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Usefulness of Amino Acid Composition To Discriminate between Honeydew and Floral Honeys. Application to Honeys from a Small Geographic Area

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With the aim of finding methods that could constitute a solid alternative to melissopalynological and physicochemical analyses to determine the botanical origin (floral or honeydew) of honeys, the free amino acid content of 46 honey samples has been determined. The honeys were collected in a small geographic area of ∼2000 km^2 in central Spain. Twenty-seven honey samples were classified as floral and 19 as honeydew according to their palynological and physicochemical analyses. The resulting data have been subjected to different multivariant analysis techniques. One hundred percent of honey samples have been correctly classified into either the floral or the honeydew groups, according to their content in glutamic acid and tryptophan. It is concluded that free amino acids are good indicators of the botanical origin of honeys, saving time compared with more tedious analyses.

KEYWORDS: Floral honey; honeydew honey; free amino acids

INTRODUCTION

Honey is perhaps one of the most complex foodstuffs produced by nature and certainly the only sweetening agent that can be used by humans without any processing. Honey is produced by honeybees from carbohydrate-containing exudates produced by plants. Honeybees add several components, mainly enzymes, that act as catalysts of biochemical changes and process the exudates to reduce simultaneously its water content. Honey is then stored for ripening in open cells in the comb. Thus, some components of honey composition come from the plants, others are added by honeybees, and yet others are due to biochemical reactions during honey maturation. With regard to the original sugar-containing raw plant material, honey may have two different botanical origins: (i) nectar, contained in specialized botanical structures, in the flowers of blossoming plants, and (ii) exudates produced by certain trees and other plants (i.e., genera *Pinus*, *Abies*, *Castanea*, and *Quercus*, among others), usually with the concourse of insects, mainly from the family Aphididae. Honey composition is thus tightly associated to its botanical origin and, to some extent, also to the geographical area in which it originated, because soil and climate characteristics determine melliferous flora.

Directive 2001/L10 from The Council of European Communities (*1*) expresses the need for methods that allow for verification of honey botanical origin. Melissopalynology, that is, the analysis and identification of pollens contained in honey, has traditionally been the method of choice to ascertain honey botanical origin and remains the reference method. Nevertheless, because palynology methods, besides not being absolutely conclusive, are extremely tedious and time-consuming and require trained analysts, alternative methods to establish honey origin are currently under active research. The aim is that these alternative methods should be easy and possible to set up in any laboratory. Among the methods tested to date, sugar composition (*2*−*5*), phenolic compounds (*6*−*8*), and/or physicochemical characteristics (*9*, *10*) have been assessed without highly conclusive results. According to several authors, amino acid composition may also be a suitable method to determine honey botanical origin. For instance, Pirini et al. (*11*) say that the presence of arginine is an important discriminating factor useful for the characterization of chestnut honey, whereas only acacia honey contains tryptophan. In that work it was also reported that the proline content of chestnut honey is higher than that of the other honeys analyzed. Bouseta et al. (*12*) found higher concentrations of proline and phenylalanine in lavender honeys than in eucalyptus ones. Conte et al. (*13*) found a higher content of amino acids in thyme honeys than in those derived from castanea, which in turn were richer in amino acids than those from eucalyptus.

The two ample groups of honeys, floral ones and honeydews, have quite different acceptances by consumers. Some attempts have been directed to characterization and differentiation of these two honey types. Thus, for instance, Nozal et al. (*14*) carried out the determination of sulfate anion in both kinds of Spanish

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honeys, with a higher content for honeydews; Campos et al. (*15*) proposed relationships between several physicochemical variables, without conclusive results; on the other hand, Terrab et al. (*10*) successfully used conventional physicochemical variables to discriminate three honeydews from 36 floral honeys from Morocco.

In any case, the literature on the composition of honeydew honeys is not as comprehensive as that from floral ones, making it necessary to analyze and collect much more data in order to characterize and differentiate them. The aim of this work is to assess the usefulness of the amino acid composition of honey as a marker for floral or honeydew origin. To this purpose, 46 artisan honeys from the same geographic area (Madrid, central Spain) were classified as floral or honeydew according to an extensive study of their physicochemical and melissopalynological data. Amino acid composition was determined in each of the selected samples, and all data were subjected to different multivariate statistical techniques.

MATERIALS AND METHODS

Honey Samples. Honey samples were provided by local beekeepers with hives settled in a small geographic area of ~2000 km^2 in central Spain. All samples were artisanally produced, obtained by centrifugation and unpasteurized. Forty-six honey samples were collected for the study, 14 from the 2000 harvest and 32 from the 2001 harvest. Analyses were made within 6 months after harvesting.

Pollen Analysis. Melissopalynological analysis of honey samples was essentially performed according to the method of Louveaux et al. (*16*) using a nonacetolytic technique in order to preserve honeydew elements (fungal spores and mycelia, microalgae, others). The modifications proposed by Terradillos et al. (*17*) for exine cleansing and staining were incorporated. Microscopical observations were carried out in a Leica DMR light microscope fitted to a digital camera and to an Image Analysis System (Leica QWin Std. software). For each honey sample two independent slides were prepared and 400 pollen grains identified on each. Palynomorphs were identified according to a specifically prepared collection of reference pollens from the Madrid area (*18*). Due to the fact that in honeydew honeys derived from *Quercus* sp., such as those from Madrid, the number of microscopic honeydew indicators (honeydew elements) is scarce or even extremely low (*19*), both the presence of honeydew elements and the abundance of *Quercus* sp. pollen were recorded and taken into account as an additional fact indicating a honeydew honey.

Physicochemical Parameters. All physicochemical determinations were essentially carried out according to the European Honey Commission methods (*20*). Four physicochemical parameters were chosen, together with melissopalynology results, for initial assignation of honey samples as floral or honeydew: pH, electrical conductivity, ash content, and glucose plus fructose content. pH was determined in a 10 g 75 mL^{-1} solution of honey in deionized water (*21*) and ash content by calcination at 550 °C until constant weight was reached, with the precaution to include a previous step of caramelization on a heating plate to control production of foams from honeys; conductivity was determined in a solution of 20% honey/dry weight in deionized water. Glucose and fructose contents were determined by HPLC with a RECEX monosaccharide precolumn and column, at 90 °C and using H_2O (HPLC grade) as eluent at a flow rate of 1.0 mL min^{-1}, and a refractive index detector.

Some other physicochemical and biochemical determinations were carried out to obtain a more complete characterization of the honey samples and also to observe if statistically significant differences sustained the differentiation between the two botanical origins (floral or honeydew). Free, lactonic, and total acidities were titrated in the same solution used for pH measurement (*21*); water content was determined by refractive index and correlation with Chataway charts. Diastase activity was determined by UV−vis spectrophotometry. α-Glucosidase and β-glucosidase were respectively assayed according to the methods of Siegenthaler (*22*) and Low et al. (*23*). Total phenols content was assayed with Folin−Ciocalteu reagent. CIE *L*a*b** chromatic coordinates were measured with a Minolta CR-200 tristimulus colorimeter, using special cuvettes made from optic glass, a D65 illuminant, and a standard observant of 2°. Net absorbance was defined as the difference between spectrophotometric absorbance measurements at 560 and 720 nm according to the method of Huidobro and Simal (*24*).

Free amino acids were quantified by the Cd−ninhydrin method (*25*). Protein content was determined according to the Bradford dye-binding assay (*26*). Total nitrogen was determined according to the Kjeldahl method with a Tecator digestion system and a Kjeltec 1030 autoanalyzer (Tecator AB, Höganäs, Sweden).

Amino Acid Analysis. Free amino acids were determined in the filtered solution (Millipore, Bedford, MA; 0.45 μm filter) of 1.25 g of honey/25 mL of water. Analyses were carried out by HPLC using a Waters (Milford, MA) liquid chromatograph controlled by a Millenium^{32} system (Waters). Samples were submitted to an automatic precolumn derivatization with *o*-phthaldialdehyde (*27*) to determine primary amino acids. The separation of amino acids was performed on a Waters NovaPak C-18, 60 Å, 4 μm column (3.9 × 150 mm). Detection was by fluorescence using wavelengths of excitation and emission at 340 and 425 nm, respectively. All reagents used were of HPLC grade. Proline was determined according to the colorimetric method of Ough (*28*).

Statistical Analysis. The statistical methods used for data analysis were cluster analysis (Ward's method from standardized variables), to discover natural groupings of the samples of honeys; two-sample *t* test, to determine if there were significant differences between the two types of honeys; and stepway discriminant analysis, to select the variables most useful in differentiating the two groups. The STATISTICA (*29*) program was used for data processing. This program was run on a personal computer.

RESULTS AND DISCUSSION

An a priori assignation of 46 Madrid honey samples to either of the groups floral (FL) or honeydew (HD) was made according to data from physicochemical and palynological analyses. **Table 1** shows the relationship of honey samples together with the harvesting year, palynological classification and identification of predominant palynomorphs, and the key assigned. As indicated under Materials and Methods, four physicochemical parameters were taken into account for assignation of a floral or honeydew character. Conductivity, ash content, and glucose plus fructose content were chosen due to their utility as quality standards differentiating both botanical origins (*30*). A high pH value (>4.3) is reported to be common in honeydew honeys (*5*).

Physicochemical and Biochemical Characteristics. A total of 21 physicochemical and biochemical characteristics have been determined. In an attempt to obtain a preliminary view of the main causes for the differentiation between the data of the samples, cluster analysis was carried out on the data of the global composition of the 46 honeys studied (variables in **Table 2**). The squared Euclidean distance was taken as a measure of proximity between two samples, and Ward's method was used as the linkage rule. The variables were previously standardized. **Figure 1** shows the dendrogram obtained. As can be observed in the figure, there are two groups of samples, the first corresponding to the ones initially classified as honeydew honeys and the other with the floral honeys. Another fact to point out is that no pattern of grouping according to blossoming plant origin can be observed within the floral group. This is likely to be due to the multifloral origin of many samples (**Table 1**).

To compare the global compositions of both honey groups, a *t* test for comparison of two means was carried out. **Table 2** shows the mean values and standard deviations of the 21 physicochemical and biochemical parameters determined for the two honey groups, as well as the significance level reached.

Table 1. Relationship of Honey Samples and a Priori Assignation of the Samples to the Floral Group or the Honeydew Group; Honey Type, Predominant Palynomorphs, Harvest Year, and the Key Used Are Also Shown

floral honeys				honeydew honeys			
honey type	predominant palynomorphs	harvest year	key	honey type	predominant palynomorphs	harvest year	key
viper	*Echium* sp.	2000	FL01	honeydew	Erica multiflora	2000	HD28
viper	*Echium* sp.	2000	FL02	honeydew	*Rosa* sp., *Rubus* sp.	2001	HD29
viper	*Echium* sp.	2000	FL03	honeydew	*Erica multiflora, Rosa* sp., *Rubus* sp.	2001	HD30
viper	*Echium* sp.	2000	FL04	honeydew	*Erica multiflora, Rosa* sp., *Rubus* sp.	2001	HD31
viper	*Echium* sp.	2000	FL05	honeydew	*Rosa* sp., *Rubus* sp.	2001	HD32
multiflower		2000	FL06	honeydew	*Rosa* sp., *Rubus* sp.	2001	HD33
viper	*Echium* sp.	2000	FL07	honeydew	*Rosa* sp., *Rubus* sp.	2001	HD34
multiflower	Leguminosae	2000	FL08	honeydew	*Erica multiflora*, *Taraxacum vulgare*	2001	HD35
multiflower	Leguminosae	2000	FL09	honeydew	*Erica multiflora*	2001	HD36
heather	*Erica multiflora*	2000	FL10	honeydew	*Rosa* sp., *Rubus* sp., Leguminosae	2001	HD37
viper	*Echium* sp.	2000	FL11	honeydew	*Rosa* sp., *Rubus* sp., *Erica multiflora*	2001	HD38
multiflower	Labiatae	2000	FL12	honeydew	*Rosa* sp., *Rubus* sp., *Genista* sp., Labiatae	2001	HD39
multiflower	Labiatae	2000	FL13	honeydew	*Rosa* sp., *Rubus* sp.	2001	HD40
rosa bush	*Rosa* sp., *Rubus* sp.	2001	FL14	honeydew	*Rosa* sp., *Rubus* sp., Labiatae	2001	HD41
rosa bush	*Rosa* sp., *Rubus* sp.	2001	FL15	honeydew	*Erica multiflora*, Labiatae	2001	HD42
multiflower	*Rosa* sp., Labiatae, *Erica multiflora*	2001	FL16	honeydew	*Rosa* sp., *Rubus* sp., *Erica multiflora*, Labiatae	2001	HD43
rosa bush	*Rosa* sp., *Rubus* sp.	2001	FL17	honeydew		2001	HD44
multiflower	*Rosa* sp., *Eucaliptus* sp., *Echium* sp.	2001	FL18	honeydew	*Rubus* sp., Labiatae	2001	HD45
multiflower	*Rosa* sp., *Echium* sp.	2001	FL19	honeydew	*Rosa* sp., *Rubus* sp.	2001	HD46
multiflower	*Rosa* sp., *Rubus* sp., Labiatae, *Erica multiflora*	2001	FL20				
multiflower	Leguminosae	2001	FL21				
multifloral	Labiatae	2001	FL22				
multifloral	Labiatae	2001	FL23				
rosemary	*Rosmarinus officinalis*	2001	FL24				
multiflower	Labiatae	2001	FL25				
multiflower	Labiatae	2001	FL26				
multifloral	*Rosa* sp., Labiatae, *Echium* sp., others	2001	FL27				

Table 2. Mean and Standard Deviation Values of Physicochemical Variables of Floral Honeys and Honeydew Honeys and Result of the *t* Test for Comparison of the Two Means

variable	floral honeys		honeydew honeys		result of *t* test[b]
	mean	SD[a]	mean	SD	
pH	3.9	0.3	4.7	0.2	***
conductivity 20% (mS/cm)	0.558	0.257	1.315	0.143	***
ash (%)	0.21	0.13	0.59	0.13	***
glucose (%)	28.90	2.05	27.22	1.60	**
fructose (%)	36.35	2.66	32.80	2.50	***
glucose + fructose (%)	64.85	5.63	60.02	3.83	**
free acidity (mequiv/kg)	28.14	10.34	37.88	5.21	***
lactonic acidity (mequiv/kg)	5.08	3.08	2.17	1.65	***
total acidity (mequiv/kg)	33.23	11.70	40.05	5.15	*
water content (%)	16.0	1.3	15.8	0.9	ns
diastase activity (°Gothe)	29.7	11.9	39.2	9.5	**
α-glucosidase (units/kg/min)	153.7	79.8	198.8	39.2	*
β-glucosidase (units/kg/min)	74.5	39.4	79.7	15.1	ns
total phenols (mg of gallic acid/g)	0.67	0.31	1.01	0.22	***
chromatic parameter *L**	27.67	3.20	24.81	2.47	**
chromatic parameter *a**	0.17	1.56	–0.82	1.18	*
chromatic parameter *b**	6.09	2.55	2.79	1.53	***
net absorbance ($Abs_{560nm} - Abs_{720nm}$)	0.22	0.11	0.53	0.11	***
amino nitrogen (mg of Leu/100 g of dm)	45.72	20.02	109.78	38.35	***
protein (mg of BSA/100 g of dm)	111.70	46.61	135.56	45.66	ns
total nitrogen (mg/100 g of dm)	79.10	33.64	131.44	38.75	***

[a] Standard deviation. [b] *, significant differences ($P < 0.05$); **, significant differences ($P < 0.01$); ***, significant differences ($P < 0.001$); ns, not significant differences.

Not significant differences exist between the water, β-glucosidase, and protein contents of both types of honeys, whereas 11 of the 21 variables determined are different with a significance level of <0.001. The rest of the variables are different with a level of significance of <0.01 (two variables) or <0.05 (three variables).

To select the physicochemical and biochemical characteristics most useful to differentiate the samples of honeys, stepwise discriminant analysis was used. Values of 4.0 and 3.9 were considered for the *F* statistic to enter and to remove variables, respectively. Three variables of the 21 determined (see **Table 2**) were selected: (i) conductivity 20%, (ii) total acidity, and (iii) total phenols. A 100% correct assignment of the samples of honeys was obtained either by standard or by leave-one-out cross-validation procedures with the selected characteristics. **Figure 2** shows the two-dimensional categorized scatterplot for the two types of honeys using the two first selected variables. The corresponding population ellipses for 95% confidence are also represented in the figure. It can be seen that honeydew samples are more tightly grouped than floral ones, because their conductivity and total acidity values are far more similar. This is probably due to the fact that most, if not all, honeydew contributions come from *Quercus pyrenaica*, the main honeydew-producing species in Madrid conditions, whereas floral honeys come from a wide diversity of blossoming plants. Sample HD36 appears quite far from its group due to its higher total acidity. This is also the only honeydew with a dominance (>45% pollen grains) of heather (*Erica multiflora*) palynomorphs in its pollen spectrum.

Free Amino Acids. A total of 23 amino acids were determined (see **Table 3**). Cluster analysis was also carried out on the data of the amino acid composition of the 46 honeys studied in order to discover natural groupings of the samples. The squared Euclidean distance was taken as a measure of proximity between two samples, and Ward's method was used as the linkage rule. The variables were previously standardized. **Figure 3** shows the dendrogram obtained. Two honey groups are obtained, one with most honeydew honeys and the other with the floral ones. In this last group appears also a honeydew honey, HD31. As was the case with the physicochemical variables (**Figure 1**), no pattern of grouping according to

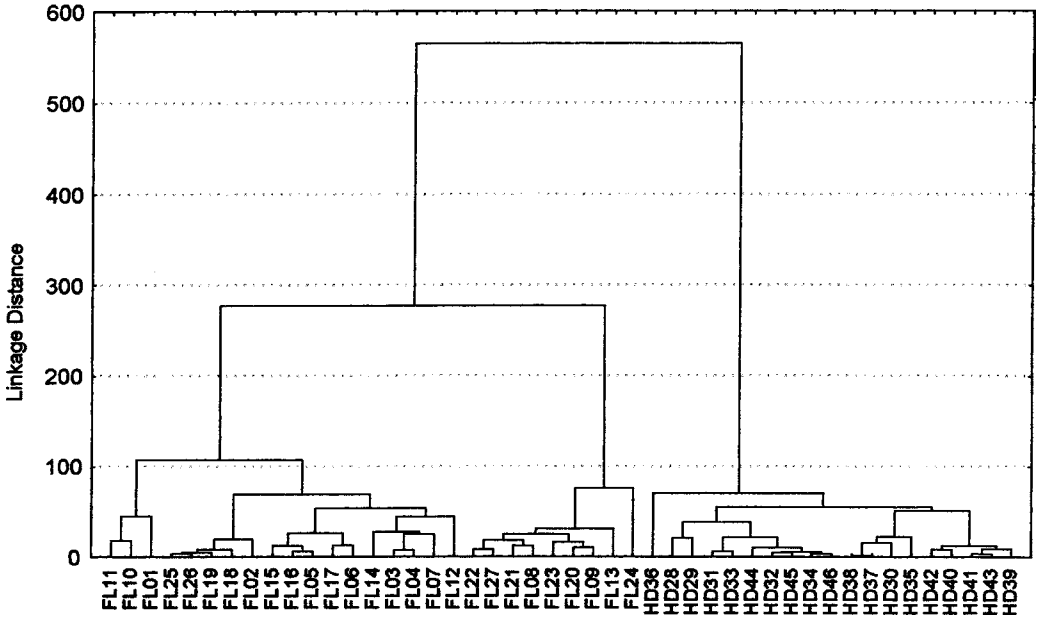

Figure 1. Dendrogram resulting from applying cluster analysis to the data corresponding to physicochemical variables (**Table 2**). For key identification, see **Table 1**.

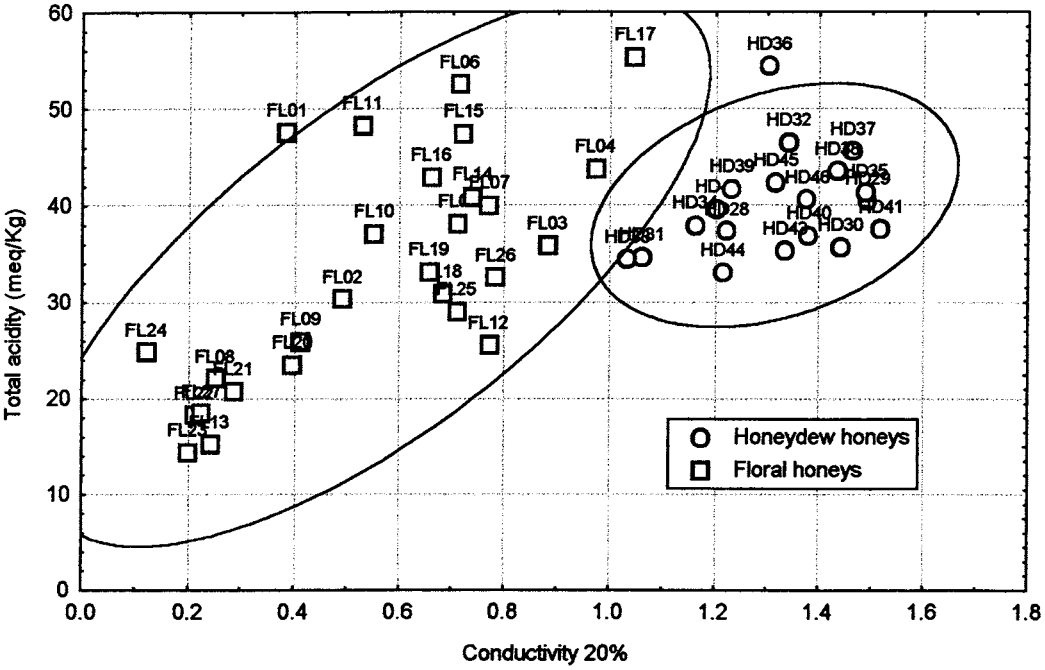

Figure 2. Representation of the 46 samples of honey on the plane defined by the variables selected by stepwise discriminant analysis of the data of **Table 2**. The 95% confidence ellipses are also shown. For key identification, see **Table 1**.

blossom origin is obtained with data from free amino acid composition. To evaluate the differences in free amino acid composition between the two groups of honey, a two-sample *t* test for comparison of two means was applied. **Table 3** shows mean values, standard deviations, and the results of the *t* test for free amino acid concentration in floral and honeydew honeys. With the exceptions of histidine, β-alanine, and lysine, there are significant differences for all of the analyzed amino acids between floral and honeydew honeys. These differences reach a significance level of <0.001 for 11 of the 21 amino acids assayed. All amino acids significantly different are found in higher concentration in honeydew honeys than in floral ones, except for tyrosine and phenylalanine. **Table 3** also shows that the major amino acid in both honey groups is proline, which comes mainly from the honeybee, as evidenced by analysis of honeys produced in sugar-fed colonies (*31*). Following a decreasing order of concentration appear, in honeydew honeys, glutamic acid, aspartic acid, asparagine, glutamine, and phenylalanine. The corresponding order for floral honeys is, after proline, phenylalanine, tyrosine, glutamic acid, asparagine, and aspartic acid. Conte et al. (*13*) also found proline and phenylalanine as the major amino acids in floral honeys. None of the analyzed honeys showed the presence of α-aminobutyric acid, methionine, or ornithine.

When stepwise discriminant analysis was applied to the amino acid data in order to select the most useful to differentiate the two types of honeys, using 3.0 and 2.9 values for the *F* statistic to enter and to remove variables, respectively, glutamic acid and tryptophan were selected. A 100% correct assignment of the samples of honeys was obtained. Glutamic acid allows the

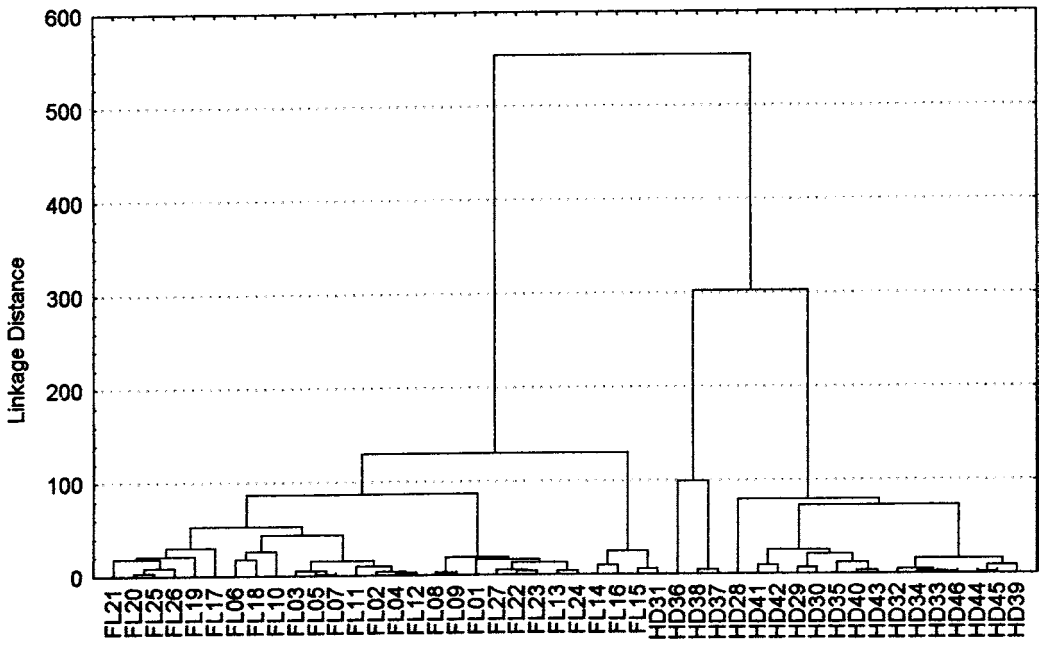

Figure 3. Dendrogram resulting from applying cluster analysis to the data corresponding to free amino acids (**Table 3**). For key identification, see **Table 1**.

Table 3. Mean and Standard Deviation Values of Free Amino Acids (Milligrams per 100 g of Dry Matter) of Floral Honeys and Honeydew Honeys and Result of the *t* Test for Comparison of the Two Means

free amino acid	floral honeys		honeydew honeys		result of
(mg/100 g dm)	mean	SD[a]	mean	SD	*t* test[b]
aspartic acid	7.12	4.49	26.70	7.66	***
glutamic acid	8.39	5.18	38.97	12.89	***
asparagine	7.77	5.42	23.94	14.05	***
serine	2.34	1.61	7.12	1.89	***
glutamine	3.16	2.32	11.63	11.65	***
histidine	1.49	1.74	1.25	3.68	ns
glycine	0.57	0.67	2.22	0.91	***
threonine	1.13	0.92	3.48	1.97	***
arginine	1.62	2.05	8.68	6.70	***
β-alanine	0.42	0.88	0.27	0.62	ns
α-alanine	3.46	2.44	8.54	1.49	***
γ-aminobutyric acid	1.19	1.50	5.61	3.88	***
tyrosine	9.69	5.43	5.50	4.27	**
α-aminobutyric acid	nd[c]		nd		
methionine	nd		nd		
valine	1.48	0.99	3.42	1.84	***
tryptophan	0.46	0.85	1.15	1.28	*
phenylalanine	23.17	16.33	11.22	13.14	*
isoleucine	1.07	0.72	2.04	1.24	**
leucine	1.07	0.88	1.62	0.70	*
ornithine	nd		nd		
lysine	1.90	2.01	2.34	2.68	ns
proline	67.15	34.43	90.46	24.68	*

[a] Standard deviation. [b] *, significant differences ($P < 0.05$); **, significant differences ($P < 0.01$); ***, significant differences ($P < 0.001$); ns, not significant differences. [c] Not detected.

correct classification of all the floral honeys, and only one honeydew, HD32, is misclassified within the floral group. For floral honeys, the concentration of glutamic acid ranges between 0.66 and 17.74 mg/100 g, with an average value of 8.39 mg/100 g. With honeydew honeys, the concentration of glutamic acid ranges from 23.62 to 67.18 mg/100 g, with an average value of 38.97 mg/100 g. The concentration of tryptophan in floral honeys ranges between 0 and 2.88 mg/100 g and in honeydew honeys, between 0 and 3.52 mg/100 g.

In summary, from the results obtained it can be deduced that the concentration of amino acids and, especially, the content in glutamic acid and tryptophan allow the differentiation between floral honeys and honeydew honeys, even from a common and small geographic area.

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