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Changes in the Free Amino Acid Contents of Honeys During Storage at Ambient Temperature

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This study was carried out to establish the changes in the free amino acid contents of floral honeys, honeydew honeys, and blend honeys during storage at room temperature and to test the capacity of the amino acids to distinguish the origin of the honeys after storage. For this purpose, 54 artisanal honeys (39 floral, 5 honeydew, and 10 blend) were studied. Samples were taken from recently collected honeys and at 3, 6, 9, 12, 16, 20, and 24 months after harvesting. The contents of most of the free amino acids were found to decrease with storage time, with the greatest reduction observed in the first 9 months. The contents of the amino acids aspartic acid, β-alanine, and proline increased in the first few months after storage, reaching maximum values at 6 months, suggesting the possible existence of enzymatic activities. The application of stepwise discriminant analysis to the free amino acid content data demonstrated that the contents of the amino acids valine, β-alanine, γ-aminobutyric acid, serine, isoleucine, α-alanine, ornithine, and glutamine correctly assigned 87% of honeys to their group of origin: floral, honeydew, or blend.

KEYWORDS: Floral honey; honedew honey; blend honey; storage; amino acids

INTRODUCTION

Honey is defined as the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature (*1*). Therefore, some honey components come from plants, others are added by honeybees, and others are due to biochemical reactions taking place during honey maturation.

The main types of honey according to their origin are as follows: blossom honey or nectar honey, honey obtained from the nectar of plants, and honeydew honey, obtained mainly from excretions of plant-sucking insects on the living part of plants or secretions of living parts of plants. The existence of honeys from nectar and honeydew is frequent. These honeys are called blend honeys. It is, therefore, necessary to be able to apply analytical methods that can ensure the authenticity of origin and the quality of the honey. European legislation (*1*) has established criteria for honey composition before it can be sold on the market. These criteria refer to the contents of sugar, water, non-water-soluble fraction, conductivity, free acidity, diastase activity, and hydroxymethylfurfural. However, these criteria cannot be used to verify the origin or freshness of a honey.

It is reported in the literature (*2*, *3*) that free amino acids are good indicators of the blossom or honeydew origin of honeys. According to several authors, amino acid composition may also be a suitable method to determine honey botanical origin of floral honeys (*4*−*7*). However, during storage and thermal treatments of honey (*8*, *9*), several compounds are formed from the reaction of the carbonyl group of a reducing sugar with the free amino group of amino acids, peptides, or proteins. Since each amino acid has a different reactivity, the proportion of the different amino acids in the honey could be affected by storage time, losing their discriminatory efficacy. This research was proposed to determine the changes in the free amino acid content in honeys during storage and to test the discriminant capacity of various different amino acids after different storage times. For this purpose, the composition of free amino acids in honey provided by artesanal beekeepers and stored for 24 months was studied by determining free amino acid contents over time.

MATERIALS AND METHODS

Honey Samples. Authentic honeys were provided by local beekeepers with hives settled in a small geographic area of about 2000 km^2 in the community of Madrid, Spain. All honeys were artisanally produced, obtained by centrifugation and unpasteurized. Thirty-nine honeys were from nectar and five from honeydew. Ten more honeys were from hives

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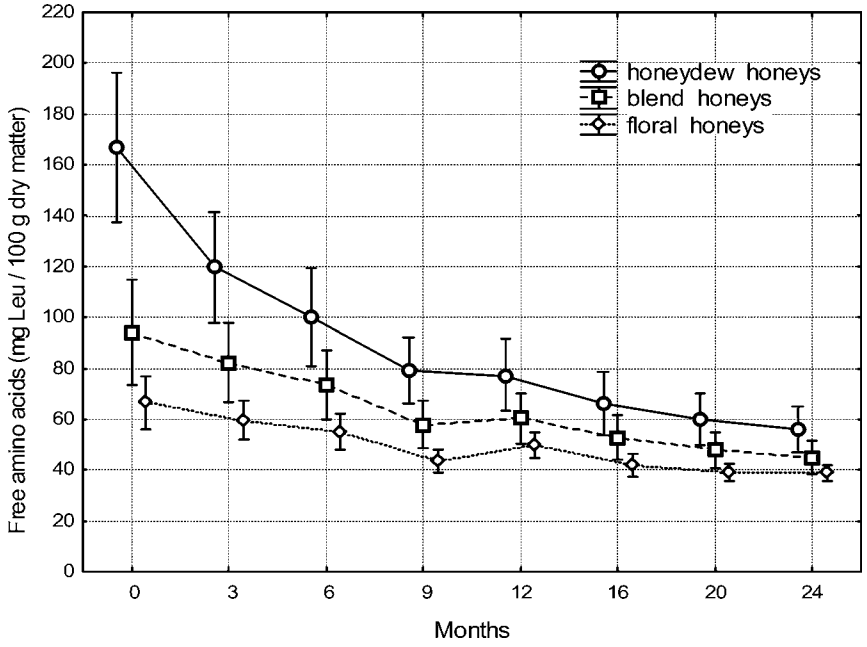

Figure 1. Mean values and 95% confidence intervals of free amino acids content (mg of leucine/100 g of dry matter, spectrophotometric method) in the three classes of honeys (honeydew, $n = 5$; blend, $n = 10$; and floral honeys, $n = 39$) during storage at ambient temperature.

Table 1. Mean Values and Standard Deviation (SD) of the Amino Acids Concentration (mg/100 g of dry matter) in Floral Honeys ($n = 39$) during the Storage at Ambient Temperature[a]

	0 months		3 months		6 months		9 months		12 months		16 months		20 months		24 months	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Asp	4.79	3.41	4.37	2.34	6.20	4.06	6.00	4.07	5.71	3.21	4.52	2.66	4.49	2.49	4.47	2.33
Glu	7.83	8.18	6.67	5.40	8.44	7.31	6.74	4.61	6.48	4.49	4.54	3.94	4.22	3.54	4.11	2.97
Asn	8.01	7.90	7.31	5.55	7.60	5.60	6.10	3.20	6.42	3.57	5.29	3.51	4.82	2.71	4.68	2.57
Ser	2.99	1.37	2.89	1.04	3.14	1.26	3.02	0.89	2.86	0.79	2.20	0.62	1.99	0.65	1.87	0.36
Gln	6.44	4.85	5.94	3.40	5.58	2.91	4.40	1.51	4.30	1.35	3.61	1.22	3.17	0.73	2.93	0.57
His	2.27	1.39	2.25	1.20	2.49	0.77	2.59	0.46	2.52	0.45	1.97	0.64	1.62	0.92	1.07	1.06
Gly	1.66	0.28	1.70	0.33	1.65	0.33	1.62	0.22	1.59	0.24	1.35	0.22	1.37	0.46	1.39	0.32
Thr	2.56	0.72	2.44	0.45	2.46	0.45	2.35	0.25	2.33	0.24	1.78	0.21	1.76	0.16	1.74	0.11
Arg	4.57	1.88	4.70	1.55	3.71	0.89	3.35	0.45	3.44	0.43	2.60	0.55	2.48	0.39	2.40	0.33
α-Ala	2.27	0.10	2.28	0.14	2.81	0.17	2.74	0.15	2.75	0.13	2.75	0.21	2.69	0.09	2.68	0.13
β-Ala	3.09	1.47	2.97	1.16	3.63	1.42	3.46	1.19	3.48	1.18	3.00	1.07	2.86	1.08	2.88	0.99
Gaba[b]	2.98	3.16	2.91	2.95	2.93	2.51	2.63	1.68	2.59	1.52	2.29	1.16	2.19	0.84	2.13	0.67
Tyr	14.41	4.21	13.68	5.98	13.92	4.57	11.95	3.74	11.84	3.76	10.37	3.76	9.03	3.21	8.73	3.09
Met	0.00	0.00	0.36	0.85	0.06	0.35	0.23	0.68	0.23	0.69	0.00	0.00	0.00	0.00	0.00	0.00
Val	2.52	0.40	2.47	0.36	2.62	0.37	2.37	0.17	2.36	0.19	1.83	0.16	1.80	0.17	1.78	0.09
Trp	3.78	0.44	3.67	0.34	3.51	0.21	3.37	0.10	3.35	0.11	2.61	0.59	2.49	0.06	2.46	0.04
Phe	28.27	13.37	26.18	16.02	27.11	13.84	22.67	10.97	22.57	11.13	20.72	10.90	18.29	9.45	17.85	9.37
Ile	2.51	0.16	2.46	0.24	2.43	0.17	2.30	0.10	2.30	0.08	1.68	0.12	1.65	0.06	1.64	0.06
Leu	2.78	0.81	2.88	1.19	2.76	0.94	2.59	0.76	2.56	0.74	2.04	0.70	1.92	0.56	1.90	0.51
Orn	2.51	0.38	2.45	0.27	1.95	0.20	1.97	0.12	1.83	0.45	2.68	0.17	2.58	0.12	2.56	0.05
Lys	4.46	0.58	4.18	0.68	3.70	0.76	3.13	0.62	3.11	0.54	2.83	0.50	2.66	0.32	2.61	0.31
Pro	67.41	16.17	63.47	14.67	84.59	24.17	74.17	19.55	64.61	13.91	62.66	13.92	64.49	14.02	63.93	14.37

[a] The mean values of concentrations of each amino acid at the different storage times are significantly different ($P < 0.05$), with the exception of Gaba. [b] Gaba = γ-aminobutyric acid

partially filled with floral honey produced from spring blossoming, and then with honeydew which bees collected mainly in the summertime from *Quercus pyrenaica* and some other tree species, referred to here as blends. Honeys were assigned to the different groups by experts by means of melissopalynological analysis, according to the method of Louveaux et al. (*10*) with the modifications proposed by Terradillos et al. (*11*) and by their physicochemical characteristics determined by the Spanish Official Methods for Honey Analysis (*12*). Honeys were stored in closed flasks at room temperature and samples were taken from the initial honey and at 3, 6, 9, 12, 16, 20, and 24 months after harvesting.

Spectrophotometric Methods. Free amino acids were quantified by the Cd−ninhydrin method (*13*) based on the reaction of primary amino group of the amino acids with ninhydrin and the measure of the absorbance at 507 nm. Proline was determined by the colorimetric method of Ough based on the reaction of secondary amino acids with ninhydrin in an acid medium and the measure of absorbance at 517 nm (*14*).

Amino Acid Analysis by HPLC. Free amino acids were determined in the filtered solution (Millipore, Bedford, MA, 0.45-μm filter) of 1.25 g of honey/25 mL water. Analyses were carried out by RP-HPLC using a Waters (Milford, MA) liquid chromatograph. Samples were submitted to automatic precolumn derivatization with *o*-phthaldialdehyde in the presence of 2-mercaptoethanol. Solvents and gradient conditions were as described by Moreno-Arribas et al. (*15*). Separations were performed on a Waters Nova-Pak C18 (150 × 3.9 mm i.d., 60 Å, 4 μm) column and the same type of precolumn. Detection was done by a fluorimetric detector (HP 1046-A) (λ excitation = 340, λ emission = 425).

Millenium32 software was employed for chromatographic control and data acquisition.

Statistical Methods. The statistical methods used for data analysis were one-way ANOVA and the Student−Newman−Keuls test to compare the three types of honey, repeated measures analysis of variance to test jointly the effects of the two factors [storage time as a within-subjects factor, with eight levels (0, 3, 6, 9, 12, 16, 20 and 24 months), and the types of honey as a categorical variable], and stepwise discriminant analysis to select the variables most useful in differentiating the three groups of honeys. The STATISTICA program for Windows, version 7.1, was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

RESULTS AND DISCUSSION

Honeys' water content at the harvest time (time 0) had a mean value of 16.1% and a standard deviation of 0.9. The decrease of the water content during the storage was very small (2% mean value) due to the fact that the storage was in closed flasks that were opened only to take the samples.

To have a global vision of the amino acids content of the honeys, total free amino acids were determined by the spectrophotometric ninhydrin method. Mean values and 95% confidence intervals of free amino acid contents of the three types of studied honeys and their evolution during the 24 months of storage are shown in **Figure 1**. The mean free amino acid contents in the honeydew honeys was 166.80 mg of leucine/100 g, significantly higher than that of the floral honeys (66.67 mg of leucine/100 g), and the blend honeys gave intermediate values (94.21 mg of leucine/100 g). These values are within the range described by Cotte et al. (*16*) in a study of 280 French floral honeys (34.19−183.16 mg/100 g) and that reported by Iglesias et al. (*2*) in floral honeys and honeydew honey from the same geographical area as those studied here. From the repeated measures analysis of variance, storage time and the type of honey factors had a significant influence ($P < 0.01$) on this variable. During the first 9 months of storage, the free amino acid contents decreases, after which the mean values of amino acid content are not significantly different in any of the honey groups studied.

In order to find out the amino acids that decrease in content during storage, individual amino acids were analyzed for the 39 floral honeys, 5 honeydew honeys, and 10 blend honeys over storage. **Table 1** shows the mean values and the standard deviation of the concentration of the different free amino acids detected in the initial samples and in samples collected at intervals during storage of the floral honeys. The values for the mean contents of each amino acid at the different storage times with the exception of γ-aminobutyric acid are significantly different ($P < 0.05$). Although the contents of free amino acids in the honeydew honeys and blend honeys are greater than that of the floral honeys, the changes in free amino acid contents over storage followed a similar pattern (data not shown).

From repeated measures analysis of variance, the storage time and the classes of honey factors had a significant influence ($P < 0.01$) on all amino acids, except for β-alanine, valine, isoleucine, leucine, and ornithine, which were only affected by storage time. The contents of most of the amino acids diminished over storage (**Table 1**). The greatest reduction was observed in the contents of glutamic acid, arginine, and glutamine, for which the content at 24 months of storage was between 45 and 52% of the initial content in floral honeys. During storage of foods with high sugar or amino acid contents, such as honeys, Maillard's reaction takes place in which the initial step produces Amadori's compounds, followed in subsequent steps by the formation of melanoidines. In honeys, the presence of Amadori's compounds derived from the amino acids lysine, proline, γ-aminobutyric acid, and arginine have been detected (*9*). Although not reported previously, it is very likely that these types of compounds are produced in honeys from other amino acids. **Figure 2** shows the changes in the contents of the amino acids lysine, γ-aminobutyric acid, and arginine in the three groups of honeys studied. It can be observed that these steadily diminish during storage, and this decrease is more pronounced in the first 9 months, as found in the global determination of free amino acids by spectrophotometry (**Figure 1**).

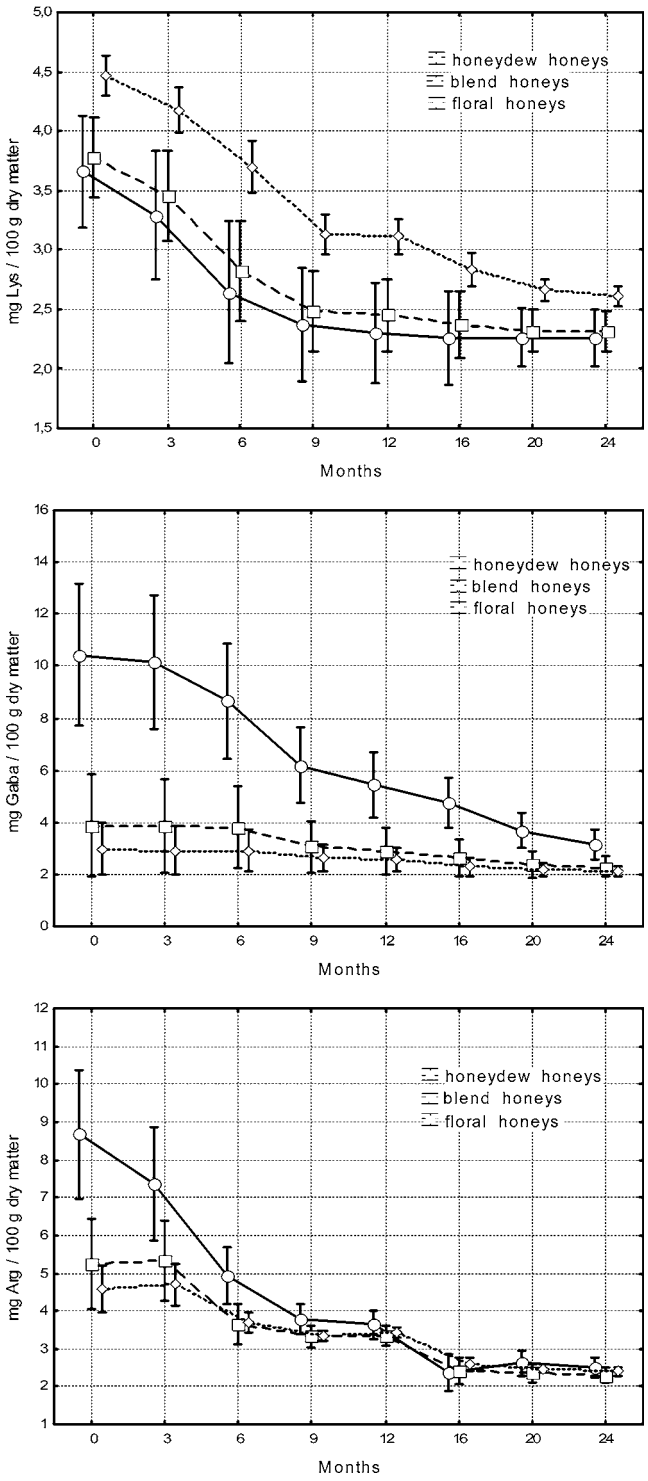

Figure 2. Mean values and 95% confidence intervals of lysine (Lys), γ-aminobutyric acid (Gaba), and arginine (Arg) content in the three classes of honeys (honeydew, $n = 5$; blend, $n = 10$; and floral honeys, $n = 39$) during storage at ambient temperature.

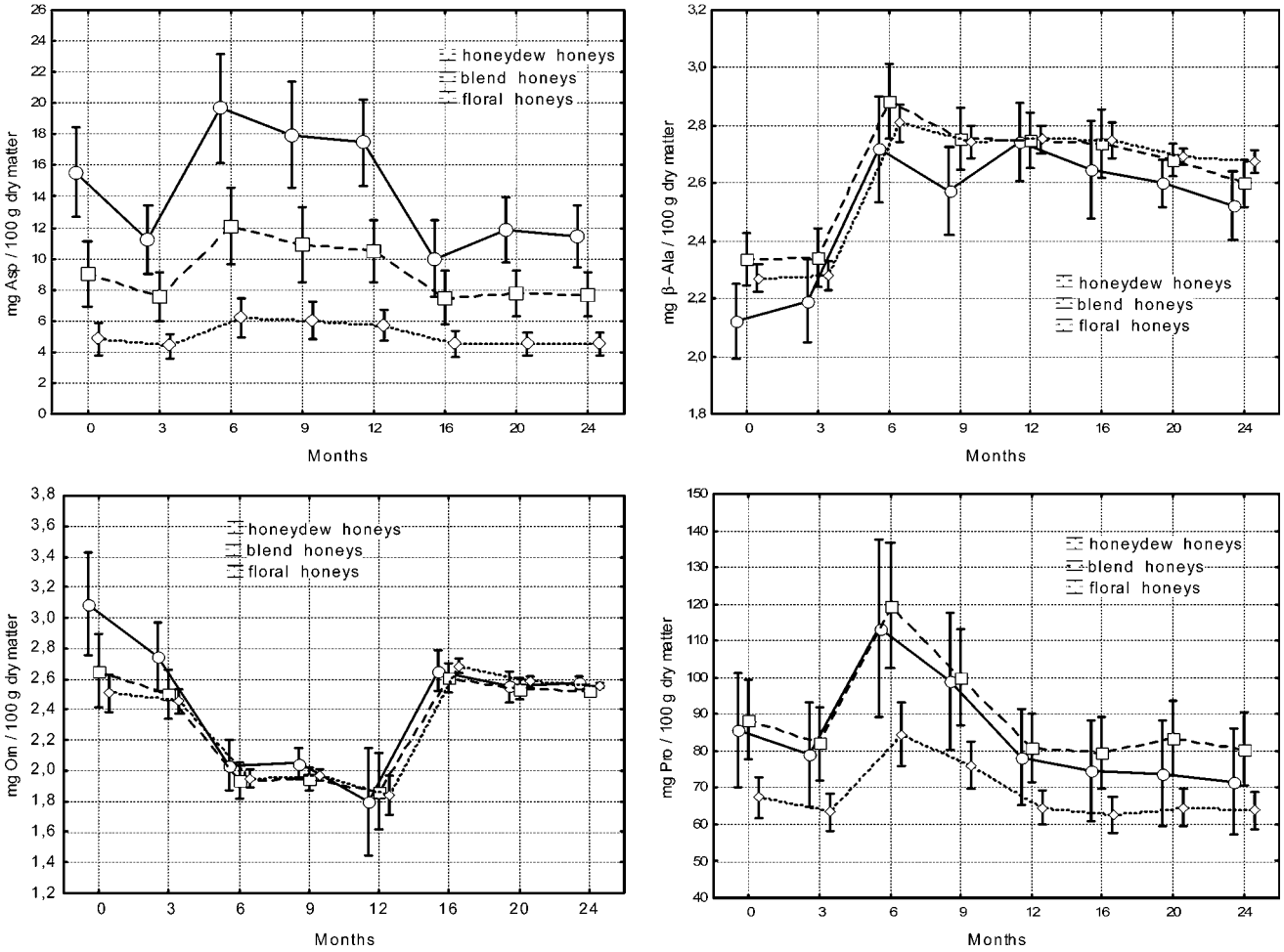

Figure 3. Mean values and 95% confidence intervals of aspartic acid (Asp), β-alanine (β-Ala), ornithine (Orn), and proline (Pro) content in the three classes of honeys (honeydew, $n = 5$; blend, $n = 10$; and floral honeys, $n = 39$) during storage at ambient temperature.

Some of the amino acids followed a different trend than the others and should be described separately: aspartic acid, β-alanine, ornithine, and proline (**Figure 3**). The amino acids aspartic acid, β-alanine, and proline increase during the first few months of storage, reaching a maximum value at 6 months. After 6 months, the concentration of proline diminishes and after 12 months the concentration of aspartic acid decreases. However, the concentration of β-alanine scarcely changes between 6 and 24 months. Sanz et al. (*9*) also did not observe a decrease in β-alanine when they studied the formation of 2-furoylmethyl amino acids in four honeys stored during 1 year at 25 °C. However, they did observe a slight decrease in this amino acid in two of four samples stored at 35 °C. This result could indicate that, although this amino acid is involved in Maillard's reaction, its reactivity is lower than that of other amino acids. The amino acids aspartic acid and proline are the majority in pollen proteins (*3*); therefore, the increase in these amino acids during the first few months of storage could be explained by the presence in the honeys of enzymes with protease and/or peptidase activity. Honey is a little studied food product and enzymes with these activities have not yet been described in this food. However, this does not mean that they are not present, since they have been found in pollen (*17*, *18*).

Ornithine follows a different trend than the other amino acids. It diminishes in the first few months, until month 12, but after this its concentration rises considerably in the three types of honeys to reach values similar to the initial ones (**Figure 3**). In some processes involving bacteria, arginine gives rise to ornithine (*19*, *20*). Since the rise in ornithine at 12 months of storage coincides with the drop in arginine (**Table 1**) and because nonpasteurized honeys, such as these, have microorganisms (*21*, *22*), the increase in ornithine could be attributed to the enzymatic action of microorganisms.

Table 2 shows the mean values and the standard deviations of all amino acids for the different samples of each type of honey. The results of the Student−Newman−Keuls test for means comparison are also included. All the amino acids have different mean values depending on the type of honey studied, except for the amino acids tryptophan, isoleucine, and ornithine.

The highest contents for most of the amino acids are found in the honeydew honey and the lowest in the floral honeys, except for the amino acids histidine, tyrosine, phenyl alanine, and leucine. It is noteworthy that the mean phenylalanine content was very different in the two kinds of honeys, 22.96 mg/100 g in floral honeys and 6.65 mg/100 g in honeydew honeys. Phenylalanine is one of the majority amino acids in pollen (*3*), which explains this result. Also, Iglesias et al. (*2*) detected a higher phenylalanine content in floral honeys than in honeydew honeys. The majority amino acid for all the honeys is proline and corresponds to approximately 33% of the total free amino acid content, with mean values of 68.42−89.32 mg/100 g in the three groups of honey studied. These values are similar to the mean values found in Spanish honeys by Gómez-Bárez et al. (*23*) and by Iglesias et al. (*2*) and in honeys from Burkina Faso, Central Africa, by Meda et al. (*24*). Proline is an amino acid mainly derived from bees' secretions (*25*), which explains why it has a similar concentration in honeys regardless of their geographical or floral origin. The majority amino acids of the

Table 2. Mean Values and Standard Deviation (SD) of Amino Acids Concentration (mg/100 g of dry matter) in Each of the Groups of the Analyzed Honeys[a]

	honeydew honeys ($n = 40$)		blend honeys ($n = 80$)		floral honeys ($n = 312$)	
	mean	SD	mean	SD	mean	SD
Asp	14.40 c	4.40	9.13 b	3.00	5.07 a	3.19
Glu	21.50 c	8.44	12.89 b	5.65	6.13 a	5.50
Asn	13.78 c	7.91	11.65 b	5.65	6.28 a	4.76
Ser	4.26 c	1.76	3.67 b	1.22	2.62 a	1.04
Gln	8.82 c	6.18	5.96 b	3.40	4.55 a	2.76
His	0.34 a	0.83	1.31 b	1.18	2.10 b	1.03
Gly	2.20 c	0.46	1.72 b	0.30	1.54 a	0.33
Thr	2.75 b	0.86	2.31 a	0.50	2.18 a	0.50
Arg	4.49 b	2.32	3.49 a	1.61	3.41 a	1.28
α-Ala	2.52 a	0.26	2.64 b	0.28	2.62 b	0.25
β-Ala	5.57 c	1.60	4.65 b	0.99	3.17 a	1.23
Gaba[b]	6.57 b	3.59	3.10 a	1.22	2.58 a	2.02
Tyr	4.66 a	1.65	7.46 b	2.74	11.74 c	4.58
Met	0.00 a	0.00	0.33 b	0.80	0.11 a	0.48
Val	2.39 a	0.58	2.34 a	0.47	2.22 a	0.42
Trp	3.28 a	0.71	3.06 a	0.52	3.15 a	0.59
Phe	6.65 a	2.59	13.68 b	4.98	22.96 c	12.50
Ile	2.05 a	0.44	2.11 a	0.42	2.12 a	0.39
Leu	1.94 a	0.33	2.13 a	0.45	2.43 b	0.88
Orn	2.44 a	0.42	2.32 a	0.36	2.32 a	0.41
Lys	2.63 a	0.53	2.75 a	0.56	3.34 b	0.86
Pro	84.41 a	19.42	89.32 a	26.83	68.42 b	18.08

[a] Means in the same row with different letters are significantly different ($P < 0.05$). [b] Gaba = γ-aminobutyric acid.

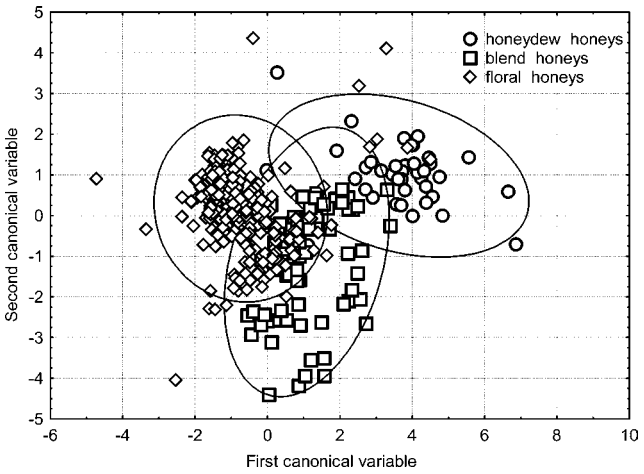

Figure 4. Representation of the 432 samples of honey on the plane defined by the two first canonical variables obtained with the nine amino acids selected by stepwise discriminant analysis and 95% confidence ellipses.

honeydew honey after proline, and with very similar values in the three types of honeys, are glutamic and aspartic acids, asparagine, and glutamine.

To verify whether amino acid content is also an indicator of the botanical origin of honeys over a long storage period, and to determine the amino acids that can best differentiate the groups, stepwise discriminant analysis was applied to the free amino acid content data. Values of 4.0 and 3.9 were used for *F*-statistics to enter and to remove variables, respectively. Nine amino acids were selected: valine, β-alanine, γ-aminobutyric acid, glutamic acid, serine, isoleucine, α-alanine, ornithine, and glutamine. With these selected compounds, 88.7% of the honeys were assigned correctly. **Figure 4** shows the honeys on the plane defined by the two canonical variables, obtained from the nine selected amino acids. The population canonical ellipses for the three groups of honeys for a 95% confidence limit are also shown in the figure. From the matrix structure, the amino acids more correlated with the first canonical variable and positively correlated were glutamic acid, α-alanine, γ-aminobutyric acid, and serine, and the most correlated with the second canonical variable are γ-aminobutyric acid (positively), and α-alanine (negatively). Higher values of the first canonical variable, i.e., glutamic acid, α-alanine, γ-aminobutyric acid, and serine, differentiate the honeydew honeys from the floral honeys, and lower values of the second canonical variable, i.e., lower values of γ-aminobutyric acid and higher values of α-alanine, differentiate some of the blend honeys from the other honeys. In the figure, it can be observed that the honeydew honeys and the floral honeys can be easily differentiated, while the blend honeys are at intermediate positions on the plane.

To summarize the results of the analysis of the free amino acid content of 54 honeys analyzed immediately after production and after 3, 6, 9, 12, 16, 20, and 24 months of storage, it can be deduced that most of the amino acid contents diminish steadily over the storage period. However, the contents of the amino acids aspartic acid, β-alanine, and proline rise in the first few months of storage, reaching maximum levels at 6 months. Therefore, possibly protease enzymatic activity could be taking place during this step, in spite of this not having been described previously. Free amino acids are good indicators of the floral, honeydew, or blend origin of honeys. To the best of our knowledge, the data presented in this paper are the first data in the literature on free amino acids content of blend honeys and the first data on changes during storage of honeydew and blend honeys.

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