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Relationship between Interannual Variation of Amino Acid Profile and Pollen Content in Honey from a Small Argentinian Region

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With the objective of evaluating the utility of the amino acid profile in the characterization of honey samples, 39 honey samples of two different harvests from a particular production zone in Córdoba, Argentina, were analyzed. Multivariate statistical techniques, such as principal component analysis (PCA), cluster analysis (CA), and multiple correspondence analysis (MCA), were applied to verify the correlation among the amino acid profiles, pollen percentages, and different harvests. PCA, CA, and MCA demonstrate the presence of differences of amino acid profiles between samples of the two harvests, such differences being mainly due to differences in pollen availability. Variation of the flora surrounding the apiary due to agricultural practices makes the analysis of amino acid profile typical for those cases with stabilized flora.

KEYWORDS: Amino acid profile; botanical origin; geographical origin; honey

INTRODUCTION

The honey market currently shows a tendency to establish geographical limits of production with the aim of protecting a production zone that has developed and marketed a particular standard of quality. Honey composition is closely associated with its botanical origin and, to some extent, also the geographical area in which it originated, because soil and climate characteristics determine melliferous floral (*1*).

Melissopalynology has been traditionally used for assessing the botanical and geographical origin of honey (*2*) and remains nowadays as the reference method in spite of several disadvantages. Noticeably, counting, identification, and interpretation of pollen analysis requires a highly trained analyst and construction of a complete pollen library and is sometimes affected by honey filtration.

Some methods alternative to melissopalynology involve measurements of physical and chemical parameters associated with honey characteristics (*3*, *4*). Other authors have looked for honey classification through the use of chemical markers such as flavonoids, amino acids, proteins, and volatile compounds (*5*−*9*).

Certain amino acids are synthesized by the bees and are common to many types of honey (*10*, *11*), whereas others originate in parts of the plants different from pollen, such as nectar and honeydew. However, the pollen of plants is by far the most important source of proteins and free amino acids for the bees (*11*, *12*).

Recently, we reported that amino acid profiles could be used as chemical markers of the botanical or geographical origin of honey (*5*). When amino acid profiles were analyzed together with chemometrics, we observed differentiation between samples according to their geographical origin. In fact, the amino acid profile was highly associated with the surrounding flora of the apiary rather than the geographical site of collection (*5*).

In this work, our objective was to verify the utility of amino acid profiles for the differentiation of the botanical/geographical origin of honeys and to investigate whether amino acid profiles remain over several years. For this purpose we have chosen samples from a particular region of the province of Córdoba, Argentina, of two different harvests. The presence of correlation among the amino acid profiles and pollen percentages of honey

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samples of the same collection zone, but different harvest year, has been evaluated by multivariate statistical analysis.

MATERIALS AND METHODS

Honey Samples. This study was carried out on 39 honey samples, from the southeastern region of Córdoba province in Argentina (latitude 31° 30′ to 32° 30′ south and longitude 62° 30′ to 63° 40′ west), which were harvested in two different years, following IRAM norms (*13*). Twelve samples were collected in apiary season 1999/2000 (samples 1−12), and 27 samples were collected in apiary season 2001/2002 (samples 13−39). In both cases the apiary season goes from October to February.

Amino Acid Analysis. Amino acid profiles were obtained following the procedure previously described (*5*). Amino acids were extracted and analyzed within 20 days of collection.

Pollen Analysis. Melissopalynology analyses were performed in all samples according to the method of Loveaux et al. (*2*). For pollen type identification, pollen slides from the CICYTTP palynotheca were used, and bibliography describing pollen taxa of areal flora have been consulted (*14*−*18*). When it was possible, pollen types were identified at species level, otherwise, at a genus, tribe, or family level.

Statistical Analysis. Because proline is the main amino acid added to honey by bees, it is necessary to evaluate its variation during two different harvests by Kruskal−Wallis analysis of variance test (*19*) to decide whether to exclude it from the analysis.

Data Matrix. Two different data matrices were constructed. First, to compare the amino acid composition among samples of two years, 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 (100%), except proline.

To compare the pollen taxa presented in the samples, a second data matrix was constructed considering pollen species having frequency in honey above 5%, at least in one sample. There were some exceptions of species, which were included in the study because they clearly permitted differentiation of groups of samples even though they did not overcome the 5% limit. These species were *Cydonia oblonga*, *Heimia salicifolia*, *Leonorus sibiricus*, *Malus silvestris*, *Parquinsonia aculeata*, *Pyracanta angustifolia*, *Schinus* sp., *Senecio pampeanus*, *Baccharis* type, *Eringium* type, *Scutellaria* type, *Vigna luteola*, and *Wedelia glauca*.

Cluster Analysis (CA). The aim of the CA is to uncover some latent structure of the objects in terms of groups of similar elements and, possibly, in terms of hierarchy of embedded groups (*20*). The procedure involves a measurement of either the distance or similarity between objects to be clustered. The clustering is represented in a dendrogram by the junction of the corresponding branches, which is called a node of the tree (*20*).

Cluster analysis was applied to both data matrices to identify groups in the samples related to free amino acid profile and to pollen species. For this analysis, the data were standardized. The complete linkage algorithm was applied using the Euclidean distance (*21*).

Principal Component Analysis (PCA). To analyze the underlying variability and to identify useful associations for different honey groups, two PCAs were performed (*21*). In the first case, as classification criterion, the groups related to free amino acid profile suggested by the CA were used. In the second case, the groups related to pollen grains frequency were used. Some graphical tools such as biplots have also been used.

Multiple Correspondence Analysis (MCA). Multiple correspondence analysis is a simple correspondence analysis carried out on an indicator (or design) matrix with cases as rows and categories of variables as columns.

For these analyses, we considered for each sample the groups suggested by cluster analyses, the amino acid percentage concentration, and the pollen grains frequency. The quantitative variables were transformed into four classes, using the quartile position. In category one were included samples belonging to the first quartile; in category two, we included samples corresponding to the second quartile; in category three, we included samples belonging to the third quartile; and, finally, in the fourth category, we included samples belonging to the fourth quartile. To get a better comprehension of the results, a biplot was performed.

All statistical tests were carried out using Infostat 1.1 software (*22*).

Table 1. Representative Pollen Taxon Percentage Concentration Mean for Each Statistical Group Obtained by Cluster Analysis and Associations Suggested by Pca between Some of These Values and the Groups

		1999/2000			2001/2002		
taxon	abbreviation	1	2	6	3	4	5
Carduus sp.	Car	4.6	2.6	7	3.4	6.1	5.4
Celtis sp.	Ce	0.8	0.1	**2.3**[b]	0.08	0.05	0
Cruciferae	Cru	13	**31.1**[a]	**19.5**[b]	12.5	15.3	11.3
Cucurbitaceae	Cu	0.1	0	0.01	**2.1**[b]	0.02	0.01
Cydonia oblonga	Co	**2.7**[b]	0	0	0	0	0
Eucalyptus sp.	Eu	2.5	4.0	4.0	3.9	4.1	1.3
Geoffroea decorticans	Gd	**5.6**[a]	0	**6.2**[a]	**0**[c]	**0**[c]	0
Gleditsia triacanthos	Gt	**3.3**[b]	**2.7**[b]	**3.5**[b]	0	0	0
Glycine max	Gm	0	**0**[c]	**0**[c]	**2.8**[b]	6.0	**34.8**[a]
Heimia salicifolia	Hs	1.1	0.3	1	**0**[c]	**0**[c]	0
Helianthus annus	Ha	**9.4**[a]	0	**2.3**[b]	1.4	0.4	0.9
Leonurus sibiricus	Leo	**1.7**[b]	0	0.5	0	0	0
Lippia turbinata	Lt	**1.9**[b]	**3.3**[a]	**1.6**[b]	0	0	0
Lotus sp.	Lo	0	0	0	**4.3**[a]	0.1	0.2
Malus sylvestris	Mar	**3.0**[b]	0	0	0	0	0
Marrubium vulgare	Mav	0	**2.1**[a]	**1.5**[a]	0	0	0
Medicago sativa	Ms	11.3	7	8.1	5.0	6.9	**12.4**[b]
Melilotus albus	Ma	12.3	17.2	20.9	**38.6**[a]	**38.4**[a]	**12.0**[b]
Moraceae	Mor	**0**[c]	**0**[c]	**0**[c]	1.08	**2.9**[b]	2.0
Parquinsonia aculeata	Pac	**2.2**[b]	0.1	**2.1**[b]	0	0	0
Prosopis sp.	Pro	1.1	0	5.1	1.0	0.9	0.3
Pyracanta angustifolia	Pya	**0.2**[b]	**1**[a]	**0.2**[b]	0	0	**0**[c]
Robinia pseudoacacia	Rp	**6.5**[a]	**6.2**[a]	**1.7**[b]	0	0	0
Schinus sp.	Sch	0	0	0.4	0	0.02	0.02
Senecio pampeanus	Sp	0.1	0	0.7	0	0	0
Styphnolobium japonicum	Sj	**7**[a]	**8.9**[a]	**1.3**[b]	0	0	0
Ammi sp. type	Tam	**0**[c]	**0**[c]	**0**	**13.7**[a]	**6.2**[a]	**6.2**[b]
Baccharis sp.type	Tba	0	0	0	0.9	0.7	0.7
Eryngium sp. type	Ter	0	0	0	**0.2**[b]	**0.2**[b]	0.2
Scutellaria type	Tsc	0	0	0	0.7	1.0	1.0
Solidago sp. type	Tsg	4.1	1.7	1	0.7	0.9	0.9
Tipuana tipu	Tt	0	5.6	0.4	0	0	**0**[c]
Trifolium sp.	Trf	0	2.7	4. 6	1.6	0.4	0.4
Vicia sp.	Vi	0.1	0	0.01	**1.5**[b]	0	0
Vigna luteola	Vl	**1.7**[a]	0	0.08	0	0	0
Wedelia glauca	Wg	0.1	0	0.7	0	0	0

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

RESULTS AND DISCUSSION

Melissopalynology Analyses. Most of the samples analyzed were multifloral with the exception of five samples (28, 29, 31, 32, 38) that were *Melilotus albus* unifloral honey and six samples (13, 14, 23−26) that were *Glycine max* unifloral honey. Representative taxon of honey samples analyzed is shown in **Table 1**.

Statistical Analysis. When a nonparametric Kruskal−Wallis ANOVA test was applied to proline concentrations, there was no significant difference in the absolute concentration means between years of harvest, so it was excluded for the comparison. Moreover, when proline was included for the calculations, a greater dispersion in the statistical comparison within years was observed.

An exploratory evaluation of the data structure was carried out by CA, searching for natural grouping (similarity) among samples related to free amino acid percenteage concentration. The results obtained are presented as a dendrogram, which is

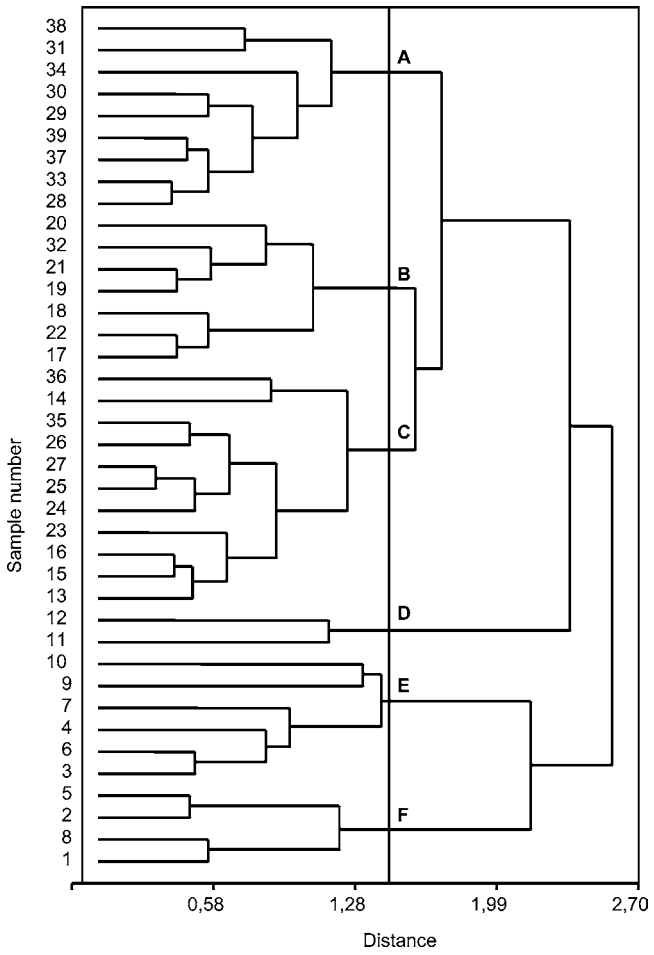

Figure 1. Dendrogram from cluster analysis of honey samples according to their amino acid profiles.

shown in **Figure 1**. It can be observed in **Figure 1** that the sample point arrangement is not random. The CA suggested six clusters **(**A−F**)** formed at a Euclidean distance of 1.39 (50% of total linkage distance). Clusters A, B, and C are composed of honey samples harvested during 2001/2002, whereas clusters D, E, and F are formed by samples collected during 1999/2000.

The associations of amino acid percentage concentration with the cluster, suggested by CA, were evaluated by PCA. The first principal component explains 46% of total variability, the second one 30%, the third one 20%, and the fourth one 2%. That is to say, 98% of total variability between samples could be explained considering only the first four principal components. **Figure 2** shows three biplots obtained from these components. It can be observed that all quantified amino acids are associated with one or more honey groups.

The 13 amino acid mean value for each statistical group was calculated (**Table 2**), and we evaluated the suggested associations by PCA between honey group and amino acids. It can be observed that the amino acids associated with each group can characterize their own amino acid profile of each group. From the results obtained by CA and PCA, differences in amino acid profiles were found between samples of different collection years, in spite of samples being of the same collection zone.

To investigate the origin of such differences, we performed the same statistical analysis but this time applied to the percentage of pollen species present in honey samples. Cluster analysis of pollen species suggested seven clusters **(**1−6**)**, as shown in **Figure 3**. In this case, we used two different cut criteria to obtain a better interpretation of association of

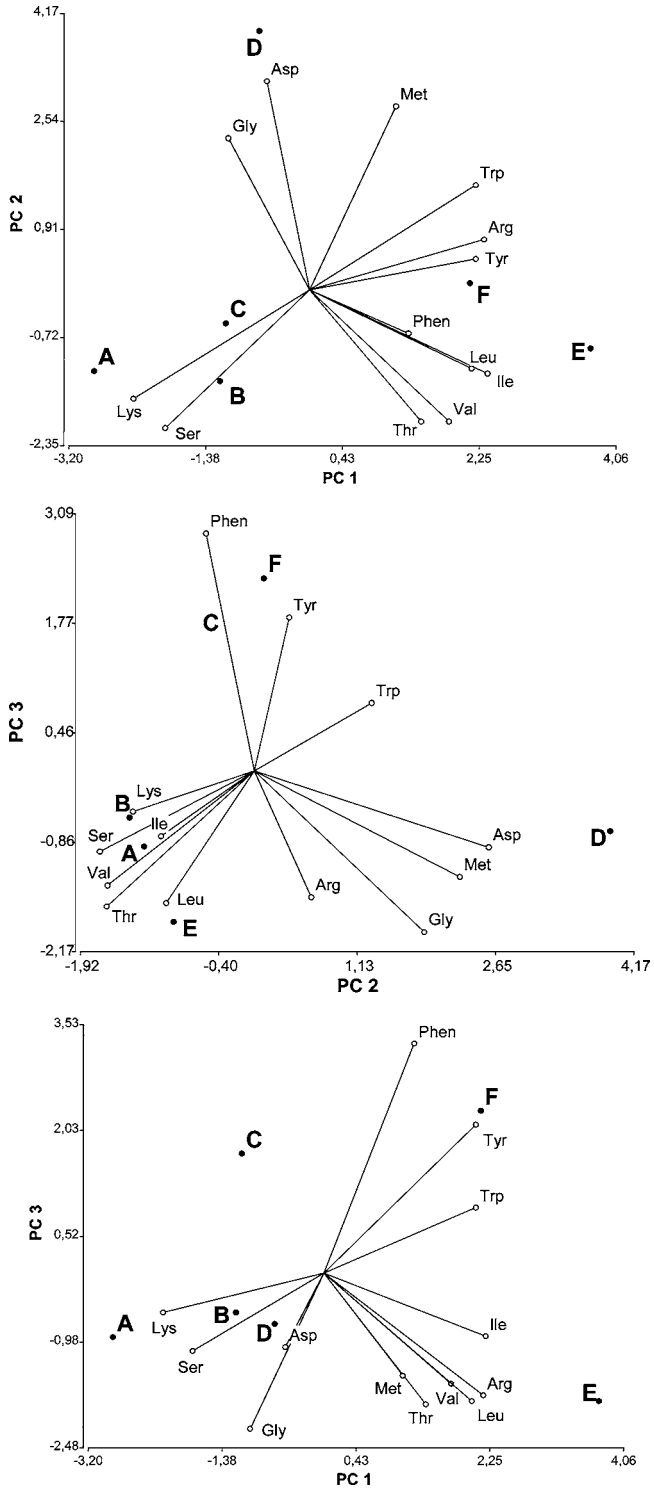

Figure 2. Biplots (PCA) with the first three principal components according to amino acid profiles. Capital letters indicate cluster analysis group.

samples: the first one at a distance of 1.24 (45% of total linkage distance) and the second one at a distance of 1.9 (72% of total linkage distance). Clusters 1, 2, and 6 are formed by samples harvested during 1999/2000, whereas clusters 3−5 are formed by samples collected during 2001/2002. Samples 37, 32, 38, and 19 are included in cluster 3. To identify the associations of pollen percentage content with the cluster suggested by CA, a new PCA was performed. Ninety-three percent of total variability between samples could be explained by looking at the first four principal components. The first principal component

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

	2001/2002			1999/2000		
amino acid	A	B	C	D	E	F
aspartic acid	6.47	4.02	4.30	**19.72**[a]	4.50	4.15
serine	**5.31**[a]	**4.80**[a]	4.19	2.05	2.84	**1.47**[c]
glycine	**11.73**[b]	**12.66**[b]	7.53	**19.72**[a]	9.27	**5.53**[c]
arginine	1.79	1.74	**1.29**[c]	**6.63**[a]	**12.68**[a]	**5.23**[a]
threonine/alanine	3.43	2.38	1.75	1.11	5.16	2.60
tyrosine	**2.99**[c]	**5.37**[c]	**8.46**[b]	7.85	**11.28**[a]	**17.24**[a]
valine	5.22	6.34	4.46	3.87	**8.23**[a]	5.75
methionine	**0.39**[c]	**0.42**[c]	**0.36**[c]	**3.00**[a]	**1.96**[b]	**1.09**[b]
isoleucine	2.55	3.64	2.93	2.66	**4.77**[a]	**3.63**[b]
leucine	2.57	**3.47**[b]	2.59	2.59	**4.97**[a]	**3.10**[b]
phenylalanine	**16.14**[c]	24.07	**34.28**[a]	**17.78**[c]	25.23	**42.16**[a]
tryptophan/ornithine	**1.56**[c]	**2.04**[c]	3.07	3.42	3.68	**3.57**[b]
lysine	**39.84**[a]	**29.06**[a]	24.80	9.61	**6.28**[c]	**5.18**[c]

[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.

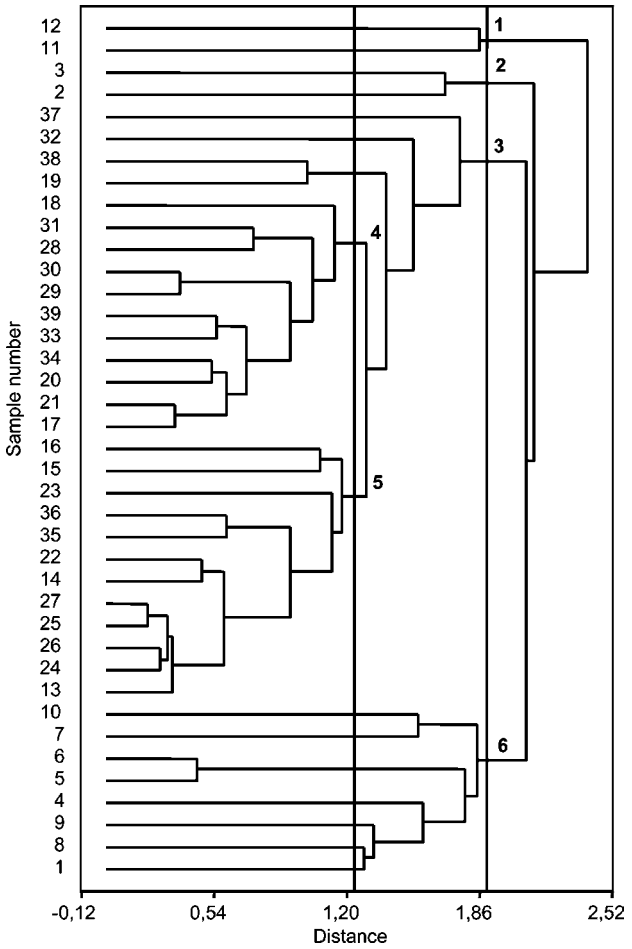

Figure 3. Dendrogram from cluster analysis of honey samples according to the pollen species present.

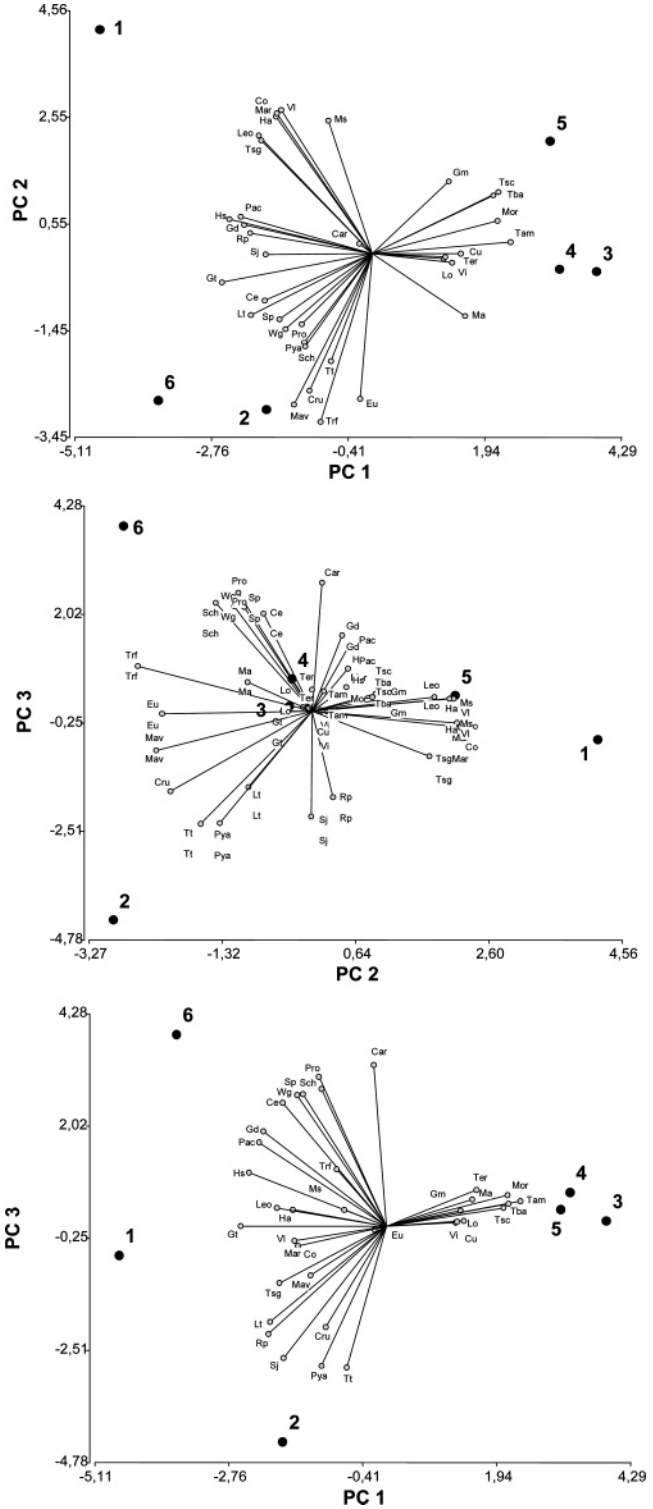

Figure 4. Biplots (PCA) with the first three principal components according to the pollen species present in the honey samples. Numbers indicate cluster analysis group.

explains 41% of total variability, the second one 21%, the third one 19%, and the fourth principal component 12% of total variability. **Figure 4** shows the biplots obtained from these components. It can be seen that several species of pollen are associated with one or more honey groups. Starting from associations suggested by PCA, the amino acids mean value for each statistical group was calculated, and the values obtained are shown in **Table 1**. It can be observed that the taxon of pollen associated with each group can characterize their own pollen profile of each group (**Table 1**).

Analyzing the groups suggested by CA, it can be said in general that samples from clusters suggested by CA from amino acid percentages coincide with samples from clusters suggested by CA from pollen percentages. For example, samples from clusters E and F (cluster group by amino acid profile; year 1999/2000) coincide with the samples of groups 2 and 6 (cluster group by pollen percentage; year 1999/2000). Likewise, samples from

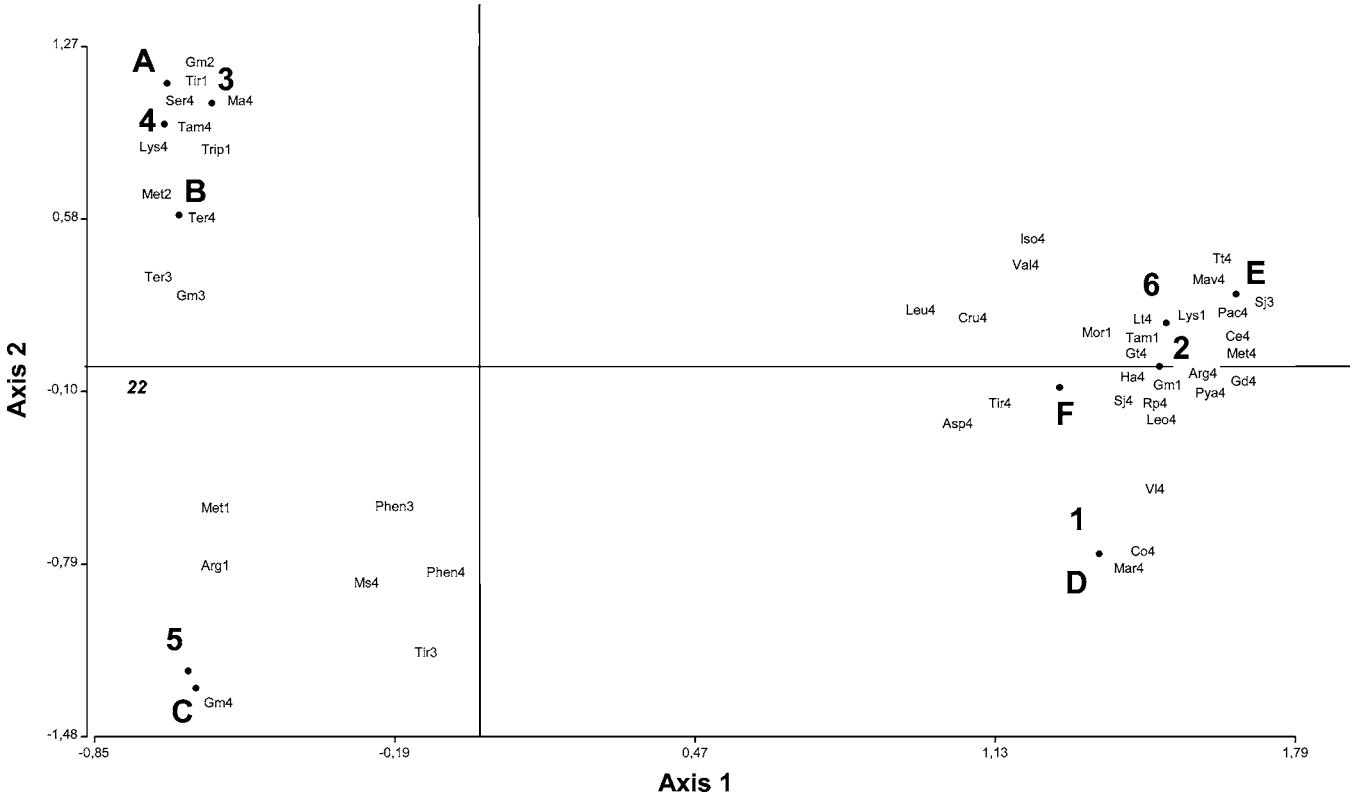

Figure 5. Factorial plane from multiple correspondence analysis. Capital letters and numbers indicate the suggested groups by cluster analysis. Small letters indicate abbreviation of vegetation species and amino acids (see **Tables 1** and **2**) and subindices correspond to (1) first quartile, (2) second quartile, (3) third quartile, and (4) fourth quartile.

cluster D coincide with samples from group 1. The same conclusion can be drawn for samples harvested in 2001/2002. Samples from A and B (cluster group by amino acid profile; year 2001/2002) coincide with the samples of groups 3 and 4 (cluster group by pollen percentage; yearr 2001/2002). Likewise, samples from cluster C coincide with samples from group 5 except for sample 22, which is included in cluster B.

Due to results obtained so far, it can be inferred that the differences found in amino acid profiles of honey samples collected in different periods could be a consequence of changes in the flora surrounding the apiaries, which is likely because the geographical region under study is mainly devoted to crops.

Finally, to understand the possible correlation between surrounding flora and honey amino acid profiles, we performed a MCA. MCA allows the construction of principal components, which optimally summarize the data, and enables the construction of graphical displays. An interesting property of these graphical displays is that associations between honey samples and amino acid profiles and pollen species percentage can be observed on various projection planes.

The results of the MCA indicated that the first two axes accumulate 48.4% of the total inertia. The results are shown as a biplot in **Figure 5**. When the group identification, amino acid, and pollen species categories of samples are projected on a factorial plane, we can observe that the first axis, which explains 32.71% of sample variance, clearly separates honey samples collected during the harvest of 1999/2000 from the ones collected during 2001/2002. This result is in concordance with the results obtained for CA for both amino acid profile and pollen percentages.

The distinct localization of groups D−F (cluster group by amino acid profile; year 1999/2000) and 1, 2, and 6 (cluster group by pollen percentage; year 1999/2000) with respect to the other groups A−C (cluster group by amino acid profile; year 2001/2002) and 3−5 (cluster group by pollen percentage; year 2001/2002) can be observed in **Figure 5**.

Additionally, the second axis, which explains 15.69% of sample variance, differentiates two groups between samples of harvest 1999/2000 and between samples collected during 2001/2002. It distinguishes C and 5 groups from A, B, 3, and 4 groups. Moreover, on the same axis we can distinguish D and 1 groups from F, E, 2, and 6 groups.

Groups D−F and 1, 2, and 6 (**Figure 5**) are positively associated with the amino acids methionine and arginine according to the MCA and PCA results (see **Tables 1** and **2** and **Figures 2** and **4**). Samples corresponding to groups E and F are also positively associated with the tyrosine, isoleucine, and leucine and negatively correlated with lysine. When analyzing cluster groups in relation to pollen percentages, these groups of samples are associated with the presence of the following pollen species: Gt, Lt, Rp, Pya, and Sj. Also, they are associated with the absence of Tam, Gm, and Mor. In particular, groups 2 and 6 are positively associated with the presence of pollen of Cru. Clusters 1 and 6 are associated with Pac, Gd, and Ha.

As can be seen in **Figure 5**, samples from cluster D-1 (bottom right of **Figure 5**) are separated from the rest according to axis 2, and this is mainly due to the association of these samples with the presence of Vl, Mar, and Co. With regard to amino acids, these samples contain high percentages of aspartic acid and glycine and low percentages of phenylalanine.

The same analysis scheme was followed for samples harvested in 2001/2002. Clusters A, B, 3, and 4 are characterized by high percentages of serine and lysine and are associated with low percentages of tryptophan/ornithine, tyrosine, and

methionine. The lack or deficiency of these amino acids is also observed for cluster C-5.

When the pollen percentages are analyzed, it can be observed that clusters A, B, 3, and 4 are characterized by their own pollen profile: high frequency of Ma (all *Melilotus albus* unifloral honeys are clustered in this group) and Tam with the presence of Ter in low proportion.

Finally, the cluster C-5 is characterized by a high proportion of phenylalanine, a moderately high proportion of tyrosine, and low concentrations of arginine and methionine.

Samples belonging to cluster C-5 are characterized by high frequency of Gm and relatively high frequency of Ms and Ma. In fact, all *Glycine max* unifloral samples are clustered in this group.

Considering that samples clustered in group 4, with the exception of sample 22, contain pollen of Gm in percentage equal to 41 ± 15%, we can assume that its amino acid profile (**Table 2**) can be an indicator of the presence of pollen of Gm in honey. In fact, even though sample 22 is included in cluster 4, in the biplot it is located at a considerable distance from all of the other samples of cluster 4 (**Figure 5**).

The correlations between the profiles of amino acids and pollen percentages found in this work are in agreement with those found by other authors (*23*−*25*). In this study multifloral honeys with a predominant specific pollen type (without being unifloral) were clustered together with unifloral honey of the corresponding flora. This confirms the hypothesis that the free amino acid profiles are mainly determined by pollen in honey.

Comparison of the free amino acid profiles of honey samples of successive harvests showed significant differences in the concentration of several of them. This situation was predictable because the sampling region is dedicated to agricultural development and the crops vary with economic factors or depending on the conditions of the soil. That is why the surrounding flora of the apiaries changes, which is correlated with the pollen species available for honey bee consumption and therefore with the free amino acids available in honey. In the 1999/2000 harvest we observed wild or implanted pollen species such as *Celtis* sp., *Geoffroea decorticans*, *Gleditsia triacanthos*, *Lippia turbinata*, *Marrubium vulgare*, *Parquinsonia aculeate*, and *Tipuana tipu*, whereas in the 2001/2002 harvest these species were replaced by other crops or weeds (e.g., *Glycine max*). This observation allows one to presume that the utilization of the amino acid profiles as indicative of the geographical origin would be applicable only to those geographical regions with stabilized flora, that is to say, where there is no changing of native flora. Thus, the amino acid profile of honey coupled with statistical analysis could be used as complementary to melissopalynology in the evaluation of the floral origin of honey.

ABBREVIATIONS USED

CA, cluster analysis; PCA, principal component analysis; MCA, multiple correspondence analysis.

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