	774.69 ± 4.18^{d}	158.25 ± 2.06^{efg}	77.20 ± 0.22^{c}	44.71 ± 2.41^{b}	14.52 ± 0.10^{c}	0.89 ± 0.010^{j}	0.79 ± 0.003^{h}	1.02 ± 0.010^{d}
S14	1013.45 ± 5.64^{b}	94.18 ± 1.95^{l}	163.38 ± 1.22^{a}	36.77 ± 1.44^{c}	7.84 ± 0.05^{i}	6.43 ± 0.073^{a}	0.74 ± 0.002^{i}	2.02 ± 0.010^{a}
S15	694.71 ± 39.56^{e}	142.85 ± 0.89^{gh}	44.01 ± 0.18^{k}	30.31 ± 1.14^{efg}	11.52 ± 0.06^{f}	1.29 ± 0.005 g	0.93 ± 0.006^{e}	0.35 ± 0.006^{lm}
S16	704.48 ± 5.23^{e}	94.65 ± 1.66^{l}	62.31 ± 0.19^{d}	32.80 ± 0.95^{de}	8.75 ± 0.12^{h}	1.69 ± 0.014^{d}	0.62 ± 0.002^{k}	0.94 ± 0.005^{e}
S17	885.73 ± 48.43^{c}	115.32 ± 1.21^{jk}	74.28 ± 0.15^{d}	37.29 ± 1.71^{c}	11.90 ± 0.09^{ef}	1.08 ± 0.008^{i}	0.62 ± 0.001^{k}	0.73 ± 0.013^{h}
S18	554.21 ± 2.18^{f}	159.95 ± 0.43^{def}	57.10 ± 0.07^{h}	28.78 ± 2.38^{fg}	17.98 ± 0.17^{a}	1.18 ± 0.053^{h}	1.07 ± 0.010^{c}	0.50 ± 0.006^{j}

Values in the same column followed by the same letter are not significant different ($p < 0.05$) by the Tukey's multiple range test.

Campos, Ruiz, & Antunes, 2015b). The ratio variability ranged from a minimum of 1.13 to a maximum of 2.13. In terms of abundance, after fructose and glucose, the evaluated sugars were classified as follow: melebiose, turanose, maltose, arabinose, trehalose and xylose. While melezitose was detected in two samples, ramnose, erlose, and sucrose were below 0.2%, the detection threshold. In the samples of the previous study, the ratio fructose/glucose was 1.48 ± 0.17 (min = 1.25 and max = 1.74), lower than the current samples. Other differences involve, mainly, the big reduction in the melezitose content from 2.00 ± 0.30 g/100 g honey, to be detected only in two samples. The relationship between this variability and the one of the pollen analyses will be probed in the multivariate analysis section.

When analysing the mineral content of Zantaz honeys, potassium prevailed in all samples, as reported in literature (Silva et al., 2016), followed by the calcium, and in a third position the sodium (Table 4). While the magnesium and iron occupied the forth and the fifth places, cupper, manganese and zinc were present at very low levels with values equal or below 2 mg/kg, except one sample which presented an amount of 6.43 ± 0.073 mg/kg cupper. Sample S9 presented the highest amount of potassium, calcium and iron, and the second highest amount of magnesium with values of 1177.47 ± 18.29 mg/kg, 50.22 ± 1.006 mg/kg, 15.61 ± 0.08 mg/kg, and 50.22 ± 1.006 mg/kg, successively. The lowest amounts were observed in honey samples S5 (448.50 ± 2.03 mg/kg), S14 (94.18 ± 1.95 mg/kg), S8 (7.61 ± 0.01 mg/kg) and S6 (20.94 ± 0.57 mg/kg), successively for potassium, calcium, iron and magnesium. For the sodium, the minimum value was 38.39 ± 0.05 mg/kg observed in sample S4, while the maximum value was 163.38 ± 1.22 mg/kg in sample S14. In overall, the mineral content, based on the assessed elements, ranged between 0.067% (S10) and 0.148% (S9), being within the reported values (0.04%–0.2%) (Silva et al., 2016).

3.2. Multivariate analysis

For honey types with well-established monoflorality percentage, authors declared the importance of identified pollen species in shaping the final characteristics of honey samples (Oddo & Piro, 2004). An example is that of lavender honey (Estevinho et al., 2016; Anjos, Santos, Paixão, & Estevinho, 2018), for which authors, after confirming that all analysed samples presented lavender pollen more than the monoflorality threshold (15%), found different clustering profiles using the pollen analysis or the physicochemical characteristics. Furthermore, when analysing the differences between the established clusters, the same authors found no significant contribution of lavender pollen, highlighting so the importance of other pollens in shaping the physicochemical features of a honey.

From the previous reference (Estevinho et al., 2016), a similar behaviour was expected to be seen in the case of Zantaz honey samples characterized by the prevalence of *Bupleurum spinosum* pollen. This expectation could be seen clearly considering Figs. 1 and 2, where specific pollen species correlate differently the assessed parameters. In other words, if we consider two honey samples presenting the same amount of *B. spinosum* pollen, but different secondary pollen species, both samples will share the characteristics provided by the main nectar, and other distinguished features matching other participating nectars. From this point of view, and as the pollen species varies greatly amongst the analysed samples, the determination of monoflorality threshold of Zantaz honey will not be a trivial task unless the maximum of parameters is considered, as well as the sensorial analysis.

Fig. 1 illustrates the distribution of the 30 Zantaz honey samples belonging to both seasons based on their melissopalynological and physicochemical analyses. To retain a good representability of the given data three components were used for plotting. The three first components represented 23.97%, 20.78% and 15.87% respectively. Considering the two first components (Fig. 1 (a)), a reasonable discrimination was seen between the 12 samples of the previous work (black circles) and the 18 honey samples of the current contribution (blue squares).

The samples of the previous season were characterized by the presence of more *B. spinosum* and *Phoenix*. ssp pollen, in comparison with the other samples, making them, based on the seen positive correlation, more acidic and darker. The current work samples presented, mainly, more *Eucalyptus* sp., *Euphorbia* sp., *Populus* sp., and so presented the opposite features: high pH values and light colour. A sub-group of those last samples, located in the bottom of the plot, was characterized by more *Eragostis* sp. and *Artocarpus* sp. Pollens and was characterized by low ash content and electrical conductivity.

Employing the first and the third PCs (Fig. 1 (b)), the conserved information did not allow the same discrimination level as in Fig. 1 (a), although it conserved the same correlation between the presence of pollen species and confirmed that there are a higher similarity between samples with higher amounts of *Eragostis* and *Artocarpus* pollens, which were significantly different from those with higher amount of *B. spinosum* pollen.

To evaluate a possible effect of pollen variability on sugar content, another PCA was made using a data matrix fusing both parameters. Also, to improve the conservation of the given data, three components were employed. Considering the first and the second PCs (Fig. 1 (c)),

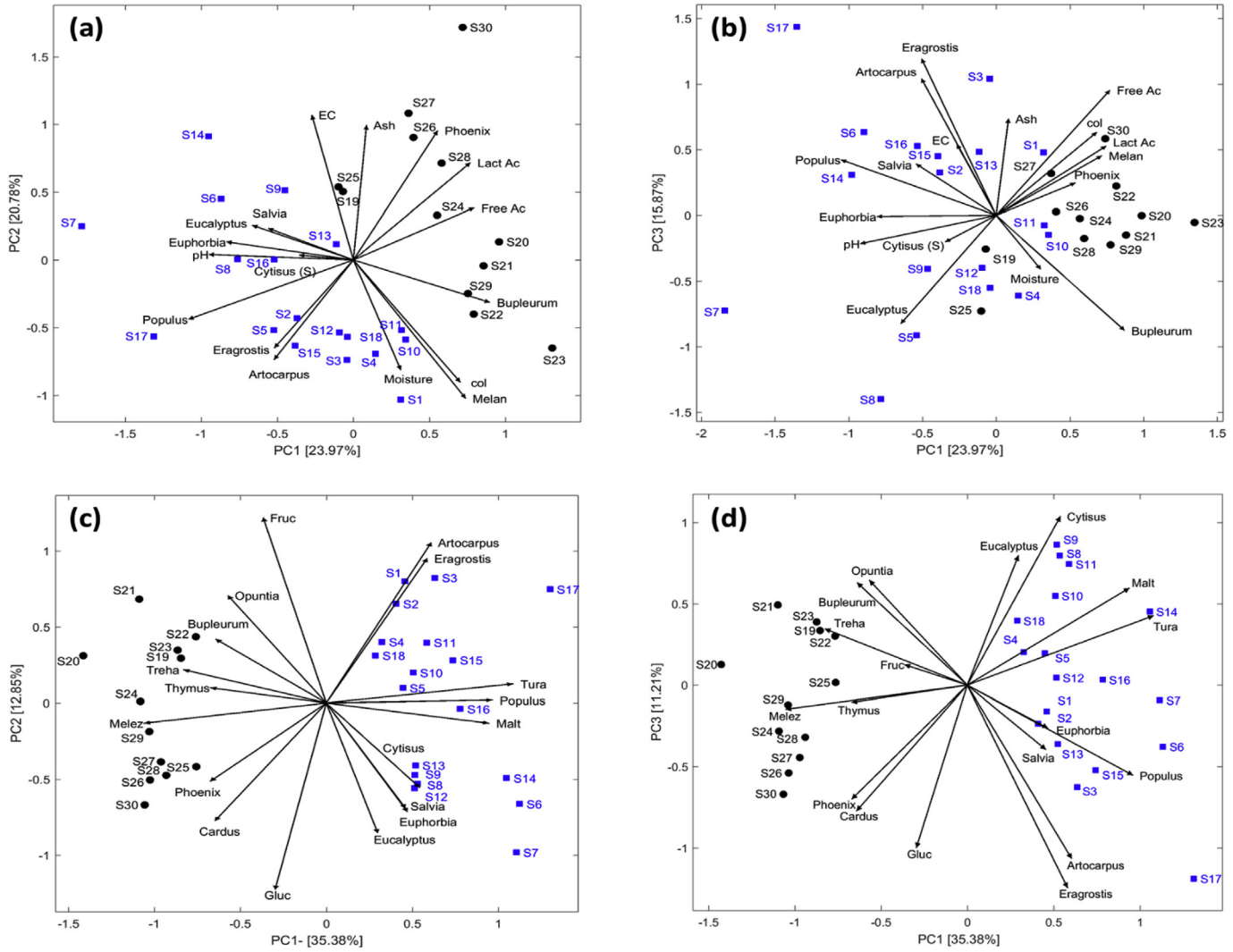

Fig. 1. PCA of the pollen analysis and the physicochemical parameters assessed in the 30 Zantaz honey samples. (a) Plot using the first and the second component. (b) Plot using the first and the third components. (c) Plot using the first and the second component. (d) Plot using the first and the third components.

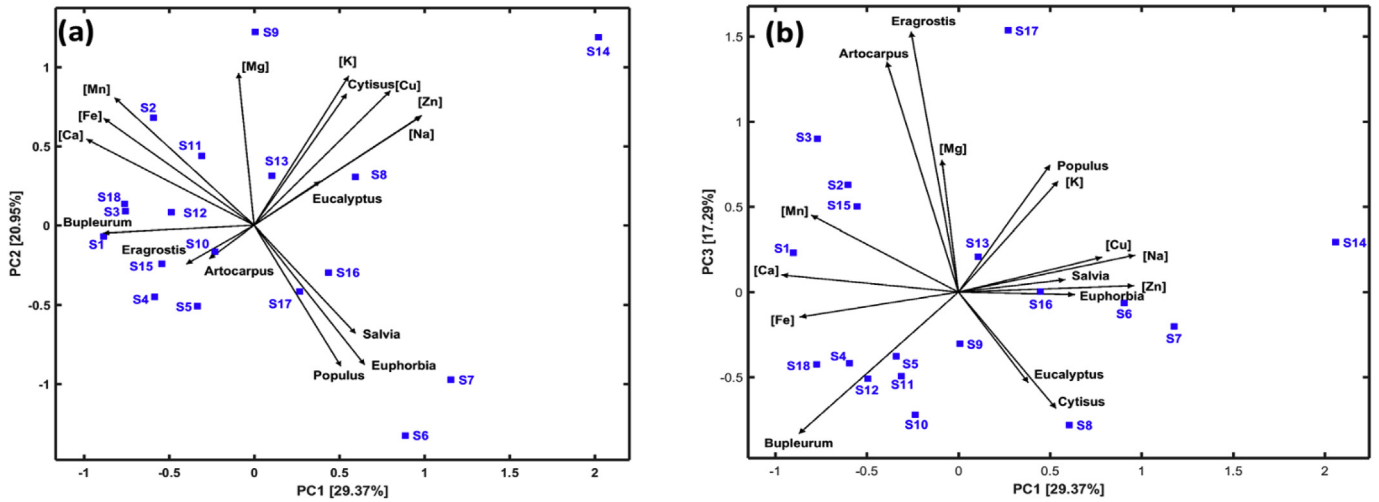

Fig. 2. PCA of the pollen analysis and the mineral content of the 18 Zantaz honey samples. (a) Plot using the first and the second component. (b) Plot using the first and the third components.

which explained 35.38% and 12.85% respectively, the samples of the two seasons were perfectly discriminated from each other by the PC1. The 12 samples of the previous works were characterized by the prevalence of *B. spinosum*, *Opuntia* sp., *Thymus* sp., *Phoenix* sp. and *Cardus* sp. pollens, and occupied the negative part of PC1. This pollen prevalence characterized the samples by higher levels in terms of trehalose and melezitose. In the 18 honey samples characterized in the present work, the *Artocarpus* sp., *Eragostis* sp., *Populus* sp., *Salvia* sp., *Euphorbia* sp. and *Eucalyptus* sp. pollens prevailed, and the samples presented higher amount of turanose and maltose. Considering the fructose and the glucose, they correlated oppositely, as observed by other authors (Anjos et al., 2015b) the PC2, presenting, so little participation to the mentioned discrimination.

Besides being a good tool for the discrimination of the geographical origin, the mineral content can also suggest the botanical origin (Pasquini et al., 2014). Therefore, a PCA was also employed to illustrate the relationship between the pollen species and the evaluated minerals. As the mineral content was only made for the 18 samples of the present work, their data was used for PCA, and the first three components conserved a sum of 67.61% of the given data. In overall no clear clustering could be seen amongst the analysed samples (Fig. 2 (a)), except the discrimination of samples S14, presenting high percentage of *Cytisus* sp. pollen and high levels of Cu, Zn, Na and K. In addition, honey samples S6 and S7 were discriminated by their high amounts of *Salvia* sp., *Euphorbia* sp. and *Popolus* sp. pollens which were linked to the prevalence of Cu, Zn and Na. the presence of high amounts of *B. spinosum* pollen was linked to higher amounts of Ca, Fe and Mn.

When PC1 was plotted against PC3 (Fig. 2 (b)), the same observation of Fig. 2 (a) were made, as the first PC was the most representative in terms of data conservation. Furthermore, the presence of *Eragostis* sp. and *Artocapus* sp. was linked to the amount of Mg and the three variables were highly represented by the third PC, which explained 17.29% from the given data. Also, the relation between *Salvia* sp. and *Euphorbia* sp. and Cu, Na and Zn became clearer.

To further understand how the presence of *B. spinosum* pollen, in addition to the other major represented pollen species, shape the final characteristics of Zantaz honey, a PCA integrating all the assessed physicochemical parameters was employed. Prior to running PCA, the data was subjected to the standardization. As the 18 samples of the present work were more characterized then those of the previous work, they were chosen to be used for the next section. The output is illustrated in Fig. 3 (a).

The first two PCs explaining 40.02% and 21.74%, successively of the given data, were used for plotting. Considering PC1, two groups could be distinguished: a group labelled as black squares presenting an average value of 58 ± 11.24% of *B. spinosum* pollen, and a second one, labelled in blue circles, presented an average of 40 ± 15.98%. three samples of this group were above the average of *B. spinosum* pollen percentage and were misplaced in the left side of the plot. The samples were, namely, S8, S9 and S16, and shared with sample S7 the presence of *Eucalyptus* pollen. The presence of *Eucalyptus* sp. pollen is linked to the production of light coloured honey, which may explain the divergence seen in the three mentioned samples compared to the remaining samples.

The observation of the variables plot showed on the onset of Fig. 3 (a) confirmed the conclusion of PCA described previously (Figs. 1 and 2): The presence of *B. spinosum* pollen correlates positively the values of colour, melanoidins, moisture, iron, manganese and calcium. However, the data concerning the sugar must be confirmed in previous work compared samples with higher amount of *B. spinosum* pollen and low or quite null percentage of these pollens.

To estimate the significance of the difference observed after the use of physicochemical parameters, the means of the major represented pollen species were compared between the two obtained groups, and the results were illustrated in Fig. 3(b). *B. spinosum* pollen showed to play an important role in this discrimination, and the difference was significant at $p < 0.05$. Another important feature is the prevalence of the *Cystus* sp. and *Populus* sp. pollens in the groups presenting low levels of *B. spinosum* pollen, although the difference was not significant.

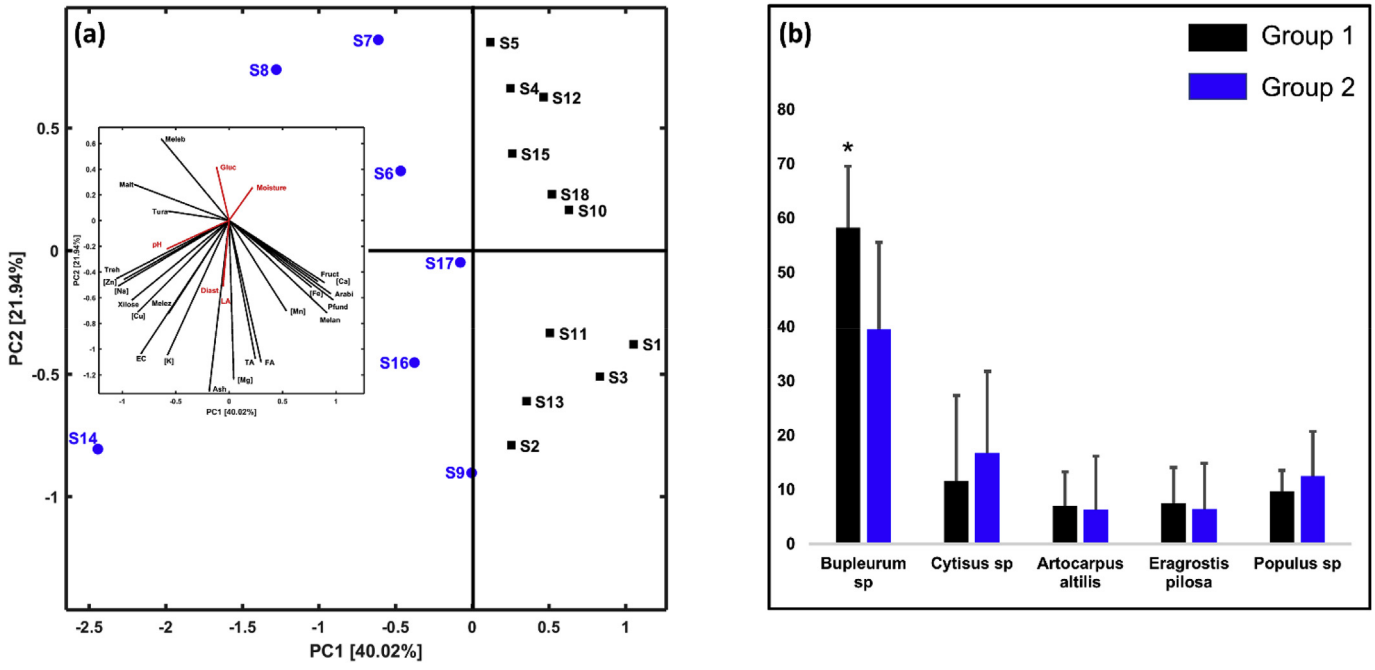

Fig. 3. The discrimination amongst the 18 samples of the present work using a PCA integrating the information of all assessed parameters (a), and the possible effect of the most abundant characterized pollen species comparing the samples discriminated by the first PC using student *t*-test (b). the onset of the figure (a) is the variable plot, and the red lines are plotted as facultative. (b) Comparison between the means of the groups discriminated in Fig. 3 (a) considering the main identified pollen species * $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusion

In this second contribution to the characterization of the Moroccan Zantaz honey, known by the predominance of *Bupleurum spinosum* pollen, 83.33% of the analysed samples presented more than 45% of *B. spinosum* pollen, which is the general monofloral threshold for honey monoflorality declaration. Besides the pollen identification, the analysis concerned mainly the sugar and mineral profiling in addition to a panel of physicochemical parameters used for honey quality control. The results showed no important aberrant values in comparison to the previous study published by our team. While comparing the data of honey samples belonging to both seasons, an attempt to understand the relationship between the identified pollen species and the routinely assessed physicochemical parameters was performed using the clustering ability of PCA. The presence of *Bupleurum spinosum* pollen was correlated to the levels of acidity, colour intensity, trehalose, melezitose, Ca, Fe and Mn.

When integrating all assessed physicochemical parameters, a PCA conserving 61.96% of the given data allowed discriminating the two groups of honey samples. The samples of the first group presented a mean of 58 ± 11.24% in terms of *B.spinosum* pollen and was significantly higher ($p < 0.05$) than the mean percentage obtained for the second group (40 ± 15.98%). No significant difference was seen in the case of other pollen species, suggesting the importance of *B. spinosum* nectar in shaping the acquired features of Zantaz honey samples. Considering the means of the two groups (58 ± 11.24% and 40 ± 15.98%), one can speculate that a Zantaz honey sample obey, at least, the general monoflorality threshold of 45%, though, a determinant conclusion can be made only after a detailed sensorial study.

Declarations of interest

None.

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