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Comparison of Free Amino Acids Profile in Honey from Three Argentinian Regions

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The physical chemistry characteristics of honey are directly related to floral origin and, as a result, to the production region. There are some available methods that can determine the botanical or geographical origin of honey such as the free amino acids profile analysis. This paper reports data on the free amino acid composition, determined by reversed-phase high-performance liquid chromatography UV detection on 56 honey samples from three different Argentine regions, with characteristic apiarian flora. To evaluate if the quantified amino acid could be used to verify the geographical or botanical origin of honey, statistical analyses were performed. The cluster analysis showed that samples were grouped in clusters related to sampling regions and more strictly to apiarian flora around apiaries. Each cluster appears associated, in accordance with the principal component analysis, to high or low concentrations of different amino acids.

KEYWORDS: Honey; HPLC; amino acid profile; honeybee; Argentina; botanical source; geographic origin

INTRODUCTION

In 2000, Argentine was the third largest honey producer and the second largest honey exporter in the world (*1*). Córdoba produced about 11% of all honey in Argentine (*2*). Both the consumption predisposition and the commercialization probabilities are related to its quality, which depends on flavor, color, fragrance, consistence, and chemical composition (*3*, *4*). All of these features are directly related to floral origin and, as a result, to the production regions. The standard procedure for assessing honey botanical origin is melissopalynology, which consists of the microscopical analysis of pollen present in the honey after filtration or centrifugation (*5*). However, melissopalinology requires previous knowledge of pollen morphology and specialized professional personnel to achieve reliable results. Besides, the melissopalynology can be difficult to apply in filtered processed honey because of pollen scarcity or a lack of pollen after filtration and mixed honey of different floral origins (*6*).

Many researchers are looking for alternative methods, which should complement the usual melissopalynology for the evaluation of the botanical origin of honey. Parameters usually examined to evaluate the quality, origin, and authenticity of honey include the measurement of standard physical and chemical parameters (e.g., pH, acidity, moisture, HMF, diastases activity, sugar profile, etc.) (*4*, *7*−*9*). Another group of chemical parameters, less frequently analyzed, includes the protein profile (*6*, *10*), flavonoids profile (*11*, *12*), phenolic acids (*13*), and $^{13}C/^{12}C$ stable isotope ratio (*14*, *15*). The analysis of amino acids has been also proposed to verify the identity of honey (*13*). The use of multiparametric studies, associated with chemometrics, yields satisfactory results for honey classification (*6*).

The amino acid content is about 20−300 mg/100 g of honey (*16*). Echigo et al. have proposed that honey amino acids can arise from different sources, i.e., nectar, honey bee, and pollen (*17*). Considering that the pollen is virtually the only source of essential amino acids in the diet of honey bees (*18*) and that the amino acid composition in pollen allows one to characterize different vegetal species (*19*−*22*), it can be inferred that the amino acid honey composition can be a floral or geographical marker.

Many researchers such as Bouseta et al. (*23*) and Pirini et al. (*24*) showed that the presence of some amino acids is characteristic of some kinds of honey from different botanical sources. Later, Conte et al. (*25*), in agreement with the above authors, proposed the amino acid analysis as a botanical origin marker in unifloral honey and the results support the fact that the amino acid component is strictly connected to the types of pollen present in honey. On the other hand, Gilbert et al. (*26*) obtained a good discrimination between samples of honey from Australia, Argentine, and Canada on 17 free amino acids studied. Previously, Davies (*20*) had obtained similar results about honey samples from 11 countries.

This paper reports data on free amino acid profiles, determined by reversed-phase high-performance liquid chromatography (RP-HPLC), of honey from three different Argentine

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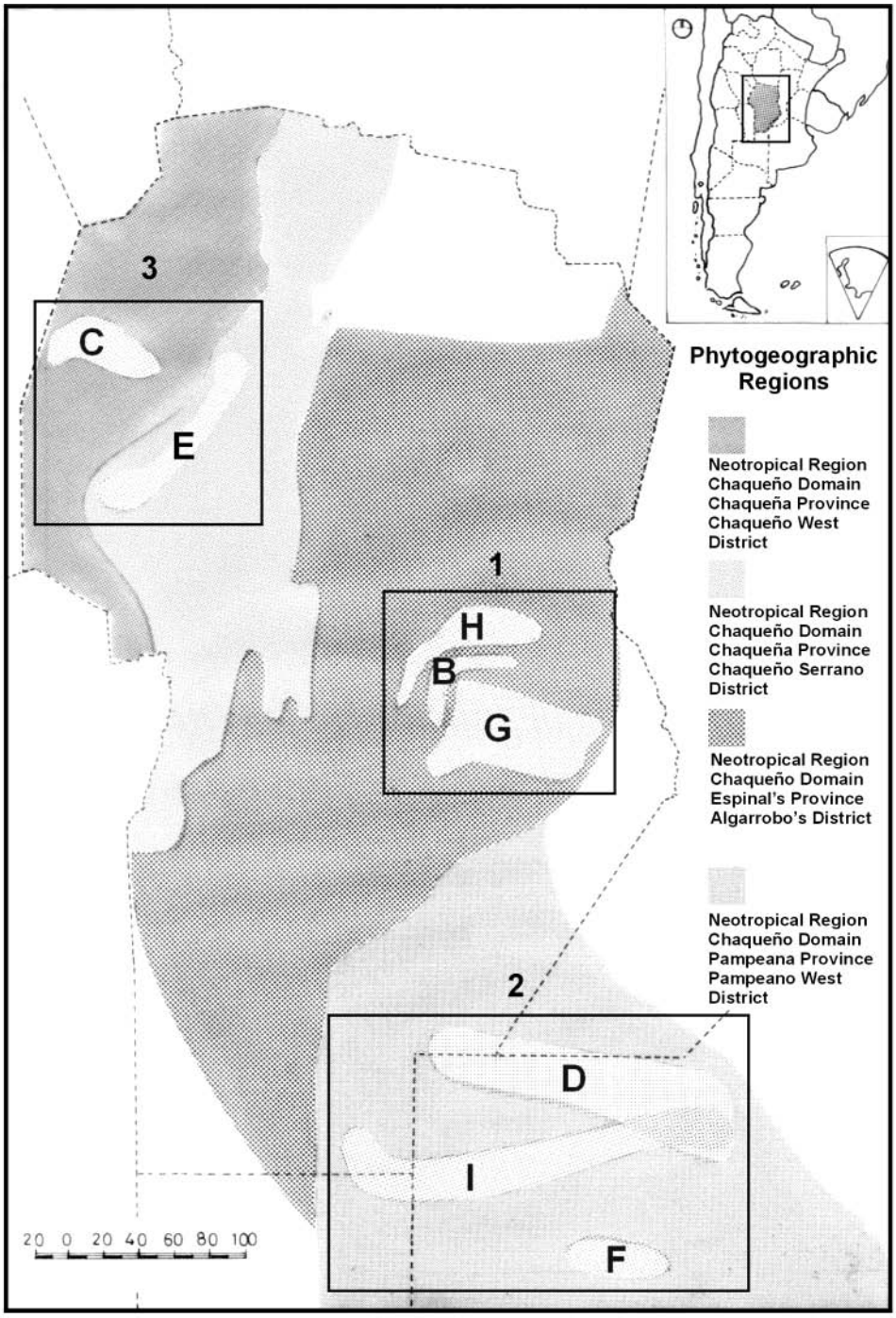

Figure 1. Phytogeographical regions, sampling regions, and regions of suggested groups by cluster analysis. (1) Córdoba Province SE (latitude 31°48′ to 33° south and longitude 62°18′ to 63°30′ west); samples 1–23. (2) Buenos Aires Province NW (latitude 34°18′ to 36° south and longitude 61°42′ to 64° west); samples 24–31, 33–34, and 37–39. (3) Córdoba Province NW (latitude 30°30′ to 31°30′ south and longitude 64°18′ to 66° west); samples 35, 43–53 (Chaqueño West district) and 32, 36, 40–42, and 54–56 (Chaqueño Serrano district). B–I are possible subregions that include suggested groups by cluster analysis. A group is not included due to corresponding sample 36.

regions. This work shows the grouping of samples relating them to their region of origin, which is associated with characteristic apiarian flora. The data matrix obtained was treated with different statistical tools to improve the identification of the geographical or botanical source and the authenticity of the product.

MATERIALS AND METHODS

Sampling. Fifty-six honey samples were harvested from three geographic regions. The three regions were included within four phytogeographic regions characterized by Cabrera (*27*). This author grouped the Argentine phytogeographic regions in accordance with taxonomic relations based on floral affinity of vegetation (**Figure 1**).

Honey samples were collected in spring/summer 1999/2000 in accordance with IRAM norm (*28*). Around each apiary, the different vegetal species of apicultural interest visited by bees were registered, considering the information given by Pelliza and Capdevila (*29*), Lütscher (*30*), Montani et al. (*31*), and local beekeepers. Melissopalynology analyses (*32*) on 40% samples of each region were carried out at random.

Reagents and Standards. Acetonitrile (Fisher, U.S.A.) was HPLC grade. Essential L-amino acids Kit (LAA-10), phenyl isothiocyanate

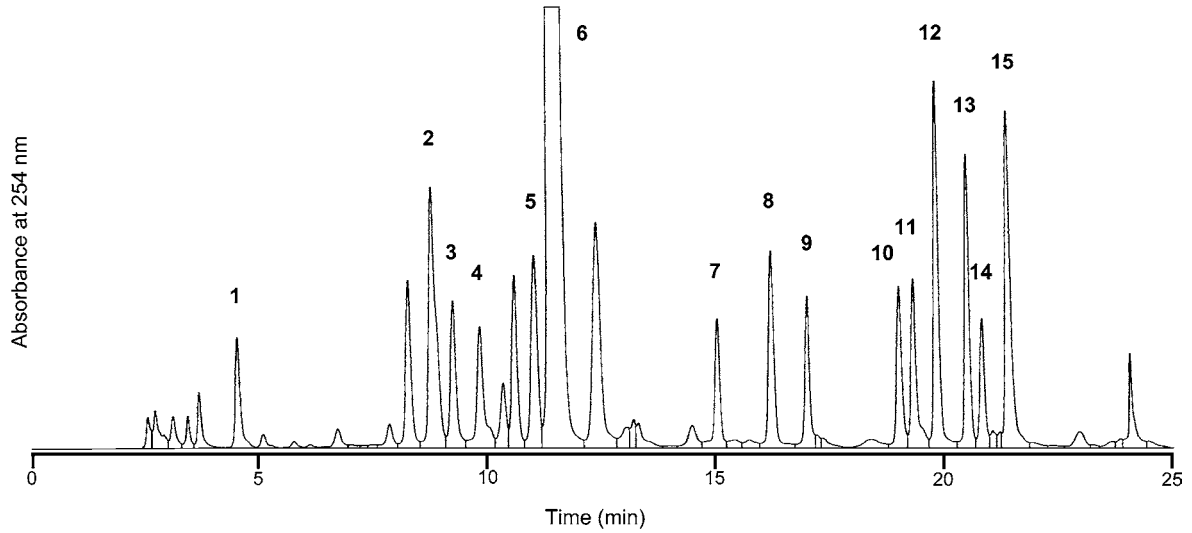

Figure 2. Chromatogram obtained from a honey sample spiked with standard amino acids. (1) Aspartic acid, (2) serine, (3) glycine, (4) arginine, (5) threonine/alanine, (6) proline, (7) tyrosine, (8) valine, (9) methionine, (10) isoleucine, (11) leucine, (12) norleucine, (13) phenylalanine, (14) tryptophane/ornithine, and (15) lysine.

Table 1. Retention Time Index, Coefficient of Linear Regression (*r*) from Calibration Curves, Recovery Percentage, and CV (%) for Each Amino Acid on Standard Mix and Honey Samples

			standard recovery and reproducibility in exchange ionic column (Amberlite IR-120) ($n = 5$)			reproducibility assays on honey samples ($n = 5$)	
AA[a]	retention time index ± SD ($n = 10$)	coefficient of linear regression (*r*)	AA (μmol/ 50 mL of solution)	% recovery	CV (%)	AA (μmol/ 100 g of sample)	CV (%)
aspartic acid	0.27 ± 0.01	0.9929	9.0	99	3.8	23	5.2
serine	0.45 ± 0.01	0.9874	10.0	96	1.9	26.3	2.0
glycine	0.48 ± 0.01	0.9929	2.0	88	2.0	8.1	2.0
arginine	0.53 ± 0.01	0.9984	2.0	78.1	0.9	9.5	1.1
threonine/alanine	0.56 ± 0.02	0.9944	2.6	89	2.6	6.6	4.3
proline	0.58 ± 0.01	0.9925	145.4	106	4.7	364	1.8
tyrosine	0.77 ± 0.01	0.9956	4.6	88	3.0	9.1	6.5
valine	0.81 ± 0.01	0.9977	3.1	94	5.7	11.9	6.3
methionine	0.85 ± 0.02	0.9948	0.9	104.7	0.7	0.64	7.2
isoleucine	0.95 ± 0.01	0.9990	3.0	97	4.6	8.4	6.9
leucine	0.96 ± 0.01	0.9990	1.2	100.8	0.8	4.8	8.7
phenylalanine	1.05 ± 0.01	0.9942	9.7	76	4.8	20	7.1
tryptophane/ornithine	1.08 ± 0.01	0.9990	2.6	80	3.4	2.3	7.9
lysine	1.11 ± 0.01	0.9973	10.5	90	2.7	5.2	5.6

[a] AA, amino acid.

(PITC), protein sequencing grade (P-1034), L-ornithine (O-2375), β-alanine (A-7752), D,L-homoserine (H-1001), 1-aminocyclopropane-1-carboxylic acid (ACPC; A-3903), and L-citrulline (C-7629) were purchased from SIGMA. D,L-Norleucine, l-α-alanine, and Amberlite IR-120 were purchased from BDH Laboratory Chemicals Group (England). L-Proline (81710) was purchased from Fluka, and L-glycine was purchased from Bio-Rad. These reagents were stored according to marker indication. All other reagents were analytical grade.

Sample Cleanup. Amino acids were extracted within 20 subsequent days of collection with the exception of sample 36, which was collected 1 year before the analysis and stored at room temperature. Isolation of amino acids from the honey samples was performed in accordance with the Adams method (*24*, *33*) with minor modifications. The activated resin (Amberlite IR-120) was packed in a glass column (11 mm × 170 mm). Twenty grams of honey and 3.05 μmol of norleucine (internal standard) were dissolved with HCl, pH 2.12. The volume was adjusted to 50 mL, and pH was adjusted to 2.12. The honey sample was passed through the Amberlite column, washed with HCl (pH 2.12) until there was a negative reaction with fehling reagent. The retained amino acids were eluted with 25 mL of 2 M NH_4OH. The entire process was carried out at a flow rate of 0.7 mL/min with a peristaltic pump. The extracts were stored in a dark bottle at 0 °C. The amino acids profile of the extracts did not undergo a change in the following 6 months.

RP-HPLC. The PITC derivatization method, the conditions gradient, and the solvents were based on many papers (*34*−*37*). A Hypersil ODS 5 μm (4.6 mm × 250 mm, Sigma-Aldrich) column was used. The gradient conditions were carried out as described by Alonso et al. (*34*) with minor modifications. Analyses were performed on a HPLC Konik-500-A and an UV-200 Konik detector. The detection was at 254 nm. The column temperature was maintained at 50 °C. The flow was 1 mL/min. Solvent A was prepared by mixing 19 g of sodium acetate trihydrate in 1 L of water. To this solution, 1.5 mL of triethylamine was added. The pH was then adjusted to 6.40 with glacial acetic acid. Finally, 79 mL of acetonitrile was mixed with 1 L of this solution. Solvent B was H_2O/acetonitrile (40:60).

Then, 300 μL of eluted extract (sample cleanup) was evaporated to dryness in a rotary evaporator (Büchi). The dried sample was derivatized in accordance with the Alonso PITC derivatization procedure (*34*), without using liquid nitrogen to dry the derivatized sample. Instead of using liquid nitrogen, we dried the sample at 37 °C by using a rotary evaporator (Büchi) connected to a vacuum pump (final vacuum, 10^{-2} Torr). We did not observe differences between the samples dried with

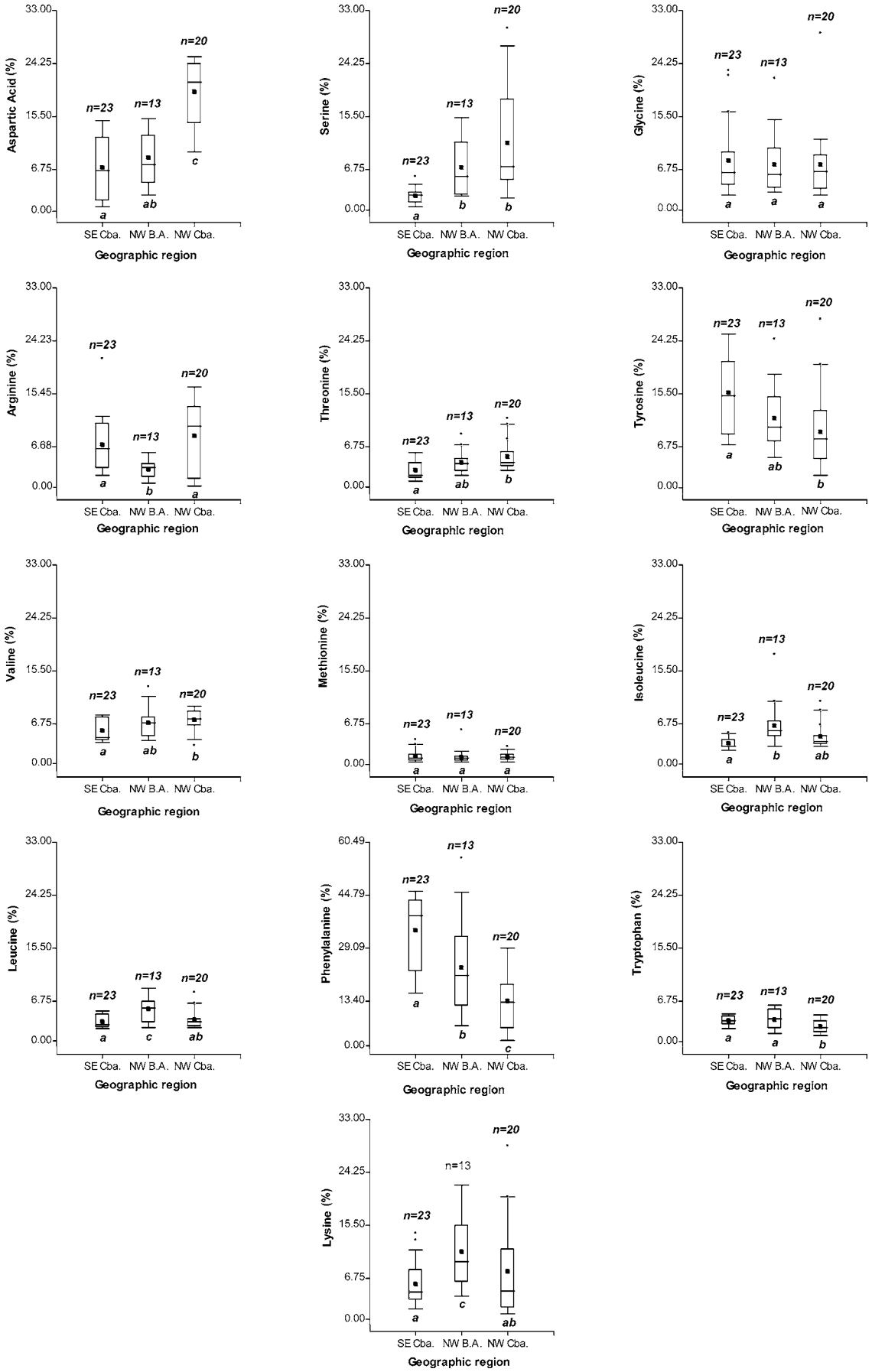

Figure 3. Box plot of 13 amino acids measured in honey samples from three geographical regions studied. Mean values (■) with different letters were significantly different ($p < 0.05$). Axis *Y*, relative composition percentage; axis *X*, sampling regions (SE Cba, Southeast of Córdoba province; NW B.A., Northwest of Buenos Aires province; and NW Cba, Northwest of Córdoba province).

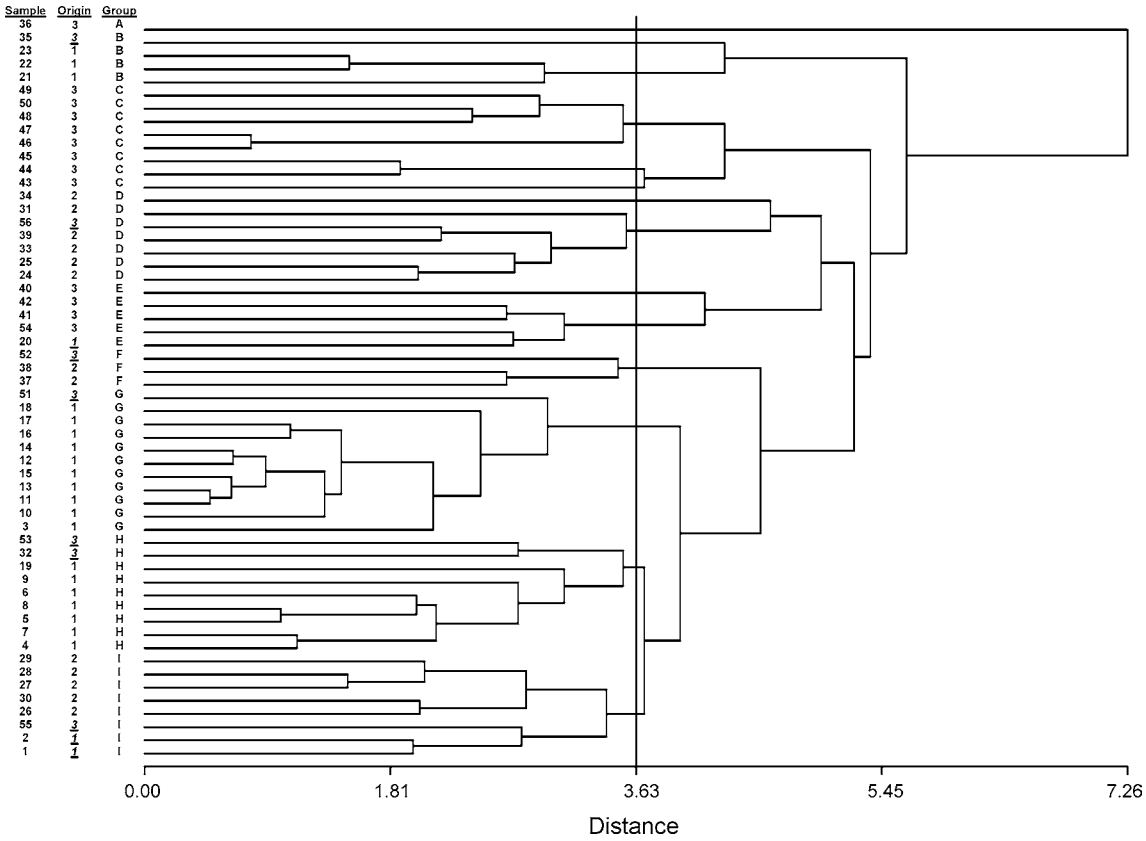

Figure 4. Dendrogram from cluster analysis. The numbers in the second column indicate sample origins (see **Figure 1**). The third column indicates the suggested statistic group of samples. Underlined italic letters indicate samples collected in a region that have been grouped with samples of others regions in the third column.

liquid nitrogen and the same samples treated with vacuum at 37 °C. The dried derivative sample was redissolved in solvent A. Finally, 20 μL of sample was then injected into the chromatographic system. All honey samples were processed in duplicate.

Reproducibility assays on honey samples ($n = 5$) were performed. Also, a recovery assay ($n = 5$) to estimate the amino acids fraction retained in the ion exchange column was performed.

Calibration. The method was calibrated using pure amino acids as external standard and norleucine as internal standard. Calibration curves were obtained from seven known amino acid concentration of standards mix. Amino acid peak area/norleucine peak area ratio vs amino acid concentration (μmol/100 g sample) was plotted. The amino acid concentration obtained from the calibration curve was called "absolute concentration" in order to differentiate it from "percentage concentration". The amino acid peak identification was carried out by the amino acid retention time/internal standard retention time ratio (retention time index). Moreover, the amino acids were identified by adding a crescent concentration standard to honey samples.

Statistical Analysis. To compare the amino acid composition among samples of the three regions, the absolute concentration for each amino acid was calculated as a percentage of total amino acid concentration. To calculate the percentage concentration, the absolute concentration of all quantified amino acids was summed up (100%), except for proline.

Data were subjected to appropriate nonparametric Kruskal-Wallis analysis of variance test (*38*) for the comparison of percentage concentration means for each amino acid. To identify groups in each region related to the free amino acid profile, a multivariate cluster analysis was tested. For this analysis, the data were standardized. The average linkage algorithm was applied using the euclidean distance (*39*). The sample size was 56. The clusters were identified at the middle point of the whole range of distance using a dendrogram.

To analyze the underlying variability and to identify useful associations for different honey groups, a principal component analysis (PCA) was performed (*40*). As classification criterion, the groups suggested by cluster analysis were used. Some graphical tools such as biplots have also used.

On the other hand, a multiple correspondence analysis was carried out, considering for each sample the group suggested by cluster analysis and the apiarian flora observed around each apiary. Species of apiarian interest were treated as binomial ("present" or "absent"). To see the results, a biplot was performed. All test were carried out using Infostat 1.1 Software (*41*).

RESULTS AND DISCUSSION

Analytical Method. By means of the method described, chromatograms with a good baseline and resolution were obtained (**Figure 2**). Both threonine/alanine and tryptophan/ornithine couples could not be separated for detection and quantification.

Vasanits and Molnar-Perl (*42*) separated and quantified 27 amino acids in apples. Using these conditions with a Hypersil ODS 5 μm, we could not separate clearly the peaks of proline/arginine, alanine/ACPC/citrulline, and homoserine/β-alanine.

Table 1 shows the retention time index, coefficient of linear regression (r) from the calibration curve, recovery percentage, and coefficient of variation (CV (%)) for each amino acid on standard mix and honey samples. The CV (%) obtained from the reproducibility assay was less than 10%. The recovery percentage averaged for the ion exchange column was 91.9% for all amino acids. The recoveries obtained for glycine, arginine, threonine/alanine, tyrosine, phenilalanine, tryptophan/ornithine, and lysine were less than 90%, although they were reproducible. The coefficient of linear regression was greater than 0.9874. Bouseta et al. (*23*) and Pawlowska and Armstrong (*16*) proposed a free amino acid procedure that showed CV values less than our results. They purified the amino acid in Dowex 50 wx 8,

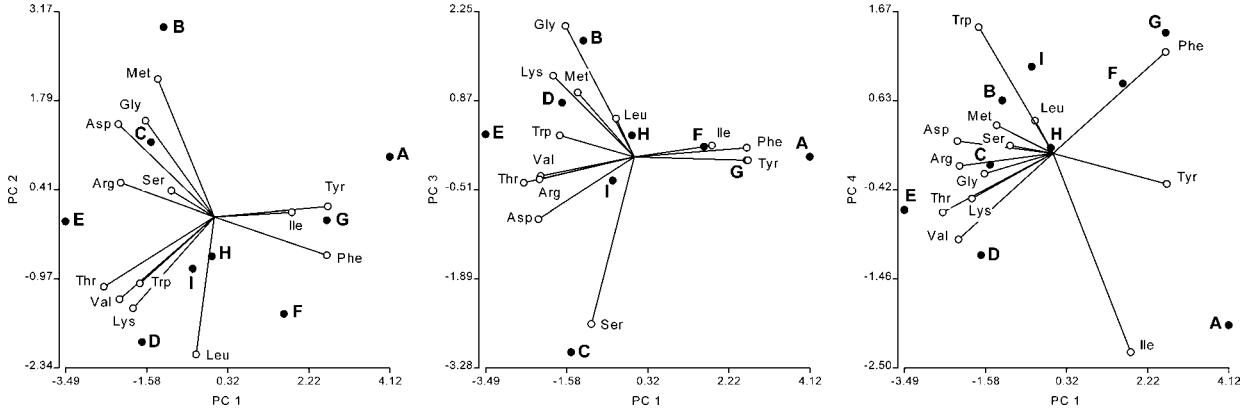

Figure 5. Biplots (PCA) with first four principal components. Capital letters indicate the cluster analysis group.

Table 2. Amino Acid Percentage Concentration Means for Each Statistical Group Suggested by Cluster Analysis and Associations Suggested by PCA between Some of These Values and the Groups

	A	B	C	D	E	F	G	H	I
aspartic acid	3.74	18.22	*22.79*[b]	9.62	19.19	7.67	5.81	4.40	10.66
serine	2.40	3.08	***18.90***[a]	5.20	3.19	3.43	2.40	3.95	8.28
glycine	5.32	20.87	3.91	10.07	13.67	4.24	4.03	9.08	4.89
arginine	1.65	6.22	8.16	2.29	17.13	***1.99***[c]	2.93	8.64	5.73
threonine/alanine	1.05	2.16	4.77	*5.70*[b]	7.33	2.80	1.76	4.49	3.53
tyrosine	***24.60***[a]	7.63	8.23	8.79	***4.69***[c]	9.38	*22.42*[b]	4.62	10.99
valine	4.17	4.91	7.40	*9.37*[b]	7.53	5.03	3.95	7.95	6.13
methionine	0.69	***3.77***[a]	1.63	1.25	1.13	0.53	0.55	1.55	1.08
isoleucine	***18.18***[a]	3.22	3.82	7.39	3.67	5.45	3.22	5.55	3.59
leucine	1.80	2.92	2.90	6.47	3.55	8.48	2.38	*4.70*[b]	3.82[b]
phenylalanine	*33.61*[b]	17.02	13.06	11.94	***4.79***[c]	***43.49***[a]	***43.86***[a]	28.78	24.99
tryptophane/ornithine	***0.61***[c]	2.61	2.25	3.31	3.72	1.93	3.30	3.49	***4.79***[a]
lysine	2.28	*7.28*[b]	2.17	***18.61***[a]	***13.84***[a]	5.57	3.97	4.77	11.53

[a] Amino acid positively correlated with column group. [b] Amino acid positively correlated with column group but in minor grade. [c] Amino acid negatively correlated with column group.

and the amino acid derivative was measured by fluorescence techniques. Because we had a lower total time procedure, we could apply our method to more samples. The above-described method affords a precise and accurate procedure to quantify the free amino acid profile in honey.

Comparison of Amino Acid Profile in Honey Samples from Studied Regions. A comparison of amino acid concentration percentage means of three regions is shown in **Figure 3**. We can see that there is a significant difference in percentage concentration of different amino acids among regions. In this way, serine, arginine, isoleucine, phenylalanine, and lysine can help us differentiate between Córdoba SE and Buenos Aires NW. Aspartic acid, serine, threonine, tyrosine, valine, phenylalanine, and tryptophan can help us differentiate between Córdoba SE and Córdoba NW. Aspartic acid, arginine, leucine, phenylalanine, tryptophan, and lysine can help us differentiate between Buenos Aires NW and Córdoba NW. Proline was excluded for the comparison, because there was no significant difference in the absolute concentration means between regions. Moreover, when proline was included for the calculations, a greater dispersion within regions was observed. Cluster analysis results are shown in **Figure 4**. It can be seen that the sample point arrangement is not random. Moreover, in accordance with grouping criterion, the statistical analysis suggested nine groups (A−I). Samples 35, 43−45, 34, and 40 are included in groups B, C, D, and E, respectively, because the separation distance is small and they are between unrandom point sequences. Sample 36 showed a very different profile as compared with the rest of honey and had different collected conditions; for these reasons, it was considered a separate group.

In general, we can consider that the suggested groups B, G, and H include mainly samples collected in Córdoba SE. A, C, and E sample groups were collected in Córdoba NW, and D, F, and I sample groups were collected in Buenos Aires NW.

Except for a few honey samples, most of them, whose apiaries are near, are grouped according to amino acid profile (**Figure 4**). Therefore, the suggested group by cluster analysis could be possible subregions within sampling regions (**Figure 1**).

There are differences between the present work and the Gilbert et al. (*26*) work, because in this study a major number of samples from Argentine were evaluated, which allowed us to see differences between honey of different regions. Considering the extensive Argentinian territory and its phytogeographical diversity, it would be difficult to dispose representative samples of all country. However, in accordance with his work and Davies (*20*), our results showed that it could be possible to determine the botanical sources by amino acid profile.

To identify the associations of amino acid percentage concentration with determined regions by cluster analysis, a PCA was performed. Eighty-three percent of total variability between samples could be explained considering only the first four principal components. **Figure 5** shows three biplots obtained from theses components. It can be seen that all quantified amino acids are strongly associated to one or more honey groups. Starting from suggested associations by PCA, the amino acid mean value for each statistical group suggested by cluster analysis was calculated and **Table 2** was made. We can see that really the associated amino acid by PCA to each suggested group by cluster analysis can characterize the amino acid profile of each group.

Table 3. Common Vegetal Species of Apicultural Interest Observed around Apiaries

vegetation	species[a]
pasture	(Ci) *Cichorium intybus* L.
	(Ls) *Lactuca sativa* L.
	(Mp) *Medicago polymorpha* L.
	(Ms) *Medicago sativa* L.
	(Ma) *Melilotus albus* Desr.
	(Mo) *Melilotus officinalis* (L.) Desr.
	(Tp) *Trifolium pratense* L.
	(Tr) *Trifolium repens* L.
mount	(Aa) *Acacia aroma* Gillies ex Hook and Arn.
	(Ac) *Acacia caven* (Molina) Molina
	(Af) *Acacia furcatispina* Burkart
	(Ag) *Aloysia gratissima* (Gillies and Hook.) Tronc.
	(Ae) *Atamisquea emarginata* Miers.
	(Br) *Bulnesia retama* (Gillies ex Hook. and Arn.) Griseb.
	(Ca) *Cassia aphylla* Cav.
	(Cp) *Cercidium praecox* (Ruiz and Pav.) Burkart and Carter
	(Cm) *Condalia microphylla* Cav.
	(Cg) *Cyclolepis genistoides* D. Don.
	(Eu) *Eucalyptus* spp.
	(Gd) *Geoffroea decorticans* (Gillies ex Hook. and Arn.) Burkart
	(Gt) *Gleditsia triacanthos* L.
	(Ld) *Larrea divaricata* Cav.
	(Ll) *Ligustrum lucidum* W. T. Aiton
	(Lt) *Lippia turbinata* Griseb.
	(Mv) *Maytenus vitis-idaea* Griseb.
	(Mc) *Mimozyganthus carinatus* (Griseb.) Burkart
	(Pt) *Plectocarpa tetracantha* Gillies ex Hook. and Arn.
	(Pm) *Porlieria microphylla* (Baill.) Descole, O'Donell, and Lourteig
	(Pa) *Prosopis alba* Griseb.
	(Pn) *Prosopis nigra* (Griseb.) Hieron.
	(Pto) *Prosopis torcuata* DC.
	(Rp) *Robinia pseudoacacia* L.
	(Sl) *Schinus longifolia* (Lindl.) Speg.
	(Sj) *Styphnolobium japonicum* (L.) Schott
	(Sd) *Suaeda divaricata* Moq.
	(Tt) *Tipuana tipu* (Benth.) Kuntze
	(Zm) *Zizyphus mistol* Griseb.
commercial cultivation	(Ha) *Helianthus annuus* L.
underbrush	(Bra) *Brassica rapa* L.
	(Car) *Carduus acanthoides* L.
	(Ct) *Carduus thoermeri* Weinm.
	(Cv) *Cirsium vulgare* (Savi) Airy Shaw
	(Hi) *Hirschfeldia incana* (L.) Lagr.-Fossat
	(Leo) *Leonurus sibiricus* L.
	(Rr) *Rapistrum rugosum* (L.) All.
	(Sp) *Senecio pampeanus* Cabrera
	(Sch) *Solidago chilensis* Meyen
fruit-bearing	(Co) *Cydonia oblonga* Mill.
	(Cr) *Citrus reticulata* Blanco
	(Mar) *Malus sylvestris* Mill.
	(Pc) *Pyrus communis* L.

[a] Letters in parentheses indicate abbreviations of species.

By means of melissopalynology analysis, it was observed that the pollen of principal apiarian species around each apiary was represented by respective honey samples. In the same way, all analyzed samples were multifloral, so we can estimate that multifloral sample proportion was larger than 0.81 with a 95% confidence interval. The variance estimation was performed with $p = 0.5$. It was calculated in the worse case because we do not have data on the real proportion of multifloral honey in these regions.

Table 3 shows apiarian species observed around the apiary. **Figure 6** shows a biplot from multiple correspondence analysis representing each floral species observed as present. The absent observations of all floral species were omitted for a better sight of the graphic.

In a factorial plane, we can observe the distinct localization of the C group on axis 1 (explain 69.41% of sample variance) with respect to other groups. On axis 2 (explain 7.7% of sample variance), the B group can be distinguished from the rest of groups. Moreover, the E and H groups can be distinguished from the F, D, I, and G groups. The ratio of total variability explained by the factorial plane was 77.15%.

Because of anthropic action, the original vegetation of each phytogeographical region has been replaced by annual or perennial cultivation of agronomic importance (*27*, *43*−*45*). However, around the visited apiaries, typical vegetable communities have been found. For example, in the C group, Mv, Ca, Sd, Aa, Ac, Pt, Zm, Br, Mc, Pto, Ae, Cp, Af, Cg, and Ld were observed. These species are Chaqueño West mount species of apiarian interest. For that reason, the C group can be good definitely and there is no crossing between other regions.

Also, in **Figure 6**, it can be observed that the B group is associated with fruit species, which differ from other groups. In the same way, each suggested group is associated with characteristic and exclusive species in the factorial plane. The H group is associated, between others species, with Sl; the I group is associated with Tp and Cv species; the F group is associated with Mp; and the G group is associated with Sch and Sp. Unlike the rest, D and E groups are not associated with exclusive species; however, they are associated with species in common with other groups. The particular combinations between species can also explain the differentiation of these groups.

When the sample identification was projected on a factorial plane, most of samples in one region were associated with characteristic vegetation of surrounding apiaries in the same region. In biplot only, the exceptions to this observation are included. These exceptions are referred to grouped samples by cluster analysis, with other samples that proceed from distant geographical regions. These samples are 1, 2, 20, 32, 35, 51, 52, 53, 55, and 56.

Sample 20, collected in Algarrobo's district, was grouped in accordance with the amino acid profile with E (Chaqueño Serrano district). This sample shows similar characteristics with flora of the E group (**Figure 6**). In the same way, samples 35 and 52 were collected in Chaqueño West district but were grouped with the B group (Algarrobo's district) and the F group (Pampeano West district), respectively.

Sample 51 was collected in Chaqueño West district, and according to amino acid composition, was grouped in G. This sample has Ms, Ma, Ha, Bra, and Car species in common with G. In a factorial plane, it appears associated to these species, which can be an explanation of crossing.

In accordance with the same criterion, samples 32 (Chaqueño Serrano district) and 53 (Chaqueño West district) were grouped with H, but these samples are associated with apiarian flora of the E group (**Figure 6**). The presence of Ms, Ma, Gd, Pa, Pn, Ag, Lt, Bra, Hi, Ct, Car, and Rr species in both groups could explain crossing. The presence of Pn, Pa, and Gd can explain the left position of 32 and 53 with respect to H (**Figure 6**).

Samples 55 and 56 (Chaqueño Serrano district) are grouped with I and D, respectively. In a factorial plane, they are associated to the F floral group. Mo, Ma, Bra, Hi, and Rr common species among three groups can explain crossing.

Samples 1 and 2 (Algarrobo's district) are grouped with I, and in a factorial plane, they are associated to Ha, Hi, Rr, and Bra instead of one particular group. Crossing could be explained by the presence of above species.

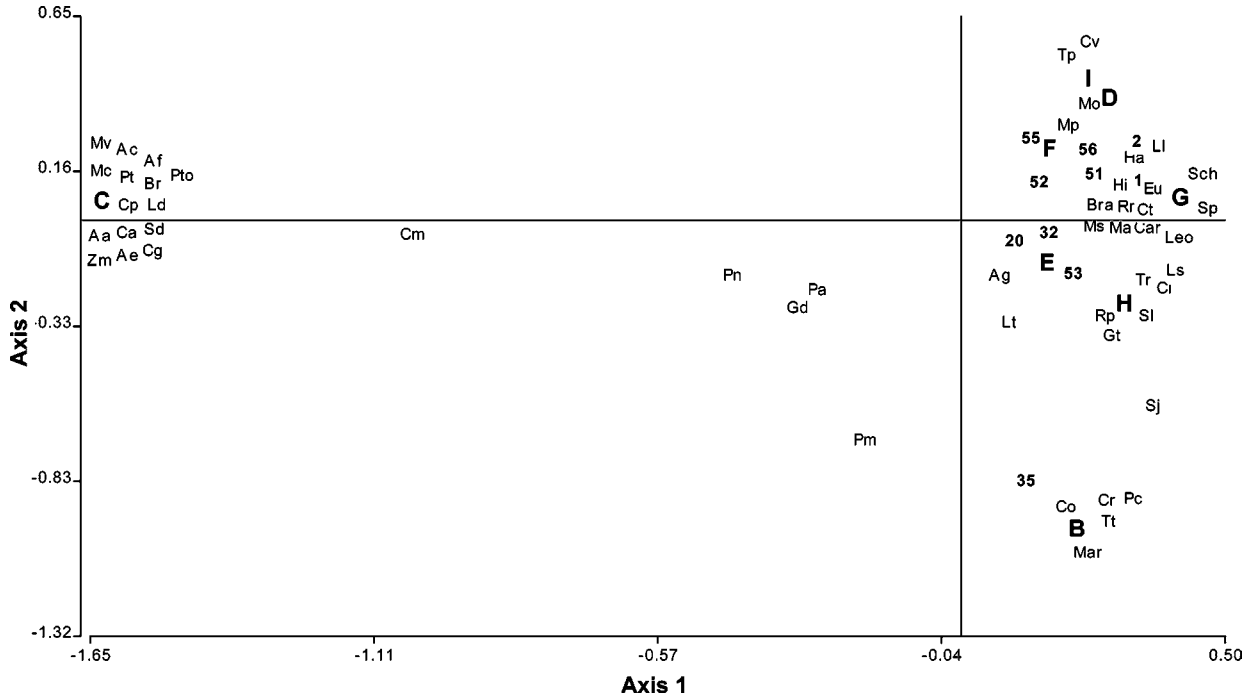

Figure 6. Factorial plane from multiple correspondence analysis. Capital letters indicate the suggested groups by cluster analysis. Small letters indicate abbreviation vegetation species (see **Table 3**). Numbers correspond to samples that have been grouped with distant geographical regions by cluster analysis.

The possible correlation between surrounding apiary flora and honey amino acid profiles agrees with the results of other authors (*23*−*25*). These authors analyzed unifloral honey, while in this work we try to improve the determination of the geographical or botanical sources of multifloral honey. The geographical differentiation could be valid for regions with origin and distinctive flora.

The sample collection method has important characteristics. Samples were collected randomly, without prior possible botanical origin studies. Multivariate statistical analysis has not previously considered samples sources, and they are grouped by similitude. The grouping was coincident with similar apiarian flora in surrounded apiaries in the same group (**Figure 6**). The apiarian flora were observed independently of amino acid profile analysis (**Table 3** and **Figure 6**), so that our results support the hypothesis that the amino acid honey profile is mainly associated with pollen of visited flora by bees. This fact must be confirmed carrying out melissopalynology analysis in all samples and comparing both methods.

Finally, sample 36 was collected in Chaqueño Serrano district (subregion E) (**Figure 1**) but was grouped in A. This fact can be explained because this sample was collected 1 year before other samples and was stored at room temperature. Therefore, to consider the amino acid profile is necessary to evaluate both state and preservation honey. Also, it is possible to think that in the same region the amino acid profile changes according to both availability and attractiveness of pollen and nectar to bees (*45*−*48*). Tellería (*49*) has described the way bees of the Buenos Aires province must adapt the collection habit of pollen and nectar available because of the changes in the same apiarian period.

Considering that the procedure described here affords good differentiation between honey of different geographical origins, we can suggest that this method can be used to verify the botanical or geographical origin of honey. Furthermore, if this analysis is repeated in consecutive harvests, most certain results could be obtained due to the possible seasonal variability. On the basis of the results, it can be deduced that the amino acid profile is associated with surrounding flora of the apiary rather than the geographical site of collection.

In this work, the geographical differentiation is based on the fact that the three chosen regions have different apiarian flora. Honey bees have different vegetal species available to extract pollen and nectar. Each suggested group corresponds to apiaries with specific surrounding flora. Grouping of honey samples from distant regions could be explained by the presence of common flora among regions. Therefore, apiaries with similar surrounding flora present similar amino acid profiles. More work is necessary to expand the scope of the present work to differentiate between either different monofloral honey or honey from very distant regions with similar flora.

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