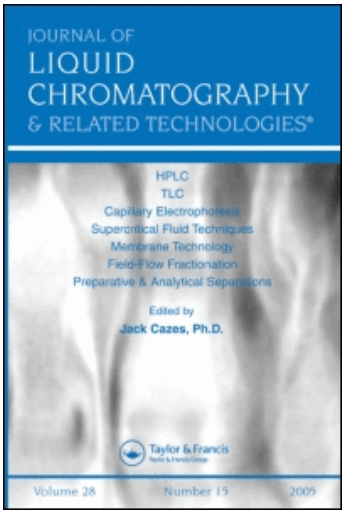

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Determination of Furosine in Honey

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ABSTRACT

A procedure is reported for the determination of ε-*N*-*L*-furoylmethyl-*L*-lysine (furosine) in honey. Furosine was quantified in acid hydrolysis by isocratic ion-pair reversed phase liquid chromatography, using C_{18} column and UV detection at 280 nm. Furosine formation by sample hydrolysis with hydrochloric acid increased with a rise in concentration from 6 N to 11.37 N. Average recovery of furosine by this method was 98.6% under our study conditions. The coefficient of variation at 2.5 mg/100 g of sample was 4.23% and the limit of detection was $\leq$ 0.15 mg/100 g of sample. Heat treatment (70°C for up to 45 min) increased the furosine content from 3.76 to 5.99 mg/100 g honey (79.7–127 mg/g nitrogen). Furosine levels in honey ranged from 0.33 to 5.49 mg/100 g honey (12–144 mg/g nitrogen.)

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Key Words: Furosine; Honey.

INTRODUCTION

Honey is a complex mixture of compounds, including enzymes that contribute to its antibacterial qualities.[1] The predominant compounds are carbohydrates (607–778 g/kg) and water (124–203 g/kg). Honey contains a large number of minor components, including proteins (≤ 5 g/kg) and free amino acids ($\cong 1$ g/kg).[2,3]

Honey manufacture involves heat treatment. Honey is pasteurized (70°C for 6–7 min) in order to delay crystallization, caused by the insolubility of crystallized sugars. Crystallization is favored by the preservation of honey at 10°C to avoid yeast proliferation and other changes,[4] and also by its storage under excessive temperature and relative humidity conditions.

Excessive heat conditions may induce changes through the caramelization of carbohydrates. Honey is an acid medium, which enhances the dehydration of carbohydrates, leading to the formation of 5-(hydroxymethyl)-2-furaldehyde (HMF) and other furfural compounds.[5] Because honey contains low amounts of protein, the Maillard reaction can also take place. The Amadori rearrangement, giving rise to Amadori compounds, is considered the key step of the Maillard reaction, and further reactions yield HMF and other products similar to those derived from caramelization.

Furosine, generated during acid hydrolysis of the Amadori compound fructosyl-lysine, is considered a useful indicator of the extent of damage during early stages of the Maillard reaction. Furosine is used to test processing and storage effects in dairy products,[6,7] baby cereals,[8] pasta,[9] tomato products,[10] and eggs,[11] among other foods. However, there is scant data on the presence of the Amadori compound in honey.[12]

The present study is aimed at developing a method for determining furosine in honey, to test the utility of furosine determination in honeys that undergo heat treatment, and to record the extent of the Maillard reaction in commercial honeys.

EXPERIMENTAL

Apparatus

The liquid chromatographic system used in this study consisted of a Perkin Elmer model 250 (Norwalk, CT) with a Waters plus 717 autosampler (Milford, MA) and Perkin Elmer diode array detector model 235 (Norwalk,

CT). Data were collected by a 1020 software data system (Perkin Elmer, Norwalk, CT).

Reagents

Analytical reagent grade chemicals were used. The furosine standard (ε-*N*-(1-deoxy-*D*-fructosyl)-*L*-lysine was obtained from Neosystem Laboratoire (Strasbourg, France). A standard stock solution containing 1.2 mg/mL of furosine in 0.1 N hydrochloric acid (HCl) was used to prepare the working standard solution (3.75 μg/mL–150 μg/mL).

Samples

The samples examined were: two samples (A and B) of raw or unheated multifloral honeys collected from the honeycomb. Six samples of commercial honeys of different flower origin: rosemary (samples C and D), orange blossom (E and F), and eucalyptus (G and H).

Sample A was heated at 70°C for 5, 15, and 45 min. All samples were sharply cooled at −18°C. Before analysis, the samples were thawed at room temperature for 30–60 min, depending on the sample size.

Furosine Determination

Furosine was determined after hydrolyzing 1.000 g of sample with 7.5 mL of 7.95 N HCl at 110°C for 24 h, in a Pyrex screw-cap vial with PTFE-faced septa. High purity nitrogen gas was bubbled through the solution for 2 min before the hydrolysis. Sample preparation for HPLC analysis followed the method of Resmini et al.[13] The hydrolyzed sample was filtered with a medium grade paper filter. A 0.5 mL portion of the filtrate was applied to a prewetted (5 mL methanol, 10 mL water) Sep-pak C_{18} cartridge (Millipore), eluted with 3 mL of 3 M HCl, and evaporated under vacuum.[13] The dried sample was dissolved in 1 mL of a mixture of water, acetonitrile, and formic acid (95 : 5 : 0.2) before the HPLC analysis,[14] and 50 μL were injected.

Chromatographic Conditions

Furosine was quantified by ion-pair reversed-phase HPLC following the method of Delgado et al.[14] using a Spherisorb ODS2 5 μm column

(250 × 4.6 mm i.d.; Phenomenex, Torrance) operating at room temperature. The mobile phase consisted of a solution of 5 mM sodium heptane sulfonate with 20% acetonitrile and 0.2% formic acid. The elution was isocratic and the flow rate was 1.2 mL/min. The UV detector was set at 280 nm. Calibration of the chromatographic system for furosine determination was by the external standard method.

Total Nitrogen Analysis

Total nitrogen content was determined using the Kjeldhal method.[15]

RESULTS AND DISCUSSION

Figure 1 depicts typical chromatograms of honey sample "B" and a standard solution of furosine. It can be observed that furosine was completely separated in 8 min. No interfering peaks were present at the furosine retention time in any honey sample.

Calibration was performed by adding increasing quantities of furosine working standard, within the expected concentration range, to a previously hydrolyzed honey that had been diluted until furosine was not detected. A calibration curve was constructed by plotting the measured absorbance, expressed in units of area versus micrograms of injected furosine. The equation for the curve was:

$$Y = 10264 + 704 \times 10^4 X$$

A linear response with a high correlation coefficient ($r^2 = 0.999$) was confirmed in the studied range (0.009–0.75 μg injected). The detection limit was 0.154 mg/100 g sample (calculated as twice the noise level).

The precision of the entire assay procedure, including acid hydrolysis, sample preparation, and RP-HPLC analysis (same day) was evaluated on "B" honey samples with low values of furosine ($n = 7$). The relative standard deviation (RSD) was 4.23% obtained on a sample with an average furosine value of 2.5 mg/100 g of sample.

Recovery (Table 1) was obtained by using a hydrolyzed acid of honey "B", to which increasing amounts of furosine standard (18.75–375 ng) were added. The recovery range was 94.8–103.7% and the average value was 98.6%. Similar recovery was obtained by Resmini et al.[13] in dairy products and by Guerra-Hernandez and Corzo[8] in baby cereals.

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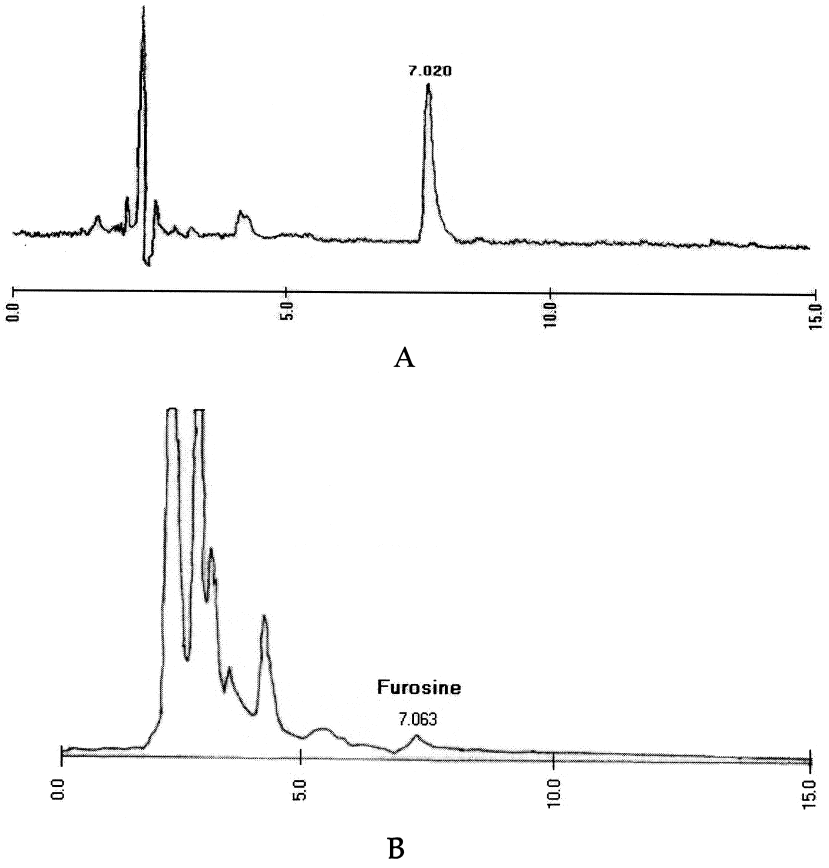

Figure 1. High performance liquid chromatography (HPLC) chromatograms of a furosine standard solution (A) and honey sample (B).

A study was undertaken of the influence on furosine formation of the 6 N–11.37 N concentration range of HCl. As observed in Fig. 2, increasing HCl concentrations lead to significantly higher furosine values. This finding is consistent with results obtained by other researchers in different foods.[16,17] The volume of hydrolyzed sample obtained with 11.37 N of HCl is inadequate for this analysis. If this concentration is eliminated, the linear regression is greatly improved (Fig. 2). Thus, HCl concentrations of either 6 or 10 N can be

Table 1. Furosine recovery in "B" honey sample[a].

Added (ng)	Detected	Recovered (%)
18.75	18.60	99.2
37.50	36.60	97.6
75.00	77.80	103.7
93.75	90.20	96.2
187.50	177.80	94.8
375.00	374.60	99.8
	Mean	98.6
	SD	3.13
	CV%	3.18

[a]Furosine content of sample: 54.7 ng.
Note: $n = 2$.

used. An HCI concentration of 7.95 N is widely used to evaluate food heat damage,[18,19] and was selected for our experiments.

The optimum amount of sample for the analysis was established by studying increasing levels (0.25–1.25 g) of honey, using 7.5 mL of 7.95 N HCl (Table 2). The average furosine content was 1.8 mg/100 g of sample, and there was a high coefficient of variation (CV) of 10.8% (Table 2). The lowest CV (3.6%) was obtained with honey samples between 0.75–1 g. One gram was fixed as the optimum amount because it gave the best chromatographic response.

In order to use furosine as an indicator of heat damage, the optimal amount of honey and HCl concentration must be established.

The method was used to analyze the effects of heat treatment on honey and to determine furosine levels in commercial single-flower honeys.

The effect of heat treatment on furosine content was evaluated in raw sample "A". The honey was heated at 70°C for 5, 15, and 45 min. The furosine content ranged from 3.76 to 5.99 mg/100 g honey (79.7–127 mg/g nitrogen) (Table 3). Furosine values during heat damage are usually expressed in mg/g nitrogen, to facilitate comparisons. Villamiel et al.[12] found similar increases when honey was heated at 90°C for 150 min. Thus, furosine assessment could be useful to monitor the pasteurization process.

Table 4 lists the furosine content of six samples of commercial single-flower honey. The range was 0.33–5.49 mg/100 g honey (12–144 mg/g of nitrogen) and the mean value obtained was 78.21 m/g of nitrogen. The lowest

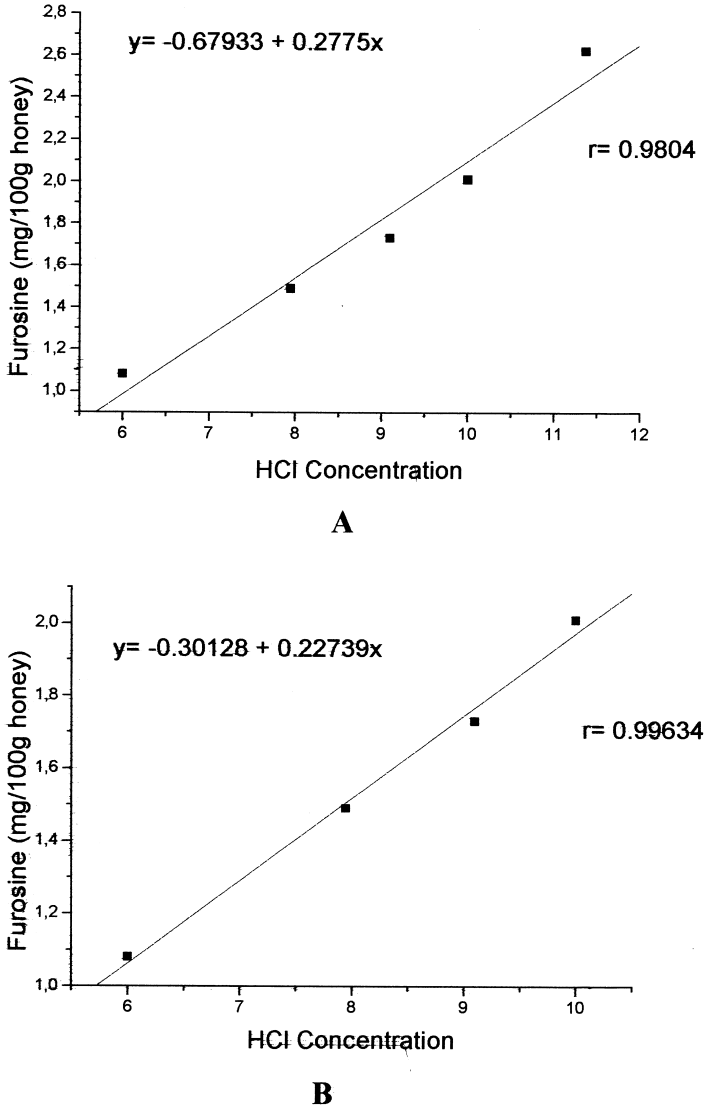

Figure 2. Effect of different concentrations of hydrochloric acid on the formation of furosine in "B" honey samples. Range 6–11.37 N (A). Range 6–10 N (B).

Table 2. Furosine values (mg/100 g of honey) in different amounts of "B" honey samples.

Honey (g)	Furosine (mg/100 g honey)
0.25	2.08 ± 0.01
0.50	1.55 ± 0.02
0.75	1.89 ± 0.03
0.85	1.77 ± 0.04
1.00	1.87 ± 0.02
1.25	1.62 ± 0.05
Mean	1.80
SD	0.193
CV%	10.76

Note: $n = 2$.

content was obtained for Orange blossom honeys and the highest for Eucalyptus honeys. Villamiel et al.[12] studied twenty-one honey samples of various types and reported a low mean furosine content of 51.43 mg/g of nitrogen.

The furosine values obtained in these honeys are considerably greater than the values reported in other processed foods, such as UHT milk (3.2–19.1 mg/g of nitrogen),[18] bread (1.2–2.1 mg/g of nitrogen),[19] and baby cereals (8.9–64.1 mg/g of nitrogen).[20]

Table 3. Evolution of furosine during heating (70°C) of "A" honey samples.

	Furosine	
Time (min)	mg/100 g honey	mg/g nitrogen
0	3.76 ± 0.02	79.7 ± 0.4
5	3.78 ± 0.02	80.1 ± 0.4
15	4.47 ± 0.06	94.8 ± 1.3
45	5.99 ± 0.06	127 ± 1.3

Note: $n = 2$.

Table 4. Furosine content in commercial honey samples.

	Furosine	
Sample	mg/100 g honey	mg/g nitrogen
Rosemary		
C	3.84 ± 0.05	108 ± 1.4
D	2.09 ± 0.13	59.4 ± 3.7
Orange blossom		
E	1.19 ± 0.02	47.6 ± 0.8
F	0.33 ± 0.01	12.0 ± 0.4
Eucalyptus		
G	3.78 ± 0.08	98.3 ± 2.1
H	5.49 ± 0.04	144 ± 1.1

Note: $n = 2$.

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