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A chemometric approach: characterization of quality and authenticity of artisanal honeys from Argentina

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The demand of honey with defined quality has increased around the world; therefore, an adequate description of the traceability and authenticity of honeys is necessary. The pollen and physicochemical characteristics of 58 honey samples collected from five different environmental units (EUs) of the Jujuy province (Argentine) were determined, in order to differentiate them by geographical origin through the application of chemometric methods. A qualitative pollen analysis was performed by microscopy. The physicochemical characteristics were determined by Association of Official Analytical Chemists methods. Correspondence analysis (CA), principal component analysis (PCA) and linear discriminant analysis (LDA) were performed. Forty-five per cent of honeys were monofloral; nearly half of them correspond to native species—among which, *Salix humboldtiana, Baccharis* sp. and *Ziziphus mistol* stood out. Physicochemical and microbiological analysis showed acceptable quality for honeys. CA characterized significantly honeys from four different EUs using all pollen data. While forward stepwise coupled to LDA identified 14 significant variables to build a discriminatory model with three significant discriminant functions, a cumulative variance of 94% was reached. Thus, 86% of the samples were correctly classified. PCA and LDA applied to physicochemical data allowed to distinguish three different groups with a significant function ($p < 0.01$) that explained 90% of the total variability. Honey corresponding to EU III Subandean Hills of Santa Bárbara was the most accurately classified by pollen and physicochemical data. The application of chemometric methods to pollen data and physicochemical parameters may be a useful tool to determine honey geographical origin. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: honey; melissopalynology; physicochemical properties; chemometric methods

1. INTRODUCTION

Over the last years, the demand for defined composition, quality, correct origin labeling and certainty of unadulteration in honey has increased around the world [1,2]. The determination of the botanical and geographical origin of honeys contributes to define its authenticity and traceability, increasing the added value of the beekeeping chain [2,3].

Argentina is one of the main honey exporters in the world; this country has excellent agroclimatic conditions and a great diversity of forages for beekeeping [4,5]. Argentina produces a wide variety of monofloral and multifloral honeys corresponding to exotic species, either introduced accidentally or cultivated, such as *Acacia* and *Eucalyptus* sp. and native species such as *Prosopis* sp., *Schinus* sp. and *Salix* sp. [6–8].

Honey's composition depends on the plant species visited by honeybees, environmental conditions, processing and storage [9]. Therefore, honey is a complex product, and several parameters need to be taken into account in order to determine and define its characteristics [10,11]. Then, statistical techniques capable of handling a set of data are required. Chemometric methods (also known as multivariate statistical techniques) are being increasingly used, because they are capable of identifying a natural clustering pattern and group variables on the basis of similarities between the samples [12–14]. These methods involve the use of mathematical models and statistical techniques to analyze and extract information from a data set [12,14,15]. Application of chemometric methods helps to reduce the complexity of large data sets and offers better interpretation and understanding of these data sets [14]. The most commonly used ones are principal component analysis (PCA) [16–18], cluster analysis [2,14] and linear discriminant analysis (LDA) [16,19–22].

The identification and quantification of pollen grains make possible the botanical origin determination of honeys, allowing differentiating and typifying monofloral and multifloral honeys [23,24]. The geographical origin of a honey may be determined by the presence of a pollen type or combination of pollen types

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characteristic of the region [23,25]. Chemometric methods are a useful tool for the characterization of the geographical origin, although there are few studies applied to pollen data [22]. For example, correspondence analysis (CA) is a type of chemometric statistical analysis [26] that has been applied in many fields [27,28] but not yet in melissopalynological studies.

The composition and quality of honey may be determined by its physicochemical and microbiological characteristics. Internationally, honey's quality criteria are specified in Codex Alimentarius [10]. The intrinsic properties of honey such as low pH and high sugar content prevent the growth and survival of microorganisms [29]. Thus, a high count of yeasts, fungi and vegetative bacteria indicates a recent contamination from a secondary source [30].

The main quality indicators are moisture, sugar content, free acidity, hydroxymethylfurfural (HMF) content, diastase activity, electrical conductivity and ash content [10,11]. The measurement of these parameters is comparatively simple and provides a good information value [3,10]. Physicochemical parameters are used in combination with chemometric analysis to differentiate honey types. Some authors discerned between flower honeys and honeydew [18,31], while others characterized different types of floral honeys [16,32–34]. Physicochemical tests were also used, complemented with mineral profiles, in order to differentiate honeys by geographical origin [19,35].

Eight environmental units (EUs) were defined in Jujuy province (Argentina) according to soil type, climatic conditions, altitude, relief, geomorphology and surface geology [35]. These conditions have determined the existence of different flora in each region capable of providing nectar and pollen to bee hives, which certainly causes variations in the properties of honey [5,32,36]. However, three EUs have adverse environmental conditions (low temperatures, rocky soils and steep terrain, low water availability and sparse nectariferous vegetation) for beekeeping activity throughout all year.

The aim of the work was to evaluate the quality and authenticity of honeys from different EUs with beekeeping aptitude of Jujuy province, through the identification of its geographical origin, by the application of chemometric methods to pollen content and to physicochemical parameters.

2. EXPERIMENTAL

2.1. Sample collection

Fifty-eight honey (*Apis mellifera*) samples were collected from the five EUs with the greatest potential to beekeeping. EUs I, IV and V were not considered in this study because their beekeeping activities were null. Figure 1 shows the geographical location of the different EUs in the province of Jujuy and describes its distinctive characteristics. All the artisanal honeys studied were of floral origin. They were collected from EUs II (18 samples), III (8 samples), VI (13 samples), VII (14 samples) and VIII (5 samples), in accordance with the beekeeping activity level. Samples were collected during the months with the greatest honey production (November–January) of years 2011 and 2012, in compliance with Codex Alimentarius guidelines to sampling for physicochemical analyses [10] and with current regulations for melissopalynological analyses. Fresh samples of approximately 1 kg were obtained directly from beekeeper's settling tanks. Samples were taken to the laboratory immediately after collection and stored in a fresh and dark place (23 ± 2 °C) until analysis, up to 2 weeks from the moment they were collected.

2.2. Pollen analysis

Pollen analysis was performed by the methodology proposed by Louveaux *et al.* with subsequent acetolysis [23]. At least 600 pollen grains were counted with the aim of ensuring the stabilization of percentages. Pollen types were identified by comparison with the Reference Pollen Collection of the Palynology Laboratory of the Faculty of Agricultural Sciences of the National University of Jujuy. Frequency classes were determined for the different pollen types: predominant pollen (more than 45% of pollen grains counted), secondary pollen (16–45%), important minor pollen (4–15%) and minor pollen (MP, 1–3%). The pollen occurrence frequency was established as the percentage of samples in which a certain pollen type appears; four groups were identified: very frequent (>50%), frequent (20–50%), infrequent (10–20%) and rare (<10%) [23].

2.3. Microbiological analysis

Yeasts and fungi were counted following the methodology of the International Commission on Microbiological Specifications for Foods (ICMSF) [37]. The average number of colonies, multiplied by a dilution factor, was considered for the counting. Results were expressed as colony forming units of yeasts and fungi per gram of honey. *Total coliforms* were investigated using the methodology of ICMSF and enumerated by the Most Probable Number technique defined in the protocol [37]. *Salmonella* spp. were investigated according to a modification of standard method suggested by the Bacteriological Analytical Manual [30]. All microbial tests were performed in triplicate.

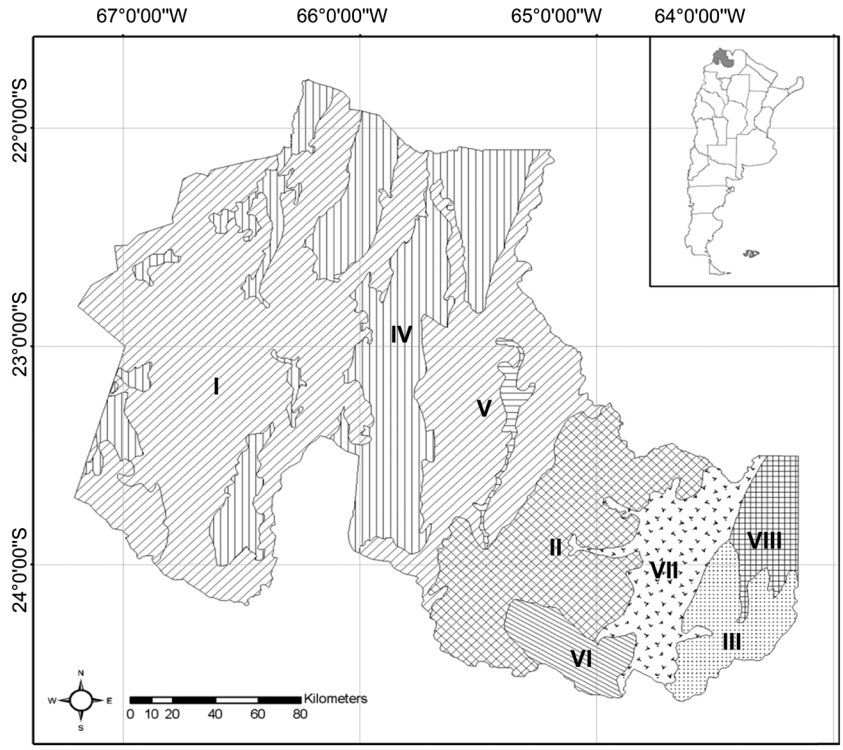
2.4. Physicochemical analysis

Moisture content was determined by refractometry at 20 ± 0.1 °C (Refractometer Abbe, Polish), reducing sugar and apparent sucrose by titration with Fehling's solution. Free acidity was determined by the titrimetric method as follows: 10 g of homogenized honey was weighed in a glass beaker, 75 mL of CO_2-free water was then added and this solution was titrated to pH 8.50 by adding 0.05 N NaOH.

The White method was used to determine the HMF content in honey samples [38]. Five grams of each sample was treated with a clarifying agent (Carrez), volume was then completed to 50 mL and the solution was filtered. Absorbance of the filtered solution was measured at 284 and 336 nm against an aliquot treated with $NaHSO_3$. Diastase activity was measured using a buffered solution of soluble starch and honey, which was incubated in thermostatic bath up to endpoint, determined photometrically in a HITACHI U-2000 device (Tokyo, Japan); diastase number was expressed in Gothe's scale. Ash content was determined by calcination at 600 °C to constant weight. pH was measured in 10% aqueous solution with a pH meter (UltraBASIC).

Analyses were executed in triplicate ($n = 3$) in accordance with the Official Methods of Analysis of the Association of Official Analytical Chemists [38].

The laboratory carried out an internal quality control procedure for each analysis. Equipment was calibrated against national standards. Calibration curves were prepared using standard solutions provided by National Institutes of Standards and Technology (NIST). All glassware used was of high metrological grade and traceable to SI units. The laboratory demonstrated its competence through regular participation in proficiency testing (PT) schemes launched by PT providers such as Food Analysis Performance Assessment Scheme.



EUs	II	III	VI	VII	VIII
	Montane Forests + Gasslands	**Subandean Hills of Santa Bárbara**	**Temperate Valleys**	**Humid Piedmonts**	**Subandean – Chacoan contact**
Relief	Hills with N-S orientation	Mountainous region	Open valleys, alluvial slopes and terraces	Varied slopes	Inclined planes. Gradients <2%
Temperature	Warm temperate to fresh	Temperate		Relatively warm all year round	Warm all year round
Vegetation	Subhumid forest. Grasslands	Forests and grasslands	Transitional forests whit xerophytic elements	Subhumid to dry forest	Subhumid-semiarid forest
Land use	Forest and livestock production	Forest and livestock production	Agricultural and livestock production	Irrigated agriculture	Agricultural and livestock production

Figure 1. Description of environmental units (EUs) of Jujuy province with the greatest beekeeping potential.

2.5. Statistical analyses

Physicochemical parameters were shown as mean value ± standard deviation and variation range for samples from each EU. Physicochemical results were analyzed using analysis of variance to determine if statistically different variables were present and whether they were due to the geographical origin of honeys. Tukey test was applied at a significance level of $p < 0.05$ and $p < 0.01$.

Chemometric methods were used to analyze and determine which variables (pollen data and physicochemical characteristic) could discriminate honeys from different EUs.

Correspondence analysis was performed on pollen occurrence frequency in honeys, to determine correspondence between them and the different EUs.

Principal component analysis was performed on physicochemical parameters to examine the grouping of honey samples and outliers, in order to visualize the relative distribution of the samples according to their geographical origin [12,17].

Linear discriminant analysis was used to determine discriminating variables (pollen data and physicochemical parameters) between two or more honey groups. This mathematical procedure was employed in order to maximize the variance between groups and to minimize the variance within each group [17]. LDA was complemented by a canonical analysis to obtain canonical score plots, which provided a visual organization of sample scores and facilitated the interpretation of the results.

SPAD software version 5.5 (Système Portable pour L'Analyse des Donees Textuelle) was used for PCA and CA. STATISTICA software version 8.0 (data analysis software system) was used for LDA.

3. RESULTS AND DISCUSSION

3.1. Pollen analysis

Figure 2 shows pollen types, frequency classes and occurrence frequency identified for the 58 honeys. In this figure, pollen types with a frequency class of <1% were not represented because of

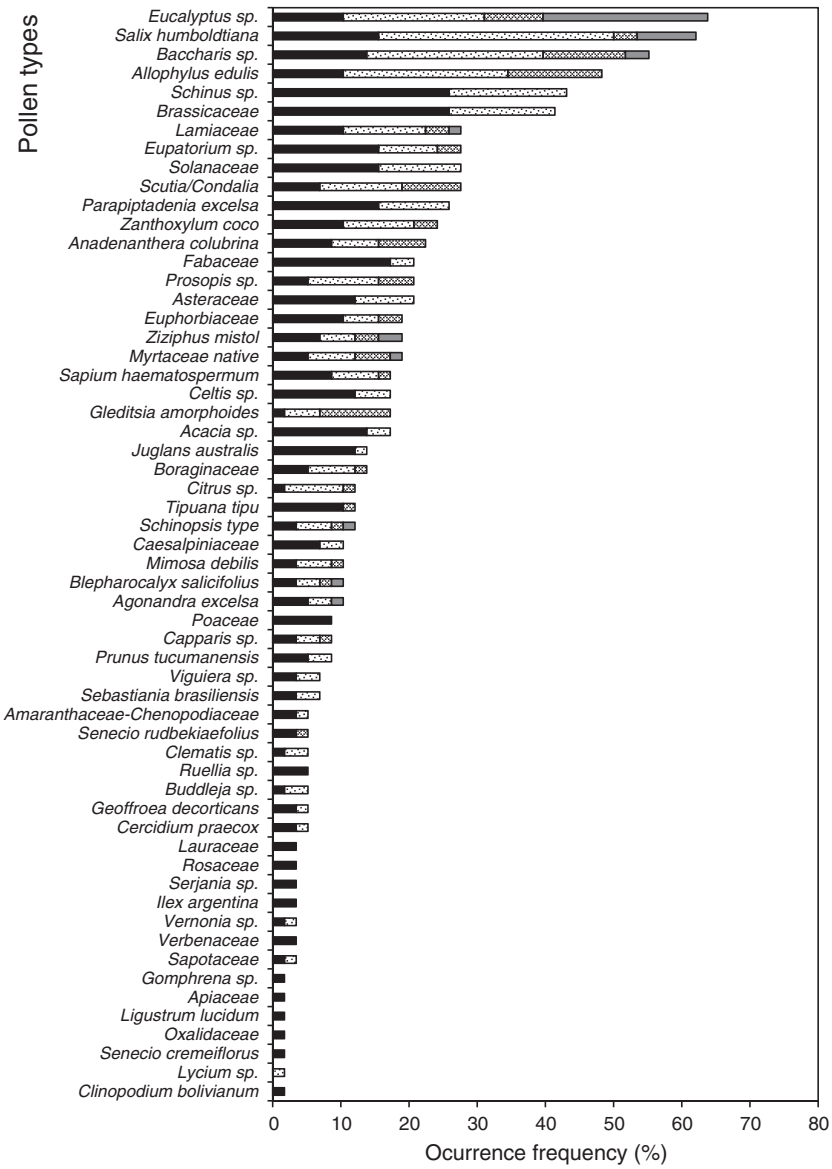


Figure 2. Pollen types: frequency classes and occurrence frequency. ■ PP, dominant pollen; ▨ SP, secondary pollen; ▩ PMI, pollen of minor importance; ▩ MP, minority pollen.

the very low content of pollen grains detected. Pollen analysis identified that 58 different pollen types correspond to 30 families. Of the 58 pollen types, 31 came from native flora, 20 from introduced flora and 7 were related to anthropogenic activities. The diversity of pollen types per sample varied in the range of 7 to 33 with 21 as the average and 24 as the most frequent value. The number of pollen types identified in each sample revealed the diversity and variability of nectariferous plants existing in different EUs in the province of Jujuy.

This number of pollen types is higher than those described for honeys in the northwest of Portugal [3] and central region of Argentina [39]. However, in other studies from ecosystems such as the Pampa region in Argentina [24], the north of Spain (Galicia) [29] and Ireland [40], the number of pollen types was higher than that determined in this study. Other authors also reported in honeys of different geographical regions the presence of pollen types from native, introduced and anthropogenic species [3,24,29,31,39]. Thirty-one of the pollen types belonged to Fabaceae and Asteraceae families. Native species *Baccharis* and *Parapiptadenia excels* from the Asteraceae and Fabaceae families were present in the highest number of honeys. These families also provided the greatest diversity of pollen types in honeys from Portugal, center of Argentina and Spain [3,7,18].

Forty-five per cent of honeys were monofloral, from which nearly half of them corresponded to native species—among which, *Salix humboldtiana, Baccharis* sp. and *Ziziphus mistol* stood out. The *Baccharis* sp. was also found in unifloral honeys from the central Argentina region [6,24,39]. The pollen spectrum of multifloral honeys revealed that 65% of the honeys contain mainly native species in secondary pollen. These results showed the importance of local vegetation in honey elaboration. Native species distributed along the five apiarian regions are herbs, shrubs and trees, which indicates that beekeeping activity occurs mainly in rural areas.

Nineteen per cent of monofloral honeys come from *Eucalyptus* sp., and a smaller percentage corresponded to other anthropogenic pollen types such as *Citrus* sp. Eucalyptus was the anthropogenic species present in a greater number of multifloral honeys (22%)

as main pollen. This species was introduced into different ecosystems. Its easy adaptation and rapid growth make it suitable for industrial use. As a consequence, there are monofloral and multifloral honeys of Eucalyptus in different countries, Argentina among them [3,7,24,39].

Concerning the distribution of pollen types according to geographical origin, 13 pollen types were found in the five EUs. The most frequent pollen type in the analyzed samples was *Eucaliptus* sp. from the Myrtaceae family, of anthropogenic origin, followed by the native species *S. humboldtiana* and *Baccharis* sp. from Salicaceae and Asteraceae families, respectively. Monofloral honeys of *Eucalyptus* sp. and *S. humboldtiana* were present in EUs II and VII, while monofloral honeys of *Baccharis* sp. were found in EUs II and VI. These nectariferous plants are common in the north and center of Argentina, and its presence is expected in artisanal honeys [7,8].

These results show the similarity of the flora across the different EUs. The fact that each EU does not contain one defined pollen type makes it more difficult to distinguish between them.

On the other hand, 15 pollen types, belonging to 14 different families, were found in a single region. Their identification could be crucial to determine the geographical origin of honeys from the melissopalynological point of view. However, these pollen types, mainly belonging to MP in samples, could probably belong to anemophilous pollinated species.

3.2. Microbial contaminations

The presence of yeasts and fungi was detected in 17% of samples, 7% of them exceeded the maximum limit set by the Argentine Food Code [11]. These values may indicate inappropriate handling during extraction and processing of honey and therefore the risk of unwanted fermentations. However, the moisture content and free acidity of honeys, according to regulation, indicated the absence of fermentation. Other authors detected the presence of yeasts and fungi in commercial Argentinean honeys obtained directly from beekeepers, with values similar to [4] or greater [30] than those permitted by national regulations [11]. Also, similar values to those described in this study were found in Portugal when other authors studied yeasts and fungi in commercial and artisanal honeys [3,9].

Total coliforms and *Salmonella* were not detected in studied honeys. Other papers reported total coliform count in Argentine honeys; however, fecal coliforms were no detected [4,30]. Although four honeys showed contamination of yeasts and fungi just above Argentine Food Code requirements [11], no alterations were detected in their physicochemical properties.

3.2. Physicochemical characteristics

Table I shows eight parameters of composition and quality of the analyzed honeys, with maximum and minimum limits established by the Codex Alimentarius for selling and consuming honey.

Table I. Physicochemical characteristics of honey samples analyzed ($N = 58$)

Parameter	Codex Alimentarius	EU II	EU III	EU VI	EU VII	EU VIII
		$n = 18$	$n = 8$	$n = 14$	$n = 13$	$n = 5$
Moisture (%)						
Mean ± SD	20 (max)	17.7 ± 1.1	16.3 ± 0.9	17.3 ± 1.3	17.7 ± 1.3	17.7 ± 1.4
Range		16.0–19.8	15.4–18.0	15.5–19.7	15.7–19.8	15.5–18.8
Reducing sugars (%)						
Mean ± SD	60 (min)	70.1 ± 2.2	70.0 ± 1.1	69.1 ± 2.9	69.1 ± 3.6	68.7 ± 2.7
Range		66.7–75.0	68.5–71.4	64.1–73.6	63.6–76.5	65.1–71.3
Apparent sucrose (%)						
Mean ± SD	5 (max)	4.3 ± 2.1	3.3 ± 1.0	4.3 ± 2.1	4.4 ± 2.3	2.6 ± 0.4
Range		1.2–7.7	1.5–4.8	0.9–7.8	1.1–7.7	2.2–3.1
Free acidity (meq/kg)						
Mean ± SD	50 (max)	20.1 ± 10.7	26.1 ± 5.6	21.5 ± 9.2	22.6 ± 9.37	22.8 ± 7.1
Range		6.0–40.0	19.8–35.2	10.0–36.7	9.2–38.0	13.5–32.0
HMF (mg/kg)						
Mean ± SD	40 (max)	$6.9 \pm 3.6^{a**}$	9.5 ± 10.2^{ab}	13.9 ± 7.9^{ab}	13.5 ± 5.2^{ab}	$21.4 \pm 14.8^{b**}$
Range		1.4–13.8	2.0–31.8	1.7–34.1	3.9–22.6	5.7–35.9
Diastase activity (ND)						
Mean ± SD	8 (min)	$14.5 \pm 3.7^{a**}$	$23.4 \pm 6.9^{b**}$	$15.2 \pm 3.6^{a**}$	$16.4 \pm 6.3^{a*}$	16.0 ± 6.1^{ab}
Range		8.0–23.0	12.7–31.3	11.0–21.7	9.0–27.0	8.0–22.5
Ash (%)						
Mean ± SD	0.6[#](max)	$0.43 \pm 0.16^{a*}$	$0.24 \pm 0.12^{b*}$	$0.43 \pm 0.13^{a*}$	0.37 ± 0.16^{ab}	0.36 ± 0.21^{ab}
Range		0.12–0.60	0.15–0.52	0.21–0.60	0.12–0.57	0.10–0.58
pH						
Mean ± SD		$4.6 \pm 0.4^{a**}$	$4.0 \pm 0.2^{b**}$	4.3 ± 0.4^{ab}	$4.1 \pm 0.4^{b**}$	4.3 ± 0.4^{ab}
Range		4.1–5.1	3.7–4.2	3.6–5.3	3.3–5.0	4.0–5.0

EU, environmental unit; HMF, hydroxymethylfurfural.
[a, b] Different letters in files indicate statistically significant differences ($p < 0.05$*; $p < 0.01$**).
[#]Maximum limit established by the Argentine Food Code.

Moisture content of analyzed honeys indicated an adequate degree of maturity according to the requirements of the Codex Alimentarius; no significant differences were observed between samples from the five EUs. Therefore, some authors determined differences in honey moisture content because of agricultural practices applied by beekeepers during extraction and storage [4,36], botanical origin [16] and the harvest season [34].

Another parameter related to maturity of honeys is sugar content [3]. Contents of reducing sugar were according to floral honey legislation [10]. Twenty-eight per cent of samples had apparent sucrose content higher than the maximum limit (5%) set by the Codex Alimentarius but less than the maximum allowed (8%) in the Argentine Food Code. The Codex only allows apparent sucrose content higher than 5% in specific honeys, for example, Alfalfa (*Medicago sativa*), *Citrus* spp. and Red Gum *(Eucalyptus camaldulensis*) [10]. Honeys with content greater than 5% originated from EUs II, VI and VII, and 12% of them were *Eucalyptus* sp. and *Citrus* sp. unifloral honeys. For this honey types, the Codex allows up to 10% sucrose [10]. Other authors reported contents higher than 5% of apparent sucrose in floral honeys from Portugal [9] and Spain [16]. Sugar content values confirmed appropriate maturity and unadulterated honeys.

All samples had free acidity values below 50 milliequivalent of acid per kilogram of honey (meq/kg), indicating no honey fermentation. No significant differences were detected. Acidity values in samples were in the range reported for floral origin honeys [3,8,32].

Honeys did not exceed both, maximum and minimum limits, for HMF and diastase activity, respectively. Significant differences ($p < 0.01$) in the HMF content and diastase activity were determined depending on their geographical origin. HMF content of honeys from EU VIII was higher than EU II, while EU III showed higher diastase activity values with respect to EUs II and VI. HMF contents in honeys of EU VIII should increase as diastase activity decreases because of the warm weather of this EU; however, there was no correlation between these two parameters ($r = 0.07$). Then, the differences in the HMF and diastase activity parameters were due mainly to the differences in handling during honey extraction and processing. Subsequently, in PCA applied to physicochemical data, HMF content and diastase activity were excluded because these parameters may not be related to geographical origin.

Ash contents did not reach 0.6% in all samples, which is a characteristic value of floral origin honeys [9,18,40]. Significant differences in ash content between EUs II, III and VI were detected. These results would suggest that ash content was modified because of the collected material by bees in different geographical regions [3,41]. Different authors described dependence between ash content and floral origin in honeys [40,41]. Therefore, ash content may be a complex function of both floral and geographical origin [41].

The pH values of samples varied from 3.3 to 5.4, with a mean of 4.3 ± 0.4, range characteristic of floral origin honeys [10,36]. These significant differences reaffirm the botanical and geographical diversity of studied EUs. In honeys from Serbian and Argentine, pH differences due to floral origin [32] and geographical origin [41] were reported. Thus, pH could be considered as an important marker of the honey's geographic origin.

Generally, physicochemical analysis of samples could indicate an acceptable quality of floral origin honeys, in accordance with the limits established by international regulations [10]. Ranges determined for all physicochemical parameters were consistent with informed values for monofloral and multifloral honeys [5,20,29,32].

3.3. Multivariate analysis

Pollen types determined as MP were not considered for chemometric analysis because of their low presence in honeys as they may come from transport and/or air mass deposition. CA was performed on 46 pollen types used as variables in the 58 samples. From CA result, applied to the frequency table for 46 pollen types of honeys, the first 10 dimensions retained represent >70% cumulative inertia.

All significant levels of the 10 dimensions were less than 0.0001, which means that there was a strong relationship between pollen types and samples from different EUs. However, different dimensions contributed with values lower than 10% of total inertia. This would suggest that pollen types associated to the samples determine profiles with similar characteristics.

Figure 3 shows a CA map with coordinates of pollen types, honey samples and the pericenter of each EUs. *Eucaliptus* sp. was placed on the positive axis extreme of dimension I (Figure 3a), while most of the other pollen types were placed on the opposite extreme. Therefore, *Eucaliptus* sp. was the major contributing species to total inertia and to the differentiation between samples, because it was one of the species with major presence in honeys. Figure 3a also shows that EU III samples were significantly characterized by dimension I. The pollen types that significantly contributed to the association and differentiation of the samples from EU III were *Gleditsia amorphoides* and *Myrtaceae native*, belonging to native species from this region. Dimension 4 allowed to characterize and significantly differentiate honeys from EUs III and VIII. Pollen types *Eucaliptus* sp. and *Blepharocalyx salicifolius* contributed significantly to the association between samples from EU VIII.

Figure 3b displays the CA map showing the dimensional axes 5 and 7, with cumulative proportions of inertia of 7% and 6%, respectively. *Citrus* sp. and *S. humboldtiana* were represented on the positive axis of dimension 5, while *Capparis*, *Schinopsis type*, *Z. mistol* and *Blepharocalys salicifolius* were on the negative.

S. humboldtiana and *Citrus* sp., associated to dimension 5, contributed to characterize significantly samples from EU VII. However, the contribution of dimension 5 to total inertia was low because these pollen types were present in monofloral and multifloral honeys from other EUs.

Dimension 7 significantly characterized several of the samples from EU II, and the pollen types contributing to their association were *Baccharis* sp. and *Allophylus eduli*. These pollen types were established as very frequent in the analyzed honeys; thus, there were samples from other groups with similar pollinic profiles. As a consequence of this, the epicenter of EU II was located near the barycenter.

According to CA results, honeys from four EUs were significantly differentiated by associations and differences between pollen types. However, a high number of dimensions were required to explain the total inertia because most of the pollen types were present in honeys from the five different EUs. This caused a lack of separation and differentiation between samples.

Because of the high number of variables, which do not contribute to the differentiation of honeys by geographical origin, a variable selection procedure forward stepwise coupled with LDA was applied to the data matrix.

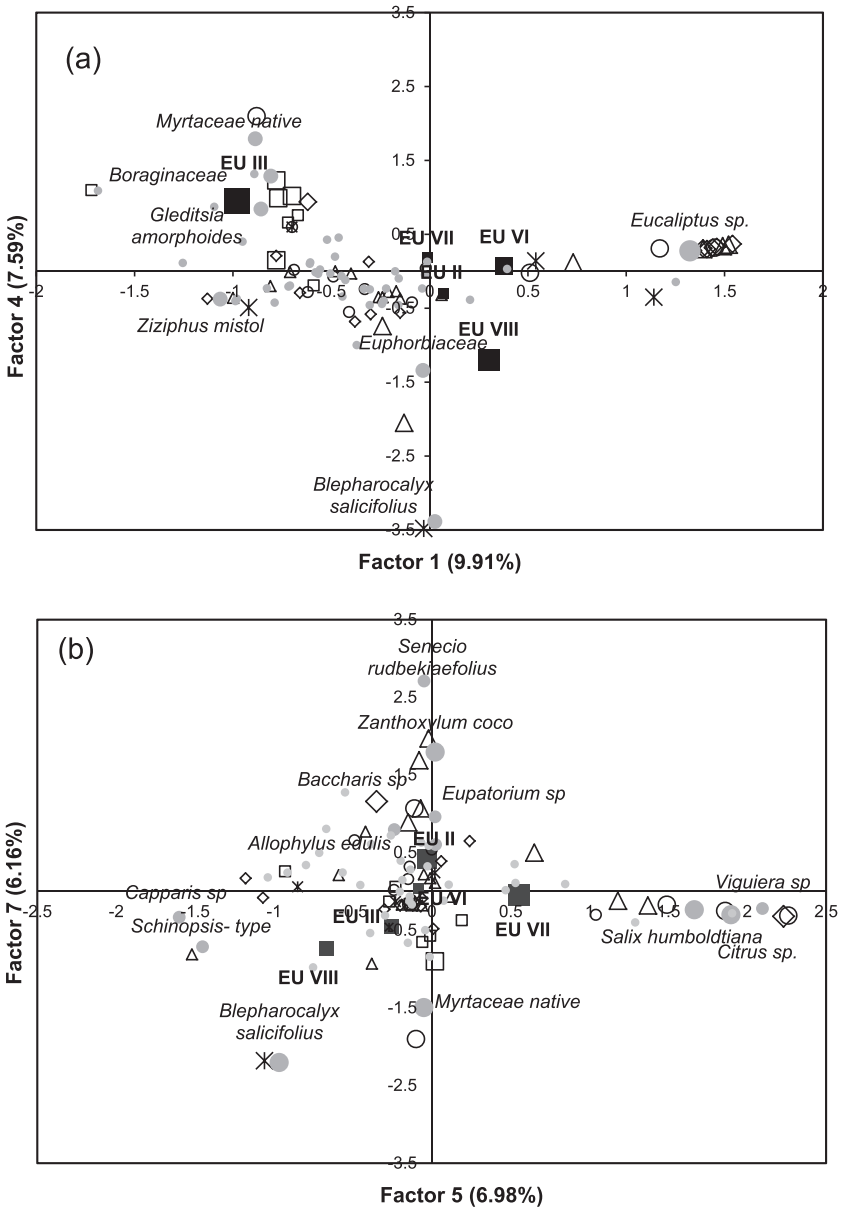


Figure 3. Correspondence analysis map for pollen types and honey samples relative to the principal axes (a) F1 and F4 and (b) F5 and F7. Increased size of each dot indicates better relative contribution (type pollen, honey and environmental units [EUs]) to the principal axes. ● Pollen types; samples: Δ EU II, □ EU III, ◊ EU VI, ○ EU VII, ○EU VIII; ■ nominal variable (EUs).

Forward stepwise analysis carried out 27 steps and found 14 significant variables ($p < 0.05$) to construct a discriminatory model. Significant variables had Wilks' lambda values <0.01 (Table II), indicating high discriminatory power for each pollen type selected, in this case mainly native species of each region.

Table III shows discriminant functions (Root) and cumulative proportion of total dispersion obtained by LDA. Three significant discriminant functions ($p < 0.05$) were determined, which explained 94% of the total variability. Pollen types *G. amorphoides, Parapiptadenia excelsa* and *Sebastiania brasiliensis* were the most discriminant variables for functions 1, 2 and 3, respectively.

Figure 4 shows score values of the first two discriminant functions. Samples from EUs III, VII and VIII were properly discriminated by these functions. However, no clear separation between samples of EUs II and VI was observed.

By using classification functions (Table IV), 86% of samples were correctly classified according to their geographical origin. Only samples from EU III were classified 100% correctly. Samples from EU III were generally multifloral honeys. Pollen types such as *G. amorphoides*, *Agonandra excelsa* and *M. native*, determined as discriminating variables, were present as secondary pollen in all honeys.

Forward stepwise method coupled to LDA applied to pollen grain count eliminated less discriminating variables and was suitable to differentiate and to classify samples according to their geographical origin. Similarly, Sancho *et al.* were able to correctly classify 93% of honeys from three provinces of Spain using discriminant analysis on pollen taxa in samples [22]. LDA results applied to pollen data validated the obtained CA outcome. According to Figures 3 and 4, honeys from EU III show a clear separation from the other EUs. Nevertheless, samples from EUs

Table II. Significant pollen types in the model selected by forward stepwise combined with discriminant analysis

Pollen types	Wilks' lambda	*F* (statistic)	*p*-level
Gleditsia amorphoides	0.0109	10.2844	0.0000
Boraginaceae	0.0081	5.9465	0.0014
Prosopis sp.	0.0079	5.6388	0.0019
Buddleja sp.	0.0079	5.6009	0.0020
Lamiaceae	0.0074	4.7507	0.0049
Prunus tucumanensis	0.0072	4.5485	0.0061
Anadenanthera colubrina	0.0071	4.4129	0.0071
Ziziphus mistol	0.0069	4.0421	0.0107
Blepharocalyx salicifolius	0.0068	3.9238	0.0122
Agonandra excelsa	0.0065	3.4759	0.0205
Parapiptadenia excelsa	0.0065	3.4191	0.0219
Myrtaceae native	0.0063	3.1464	0.0302
Schinus sp.	0.0061	2.8388	0.0436
Sebastiania brasiliensis	0.0061	2.7515	0.0485

Table III. Significant discriminant functions obtained by discriminant analysis of pollen types

Roots removed	Eigenvalue	Cumulative proportion	Wilks' lambda	Chi-square	*p*-level
1	10.06	64.31	0.004	222.94	0.0000
2	2.85	82.56	0.048	124.38	0.0006
3	1.81	94.17	0.185	69.03	0.0383
4	0.91	100	0.522	26.58	0.3240

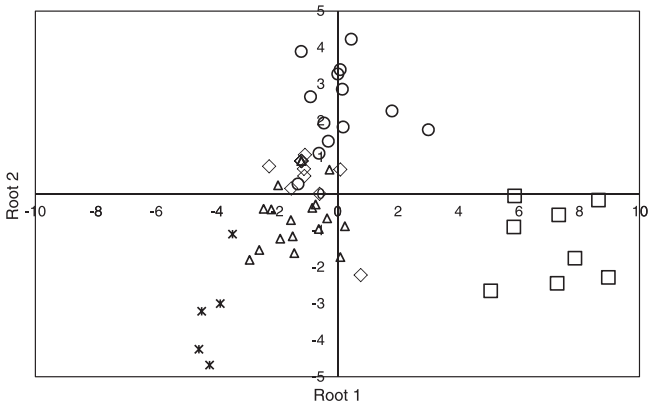


Figure 4. Scatterplot of canonical scores of discriminant analysis to pollen types of honeys. Δ environmental unit (EU) II, □ EU III, ◊ EU VI, ○ EU VII, ✱ EU VIII.

II, VI, VII and VIII were grouped with significant overlap probably because of the presence of similar pollinic profiles in honeys from these EUs.

Figure 5 shows the factor coordinates from PCA applied to the physicochemical parameters. Considering the Kaiser criterion, only factors (principal components) with eigenvalues greater than 1 were retained [19]. The first three factors explained 71% of total variance; free acidity was the most important parameter followed by reducing sugar and moisture. Honeys from EU III were grouped with small variability and were separated from

Table IV. Classification matrix of honeys on the basis of pollen types

Group	% Correct	II	III	VI	VII	VIII
II	83	15	0	3	0	0
III	100	0	8	0	0	0
VI	93	1	0	13	0	0
VII	77	1	0	2	10	0
VIII	80	0	0	1	0	4
Total	86	17	8	19	10	4

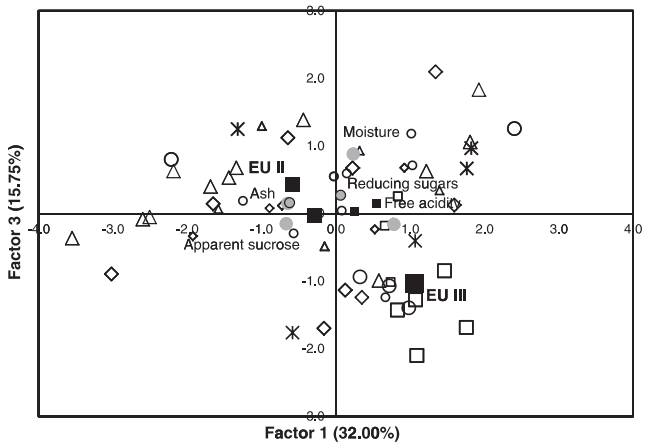


Figure 5. Plot of factor 1 and 3 coordinates from principal component analysis of physicochemical parameters. ● Physicochemical parameters; samples: Δ environmental unit (EU) II, □ EU III, ◊ EU VI, ○ EU VII, ✱ EU VIII; ■ nominal variable (EUs).

the EU II samples. Factor 1 allowed discriminating significantly samples from EUs II and III, although samples from both of these groups showed significant overlaps with samples from EUs VI, VII and VIII. Factor 1 explained 32% of total variability, and free acidity contributed significantly to its value. Free acidity showed the greatest variability among physicochemical parameters and was significantly correlated ($p < 0.05$) with apparent sucrose, ash and pH, although lineal correlations were low ($r < 0.4$) between them. Free acidity was used as the discriminant parameter with respect to botanical [14,16,20] and geographical [5] origin when PCA was applied to honeys from Uruguay, Spain and Argentine.

Factor 2 explained 23% of total data variability. Reducing sugar content was the most important parameter, although it did not contribute significantly on discrimination of the samples according to their geographical origin.

Moisture content was the physicochemical parameter with the lowest variability; however, it was the most important variable on factor 3 and contributed to differentiate significantly samples from EUs II and III. Moisture was also reported as the discriminant parameter in respect to botanical origin from honeys of Croatia, Uruguay, Spain and Turkey [14,16,17,34], while reducing sugar was determined as the discriminating variable for botanical origin and harvest season in Croatia [31]. These results could indicate that honey types from EUs II and III may be distinguished between them and from those of the other EUs, and they were clustered together because of a combination of characteristics derived from their botanical and geographical origin. Samples from EUs VI, VII and VIII could not be distinguished from one another possibly because of the presence of monofloral and

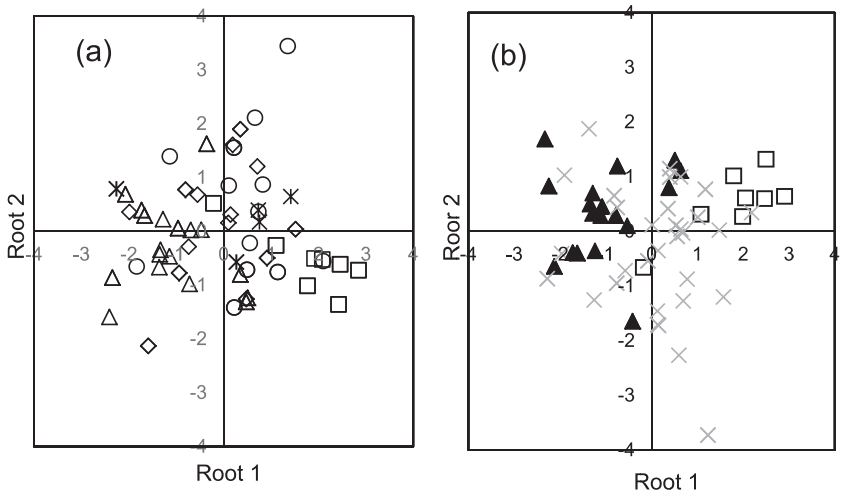


Figure 6. Scatterplot of canonical scores of physicochemical parameters. (a) Five groups: Δ EU II, □ EU III, ◊ EU VI, ○ EU VII, ✱ EU VIII; and (b) three groups: ▲ EU II, □ EU III, **x** EUs VI + VII + VIII.

multifloral honeys with similar pollen types and therefore similar physicochemical characteristics [33,34]. Lazarević *et al.* also had difficulty in discriminating monofloral and multifloral honeys by its geographical origin using PCA to the physicochemical parameters [32].

Linear discriminant analysis was applied to honey's physicochemical data to build a discrimination model; significant variables ($p < 0.05$) were pH and moisture. Wilks' lambda variable values were greater than 0.5, indicating a low discriminatory power. A significant ($p < 0.01$) discriminant function was found with Wilks' lambda of 0.4, which explained 89% of total variability; pH was the most discriminant variable. Figure 6a shows that the discriminant model was not suitable to separate the samples of the five EUs. LDA classification applied to the complete data matrix determined that only 55% of honeys were correctly classified according to geographic origin.

Taking into account the lack of discrimination of samples by geographical origin, a second LDA analysis was applied to honeys separated in three groups: (i) EU III samples; (ii) EU II samples; and (iii) EU VI, VII and VIII samples. In this case, LDA determined three significative variables: pH, moisture and ash. Only the first discriminant function was significant ($p < 0.01$), and pH was the most discriminant variable. The significant function had Wilks' lambda value of 0.48 and explained 86% of the discriminatory power of model. Figure 6b shows that the first function separated samples of EUs III and II. In this case, samples correctly classified increased up to 75%. Similar results were reported by Paramás González *et al.*, where 52% of honeys were accurately classified as originating from six different geographic areas [19]. The number of honeys correctly classified increased to 92% when samples were distributed in three geographical groups, selecting four discriminant parameters: total acidity, reducing sugars, conductivity and ash.

As a conclusion of PCA and LDA results, physicochemical parameters employed in this work were not sufficient to discriminate honeys belonging to the five EUs.

However, for results obtained for pollen data, LDA applied to physicochemical data validate the results obtained by PCA. These results could indicate that the honey types of EU III may be distinguished from those of the other EUs and could be clustered together because of a combination of characteristics derived from their botanical and geographical origin.

4. CONCLUSIONS

Correspondence analysis applied to pollen data of honeys is an exploratory technique suitable to determine the significative association and differentiation between pollinic types and honeys from different EUs (geography origin).

Furthermore, the use of a variable selection method coupled to the discriminant analysis was appropriate to determine significant pollen types and to build a discriminant model according to the geographical origin of honey samples. According to PCA and LDA applied to physicochemical parameters, only EU III samples, corresponding to Subandean Hills, were properly separated. It may be concluded that this region is suitable for obtaining honeys that may be clearly differentiated from others according to their pollinic and physicochemical characteristics.

Chemometric methods are a useful and accurate tool to distinguish honeys by geographical origin in order to contribute to its authenticity and traceability.

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