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Differentiation of argentine propolis from different species of bees and geographical origins by UV spectroscopy and chemometric analysis

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ABSTRACT

Bees collect vegetal resins that they mix with their wax and mechanical impurities to elaborate propolis, whose chemical composition is complex and variable depending on botanical/geographical origin, type of bee, time of year when it was produced and function in the hive. The presence of compounds that absorb UV radiation, such as those of the phenolic type: acids, esters, flavonoids and chalcones, largely responsible for their antioxidant, antimicrobial and anti-inflammatory biological activity has been reported. The objective of the present work was to establish if it was possible to differentiate Argentine propolis using UV spectroscopy and chemometric analysis, in the following cases: (a) Propolis elaborated by three different species of bees (*Apis mellifera*, *Tetragonisca fiebrigi*, *Scaptotrigona jujuyensis*) of the same geographical origin, and (b) Propolis produced by a species of bee (*Apis mellifera*) of four different geographical origins. UV spectrograms were performed in the 190–420 nm range for all the samples followed by analysis of principal components, hierarchical clusters and linear discriminants. The results showed that Argentine propolis could be differentiated in the two cases studied, and that *A. mellifera*, *T. fiebrigi* and *S. jujuyensis* would not use the same plant species to produce them.

1. Introduction

The native stingless bees (ANSA) (*Meliponini* Tribe) and the honey bees (*Apini* tribe: *Apis* genus), constitute a superfamily of the *Hymenoptera* order to which wasps and ants also belong. They are considered really social or eusocial and in their natural state they build complex nests inside cavities or in the open, generally formed by brood combs and cells or small pots for storage of reserves, constructed with wax, with or without the mixture of resins (Michener, 2007). The *Apini* tribe presents only the genus *Apis*, the honey bee, formed by about 9 species and the *Meliponini* tribe is represented by several genera, among which are *Tetragonisca* and *Scaptotrigona* with their species (Michener, 2007; Roig-Alsina et al., 2013). In Argentina, honey bees are found almost everywhere, while the ANSA are located in the provinces of Misiones, Corrientes, Entre Rios, Formosa, Chaco, Santiago del Estero, Santa Fe, Tucumán, Salta and Jujuy (Roig-Alsina et al., 2013).

Propolis is a product that bees make by mixing resinous substances collected from certain plants with their wax, pollen and mechanical impurities. They put it in specific places of the hive either as a sanitizing element or for structural purposes. (Bedascarrasbure et al., 2006). Different breeds of *Apis mellifera* (APIS) have different resin collection tendencies (Ghisalberti, 1979; Koo and Park, 1997; Mobus, 1972). The chemical composition of propolis is variable and complex, depending mainly on its botanical/geographical origin (Bankova, 2005; Dezmirean et al., 2017; Sforcin et al., 2000; Vera et al., 2011). Phenolic compounds like acids, esters, chalcones and flavonoids, responsible for its biological, antioxidant (Isla et al., 2001; Kurek-Górecka et al., 2013), antimicro-

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bial (Nieva Moreno et al., 1999; Salas et al., 2014) and anti-inflammatory (Ramos and Miranda, 2007; Salas et al., 2016) activities, have been identified. Diterpenes and triterpenes have also been reported (Aminimoghadamfarouj and Nematollahi, 2017; Mendes Araujo et al., 2015). Since the biological properties of propolis are closely related to its chemical composition and this, in turn, depends mainly on its botanical origin, the standardization of propolis is a complex problem, which is why an adequate method is needed to discriminate its origin (Bankova, 2005; Salatino et al., 2005). Various methods based on chromatography techniques (HPLC and GC), mass spectrometry (ICP-MS and GC-MS) and spectroscopy (NMR and IR) were used to determine the origin of food products (Luykx and van Ruth, 2008). However, these methods are laborious, slow, destructive and require prior preparation of the samples.

UV spectrometry is a widely spread, simple application method that does not destroy samples and is used in beekeeping and various agri-food products. However, its low selectivity makes it difficult to use in the analysis of complex samples such as propolis because of the large amounts of data to be dealt with. In some cases similar spectrograms are obtained, which is why it is associated with chemometric methods that differentiate them according to their geographical origin or time of production (Paganotti et al., 2014; Tomazzoli et al., 2015).

The Argentine Food Code (CAA) establishes in its physical and chemical requirements that the UV–Vis spectrogram of propolis should have a maximum absorption between 270 nm and 315 nm, regardless of the profile obtained. Currently, products differentiation is a growing strategy of adding value in Argentine beekeeping but no studies have been published up to now on the differentiation of Argentine propolis through its UV spectrograms and chemometric analysis. Hence, the objective of this study was to establish whether it was possible to achieve such a differentiation in the following cases:

a. Propolis made by three different species of bees (*Apis mellifera*, *Tetragonisca fiebrigi* and *Scaptotrigona jujuyensis*) of the same geographical origin.
b. Propolis produced by the same species of bee (*Apis mellifera*) of four different geographical origins.

2. Materials and methods

2.1. Propolis samples

ANSA propolis samples of the species *Tetragonisca fiebrigi* (Yateí) [n = 4 samples] and *Scaptotrigona jujuyensis* (Peluquerito) [n = 4] were obtained from hives under the rational breeding of the INTA EEA Famaillá meliponary (Famaillá, Tucumán) between 2013 and 2014.

APIS propolis were harvested from beehives with stamped out plastic meshes in apiaries that apply the INTA-PROAPI technological path in different places of Argentina: INTA EEA Famaillá [n = 9] and Leales [n = 11] (Tucumán), Andalgalá [n = 12] (Catamarca), Calingasta [n = 15] (San Juan) and General Obligado [n = 5] (Santa Fé) from 2001 to 2014 (Fig. 1). Once obtained, the samples were stored at −20 °C until processed. The vegetation surrounding the apiary/meliponary in each site was composed mainly of:

Famaillá: eucalyptus (*Eucaliptus grandis*, *Eucalyptus camaldulensis*), pine (*Pinus taeda*), fresno (*Fraxinus* sp.), ibirapitá (*Peltophorum dubium*), lemon, orange, grapefruit (*Citrus* sp.) and espinillo or aromo (*Acacia* sp.). There were sugar cane (*Saccharum officinarum*) and strawberry (*Fragaria ananassa*) plantations too (J. Grignola, 2017, personal comment).

Leales: vil-vil (*Myrcianthes cisplatencis*), lecherón (*Sapium haematospermum*), viraró (*Ruprechtia laxiflora*), molle (*Schinus fasciculata*), tusca (*Acacia aroma*), algarrobo negro (*Prosopis nigra*), cochucho (*Fagara coco*), pacará (*Enterolobium contortitsilicum*) and tala (*Celtis tala*). (Asoc.Coop. INTA Leales, 2016, unpublished data).

Fig. 1. Geographical location of propolis samples from APIS and ANSA.

Andalgalá: algarrobo (*Prosopis* sp.), jarillas (*Larrea* sp.), garabato (*Acacia praecox*), tintitaco (*Prosopis torquata*), chañar (*Geoffroea decorticans*), tusca (*Acacia aroma*), molle (*Schinus* sp.) (Maldonado et al., 2018).

Calingasta: jarillas (*Larrea* sp.), brea (*Cercidium* sp.), eucalyptus (*Eucalyptus* sp.), álamo (*Populus* sp.) and *Baccharis* sp. (Isla et al., 2009).

General Obligado: quebracho (*Schinopsis* sp.), chilca (*Baccharis salicifolia*), chañar (*Geoffroea decorticans*), algarrobo negro (*Prosopis nigra*), ñandubay (*Prosopis affinis*), curupí (*Sapium haematospermum*) (Sandrigo et al., 2014).

2.2. UV spectrograms obtention

APIS (2 g) and ANSA (10 g) propolis samples were processed in duplicate, according to the Norma Argentina IRAM-INTA 15935-1, 2008 standard, making successive extractions with n-hexane and ethanol to get standardized ethanolic extracts.

Absorption spectra in the UV region were acquired from aliquots of 50 μL or 250 μL of the APIS or ANSA standardized extracts respectively. They were placed in 25 mL volumetric flasks and completed in volume with ethanol. A Hewlett-Packard 8542A spectrophotometer and quartz cuvettes of 1 cm optical path were used, the absorbances being recorded in triplicate against an ethanol blank, in the range between 190 and 420 nm, with increments of 2 nm.

2.3. Chemometric analysis

The absorbances for each wavelength in the range considered and for each sample were exported to Excel 2016, obtaining a matrix of 116 columns and 13 rows for Case a, and 116 columns and 43 rows for Case b, where each column represents a variable that indicates the absorbance at a certain wavelength and each row represents a

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sample. To reduce dispersion, the original spectra were modified, centered and scaled by their own standard deviation using the standard normal variation algorithm (SNV) applying Eq. (1):

$$A_{(SNV)} = \frac{(A - A_m)}{sd} \quad (1)$$

where $A_{(SNV)}$: absorbance modified; A: absorbance read; A_m: average value of spectrum absorbances; sd: standard deviation of spectrum absorbances. This process was done in Excel 2016. Two other matrices of the same dimensions as the original ones were obtained and used for the chemometric analysis. Unsupervised techniques of principal components analysis (PCA) and hierarchical clusters (HCA) and supervised techniques of linear discriminant analysis (LDA) using the Infostat software version 2013 (Di Rienzo et al., 2013) (Universidad Nacional de Córdoba, Argentina. http://www.infostat.com.ar) were applied. PCA was exploratory to establish if the samples could be separated according to their spectra, especially in those cases where the differences were not so evident, and to analyze the relationship between samples and wavelengths. HCA facilitated their classification in groups according to their degree of similarity, while LDA established the capacity of differentiation of the UV spectra with respect to the type of bee or geographical origin of the samples.

3. Results and discussion

3.1. Case a. Propolis elaborated by three different species of bees (Apis mellifera, Tetragonisca fiebrigi and Scaptotrigona jujuyensis) of the same geographical origin

3.1.1. UV spectrograms

APIS propolis spectrograms were similar to each other and congruent with those reported for Tucumán propolis (Isla et al., 2005). For wavelengths greater than 250 nm, they showed a shoulder at 270 nm preceding the maximum absorption at 290 nm, thus meeting CAA requirements presenting a maximum between 270 and 315 nm. Those of Peluquerito showed an absorption peak at 210 nm, decreased to 250 nm where they presented a wide absorption band up to 300 nm, and then went down to zero. One of the samples presented a different spectrogram with a maximum at 290 nm, idem to APIS propolis. This could be due to the fact that Peluquerito collected propolis from an APIS hive, instead of elaborating it from vegetable resins. This behavior is known as "pillage". The Yateí also presented a peak at 210 nm, but gradually decreased to zero, at approximately 420 nm (Fig. 2). As a whole, the spectrograms showed two absorption bands: one from 190 to 250 nm and another from 252 to 420 nm defined by the vertical lines at 250 and 340 nm. They were thus divided into three wavelength ranges: 190 to 250 nm, 252 at 340 nm and 342 at 420 nm. An exploratory analysis by PCA was carried out in each to determine if a better propolis separation was achieved by any and to analyse the relationship between samples and wavelengths. The complete range from 190 to 420 nm was also included in the analysis. The bands observed in the spectra are attributed mainly to the phenolic acids and flavonoids that constitute them, so that the band near 210 and 230 nm is due to the bathochromic change caused by the different substituents such as hydroxyl, methoxyl and polynuclear compounds that displace the primary benzene band of 202 nm towards longer wavelengths. Another common band in most samples is in the range of 310 nm, which corresponds to the displacement of the secondary band of 255 nm for benzene. The band around 270 nm may be due to the conjugated double bonds present in the structure of some propolis compounds which are not always present in all samples. Absorption in the region around 380 nm is in the UVA zone near the absorbance at 400 nm corresponding to the violet zone of the visible spectrum, responsible for the

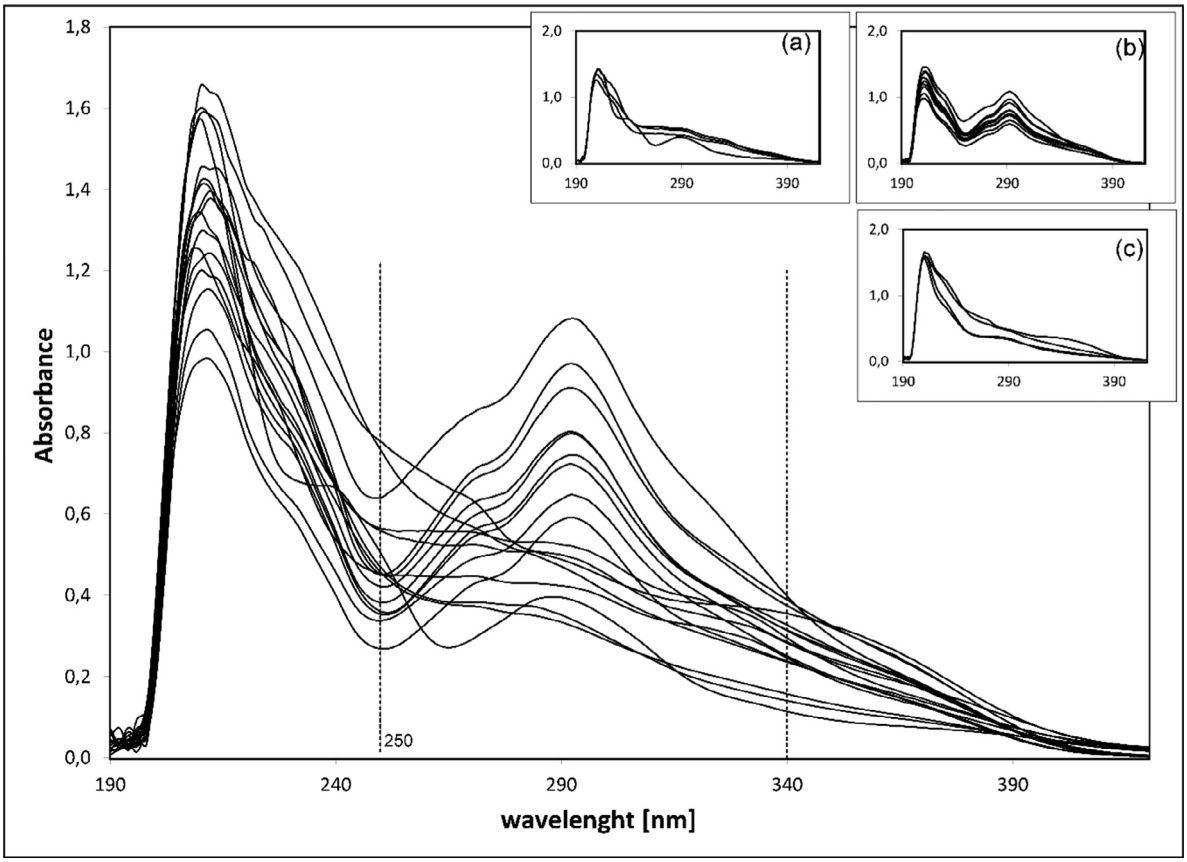

Fig. 2. UV absorption spectra of Argentine propolis extracts produced by different species of bees. (a) *Scaptotrigona jujuyensis*, (b) *Apis mellifera*, (c) *Tetragonisca fiebrigi*.

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yellow colour of most propolis (Barbeira et al., 2013; Paganotti et al., 2014).

3.1.2. Principal component analysis

The best separations of APIS and ANSA propolis were achieved in the ranges from 252 to 340 nm and from 190 to 420 nm (Fig. 3). In both cases with two components, 100% of the observed variability was explained. Those of APIS were strongly associated with component 1 and with wavelengths between 280 and 340 nm, corresponding to the absorption zone of phenolic compounds reported for propolis from Tucumán (Isla et al., 2005). The variability that is not explained by component 1, was achieved through component 2, so that the Yatei and Peluquerito propolis would be more associated with lower than 250 and higher than 360 nm wavelengths, which would correspond to compounds different of phenolics ones (Patricio et al., 2002). However, they were also reported in *Tetragonisca fiebrigi* propolis (Brodkiewicz et al., 2014; Campos et al., 2015). This would indicate that, although the supply of propolis producing plant species is the same, those used by APIS differ from the ones preferred by Yateí or Peluquerito, and differences could be found between them as suggested in Fig. 3 (a) and (c). The 190–420 nm wavelength range was used for subsequent analyses since, as indicated, sufficient separation/differentiation between the samples to be analysed was also achieved.

3.1.3. Hierarchical cluster analysis

A cluster or group consists of a data set that are more similar to each other, compared to the data that make up other groups. The clusters analysis is linked to the classification and can answer different questions but focused on the search of patterns (associations between species, taxa, classes, etc). Generates groupings of different level of similarity (hierarchical) like a tree (dendrogram) that can indicate relationships between cases and between variables. As seen in Fig. 4, the resulting dendrogram divides the samples in two main groups: that of APIS propolis and the one conformed by Yateí and Peluquerito propolis. The cophenetic correlation coefficient, in the framework of the reported PCA, calculates the correlation between the Euclidean distances in the reduced space with respect to the same distances in the space of dimension given by the number of original variables and can be used as a measure of the quality of the dimensional reduction achieved with the proposed model. In this case, the cophenetic correlation coefficient obtained was 0.97. Values equal to or greater than 0.75 indicate that the original distances were efficiently preserved.

3.1.4. Linear discriminant analysis

Two canonical discriminant functions were used that completely explained variability (canonical axis 1 explained 98%). All samples were correctly classified since the apparent error rate of classification was equal to 0%. Propolis separation in the discriminating space defined by the two functions is shown in Fig. 5. There are three clearly differentiated groups, corresponding to the propolis studied in this case, produced by each species of bee. Hence, propolis made by different bee species from the same geographic origin may be differentiated from their UV spectrograms by applying chemometric tools.

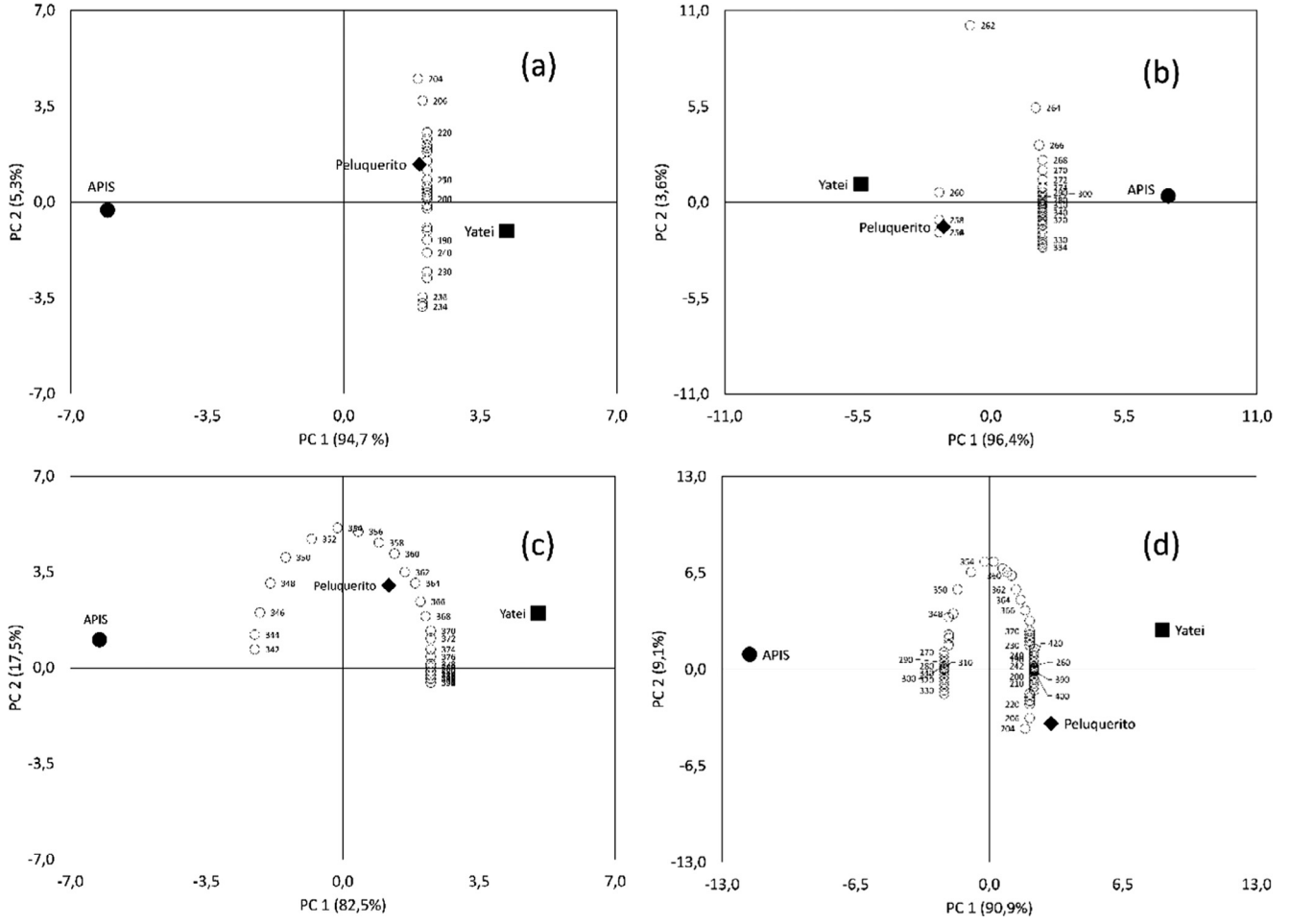

Fig. 3. Biplots that represent propolis of different species of bees and wavelengths in different ranges: (a) 190–250 nm, (b) 252–340 nm, (c) 342–420 nm, (d) 190–420 nm.

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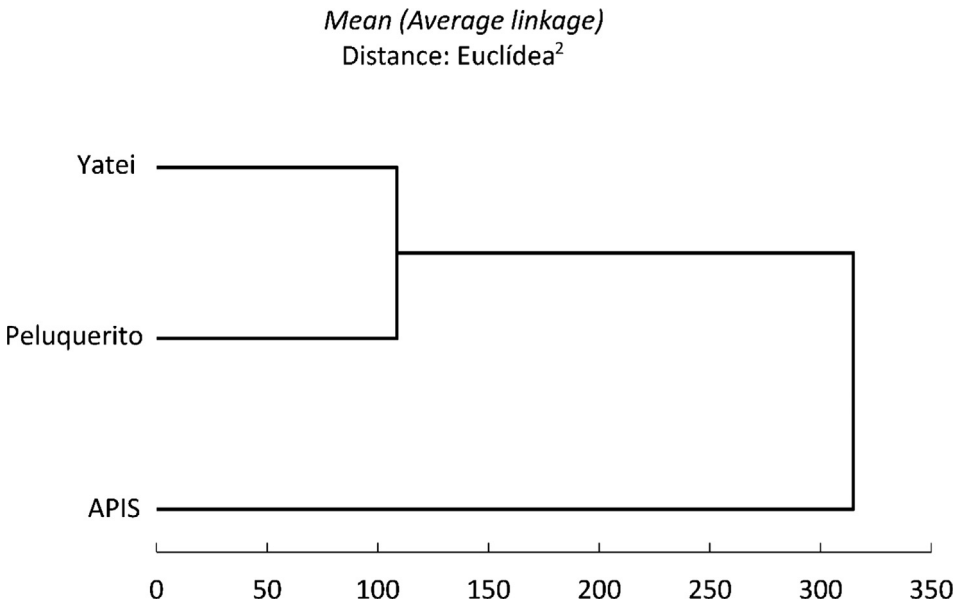

Fig. 4. Dendrogram indicating the separation of Argentine propolis from different species of bees into groups.

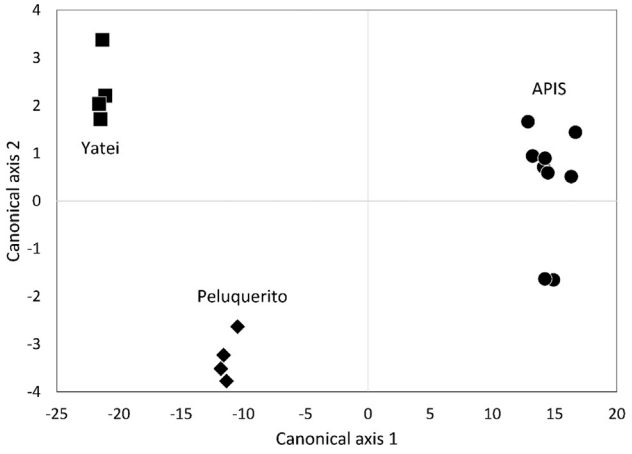

Fig. 5. Differentiation of Argentine propolis from different species of bees in groups.

3.2. Case b. Propolis produced by the same bee species (*Apis mellifera*) of four different geographical origins

3.2.1. UV spectrograms

The original spectrograms of the propolis obtained in the four localities considered showed two absorption bands in the 190–250 nm and 252–420 nm wavelength ranges (Isla et al., 2005). In the second band, maxima between 286 and 290 nm were observed with a small anterior or posterior shoulder, typical of APIS propolis produced in different regions of Argentina (Bedascarrasbure et al., 2004). The CAA requirement of exhibiting a maximum absorbance between 270 and 315 nm was met in all cases. Beyond the differences in absorption intensity, the spectrograms did not provide enough information to differentiate at first glance, propolis samples by their geographical origin, but chemometric tools facilitated into homogeneous groups (Tomazzoli et al., 2015). PCA, HCA and LDA analyses were carried out as in Case a (see Fig. 6).

3.2.2. Principal component analysis

As shown in Table 1, 94% of total variance was explained with two components through this analysis. Calingasta propolis samples were associated with component 1, while those of Andalgalá and Leales were identified with component 2, but in the opposite direction (Fig. 7). Those that showed a closer relationship were those of Leales and General Obligado. Although different contents of phenolic compounds were reported for propolis samples from all the places considered (Bedascarrasbure et al., 2006; Isla et al., 2009; Sandrigo et al. (2014); Maldonado et al., 2018), those of Calingasta showed the closest association with this type of compounds, as shown in Fig. 7.

3.2.3. Hierarchical cluster analysis

Samples of APIS propolis were separated into 3 geographical groups: Calingasta, Andalgalá and Leales-General Obligado (as suggested by the PCA) (Fig. 8). The cophenetic correlation coefficient was 0.76. In this way, it is clear that the use of HCA as a grouping criterion was efficient.

3.2.4. Linear discriminant analysis

Three canonical discriminant functions were determined and the first two represent 89% of the variance (Table 2). In Fig. 9, propolis separation into four groups corresponding to their origin may be observed. All samples were correctly classified since the apparent error rate of classification was equal to 0%. Hence, chemometric analysis applied to *Apis mellifera* propolis UV spectrograms differentiated them according to their geographical origin.

4. Conclusions

The results obtained in this study, established that it is possible to differentiate Argentine propolis made by different species of bees of the same geographic origin and propolis produced by a bee species of different geographical origins using a simple tool of fast application like UV spectroscopy complemented with chemometric analysis. It could also be inferred that, given the same availability of plant species to produce propolis, *Apis mellifera* preferences differ from those of *Tetragonisca fiebrigi* and *Scaptotrigona jujuyensis*, and there could even be different choices between the latter two. *Apis mellifera* propolis samples would be more closely associated with phenolic type compounds, while those of *T. fiebrigi* and *S. jujuyensis* would do so with other types of molecules. This difference might be used in a complementary way to have a larger spectrum of biomolecules, with potentially greater possibilities of application by achieving a better use of renewable natural

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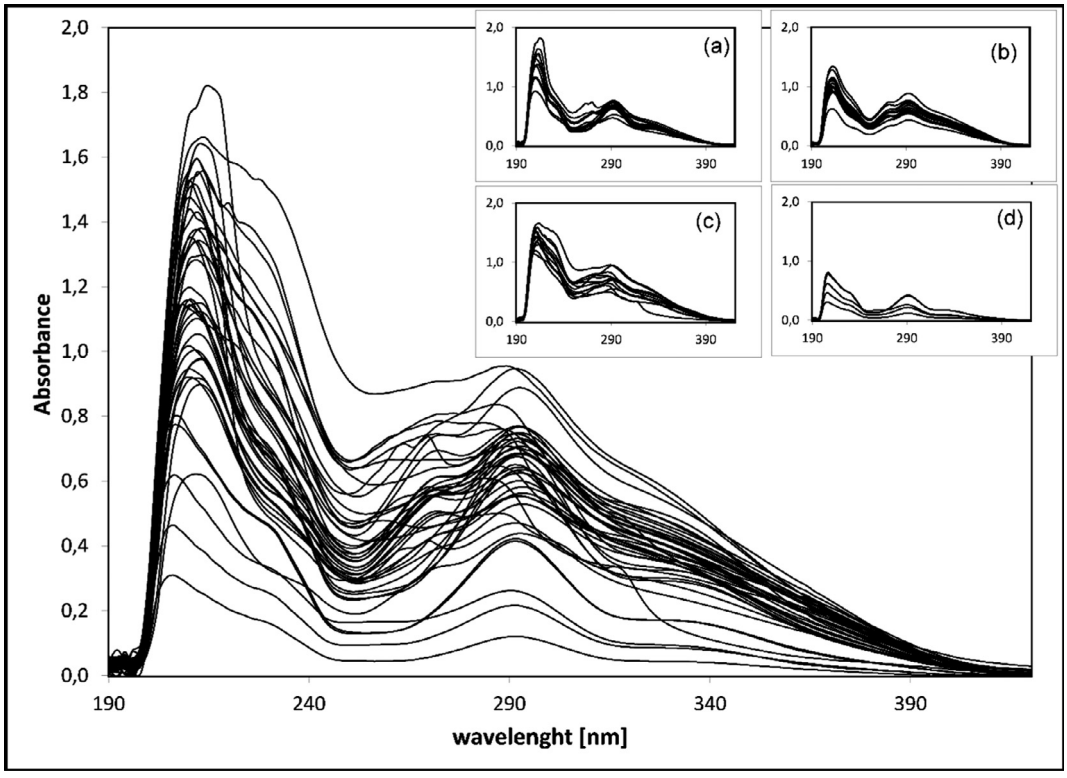

Fig. 6. UV absorption spectra of Argentine propolis extracts produced by *Apis mellifera* in different locations: (a) Leales, (b) Calingasta, (c) Andalgalá, (d) General Obligado.

Table 1
Importance of the principal components.

Component	Value	Proportion	Cumulative proportion
1	68.26	0.59	0.59
2	41.18	0.36	0.94
3	6.56	0.06	1.00

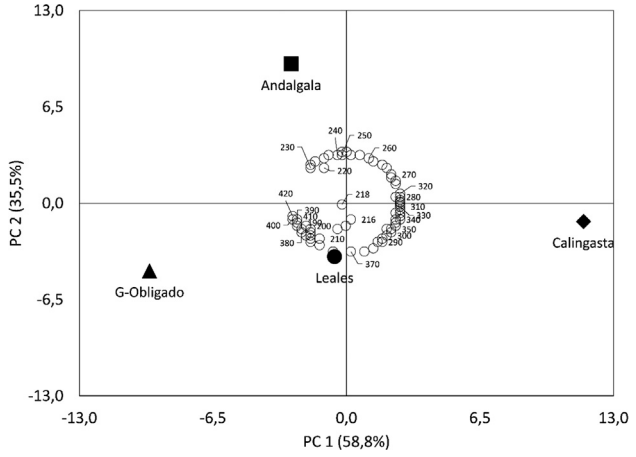

Fig. 7. Biplot representing propolis of *Apis mellifera* obtained in different localities of Argentina and the wavelengths as variable.

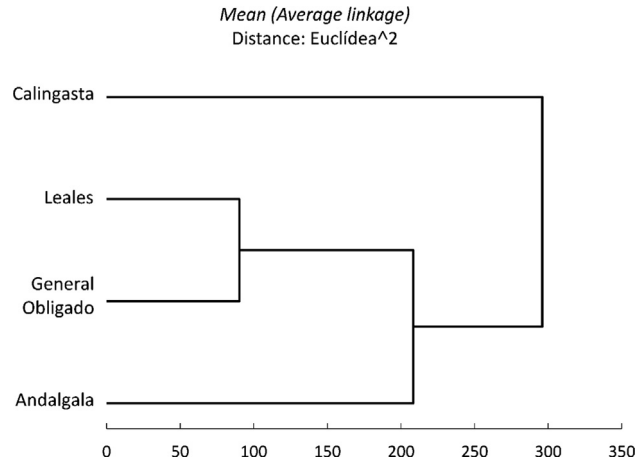

Fig. 8. Dendrogram that indicates the separation into groups of propolis of *Apis mellifera* produced in different localities of Argentina.

Table 2
Summary of canonical discriminant functions.

Function	Eigenvalues	% Variance	% Cumulative variance
1	151.79	57.30	57.30
2	85.10	32.13	89.43
3	28.00	10.57	100.00

resources; however, additional studies would be necessary to verify these inferences.

Author contributions

Luis Maldonado: conceived the study idea, analyzed the results of chemometrics analysis, and wrote the manuscript.

Karenina Marcinkevicius: performed the experiments (ANSA) and wrote the manuscript.

Gerardo Gennari: responsible for the Famaillá meliponary and apiary, wrote the manuscript.

Virginia Salomón, Romina Borelli: performed the experiments (APIS).

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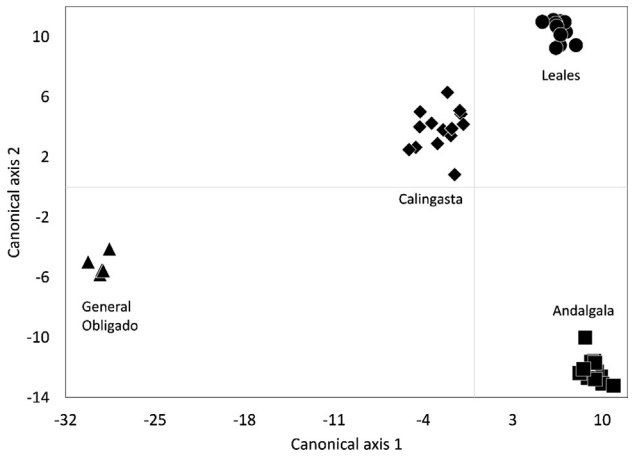

Fig. 9. Separation of Argentine propolis in groups according to their geographical origins.

María I. Isla, Nancy Vera: reading, editing and revision of the manuscript.

Valeria Borelli: performed and analyzed the results of chemometrics analysis.

All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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