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2-Furoylmethyl Amino Acids and Hydroxymethylfurfural As Indicators of Honey Quality

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Determination of changes in 2-furoylmethyl amino acids and hydroxymethylfurfural during the storage of four honey samples at 25 and 35 °C during 12 months was achieved to assess the potential use of both parameters, singly or in combination, as quality indicators. 2-Furoylmethyl amino acids increased during storage at both temperatures, whereas hydroxymethylfurfural only presented slight variations during storage at 25 °C but increased noticeably at 35 °C. The study of 2-furoylmethyl amino acids in 49 commercial honeys revealed that 2-furoylmethyl lysine (furosine) was present in all samples, whereas 2-furoylmethyl derivatives of arginine, GABA, and proline were only present in seven samples. Hydroxymethylfurfural can be considered as a good indicator of heat treatments applied to honey samples, whereas 2-furoylmethyl amino acids can be used as suitable markers of the storage period. The use of both parameters can be useful to detect adulteration with invert syrups, excessive heat treatments, or prolonged storage of honey samples.

KEYWORDS: Furosine; 2-furoylmethyl amino acids; hydroxymethylfurfural; honey; quality indicator

INTRODUCTION

Honey is a natural product in which many variations in composition must be expected; moreover, it may undergo various changes on storage, so that the determination of authenticity in honey is a difficult task. Honey, composed primarily of sugars, has traditionally been a target for adulteration with syrups. This material can be produced inexpensively, and the resulting major carbohydrate profile (glucose, fructose, and sucrose) can be manipulated easily to resemble major carbohydrate profile of honey (*1*), which makes it difficult to detect the syrup addition to honey on the basis of carbohydrate composition.

Since hydroxymethylfurfural (HMF) is formed during acid hydrolysis of sucrose, the presence of high levels of this compound suggests the possibility that the honey has been adulterated with invert syrup (*2*). The drawback with this test is that HMF is also naturally present in honey, especially if it has been subjected to heat or abusive storage (*3*, *4*), so it is impossible to use HMF alone for detecting syrup adulteration of honey.

2-Furoylmethyl lysine (furosine), formed by acid hydrolysis of the Amadori compound fructosyl-lysine (**Figure 1**), is considered a useful indicator of the extent of damage in processed or long-time stored foods (*5*−*9*). Similarly, other 2-furoylmethyl amino acids (2-FM-AA) originated from free amino groups present in foods, have been recently proposed as quality indicators of orange juice, processed tomato products, and dehydrated fruits (*10*−*12*).

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Villamiel et al. (*13*) have recently reported the presence of furosine in hydrolyzates of honey samples. Amadori compounds can be present in freshly collected honey samples but can be also formed during prolonged storage before consumption, not only from lysine but also from other free amino acids present in considerable amounts. However, neither the formation of different 2-FM-AA nor changes in furosine levels during storage have been studied.

Simultaneous formation of furosine and HMF has been reported in a number of foods (*6*, *14*, *15*). These indices presented different evolution during storage of tomato products (*7*), whereas they showed a high correlation during UHT treatment of milk (8).

In the present work, a study of the simultaneous formation of furosine and other 2-furoylmethyl amino acids (2-FM-AA) and HMF during storage of honey was undertaken to assess their usefulness as indicators of quality in commercial honey samples.

MATERIALS AND METHODS

Samples. Thirty three nectar honey and eight honeydew honey samples were purchased from local markets. Eight honey samples were directly obtained from beekeepers.

Honey samples were kept at room temperature in the laboratory and analyzed within the first week after purchasing. All the samples were analyzed before the end of their shelf life date.

Synthesis of 2-Furoylmethyl Amino Acids. 2-Furoylmethyl derivatives of γ-amino butyric acid (GABA), arginine, and proline were synthesized as it was indicated in a previous work (*12*). With this purpouse, Amadori compounds were obtained by mixing 6 mmol of glucose and 1 mmol of the corresponding L-amino acid with 1 g of

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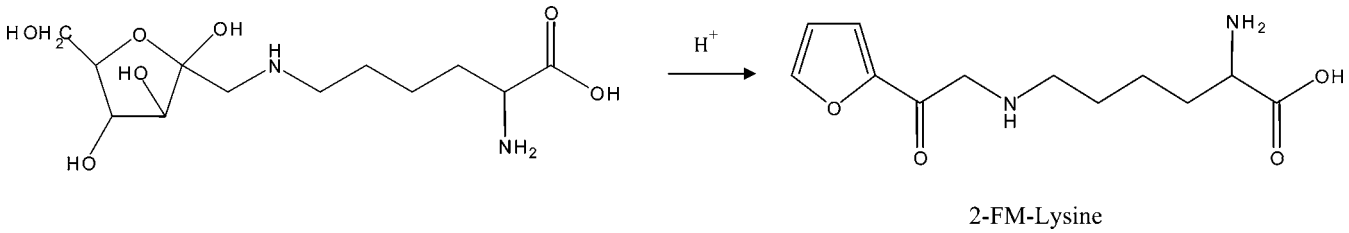

Figure 1. General scheme of 2-FM-Lys formation by acid hydrolysis of Amadori compounds.

microcrystalline cellulose (Merck). These mixtures were dissolved in 5 mL of 0.01N phosphate buffer (pH 7) and lyophilized. Samples were stored at 50 °C in a dessicator with a saturated K_2CO_3 solution (a_w = 0.44) and the storage was stopped when a yellow-brown color appeared. The 2-furoylmehtyl amino acids were obtained by hydrolysis of the corresponding Amadori compounds in 7.95 N HCl for 24 h at 110 °C.

Storage Assays. Two fresh samples obtained from beekeepers (samples A and B), one commercial nectar honey sample (C) and one commercial honeydew honey sample (D) were selected for storage assays. Aliquots of honey samples (8 g) were stored in closed vials of 5 mL of capacity at room temperature (25 °C) and at 35 °C, during 12 months. Sampling was carried out in duplicate at 0, 0.5, 1, 1.5, 2, 3, 5, 7, and 12 months of storage.

Analytical Methods. All analyses were carried out in duplicate.

Determination of total nitrogen content was carried out following the AOAC Official Method 962.18 (*16*). Protein levels were calculated using 6.25 as conversion factor.

Water content was determined following the AOAC method n° 969.38 (*16*) using a refractometer Carl Zeiss 22276 thermostatized at 20 °C.

Chromatographic Analysis. Acquisition of data was carried out using a System Gold software data system (Beckman Instrument).

Determination of Free Amino Acids. A 0.5-g sample of honey was diluted with 10 mL of distilled water. A 2 mL sample of these solutions was mixed with 1 mL of 0.4M borate buffer (pH 10), for primary amino acids, and with 1 mL of water, for secondary amino acids. Samples were filtered through a 0.22-μm membrane filter (Millipore), and 50 μL was injected in the chromatograph. Analysis was carried out by HPLC using a Beckman liquid chromatograph with a Triathlon automatic injector (Spark, Netherlands). Samples were submitted to an automatic precolumn derivatization with *o*-phthaldialdehyde (OPA) to determine primary free amino acids (*17*) and with fluoroeniloxycarbonil chloride (FMOC) to detect secondary amino acids (*18*). The separation of amino acids was carried out in a Novapak C_{18} 60 Å 4-μm column (150 × 3.9 mm i.d.), with a linear gradient at a flow rate of 1.5 mL/min. The mobile phase was constituted by:

Solvent A, methanol/10 mM phospate buffer (pH 7.3)/tetrahydrofurane (19:80:1) (v/v/v).

Solvent B, methanol/10 mM phospate buffer (pH 7.3) (80:20) (v/v).

Detection was performed by fluorescence using wavelengths of excitation and emission at 340 and 425 nm, respectively, for OPA derivatives. For FMOC derivatives the excitation and emission wavelengths were 250 and 335 nm, respectively. Quantitation was performed by external standard method, using a commercial amino acid mixture (Sigma Chemical Co) in a concentration range of 0.25 μg/mL and 50 μg/mL, being the precision of the method below 6% for each amino acid.

Determination of HMF. A 0.5-g sample of honey was diluted with 10 mL of distilled water. Solutions were filtered through a 0.22-μm filter (Millipore), and 50 μL were injected in the chromatograph. Analysis of HMF was performed by HPLC at room temperature using a Beckman liquid chromatograph. Separation was carried out using a Novapak C_{18}, 60 Å 4-μm column (150 × 3.9 mm i.d.) and a gradient elution technique with mobile-phase methanol−water and a flow rate of 1 mL/min, following the method proposed by Viñas et al. (*19*). UV detection was performed at 280 nm. Quantitation was performed by external standard method, using commercial HMF (Sigma), and the calibration curve was linear in the range studied (0.1−100μg/mL). The precision of the method was below 6%.

Determination of 2-FM-AA. Determination of 2-FM-AA was carried out, as it was previously indicated by Villamiel et al. (*13*) for furosine. A 0.5-g sample of honey was mixed with 6 mL of 7.95 N HCl and bubbled with helium. Hydrolysis was carried out at 110°C during 23h, to obtain the possible 2-FM-AA.

Analysis of these compounds was performed by ion-pair RP-HPLC (*9*), using a chromatographic system composed of a pump 250 model (Perkin-Elmer), an oven (Kariba), a UV detector SM 4000 (LDC Analytical), and an interface 406 model (Beckman). Compounds separation was carried out using a C_8 (Alltech furosine-dedicated) column (250 × 4.6 mm i.d.) and a linear binary gradient at a flow rate of 1.2 mL/min. Mobile phase was constituted by solvent A, 0.4% acetic acid, and solvent B, 0.3% KCl in phase A. Detection was performed using a variable wavelength UV detector at 280 nm (LDC Analytical, SM 4000). Calibration was performed by the external standard method, using a commercial standard of pure furosine (Neosystem Laboratories, Strasbourg, France) in a range of 0.21−3.6 mg/L. Quantitative determination was performed, assuming the same response factor for all 2-FM-AA (*11*) being the precision of the method below 10% for each compound.

Identity of 2-FM-AA in honey samples was confirmed by API-ES HPLC-MS, following the method described in previous works (*12*, *20*).

RESULTS AND DISCUSSION

Storage Assays. Honey samples A, B, C, and D presented a water content of 16.84, 14.38, 16.28, and 17.25%, respectively. No variations were observed during the storage at both temperatures.

HPLC analysis of acid hydrolyzates of honey samples showed the presence of furosine in the four studied honeys. In sample D, three other peaks were also observed (**Figure 2**). HPLC-MS identification of these peaks revealed the presence of 2-furoylmethyl-proline (2-FM-Pro) (198 *m*/*z*), 2-furoylmethyl-γ-aminobutyric acid (2-FM-GABA) (212 *m*/*z*) and 2-furoylmethyl-arginine (2-FM-Arg) (283 *m*/*z*). Traces of these compounds were also detected in sample B. Also, traces of 2-FM-GABA were observed in sample C.

In most foods rich in protein such as milk, cheese, pasta, etc., the Amadori compounds formed during Maillard reaction are mainly derived from the interaction of glucose or glucose-containing carbohydrate with the free ϵ-amino group of lysine linked to the protein. These Amadori compounds give rise to furosine during acid hydrolysis. However, in foods containing considerable amounts of free amino acids able to take part in Maillard reactions, different Amadori compounds can be formed, and 2-furoylmethyl amino acids other than furosine have been reported in the acid hydrolyzates of orange juice, tomato products, and dehydrated fruits (*10*−*12*)

Figure 3 shows the evolution of furosine values during storage at 25 and 35 °C in the four hydrolyzates of honey samples studied. Samples A, B, and C showed low initial furosine contents (between 250 and 600 mg/100 g protein), while in sample D, it was near 1200 mg/100 g protein. This high level of furosine in sample D indicates advanced stages of Maillard reaction.

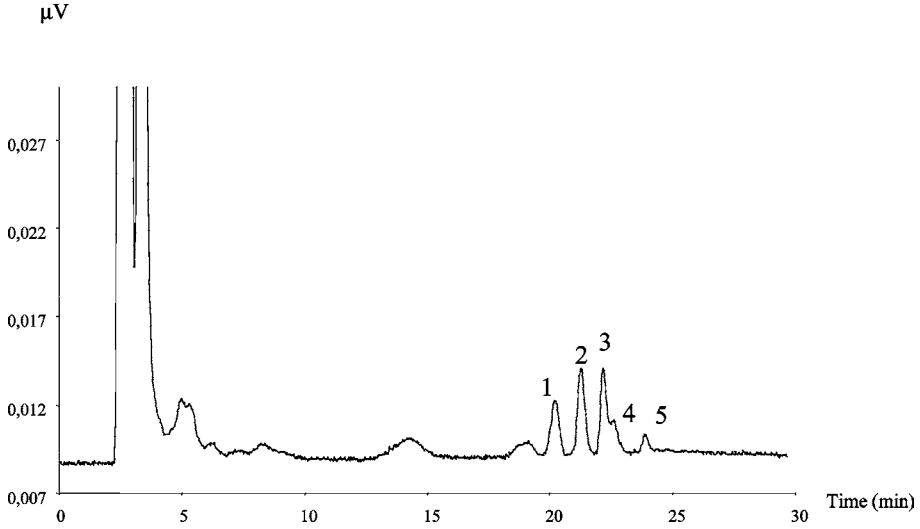

Figure 2. HPLC chromatographic profile of the acid hydrolyzate of honey sample D. (1) 2-FM-Pro, (2) 2-FM-GABA, (3) furosine, (4) 2-FM-Arg, (5) unknown.

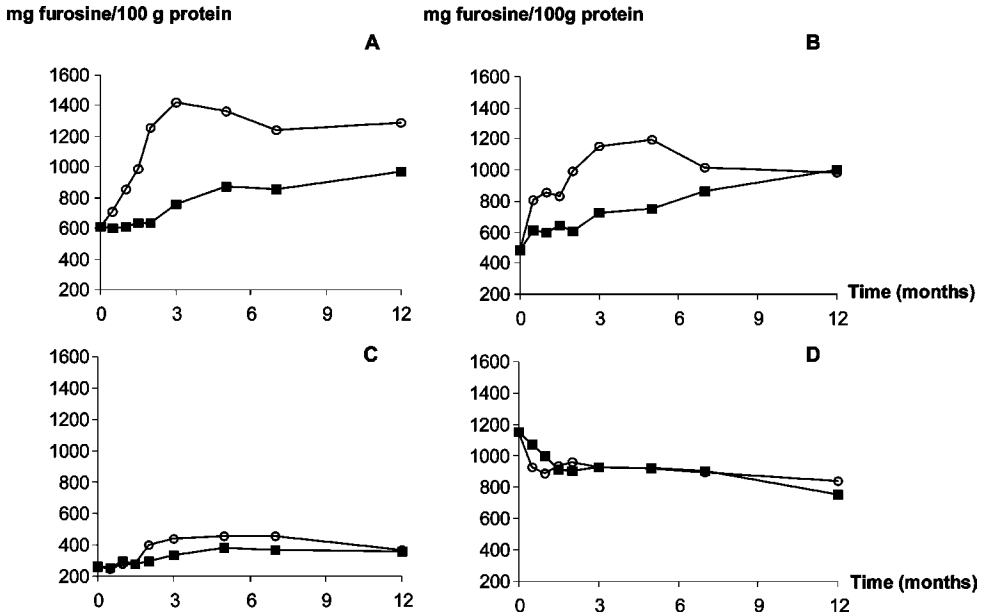

Figure 3. Evolution of furosine content (mg/100 g protein) of honey samples during storage at 25 and 35 °C. Closed symbol = 25 °C, open symbol = 35 °C.

Samples A and B stored at 35 °C showed a marked increase in furosine content during the three initial months, reaching values of 1400 mg/100 g protein and 1200 mg/100 g protein, respectively. Slight decrease of furosine contents were observed during further storage, which may be attributed to the progress of advanced stages of Maillard reaction. During storage at 25 °C, formation rate of furosine was slower than at 35 °C, but a linear increase during all the storage period was observed. Sample D shows a high level of furosine; however, during storage, a decrease was observed at both temperatures studied. The formation of furosine at early stages of storage followed by a decrease during prolonged periods is in agreement with previous studies on the changes in furosine content during food storage, showing that furosine increased to a maximum value followed by a decrease due to the degradation of the Amadori compounds at advanced stages of Maillard reaction (*6*). According to this, the low level of furosine content in sample C and the observed slight variations during storage at both temperatures may indicate that, before the storage study, the sample had reached an advanced stage of Maillard reaction, causing a considerable decrease of the Amadori compound precursor of furosine.

The evolution during storage of 2-FM-AA other than furosine is shown in **Figure 4**.

In sample D, with high initial 2-FM-AA content, a considerable decrease during the first two months of storage followed by slight changes during prolonged storage was detected. In sample B, the formation of 2-furoylmethyl derivatives of GABA and proline increased during storage at both temperatures, as was observed for furosine. In sample A, a considerable formation of 2-FM-GABA was observed after the first 2 months of storage, and the formation of 2-FM-Pro was observed after 6 months of storage. Only traces of these compounds were observed in sample A at the end of the storage at 25 °C. Traces of 2-FM-GABA were also detected in sample C for all studied periods at both temperatures, which could confirm the possibility of the advanced stages of Maillard reaction in this sample. Taking into account that formation of 2-FM-AA has been detected in all samples during their storage at both temperatures,

Table 1. Major Free Amino Acid Contents (mg/100 g product) of Control Honey Samples and Stored during 12 Months at 25 and 35 °C

	Sample A			Sample B			Sample C			Sample D		
	control	25 °C	35 °C	control	25 °C	35°C	control	25 °C	35 °C	control	25 °C	35 °C
aspartic acid	3.43	3.17	2.16	13.41	10.33	2.03	0.85	0.59	0.44	9.48	7.95	3.01
glutamic acid	2.69	1.58	0.26	13.78	9.62	0.34	0.96	0.49	0.14	8.41	5.98	0.44
asparagine	4.29	3.12	1.54	8.45	4.74	0.78	1.66	1.60	0.79	9.18	6.63	2.06
serine	1.23	0.93	0.71	3.56	2.66	0.41	0.98	1.10	1.29	2.99	2.40	0.95
glutamine	2.69	1.68	0.37	13.06	5.83	0.23	1.54	1.21	0.18	4.23	2.33	0.67
arginine	0.38	0.25	0.11	2.65	1.24	0.25	0.19	0.19	0.10	1.96	1.53	0.37
β- alanine	1.91	1.60	1.43	6.31	5.61	1.77	1.29	1.33	1.35	5.62	5.50	2.50
gaba	0.52	0.48	0.27	4.02	2.67	0.32	0.26	0.29	0.16	4.55	3.26	0.31
phenylalanine	2.66	1.92	1.20	5.26	3.06	0.95	2.38	2.34	1.63	2.36	1.98	1.55
lysine	2.32	1.00	0.00	1.46	0.36	0.00	0.73	0.75	0.62	0.20	0.09	0.02
total primary amino acid	27.91	19.48	10.00	82.05	52.49	8.87	13.54	12.58	8.93	56.99	44.12	15.43
proline	66.60	66.02	47.75	94.39	87.09	61.64	41.47	37.42	30.61	65.61	65.89	47.54

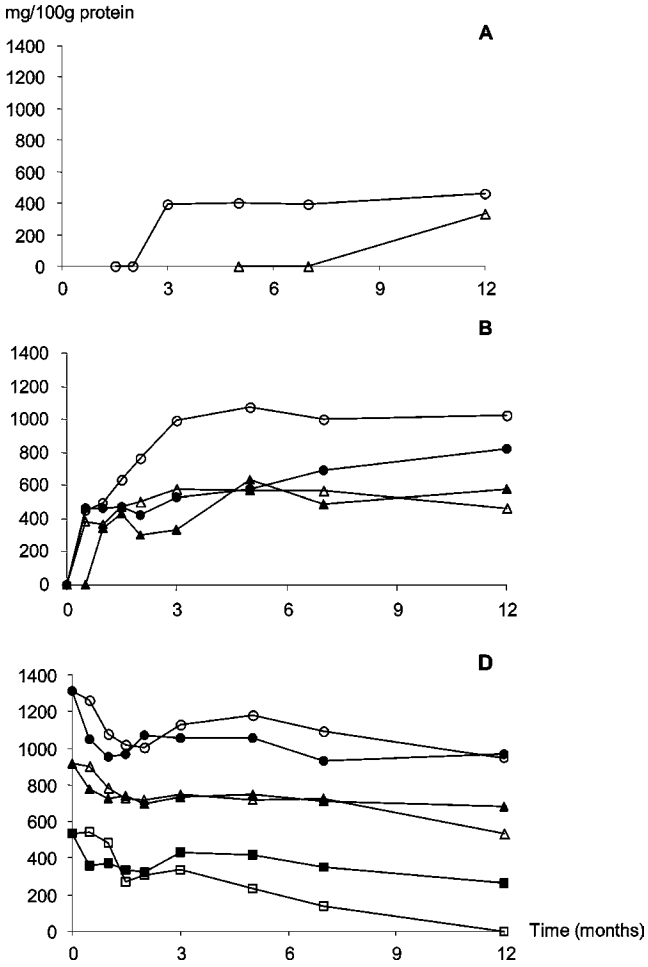

Figure 4. Evolution of 2-FM-AA content (mg/100 g protein) of honey samples during storage at 25 (closed symbol) and 35°C (open symbol). 2-FM derivatives of: ●, GABA; ■, arginine; and ▲, proline.

these compounds could be considered as indicators of honeys submitted to prolonged storages.

Differences on the evolution of 2-FM-AA among samples may be in part attributed to their free amino acids content. As is shown in **Table 1**, initial contents of free arginine, GABA, and proline in samples B and D were considerably higher than in samples A and C. The noticeable loss of these amino acids during storage of sample B may explain the formation of the corresponding 2-furoylmethyl derivatives. During storage of samples A and B, free lysine content decreased considerably at 25 °C and was completely lost at 35 °C. These results are in agreement with the noticeable increase of furosine observed at

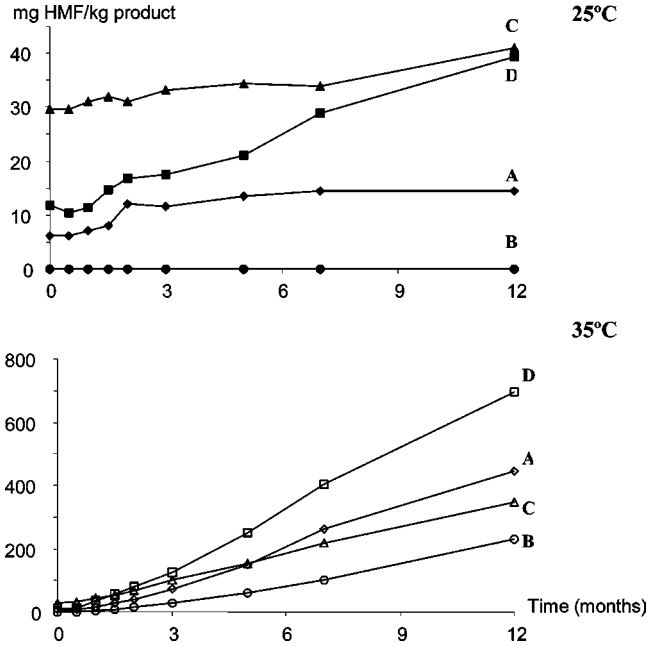

Figure 5. Evolution of HMF content (mg/kg product) of honey samples stored for 12 months at 25 (closed symbol) and 35 °C (open symbol).

both temperatures. In sample D, the very low initial content of lysine was probably due to the high content of furosine, indicative of the progress of Maillard reaction. Sample C showed a small loss of lysine during storage, in agreement with the low increase of furosine detected. The low free amino acid values of this sample could be due to the development of Maillard reaction prior to the storage assays.

The initial HMF contents of stored samples were below the limit of 40 mg/kg: 4.32 mg/kg (sample A), traces (sample B), 28.34 mg/kg (sample C), and 9.37 mg/kg (sample D). The presence of amounts greater than 40 mg/kg HMF in honeys is considered an indication about either deterioration of the honey due to faulty or prolonged storage, or presence of invert sugar as an adulterant (*21*). **Figure 5** shows the evolution of HMF content in these honey samples during the storage at 25 and 35 °C. No important changes in HMF values were detected in samples A, B, and C at 25 °C, whereas a slight increase was observed in sample D along the studied period. As indicated above, sample D showed a decrease in its 2-FM-AA content during storage, being an indication of advanced stages of Maillard reaction, which may cause an increase in HMF content. A great increase in HMF was observed at 35 °C in all samples. The highest values at the end of the studied period was attained in sample D (800 mg/kg of product), whereas the rest of stored

Table 2. Mean, Maximum, and Minimum Values of Protein (g/100 g product), Furosine (mg/100 g protein), and HMF (mg/kg product) of Commercial Honey Samples

	protein (mg/100 g product)			furosine (mg/100 g protein)			HMF (mg/kg product)		
samples	mean	min	max	mean	min	max	mean	min	max
fresh honey from beekeepers (*n*=8)	0.46	0.32	0.58	590	350	830	6.97	0.00	22.75
nectar honey (*n*=33)	0.34	0.14	0.79	880	260	1640	32.24	5.15	151.52
honeydew honey (*n*=8)	0.55	0.34	0.75	1130	730	1840	12.90	7.09	18.48

Table 3. 2-FM-AA (mg/100 g protein) and HMF (mg/kg product) Contents of Commercial Honey Samples

samples	2-FM-Lys (Furosine)	2-FM-Pro	2-FM-GABA	2-FM-Arg	HMF
1	1032.12	270.80	408.47	tr[a]	7.72
2	1075.98	373.95	407.56	tr	5.15
3	854.66	117.05	400.76	tr	13.46
4	1841.21	441.01	835.59	tr	18.48
5	1130.61	924.80	1372.03	530.34	9.37
6	1052.54	253.57	382.14	tr	11.22
7	1507.03	351.20	939.00	tr	7.09

[a] tr = traces.

samples reached values lower than 500 mg/kg of product. Previous studies on formation of HMF in honeys have reported slight modifications during the storage at room temperature and considerable increase during heat treatment (*22*−*25*).

In a previous study, it was observed that severe heat treatments also increase furosine contents of honey (*13*) so that high levels of HMF and furosine in commercial samples may be indicative of excessive heating. On the other hand, samples with high levels of HMF, low levels of furosine and absence of other 2-FM-AA are suspected of being adulterated with invert sugar. The presence of low amounts of HMF and high amounts of furosine may be an indication that honey has been treated under appropriate temperature conditions but submitted to prolonged storage.

Fresh and Commercial Samples. Table 2 reports the protein, HMF, and furosine contents found in honey samples. As expected, in fresh samples obtained directly from reliable beekeepers, mean values of furosine were lower than those found in the rest of the commercial samples studied. On the other hand, the low values of HMF in fresh samples indicate that they are unheated or low-heat treated honeys. Nectar and honeydew honey samples showed a wide variation on both furosine and HMF contents.

Besides furosine, variable amounts of 2-FM derivatives of arginine, proline, and GABA were detected in seven of the studied commercial samples, and the results are shown in **Table 3**. Considering the low amounts of HMF present in these samples, the presence of 2-FM-AA other than furosine may be an indication of prolonged storage.

A plot of furosine content versus HMF content enables commercial honey samples to be distributed according to the relationship between both parameters (**Figure 6**). Most of the samples showed HMF contents lower than 40 mg/kg, relatively low values of furosine, and absence of other 2-FM-AA. According to the HMF, samples may be considered to be properly heat treated, and the low values of furosine and the absence of 2-FM-AA could indicate that samples are fresh or submitted to a short period of storage under adequate conditions. High content of furosine and low values of HMF in samples could be attributed to prolonged storage, whereas the three samples with HMF content greater than 40 mg/kg and low furosine values might be suspected of being adulterated by invert sugar. High levels of furosine and HMF in samples may be due to excessive heating or storage under adverse conditions.

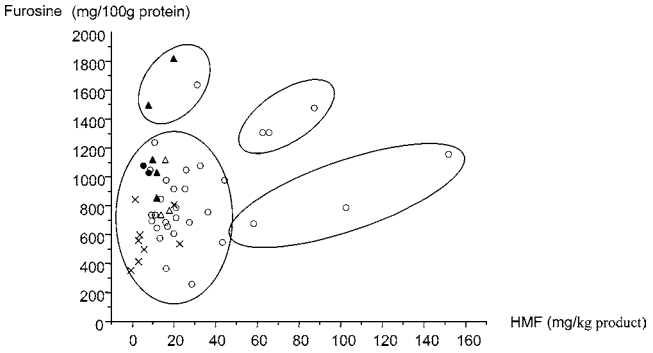

Figure 6. Graphical representation of HMF versus furosine contents of commercial and fresh samples. ×, fresh samples from beekeepers; ○, nectar honey samples; △, honeydew honey samples; ●, ▲, nectar and honeydew honey samples containing furosine together with other 2-FM-AA.

With respect to the samples containing 2-furoylmethyl derivatives of GABA, arginine, and proline, above results in stored samples suggest that prolonged storage of honey may cause a decrease of the furosine formed during the early stages of storage together with formation of appreciable amounts of these 2-FM-AA. Thus, the presence of these compounds in commercial honeys containing low levels of HMF and furosine may be an indication of prolonged storage.

These results seem to indicate that the combination of furosine and other 2-FM-AA with HMF may be useful for evaluating honey quality. However, more studies on 2-FM-AA formation in honeys are needed for a realistic approach in establishing regulations on 2-FM-AA content requirements for high quality honeys.

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