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Free amino acid composition and botanical origin of honey

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Abstract

The main amino acids found in 31 Spanish honeys of five different single botanical origins, were proline, phenylalanine, tyrosine and lysine, followed by arginine, glutamic acid, histidine and valine. Principal component analysis explained 64% of the variance with the first three PC variables, the lavender honeys being the only group well differentiated. Although the best grouped honeys were those from orange blossom, overlapping with eucalyptus honeys, on the one hand, and with the indistinguishable group formed by rosemary and thyme honeys, on the other hand, was observed. The Student–Newman–Keuls test allowed the grouping of rosemary, thyme and orange blossom honeys, whereas eucalyptus and lavender honeys showed specific amino acid compositions which made them different when compared with this group and also between themselves. Lavender honeys had the highest concentrations of tyrosine. The results obtained for the former honeys together with those obtained for another set of 17 samples, were used to establish a range for amino acid composition of Spanish honeys.

Keywords: Honey; Botanical origin; Amino acids; Eucalyptus; Lavender; Orange blossom; Rosemary; Thyme

1. Introduction

European Community food laws establish compositional and quality parameters for honey, such as hydroxymethylfurfural content, humidity, enzymatic activities, and pesticide levels, but such parameters have no relationship to geographical or botanical origin of honey. Pollen recognition has been the traditional method to determine the floral origin of the honey, but this technique is tedious and has some limitations. Many studies have sought analytical markers of botanical origin for honey, based on aroma compounds, sugar profile, flavonoid pattern, non-flavonoid phenolics, organic acids, isotopic relations, and protein and amino acid compositions (Anklam, 1998).

Proteins and amino acids in honeys are attributable both to animal and vegetal sources, the major of these being pollen. Amino acids account for 1% (w/w), and proline is the major contributor with 50–85% of the total amino acids. Besides proline, there are 26 amino acids in honeys, their relative proportions depending on the honey origin (nectar or honeydew). Since pollen is the main source of honey amino acids, the amino acid profile of a honey could be characteristic of their botanical origin.

Free amino acid composition is more likely than protein composition to determine the botanical source of a honey. It has also been proposed that better and more useful information can be provided by increasing the number of analysed samples, by combination of these and other analytical data, and by statistical treatment of the results (Anklam, 1998). However, the determination of the botanical origin of a honey is a hard task, due to the complexity of the matrix, and because composition is affected by many factors, including climatic or soil conditions (Shuel, 1975).

Davies (1975, 1976) found that the ratios between certain honey amino acids were different, depending on geographical origin, and that these differences increased when comparing honeys from the same region but of different floral origins. The application of lineal discriminant analysis to the 16 proteic amino acids found in Spanish honeys allowed both geographical and botanical differences to be established (Pérez Arquillue & Herrera Marteache, 1987). Gas chromatography analyses of free amino acids present in honeys were not the same when honey came from the United Kingdom,

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Argentina, Australia or Canada (Gilbert, Shephard, Wallwork, & Harris, 1981). HPLC analysis of proline, leucine and phenylalanine, and their enantiomeric ratios, has been proposed to assess the effect of storage, ageing and processing techniques on honey quality, leucine being the most variable amino acid found in the analysed samples (Pawlowska & Armstrong, 1994).

Proline is unique, because this amino acid come mainly from the honeybee during the conversion of nectar into honey. The amount of proline in honey has been proposed (Von der Ohe, Dustmann, & Von der Ohe, 1991) as an indicator of honey ripeness, together with other determinations also related to honeybee, such as sacharase and glucose oxidase activities. Proline in honey must account for more than 200 mg/kg, and at least 66% of the total free amino acids (usually 80–90%).

Since some amino acids are precursors of volatile compounds, attempts to find useful relationships between amino acid composition and characteristic aromas for eucalyptus and lavender honeys, have been made (Bouseta, Scheirman, & Collin, 1996). Eucalyptus honey could be characterized by means of 7 volatile compounds, whereas lavender honey had only 5 characteristic volatile compounds, the amounts of phenylalanine and tyrosine found in these honeys being much higher than other amino acids (1238 and 440 ppm, respectively). Proline was always the main amino acid in eucalyptus honey, but not lavender honey.

In a study carried out with 92 samples of honeys from 17 botanical and 4 geographical different sources (Conte, Miorini, Giomo, Bertacco, & Zironi, 1998), amino acids were isolated and, after derivatization, analysed by gas chromatography. The majority of the samples showed proline as the main amino acid, and important amounts of phenylalanine, aspartic acid + asparagine (ASX), and glutamic acid + glutamine (GLX). For thyme honey, higher levels of serine, tyrosine and lysine were found. For rosemary honey, tyrosine was the main amino acid, and also high amounts of proline and phenylalanine. After application of lineal discriminant analysis to the data, it only was possible to differentiate thyme honey from chestnut honey. These authors suggested that the combination of amino acid analysis together with the determination of water activity, the pH value, the sugar content, the sensory evaluation, and the use of statistics, could be the best method for distinguishing botanical origins of honeys.

An analytical method, based on derivatization of isolated amino acids with diethyl ethoxymethylenemalonate and subsequent HPLC separation (Alaiz, Navarro, Girón, & Vioque, 1992), has been modified to allow the simultaneous determination of 22 amino acids, including proline, asparagine and glutamine, as well as the ammonium ion (Chicón, Hermosín, & Cabezudo, 2001). This method can be applied to honey samples, so increasing the number of amino acids analysed. Other HPLC methods can only analyse 15 (Conte et al., 1998) or 17 amino acids (Bouseta et al., 1996; Pirini & Conte, 1992), and usually they give data for ASX and GLX because of hydrolysis of asparagine and glutamine under derivatization conditions.

The aim of this work was, first to contribute to the scarce knowledge about the free amino acid composition of Spanish honeys. Secondly, the information obtained from this analysis was examined in order to establish whether free amino acid composition of a honey can explain its botanical origin. To carry out this study, 48 samples of honeys, from six different regions of Spain and 10 botanical sources, were analysed.

2. Materials and methods

2.1. Honey samples

Forty-eight honey samples were purchased in Spanish markets. Their commercial certified botanical origins were: a first set of 31 honey samples from eucalyptus (6 samples), lavender (4), rosemary (11), orange blossom (7), and thyme (3); a second set of 17 honey samples consist of heather (3), holm oak (2), forest (2), oak (1), and multifloral (9). The geographical origins of the honey samples were mainly from the regions of La Alcarria (middle east) and Cabañeros (middle sudwest), but also from Cáceres (middle west), Ávila (middle northeast), Valencia (east) and Cantabria (north).

2.2. Sample preparation

Amino acids and ammonium ion were isolated from honey samples according to the method described by Bouseta et al. (1996), starting from 12.5 g of honey treated with 375 μl of a solution of α-aminobutyric acid (Sigma-Aldrich) used as internal standard (1 g/l in 0.1 *N* HCl). After isolation, the final volume sample was 10 ml, the pH was adjusted to 3.2, and the sample was refrigerated prior to derivatization.

The derivatization procedure was the same as used by Chicón et al. (2001) for wine and must samples: 30 μl of diethyl ethoxymethylenemalonate (Sigma-Aldrich), 1.5 ml of methanol, 1 ml of the solution of isolated amino acids, and 3.5 ml of borate buffer (1*M*, pH 9.0) were placed in a 10 ml tube with screw tap. After closing, the tube was placed in an ultrasound bath for 30 min at room temperature. The derivatizated sample was kept at room temperature until chromatographic analysis.

2.3. Analytical methods

Direct injection of the derivatizated samples, after filtration through a nylon membrane of 0.45 μm, was

made on a Waters 2690 liquid chromatograph, fitted with a Novapack C18 column (4 μm, 300×3.9 mm) thermostatted at 16 °C. Detection was at 280 nm with a PDA Waters 960 detector. Elution solvents were acetonitrile (A) and acetate buffer (25 mM) at pH 5.8 (B), with the next solvent programmes: 6% A; 16% A (13 min); 18% A (13.5 min); 18% A (17 min); 22% A (20 min); and 32% A (32 min). Identification was by means of the retention times obtained from pure compounds. Quantification was achieved by using calibration curves obtained from amino acid and ammonium solutions of known concentrations containing the same amount of internal standard as added to samples.

The principal component analysis and the Student–Newman–Keuls test were used for data analysis (SPSS pack, version 7.5 2S, SPSS Inc.).

3. Results and discussion

HPLC allowed the separation and quantification of 22 amino acids plus ammonium ion in the analysed honeys, as can be seen in Fig. 1 for a sample of lavender honey. Table 1 shows the concentration of every amino acid and the ammonium ion obtained for the first set of samples, constituted by the most common types of single floral origin honeys: rosemary, eucalyptus, lavender, thyme, and orange blossom. The main amino acids found for these honeys were proline, phenylalanine, tyrosine and lysine. Lower but also important amounts of arginine, glutamic acid, histidine and valine were present. Sulphur-containing amino acids (methionine and cysteine) were a minority and not found in some samples. Pirini and Conte (1992) showed that arginine was present in chestnut honey (mean value of 0.35 mg/100 g) but not in orange blossom, acacia, rosemary, and lime tree honeys; they also only found tryptophan (mean value of 0.43 mg/100 g) in acacia honey. In contrast, Bouseta et al. (1996) analysed eucalyptus and lavender honeys in which arginine was detected. In the present study arginine and tryptophan were found in all the samples, arginine being one of the amino acids that accounts for a relatively high amount.

In order to confirm whether these results showed significant differences according to the botanical origin of the honey, the data were subjected to principal component analysis. Table 2 contains the first three principal components (PC), as well as the amino acids most closely correlated to them and the percentage of variance explained after the Varimax rotation. Fig. 2 plots the 31 samples on the coordinate grid defined by the first three PC and shows that PC 2 separated the lavender honey samples from the rest. Eucalyptus honey samples were grouped in the negative axis of PC 2. The samples of rosemary and thyme honeys were mixed and overlapped with those of orange blossom honeys, which constituted the most compact group and was situated in the gravity centre of the coordinate grid.

The amino acids most closely correlated with the first two PC variables indicated that they were in different amounts in at least one of the botanical origins. The Student–Newman–Keuls test for the multiple comparison of the mean values was then applied to these amino acids in order to show which honey samples were different according to their botanical origins (Table 3). Results showed that rosemary, thyme and orange blossom honey

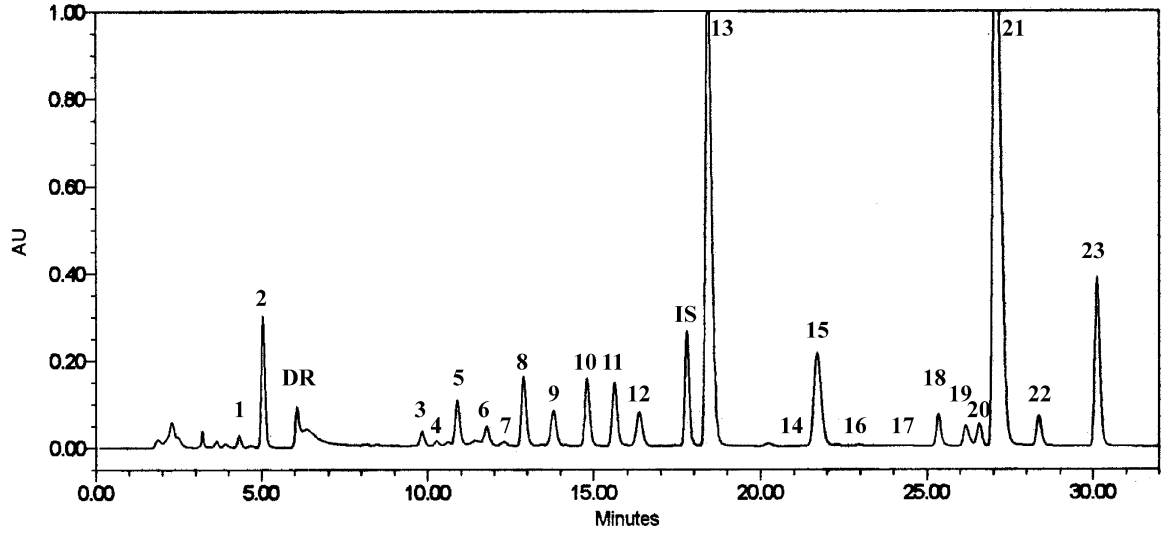

Fig. 1. HPLC chromatogram of the amino acids and the ammonium ion isolated from a sample of Spanish certified lavender honey. Peak identification: 1, glutamic acid (Glu); 2, aspartic acid (Asp); DR, derivatization residue; 3, asparagine + serine (Asn + Ser); 4, glutamine (Gln); 5, histidine (His); 6, glycine (Gly); 7, threonine (Thr); 8, β-alanine (β-Ala); 9, arginine (Arg); 10, α-alanine (α-Ala); 11, γ-aminobutyric acid (Gaba); 12, proline (Pro); IS, α-aminobutyric acid used as internal standard; 13, tyrosine (Tyr); 14, valine (Val); 15, ammonium ion (NH_4^+); 16, methionine (Met); 17, cysteine (Cys); 18, isoleucine (Ile); 19, leucine (Leu); 20, tryptophan (Trp); 21, phenylalanine (Phe); 22, ornithine (Orn); 23, lysine (Lys).

Table 1
Distribution of amino acids and ammonium ion concentrations (as mg/100 g of honey) for Spanish single botanical origin honeys: mean value (MV); standard deviation (S.D.); maximum value (Max); minimum value (Min)

	Rosemary ($n=11$)				Eucalyptus ($n=6$)				Lavender ($n=4$)				Thyme ($n=3$)				Orange blossom ($n=7$)			
	MV	S.D.	Max	Min	MV	S.D.	Max	Min	MV	S.D.	Max	Min	MV	S.D.	Max	Min	MV	S.D.	Max	Min
Asp	0.23	0.09	0.37	0.10	0.60	0.42	1.24	0.13	0.77	0.48	1.40	0.27	0.27	0.18	0.46	0.10	0.36	0.20	0.69	0.16
Glu	1.04	0.33	1.41	0.53	4.29	4.95	13.9	0.48	3.76	2.65	7.01	1.06	1.74	1.52	3.48	0.71	1.28	0.34	1.77	0.77
Asn + Ser	0.17	0.10	0.42	0.06	0.38	0.32	1.00	0.13	0.44	0.48	1.15	0.07	0.22	0.14	0.38	0.11	0.35	0.30	0.82	0.14
Gln	0.11	0.07	0.26	0.04	0.22	0.15	0.39	0.03	0.16	0.11	0.25	0.03	0.07	0.04	0.12	0.05	0.19	0.13	0.39	0.06
His	1.52	0.59	2.54	0.79	1.44	0.70	2.64	0.54	2.21	0.15	2.37	2.02	1.64	0.40	2.08	1.30	1.43	0.48	1.99	0.63
Gly	0.51	0.21	0.80	0.21	0.96	0.48	1.54	0.38	1.00	0.48	1.54	0.53	0.55	0.18	0.73	0.37	0.69	0.11	0.86	0.53
Thr	0.08	0.06	0.20	0.02	0.14	0.10	0.30	0.05	0.21	0.14	0.42	0.11	0.19	0.09	0.29	0.11	0.19	0.08	0.27	0.07
β-Ala	1.08	0.31	1.49	0.66	1.51	0.50	2.28	0.77	2.03	0.11	2.20	1.96	1.32	0.12	1.45	1.24	1.18	0.19	1.41	0.84
Arg	1.09	0.79	2.65	0.42	1.44	0.36	2.10	1.15	1.68	0.31	2.12	1.47	2.58	2.82	5.82	0.73	0.92	0.16	1.16	0.74
α-Ala	0.80	0.27	1.16	0.41	1.79	1.04	3.51	0.58	2.13	0.51	2.49	1.38	1.03	0.29	1.28	0.72	1.08	0.46	2.08	0.81
Gaba	0.53	0.19	0.79	0.28	0.77	0.45	1.44	0.25	1.70	1.17	3.18	0.34	0.59	0.52	1.12	0.08	0.63	0.28	0.80	0.01
Pro	28.0	14.8	55.6	10.1	49.3	26.8	81.4	13.8	55.6	29.7	87.9	27.4	32.1	7.02	40.1	26.9	35.0	11.7	48.4	11.6
Tyr	11.2	9.49	26.0	2.36	3.48	1.43	5.71	1.40	29.9	10.6	40.4	16.7	17.10	5.12	21.1	11.31	3.15	1.62	5.46	1.79
Val	0.85	0.28	1.23	0.42	1.75	0.95	3.41	0.58	1.90	0.36	2.30	1.43	0.87	0.18	1.04	0.69	0.98	0.18	1.23	0.66
NH_4^+	1.14	0.52	2.18	0.43	1.51	0.38	2.06	1.04	1.48	0.33	1.84	1.05	0.82	0.53	1.43	0.48	1.43	0.25	1.80	0.99
Met	0.03	0.02	0.06	0.00	0.08	0.07	0.17	0.00	0.03	0.01	0.04	0.01	0.06	0.08	0.16	0.01	0.02	0.02	0.06	0.00
Cys	0.005	0.002	0.01	0.00	0.006	0.006	0.01	0.00	0.012	0.070	0.03	0.00	0.009	0.007	0.02	0.00	0.006	0.005	0.01	0.00
Ile	0.85	0.28	1.44	0.47	1.15	0.50	1.92	0.38	1.56	0.30	1.85	1.20	0.86	0.20	1.06	0.66	0.71	0.15	0.91	0.41
Leu	0.53	0.20	0.84	0.25	0.91	0.31	1.19	0.47	0.86	0.21	1.06	0.65	0.77	0.09	0.83	0.66	0.59	0.21	0.96	0.30
Trp	0.30	0.21	0.68	0.10	0.20	0.05	0.25	0.14	1.10	0.62	1.68	0.54	0.60	0.08	0.65	0.50	0.19	0.07	0.30	0.06
Phe	28.4	32.7	88.5	2.85	10.7	4.22	15.5	3.29	61.6	1.81	64.2	60.1	41.0	22.6	56.2	15.0	12.2	7.62	23.9	5.29
Orn	0.17	0.06	0.28	0.10	0.24	0.11	0.44	0.14	0.52	0.14	0.69	0.38	0.24	0.11	0.34	0.13	0.28	0.06	0.36	0.17
Lys	3.16	1.16	5.00	1.70	2.94	1.09	5.00	2.05	3.90	0.73	4.75	3.04	4.19	0.92	4.92	3.15	3.18	0.55	3.76	2.05

Table 2
Correlation coefficient values for the amino acid and ammonium contents of Spanish honey samples from five different single botanical origins: rosemary, eucalyptus, lavender, thyme, and orange blossom, against the first three principal components (PC)

PC	Coefficients and variables most correlated to each PC		Explained variance (%)	Accumulated variance (%)
1	0.96	Val	31.07	31.07
	0.93	Glu		
	0.92	α-Ala		
2	0.92	Tyr	18.9	49.97
	0.88	Phe		
3	0.91	Asn + Ser	13.7	63.70
	0.85	Thr		

samples were very similar in their amino acid compositions. Significant differences were found between lavender and eucalyptus honey samples, the former accounting for more tyrosine and phenylalanine. Lavender honey samples are distinguishable from those of rosemary and thyme, due to the amino acids valine, α-alanine and tyrosine. Furthermore, lavender honey samples had significantly different amounts of valine, α-alanine, tyrosine and phenylalanine from the orange blossom honey samples. Eucalyptus honey samples showed significant differences in valine contents from

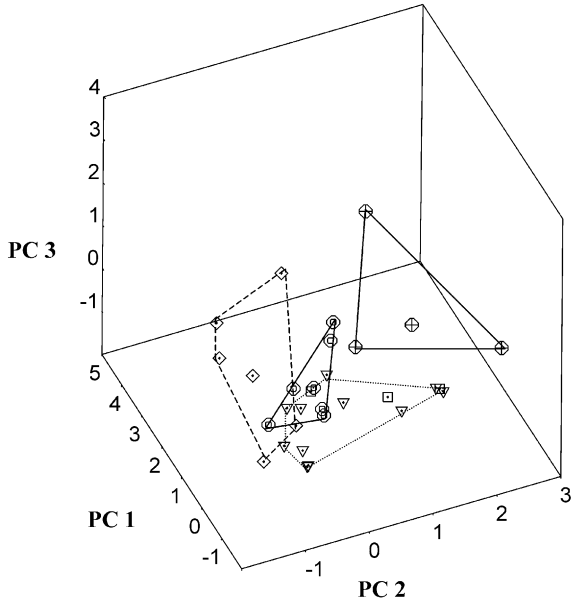

Fig. 2. Plot of the single botanical origin honey samples on the coordinate grid by the first three principal components (PC). Amino acids most closely correlated with each PC: PC 1 (Val, Glu, α-Ala); PC 2 (Tyr, Phe); PC 3 (Asn + Ser, Thr).

Table 3
Comparison of the different groups of single botanical origin honeys, according to the content (as mg/100 g of honey; mean values and standard deviation) of the amino acid most closely correlated with the principal components 1 and 2

	Rosemary ($n=11$)		Eucalyptus ($n=6$)		Lavender ($n=4$)		Thyme ($n=3$)		Orange blossom ($n=7$)	
	MV	S.D.	MV	S.D.	MV	S.D.	MV	S.D.	MV	S.D.
Val	0.85a	0.28	1.75b	0.95	1.90b	0.36	0.87a	0.18	0.98a	0.18
Glu	1.04	0.33	4.29	4.95	3.76	2.65	1.74	1.52	1.28	0.34
α-Ala	0.80a	0.27	1.79b,c	1.04	2.13b	0.51	1.03a,c	0.29	1.08a,c	0.46
Tyr	11.2a,b	9.49	3.48b	1.43	29.9c	10.6	17.1a	5.12	3.15b	1.62
Phe	28.4a,b	32.7	10.7a	4.22	61.6b	1.81	41.0a,b	22.6	12.2a	7.62

Different letters in the same row indicate statistical differences at the 0.05 level according to the Student–Newman–Keuls test.

thyme and orange blossom honey samples, as well as in valine and α-alanine contents when compared with rosemary honey samples. The significantly highest tyrosine content found in lavender honey samples differentiated this botanical origin from the others.

Single botanical origin honeys are well appreciated, but they constitute only a small share of total commercial production. However, there is a general interest in knowledge of the chemical characteristics of certificate honeys in order to defend them against modified or falsified honeys. The composition of the second set of Spanish honey samples showed an amino acid composition that can be considered in the same way as found for the first set of honey samples. Although the results show that amino acid composition was not absolutely able to distinguish botanical origins of honey samples, at least the concentration of amino acids and ammonium ion for Spanish certificate honey samples ranged between the limits derived from the analysis of all the samples studied (Table 4).

Table 4
Distribution (maximum and minimum values) of the concentrations of amino acids and ammonium ion (as mg/100 g of honey) obtained from the analysis of 48 samples of certified Spanish honeys of diverse botanical and geographical origins

Amino acids + ammonium ion	Honey samples ($n=48$)	
	Maximum value	Minimum value
Asp	3.39	0.10
Glu	15.3	0.18
Asn + Ser	2.52	0.05
Gln	0.60	0.03
His	3.57	0.54
Gly	2.11	0.21
Thr	0.65	0.02
β-Ala	2.28	0.66
Arg	9.64	0.42
α-Ala	3.76	0.41
Gaba	8.64	0.01
Pro	87.9	10.1
Tyr	40.4	1.40
Val	3.41	0.42
NH_4^+	2.59	0.35
Met	0.26	0.00
Cys	0.05	0.00
Ile	2.26	0.38
Leu	2.79	0.25
Trp	1.68	0.06
Phe	88.6	2.85
Orn	1.70	0.10
Lys	3.31	1.58

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