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ORIGINAL ARTICLE

Determination of amino acid content and its enantiomeric composition in honey samples from Mendoza, Argentina

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Abstract

The amino acid (AA) content in honeys from Mendoza (Argentina) was determined by high-performance liquid chromatography and the relative quantities of D- and L-AAs were determined by chiral gas chromatography coupled to mass spectrometry. The results showed that proline was the most abundant AA in all analyzed samples, followed by phenylalanine. Based on the AA content, different chemometric tools were assessed for provenance differentiation. The unsupervised chemometric methods, however, could not differentiate unquestionably the geographical origin of honey based only on their AA content. Enantiomeric ratio demonstrated that D-proline amount was lower than D-phenylalanine levels in practically all honey samples. In addition, D-enantiomers of alanine, valine, glutamic acid, leucine, and isoleucine were found in most samples. The study demonstrated that certain D-AAs can occur naturally in this foodstuff, probably, as a consequence of the Maillard reaction, which is not dependent on microorganism actions.

Novelty impact statement

- The amino acid content in honey samples from Mendoza was determined by high-performance liquid chromatography. Proline and phenylalanine were the more abundant amino acid.
- Amino acid enantiomeric ratio was assessed by chiral gas chromatography coupled to mass spectrometry. D-amino acids can occur naturally in the honey samples from Mendoza (Argentina).
- Chemometric tools were applied to discriminate samples from the geographical origin.

1 | INTRODUCTION

Argentina produces about 60,000 tons of honey per year (Salgado-Laurenti et al., 2017). However, in this country—like China—the annual consumption rates range between 0.1 and 0.2 kg per capita (Alvarez-Suarez et al., 2010), whereas in developed countries the annual consumption per capita is about 1 kg. Thus, almost 95% of the local honey production goes to the export market (Mujica et al., 2016). According to Food and Agriculture Organization of the United Nations (FAO) statistics (FAO, 2011), Argentina is ranked as one of the three main exporters of honey in the world, after China and Turkey, and most of their productions are destined to the United States, Germany, and Japan (Fernández et al., 2017). Particularly, 90% of the beekeeping production of Mendoza province—one of the most important producers in Argentina—is destined for export, whereas the remaining 10% is sold in the domestic market (Prensa Gobierno de Mendoza, 2019). The National Registry of Beekeepers Producers (RENAPA, updated to February 2019) has registered about 400 producers, handling approximately 70,000 hives that are distributed throughout the provincial territory. Just more than 70%

of the hives are concentrated in the South, San Rafael and Alvear, the rest is distributed between the North, Valle de Uco, and the East.

The National Food Code (CAA, Chapter X, Sugar foods, Article 782, Res 2256, 2010) defines the honey or bee honey as "the sweet product elaborated by the worker bees from the nectar of the flowers or of exudations of other alive parts of the plants or present in them," that is, bees collect, transform, and combine with specific substances of their own, storing it in honeycombs, where it matures until completing its formation. Honey is probably one of the most complex food products that can be consumed by humans without prior treatment (Iglesias et al., 2004). Its diverse composition is affected by many factors such as the botanical source and geographical area, along with the season in which the honey is produced, time and storage conditions (Burns et al., 2018). The geographical classification is relevant for monitoring the chemical characteristics of honey (Azevedo et al., 2017). It is of general interest in terms of their authentication, one of the most important food quality assurances (Kečkeš et al., 2013). In this sense, the expansion of the honey market shows a tendency to establish geographical limits of production with the aim of preserving a production zone that has developed a particular standard of quality (Baroni et al., 2009).

Honey contains between 20 and 300 mg of amino acids (AAs) in 100 g of dry matter (Biluca et al., 2019). There are approximately 26 AAs in honeys, and usually proline (Pro) represents about 50%–85% of the total AA content (del Campo et al., 2016). The source of AAs in honey is attributable to both animal (bee secretions) and vegetal (nectar, honeydew, and mainly pollen) origins (da Silva et al., 2016). The AAs are nutritionally important, but, also, their analysis has proven to be a good indicator of both botanical and geographical origin of honey (Azevedo et al., 2017; Sun et al., 2017). Other authors found that AA content could be a criterion for distinguishing adulterations (del Campo et al., 2016). In this context, Pätzold and Brückner (2006) demonstrated that certain D-AAs are naturally found in honey. According to the authors, relative quantities and kinds of D-AAs detectable therein depend on samples and the amount of D-enantiomers increases by heating. Therefore, the presence and relative content of D-enantiomers found in honeys could be used as a test for the long-term storage and the nature of the processing of the honey (Pätzold & Brückner, 2006; Pawlowska & Armstrong, 1994).

Most studies reported data of free total AAs in honey determined by chromatographic methods (Biluca et al., 2019; Chen et al., 2017; del Campo et al., 2016) and only a few published works discriminate between L and D-AAs (Pawlowska & Armstrong, 1994). To the best of our knowledge, there are no studies related to determination of AAs, along with their enantiomeric separation in honey samples from Mendoza, one of the most important provinces of Argentina in terms of apiculture. Then, the main objectives of this study were as follows: (i) to evaluate the concentration of AAs in honey samples from different regions of Mendoza province; (ii) to determinate the ratio between L and D-AAs, and (iii) to assess if the AAs content can be used as a feature to achieve a geographic grouping of the samples.

2 | MATERIALS AND METHODS

2.1 | Reagents and materials

All chemicals and reagents were of analytical grade. The racemic AAs used, alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glycine (Gly), glutamic acid (Glu), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), ornithine (Orn), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and from The British Drug Houses (Poole, England). Acetonitrile (ACN) and methanol (MeOH, analytical-grade purity, 99.8%) were from Baker (Mexico City, Mexico), hydrochloric acid (HCl) from Merck (Darmstadt, Germany) and ammonium hydroxide solution (NH_4OH, liquid 28%) from Baker. 1-fluoro-2,4-dinitrobenzene (FDNB) and trifluoroacetic anhydride were purchased from Fluka (Bucks, Switzerland). Acetyl chloride and dichloromethane (CH_2Cl_2) were purchased from Sigma-Aldrich (St. Louis, MO, US). Sodium borate decahydrate $Na_2B_4O_7.10H_2O$ and formic acid were from Anedra (Argentina). The Dowex 50W-X8 cation exchange resin (200–400 mesh) was purchased from Sigma Aldrich. Deionized water was obtained from a purification Milli-Q system (Simplicity, Millipore, MA, USA).

2.2 | Instrumentation and chromatographic conditions

The high-performance liquid chromatography (HPLC) studies were performed on an Agilent 1200 series LC system (Agilent, CA, USA) equipped with a binary pump, vacuum degasser, temperature-controlled column compartment, autosampler and photodiode array detector (DAD) mounted with a 1-μl flow cell. The detector was set at 365 nm for all analytes. A ZORBAX Eclipse XDB-C8 column (150 mm × 3.0 mm i.d., 3.5 μm) was used. Separations were carried out by a gradient elution as follows 0–10 min 5% B, 10–47 min 5%–44% B, 47–53 min 44% B, 53–58 min 44%–64% B and 58–70 min 64% B; A = 60 mM ammonium formate buffer pH 3.5 and B = MeOH and the flow rate was set at 0.85 ml/min. The injection volume was 1–10 μl depending on the solution. Column temperature was set at 40°C.

A Shimadzu gas chromatography coupled to mass spectrometry (GC-MS) model QP2010 Ultra was used for the AAs enantiomeric separation. The carrier gas was hydrogen, and operation was conducted at constant pressure of 6.8 psi. Injections were made with a split ratio 30:1–60:1 depending on the sample. The injector temperature was set at 200°C and the ion source at 210°C. A chiral column octakis (6-O-tert-butyldimethylsilyl-2,3-di-O-acetil)-γ-cyclodextrin in OV1701 (10 m × 250 μm, 0.1 μm) fabricated in the laboratory was used (Menestrina et al., 2016). Separations were carried out at 55°C for 2 min, 2°C/min up to 150°C and finally, 150°C for 15 min. The injection volume was 1 μl. The mass spectrometer was operated in electron ionization mode and detector voltage was set relative to the tuning result. Selected ion monitoring mode was used for the

quantitation process. Appropriate ions were selected for each AA, not always they were not the most abundant. The characteristic mass fragments (*m*/*z*) of the AA derivatives are indicated in Table 3.

2.3 | Amino acid derivatization procedures

A standard stock solution containing racemic mixtures of 18 AAs plus Gly, was prepared by dissolving a known amount of each amino (~1.7 mg) to a final volume of 1 ml with 0.1 M HCl. The solution was stored at 4°C and diluted before using.

AAs were derivatized with 1-fluoro-2,4-dinitrobenzene (FDNB) to form dinitrophenyl amino acids (DNB-AAs) by a protocol optimized (Paraskevas et al., 2002). Briefly, 180 µl of AA_T standard solution (where the subscript "T" refers to the total concentration of AAs) or honey sample was transferred into Eppendorf tubes where 70 µl of 0.032 M FDNB solution in ACN, 180 µl of 0.050 M borate buffer pH 9.2, and 720 µl of ACN were added. The tubes were protected from light and kept in a water bath at 60°C for 45 min. After cooling, each solution was acidified with 50 µl of 0.56 M HCl solution. A blank reaction was also prepared following the same procedure. All final solutions were filtered through 0.22-µm Nylon membrane (Micron Separations, Inc., Westborough, MA, USA).

For GC-MS enantiomeric analysis, a methylation followed by acetylation procedure was carried out (Menestrina et al., 2016). About 1 mg of AAs (or 100 µl of sample extract dissolved in MeOH) in 200 µl of methanol solution of HCl (15%) was heated at 120°C for 20 min into a vial. After cooling, the solvent was removed in a stream of dry nitrogen. Then, dichloromethane (110 µl) and trifluroacetic anhydride (100 µl) were added, and the vials were heated at 120°C for 10 min followed by cooling and evaporation of the solvents at room temperature in a stream of dry nitrogen. Finally, residues were dissolved in dichloromethane before injection.

2.4 | Honey samples

Fourteen honey samples were collected between 2017 and 2018 directly from the hive by beekeepers, from different departments or regions of Mendoza province: (1) Luján de Cuyo: samples from Potrerillos (S1, S2 and S4), Chacras de Coria (S5), Mayor Drummond (S6) and Perdriel (S13); (2) Valle de Uco: samples from San Carlos (S7 and S12); (3) Gran Mendoza: samples from Maipú (S9 and S10) and Guaymallén (S11); (4) East region: samples from Rivadavia (S3) and Lavalle (S14); and (5) A honey sample from Malargüe (S8).

2.5 | Sample pretreatment

Honey samples were treated as follows (Paramás et al., 2006): 1 g of each sample was transferred to a 5 ml volumetric flask with 0.01 M HCl pH 2.0. Separation of sugars and preconcentration of AAs were performed in an anion-exchange column. For that, 3 g of cation exchange resin Dowex 50 WX8-200 was activated with 50 ml of 2 M NaOH, water until neutral pH, 50 ml of 2 M HCl, and finally water again until neutral. The treated resin was introduced into plastic columns, and samples were passed through them. Sugars were eluted with 10 ml of water (three times). Then, the AAs were eluted with 2 ml of 8 M NH_4OH followed by 1 ml (50:50) of 8 M NH_4OH : MeOH solution for eluting more hydrophobic AAs. The collected solutions were dried at room temperature in a stream of dry nitrogen and reconstituted in either, 200 µl of water prior to derivatization for HPLC analysis or in HCl/MeOH prior to chiral GC analysis.

2.6 | Statistical data analysis

Eighteen AAs were determined in the present work. Calibration curves for each DNB-AAs were obtained by least-squares linear regression with standard solutions. The statistical assumptions such as residues normality test (p-value ≥ .05 by Shapiro–Wilk test) and homoscedasticity test (p-value ≥ .05 by Bartlett's test) was verified. The calibration curve obtained for each AA was evaluated by an ANOVA test (Danzer & Currie, 1998; Olivieri, 2015), and no lack of fit for the linear models was observed within the 95% confidence interval (p-value ≥ .05). The linear range was between 0.70 and 177 nmol injected. LODs and LOQs were determined according to the definition recommended by IUPAC (Danzer & Currie, 1998; Olivieri, 2015). Table 1 gathers the slope and intercept of each calibration curve and its associated errors and LODs and LOQs.

All honey samples were quantitatively analyzed by HPLC-DAD, and 12 descriptors (AA concentrations) were used for honey geographical characterization (Pro, Lys, Ala, Val, Ile, Leu, Thr, Glu, Ser, Phe, Asn, Arg, and Gly). The data matrix for the chemometric treatment contained 14 rows and 13 columns. The matrix rows represented the number of samples analyzed, and the columns corresponded to the AA concentrations, and one column represented the honey geographical origin as the dependent categorical variable. The preprocessing of the data set in the matrix was auto scaled due to differences in data dimensionality. Values that were found lower than the LODs for some samples were treated by assuming these LODs values in the corresponding calculations for the multivariate statistical techniques. For further chemometrical processing, honey samples of the different producing locations were grouped in four regions according to their similar conditions for the apicultural activity, referred to the climate and the vegetation cover. The honey regions were identified as Luján de Cuyo (n = 6), Valle de Uco (n = 2), Gran Mendoza (n = 3), and East Region (n = 2). The sample from Malargüe (n = 1) was not taken into account for statistical analysis of geographical classification due to it cannot be grouped in any region.

To identify similarities and differences among the honey samples produced in the different Mendoza regions, basic

TABLE 1 Results of HPLC-DAD calibration

AA	Slope (S_s)[a]	Intercept $\times 10^{-2}$ ($S_i \times 10^{-2}$)[a]	LOD (nmol/ml)	LOQ (nmol/ml)
Pro	4.0 (0.1)	4 (1)	0.9	2.6
Lys	11.9 (0.3)	9 (2)	0.2	0.5
Ala	6.6 (0.2)	10 (2)	1.1	3.3
Val	6.7 (0.2)	8 (2)	1.2	3.7
Ile	11.4 (0.5)	10 (3)	0.6	1.8
Glu	3.8 (0.1)	8 (2)	1.2	3.6
Thr	3.8 (0.1)	8 (2)	1.2	3.6
Ser	6.7 (0.2)	8 (2)	0.7	2.1
Phe	6.4 (0.2)	3 (1)	0.4	1.1
Asx[b]	6.4 (0.3)	5 (2)	0.8	2.3
Arg	7.1 (0.3)	5 (1)	0.4	1.1
Leu	3.9 (0.1)	4 (1)	0.7	2.0
Gly	6.0 (0.2)	9 (2)	0.9	2.7
Trp	6.5 (0.2)	2 (0.8)	0.3	1.0
Tyr	7.7 (0.2)	4 (1)	0.5	1.6
Met	6.8 (0.2)	6 (2)	1.5	5.1
Orn	4.0 (0.1)	4 (1)	0.9	2.6

Note: Slope and intercept of the linear regression corresponding to amino acid standard solutions, LOD and LOQ.

[a]S_s, standard deviation of the slope; S_i, standard deviation of the intercept;

[b]Asn + Asp.

exploratory analysis was made by principal component analysis (PCA) and cluster analysis (CA). PCA is a mathematical tool that allows to reveal groups of observations, trends, and outliers and uncover the relationships between observations and variables in the experimental data. This technique reduces the dimensions of the original data matrix by explaining a large part of the variance using synthetic factors, called principal components (PCs). Moreover, permits the visualization of the original arrangement of honey samples in an n-dimensional space, principally of two or three dimensions, by identifying the directions in which maximum variability is retained and displaying the relationship between variables and observations (Granato et al., 2018; Varmuza & Filzmoser, 2016). In this work, the concentration data of 12 AAs were used to perform the PCA. The total information content of the given number of PCs was expressed by cumulative percent (cum.%) value of the total variance.

The CA method is also an exploratory technique whose purpose is to find patterns or groups (clusters) within a set of observations. CA was performed to further characterize the role of AAs in classifying honey origin. Is this work hierarchical CA was used for grouping experimental samples into clusters, based on similarity within class and dissimilarity between different groups, according to the region of origin. The result of hierarchical CA is usually presented in a dendrogram, a plot which shows the organization of samples and its relationships in tree form (Granato et al., 2018).

All statistical chemometrics procedures were computed using the statistical R software version 3.6.0 (R Core Team, 2012).

3 | RESULTS AND DISCUSSION

3.1 | Amino acid determination

AAs amount of 14 samples of honey from Mendoza are summarized in Table 2. Large variation in AAs content can be observed, probably due to the wide variety of food sources and the environmental conditions that affect the chemical content of honey. Figure 1 shows the HPLC chromatogram of S11. Most of the AAs were detected in all analyzed honey samples. However, Met, Orn, and Trp were not found in any samples, and Tyr was detected in only two ones (S8 and S11). These findings agree with results previously reported. Several authors demonstrated that sulfur containing AAs (Met and Cys) were absent, whereas others such as Trp and Orn were present in minor amounts in several sample honeys (Kečkeš et al., 2013; Rebane & Herodes, 2008; Sun et al., 2017). Pro was, by far, the most abundant AA found in all samples, representing between ~40% and 80%, except for sample S3 where it represented about 18% of total AAs. In this last sample, Phe was the prevalent AA (~64% of the total AAs). With the exception mentioned above, Phe and Glu rank second in prevalence of the total AAs in the analyzed honey samples. Phe ranged from ~4% (S1) to 80% (S3), and Glu were found between 2% (S7) and 50% (S13). In S4, the only one where Phe was not detected, important amounts of Lys (25%) were found, being this the second AAs in abundance. Lower but still substantial amounts of Asx, Arg, and Ser were present in the samples analyzed. Unfortunately, scarce information about AAs in Argentinian honeys is available (Baroni et al., 2009; Cometto et al., 2003) and

TABLE 2 Amino acid concentration (expressed as mg/100 g sample) in honey samples of Mendoza

Honey samples[a]	Pro	Lys	Ala	Val	Ile	Glu	Thr	Ser	Phe	Asn	Arg	Leu	Gly	mg AA_T[b]
S1	61.1	2.72	–[c]	–	–	5.54	–	–	4.21	0.97	–	–	–	76
S2	93.4	9.67	3.43	1.12	11.1	3.95	–	1.21	24.1	–	–	2.52	0.98	151
S3	23.1	3.76	3.96	1.55	9.76	–	–	–	80.2	–	–	2.06	–	124
S4	50.5	25.3	3.95	1.60	1.90	4.25	–	1.24	–	4.68	–	–	–	94
S5	70.1	15.1	5.99	4.15	13.3	27.1	1.42	2.65	9.47	1.42	1.94	8.29	0.80	162
S6	82.4	4.65	–	2.42	3.56	3.30	–	1.58	36.1	2.05	1.94	2.99	–	141
S7	83.2	–	–	–	–	2.26	–	20.1	6.89	–	–	1.11	–	114
S8	185	–	3.91	1.84	20.0[d]	4.40	2.08	–	15.9	1.07	–	1.06	–	235
S9	200	–	8.44	–	4.13	16.4	2.62	3.90	17.1	1.62	2.71	5.12	–	262
S10	201	–	27.0	4.45	9.22	11.8	1.43	11.2	21.8	2.49	2.89	19.8	–	313
S11	211	1.25	15.4	3.88	10.9[d]	4.21	7.94	12.8	51.5	1.15	3.09	1.83	2.25	339
S12	112	6.10	9.47	6.85	18.4	20.1	1.78	2.89	19.8	4.37	1.12	4.47	1.26	299
S13	81.4	50.0	11.3	8.73	24.2	54.8[e]	–	4.07	20.1	4.80	1.31	12.9	3.26	360
S14	35.6	1.43	–	6.26	9.79	27.2[e]	–	6.41	53.8	–	2.52	1.69	1.75	254

[a]Sample geographical origin: S1, S2 and S4: Potrerillos, S3: Rivadavia; S5: Chacras de Coria, S6: Mayor Drummond; S7: El Cepillo; S8: Malargüe; S9: Lunlunta; S10: Barrancas; S11: Colonia Segovia; S12: Pareditas; S13: Perdriel and S14: Lavalle

[b]AA_T = Total amino acids;

[c] The dash indicate signals below the LOQs tabulated in Table 1;

[d]Ile + small signal from Tyr;

[e]Glu + Met.

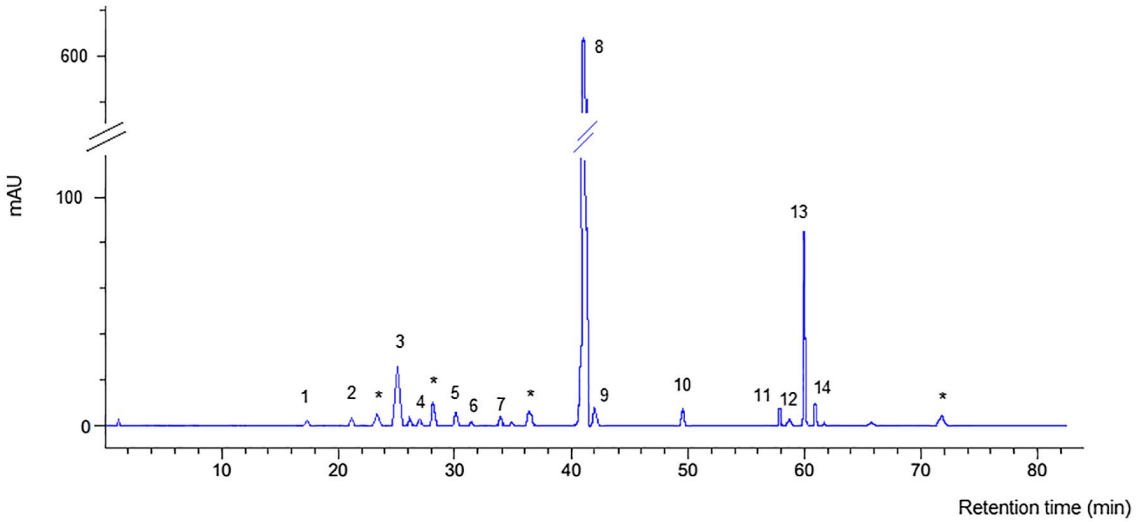

FIGURE 1 Chromatogram of high-performance liquid chromatography separation of DNF-amino acids in honey sample S11. Column: Zorbax Eclipse XDB-C8 (150 × 3.0 mm i.d., 3.5 μm). Gradient: 0–10 min 5% B, 10–47 min 5%–44% B, 47–53 min 44% B, 53–58 min 44%–64% B and 58–70 min 64% B; A = 60 mM ammonium formate pH 3.5 and B = MeOH. Flowrate = 0.85 ml/min. Temperature = 40°C. Peak identification: 1 = Asx; 2 = Arg; 3 = Ser; 4 and 13 = Lys; 5 = Gly; 6 = Leu; 7 = Thr; 8 = Pro; 9 = Ala; 10 = Glu; 11 = Val, 13 = Phe and 14 = Tyr + Ile. * = not identified

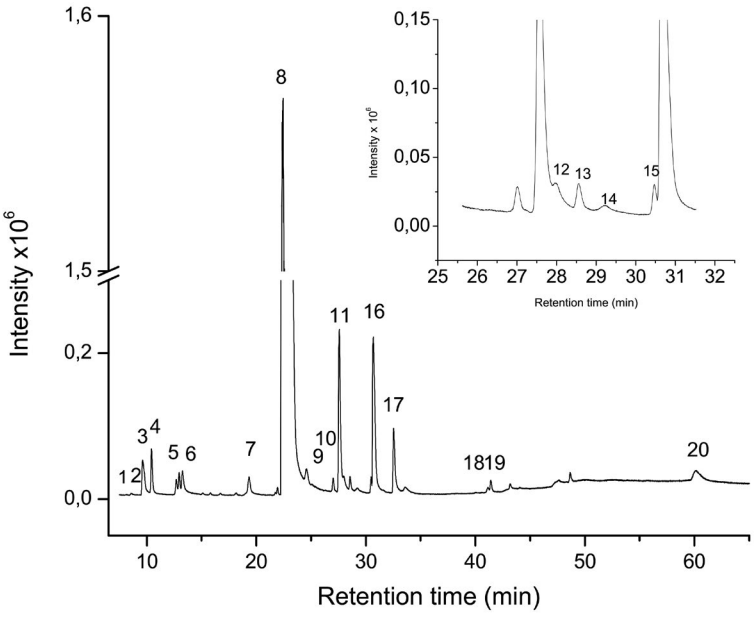

FIGURE 2 Determination of enantiomeric composition of amino acids in honey sample S1 by GC-MS. Chiral column: octakis(6-O-tert-butyldimethylsilyl-2,3-di-O-acetil)-γ-cyclodextrin in OV1701 (10 m × 250 μm, 0.1 μm); carrier hydrogen at constant 6.8 psi; split ratio 60:1; injector and ion source temperatures 200°C and 210°C, respectively. Column temperature, 55°C for 2 min, 2°C/min up to 150°C, 150°C for 15 min.; injection volume 1 μl. Peak identification: 1 = D-Val; 2 = D-Ala; 3 = L-Ala; 4 = L-Val, D-Leu, D-Ile; 5 = L-Leu; 6 = L-Ile; 7 = D-Pro; 8 = L-Pro; 9 = D-Thr; 10 = L-Thr; 11 = D-Ser, L-Phe; 12 = D-Phe; D-Asn; 14 = D-Glu; 15 = L-Ser; 16 = L-Asn, L-Met; 17 = L-Glu; 18 = D-Lys; 19 = L-Lys; 20 = L-Lys2

even less in Mendoza samples making comparisons difficult. It is possible to mention, however, that the predominant presence of Pro and Phe in these honey samples is coincident with the results observed for samples from Cordoba province (Cometto et al., 2003) as well as honey samples from other countries (Biluca et al., 2019; Cotte et al., 2004; Nozal et al., 2004; Rebane & Herodes, 2008; Sun et al., 2017). Pro has an important role in insect flying metabolism (Darvishzadeh, 2015; Teulier et al., 2016). Insects can detect Pro in their food and use it as a fuel for flight. Some bee species have a feeding preference for nectars with higher concentrations of this AA, which would explain the prominence of this AA in most honeys (Cotte et al., 2004). From Table 2, it is possible to observe that the lowest levels of Pro are found in samples from the East region of Mendoza, that is, S3 and S14, and the higher amounts (around 200 mg Pro/100 g samples) were obtained in the Gran Mendoza area (S9, S10, and S11 samples). Sun et al. (2017) determined AA contents of five floral sources of Chinese honey and the researchers found Pro levels between 175 and 457.5 mg for 100 g of sample. To set the total content of AAs of Mendoza honey in the worldwide context, the actual data were contrasted against the literature reported. As expected, local levels (75.6–360 mg/100 g of sample) were within the order reported by other researchers of several countries, mainly ones of Spain (Cotte et al., 2004; Kečkeš et al., 2013; Nozal et al., 2004; Zhao et al., 2018), though, the

TABLE 3 Relative abundance of D- to L-amino acids determined in honey samples

Honey samples[a]	Pro (166)[b]	Lys (180)	Ala (140)	Val (168)	Ile (168)	Glu (180)	Thr (152)	Ser (138)	Phe (162)	Asx[c] (198)	Leu (168)	Tyr (152)	Met (185)
S1	1.55	–[d]	4.68	0.89	3.02	2.24	3.59	3.59	6.41	8.36	3.94	n.d.	n.d.
S2	0.47	–	4.55	2.36	0.89	–	n.d.	–	6.01	7.04	4.03	n.d.	n.d.
S3	10.4	–	4.81	1.09	2.38	4.46	–	–	6.98	6.47	1.75	n.d.	n.d.
S4	0.28	–	8.49	2.43	–	–	–	–	7.41	4.86	–	n.d.	n.d.
S5	3.71	–	10.0	5.31	–	5.26	–	–	4.33	8.70	–	n.d.	n.d.
S6	0.64	–	4.07	0.88	3.40	–	–	–	7.38	3.92	2.91	–	n.d.
S7	0.86	–	8.40	0.99	2.48	1.05	–	–	–	4.56	2.33	–	n.d.
S8	0.36	–	3.09	–	–	1.26	–	–	4.71	2.88	–	–	n.d.
S9	0.26	n.d.[d]	2.43	2.30	6.14	2.04	–	–	–	2.41	3.59	n.d.	n.d.
S10	0.20	n.d.	2.07	4.91	5.95	1.54	–	–	4.58	2.70	3.65	n.d.	n.d.
S11	0.35	–	4.47	0.92	3.23	1.16	–	–	0.64	1.34	2.01	–	n.d.
S12	–	–	–	18.2	–	–	–	–	–	5.00	–	–	n.d.
S13	0.34	–	1.49	3.23	2.21	0.27	–	–	–	2.08	3.95	–	–
S14	0.23	–	4.65	5.71	4.58	0.77	–	–	3.63	3.15	3.61	n.d.	–

[a]Sample designation, seeTable 2;

[b]Characteristic *m/z* ion of each amino acid;

[c]Asn + Asp;

[d]–, Only the L-enantiomer was detected; n.d.: any enantiomer was detected. Percentage relative was calculated as D/L%.

results were lower than those found in some Chinese honeys (Sun et al., 2017).

3.2 | Enantiomeric determination of amino acids

The degree of racemization, or the D/L ratio, has been considered a significant marker of processing conditions, and for assessing food quality (Wehmiller, 2013). Particularly, in honey, it could serve as indicator of age, processing, and honey storage (Friedman, 2010). This ratio found in fourteen honey samples is shown in Table 3. According to our knowledge, enantiomeric analysis of AAs in local honeys has not been carried out before. The chromatogram in Figure 2 illustrates the enantiomeric analysis of S1.

Orn and Met, which were not detected in honey samples analyzed by HPLC, were observable in the GC-MS chromatograms corresponding to a few samples. Orn, however, is not reported since their derivatives were not enantioseparated. On the other hand, Arg did not elute from the GC column. The D-Pro enantiomer was detected in all samples except in S12. In samples S5 and mainly S3, the D-Pro amount was far greater than in the other samples. The exceptionally high D-Pro level in sample S3 can be associated to the origin of honey (Pawlowska & Armstrong, 1994). Regarding the second most abundant AA (Phe), its D-isomer was found in significant larger proportion than D-Pro in practically all samples. Similar behavior for Pro and Phe was reported in other honey samples (Pawlowska & Armstrong, 1994). Additionally, in all samples, only the L-isomer of the Lys was detected. This also happened with the AAs Ser and Thr except for samples S1. In general, Ala and Phe showed the highest degree of racemization. It can also be seen that S12 has mostly L-enantiomeric forms, however this sample has the highest amount of D-enantiomers of Val (18% for D-Val) and Asx.

Few works studied the possible occurrence of enantiomeric L- and D-AAs in honeys (Kim & Lee, 2008; Pätzold & Brückner, 2006; Pawlowska & Armstrong, 1994). Because this foodstuff was not subject of intensive technological treatment, the presence of D-AAs might have other origin. Nowadays, it is known that certain D-AAs can naturally occur (Pätzold & Brückner, 2006). Honey has a high glucose and fructose content, low water activity, and pH neutral or weakly acidic (Pawlowska & Armstrong, 1994); consequently, it is an excellent food in which the Maillard reaction can develop at room temperature, and in the absence of microorganisms (Kim & Lee, 2008). Therefore, it has been proposed that D-AAs could be generated from stable intermediates of the Maillard reaction, named Amadori compounds or fructose-AAs (Pätzold & Brückner, 2006, 2009). Mainly, steric and electronic properties of AA side chains are particularly important in the extent of these racemizations (Kim & Lee, 2008).

3.3 | Chemometric analysis

Multivariate analysis can extract useful information from several data and make complex analysis become simple. Its combination with HPLC analysis has been used in the determination of certain chemical fingerprints in honey samples (Sun et al., 2017). PCA and CA were used to discriminate Mendoza honeys according to their origin, by identifying similarities and differences between samples.

In addition, linear discriminant analysis (LDA) was used to assess the classification of honey samples in accordance with their geographical origin.

As a first exploratory step, the distribution of each numerical variable (AAs concentrations) from the different groups or categories (region of origin) was displayed in box-plot figures. The range and distribution of AAs concentrations expressed as mg/g for samples from the four regions can be compared in Figure 3. It can be observed clear differences in the concentration of Ala, Arg and Pro between the different origin regions, Valle de Uco and Gran Mendoza honeys present higher concentration of Ser than Luján de Cuyo and East region. It can also be observed that the concentration of Gly presents variability between regions. The remaining AAs show different grade of dispersion within the regions, nevertheless differences are not so evident.

For PCA, the results show that for instance, when all 12 variables were used, the first two PCs represented 59.5%. Figure 4 shows the most important PCA graph, PC2 versus PC1 biplot. It can be possible identify the relationship between the AA contents with the samples identified according to their origin. It can be observed that the scores corresponding to different samples identified according to East, Gran Mendoza and Valle de Uco regions are grouped in a quadrant of the biplot. Samples of East region showed principally negative scores on the PC1 and presented positive and negative score on PC2, all honeys from Gran Mendoza are grouped with positive scores on the PC1 and negative scores on PC2, and samples from Valle de Uco region showed scores along the PC1 axis. Scores of samples from Luján de Cuyo show more dispersion in the plot; therefore, a natural grouping of these samples is difficult. The results obtained by PCA showed that only the samples from Gran Mendoza could be slightly differentiated considering their AA profiles, but other honey samples with different geographical origin could not be solved by this unsupervised chemometric method.

With respect to the orientation of the variables (concentrations of AAs) on the most informative PCs, PC1 and PC2, it can be observed that the first PC was strongly associated with the values of Val, Thr, Glu, Leu, and Val, indicating higher concentrations in samples that showed positive scores (Gran Mendoza samples). On the other hand, Lys, Gly and Asn were the dominant variables in the second PC. Positive scores on PC2 correspond to high concentrations of these elements and samples with negative scores to high concentrations of Arg, Pro, and Phe.

Regarding CA analysis, the Ward's method with Euclidean distance as a similarity measurement was used. The dendrogram after applying the hierarchical CA is presented in Figure 5. It can be seen

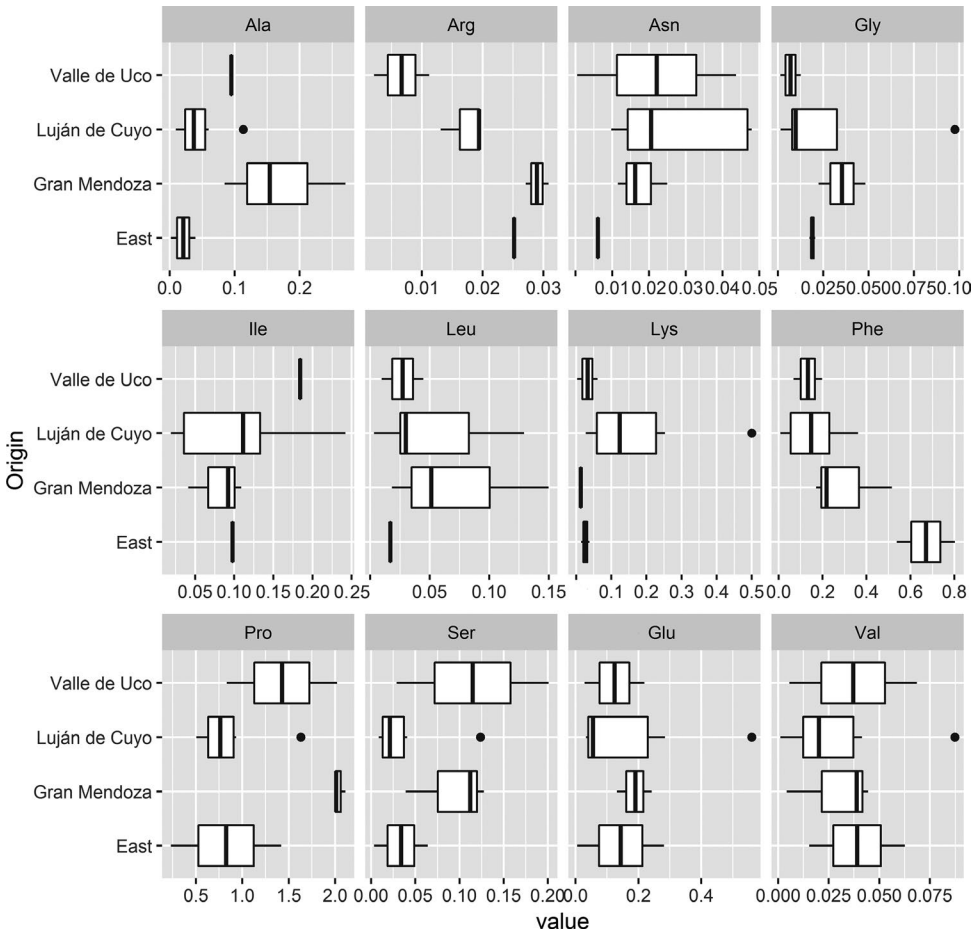

FIGURE 3 Box plot of the thirteen amino acids selected to assess honey provenance (geographical origin vs AAs concentration)

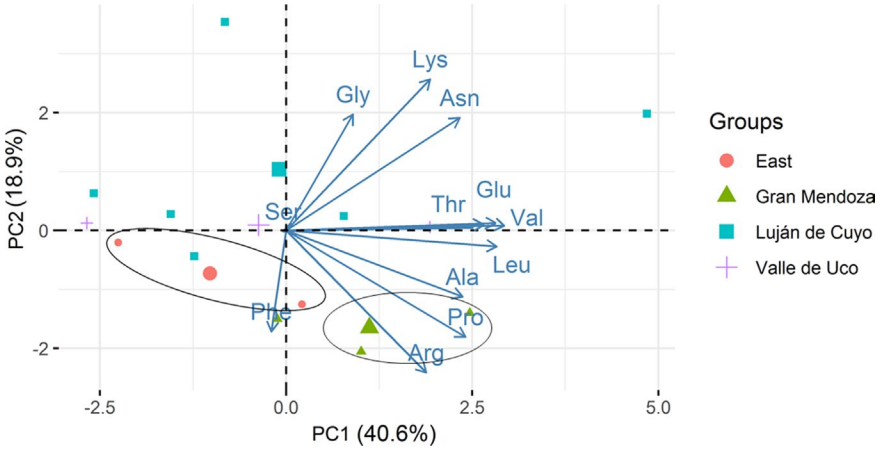

FIGURE 4 Score and loading plots of the first principal component (PC1) versus the second principal component (PC2)

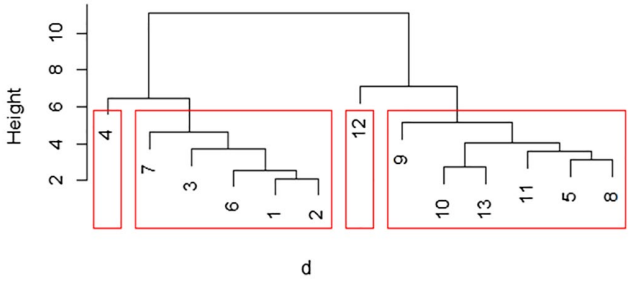

FIGURE 5 Dendrogram of the cluster analysis for the studied honey samples

TABLE 4 Results of the classification of honey samples in two groups of geographic origin using linear discriminant analysis based on the amino acid composition data matrix

	Prediction		
Region	**LC-VU**	**GM-E**	**Total**
LC-VU	7	1	8
GM-E	1	4	5
Overall accuracy = 85%			

Note: Categories: LC-VU: Luján de Cuyo and Valle de Uco; GM-E: Gran Mendoza and East.

draws of four rectangles around the branches of a dendrogram highlighting the corresponding clusters. In this study, all samples were divided into four minor categories that were grouped in two major groups. In the first major cluster, four samples from Luján de Cuyo, one from East region, and Valle de Uco were grouped. The second major cluster presented two honey samples from Luján de Cuyo, the three samples from Gran Mendoza, and one from Valle de Uco and East region. After applying CA to the AA data from all samples, only the samples from Gran Mendoza were grouped together, but the rest of the samples from the three regions remaining were divided between the two major groups. If we consider the four minor groups no good differentiation between the four regions of honey origin was observed.

Based on the previous results, we conclude that the unsupervised chemometric methods could not clearly differentiate the geographical origin of honey samples based on their AA content, only honeys from Gran Mendoza were slightly differentiated from samples of the other regions. Since PCA and CA cannot be properly considered as classification methods, LDA was performed to further investigate the possible classification of the honey samples based on its region of origin and propose a predictive model. LDA is a multivariate classification procedure, which needs an initial sample classification into predefined groups or classes to identify the respective type of a sample according to various values (Sun et al., 2017). Due to the small number of samples available, cross-validation (CV) approach was used to determine the predictive capacity of the model, avoiding the possible overfitting of the proposed model. In k-fold CV, the dataset is randomly split into k mutually exclusive subsets (the folds) of approximately equal size. The model is trained and tested k times, and to estimate the accuracy of the models, the overall number of correct classifications is divided by the number of instances in the data set. In this work, k-fold CV was used with $k = 3$ for the model. The method was applied considering only two regions. Honey samples from Luján de Cuyo and Valle de Uco (LC-VU) and from Gran Mendoza and East (GM-E) regions were grouped together attending to its nearby location and to obtain a reliable model for predictive origin. Table 4 shows the results of the samples classification on the two regions considered. The model discriminates 87.5% (7 of the samples) of the LC-VU honeys and 80% (4 of the samples) of the GM-E honeys. The average of correctly classified samples by CV reached 85.0%. Although the classification achieved was not perfect, the level of success reached was considered satisfactory, suggesting that each region share the same phytogeography, which influenced honey composition. These results were in agreement with those obtained in CA analysis, in which two major clusters were obtained, one with the majority of the samples from Luján de Cuyo, and the other group, with all honey samples from Gran Mendoza. Finally, these data can be used in allocating new observations in either of the two groups.

4 | CONCLUSION

The AAs composition of 14 honey samples from different regions of Mendoza was determined by HPLC-DAD. In addition the AAs enantiomeric ratio of all these samples was assessed by chiral GC-MS. According to our knowledge, it is the first time that the D/L enantiomeric ratio of AAs in honey samples of Mendoza was determined. For the main AAs found in the honey samples studied, i.e. Pro and Phe, L-isomers were prevalent. Furthermore, in many samples D-Phe was found in greater proportion than D-Pro. The racemization mechanism based on the formation of Amadori compounds has been used to explain the levels of D-AA found in honey samples. Finally, multivariate statistical tools were applied to samples containing diverse AA amounts did not show a clear classification according to their origin. The PCA method resulted in a reduction of dimensions and complexity of the data matrix and was able to slightly differentiate samples from the Gran Mendoza region. By LDA method was possible to discriminate the geographic origin of the honey samples by their AA concentration, with an average prediction capacity of 85%.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Pamela Yanina Quintas: Conceptualization; Data curation; Formal analysis; Investigation; Writing-original draft; Writing-review & editing. **Sonia Keunchkarian:** Data curation; Formal analysis; Investigation; Methodology; Supervision; Writing-original draft; Writing-review & editing. **Lilian Romero:** Formal analysis; Methodology; Software; Validation; Writing-original draft; Writing-review & editing. **Brenda Canizo:** Data curation; Formal analysis; Software; Writing-original draft. **Rodolfo Wuilloud:** Funding acquisition; Writing-original draft; Writing-review & editing. **Cecilia Castells:** Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Supervision; Writing-original draft; Writing-review & editing.

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