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Effects of conditioning on HMF content in unifloral honeys

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Abstract

HMF (5-hydroxymethylfurfural) is considered an important quality parameter for honey and maximum values are fixed by the European Directive no.110 (2001). In this paper, the HMF levels during the heating process of four unifloral Sicilian honeys (Orange, Eucalyptus, Sulla, Chestnut) were determined; the kinetics of HMF formation were also investigated. The HMF formation was correlated with chemical characteristics (pH, free acids, total acidity and lactones) of the different honeys. The data obtained were statistically elaborated. The results indicate non-equivalence among different honey types with regard to the heating treatment and the importance of reviewing the directive. Thus, the present standards for honey HMF content seem too large in some cases (Chestnut honey) and too restrictive in others (Citrus honey).

Keywords: Unifloral honeys; Food standards; HMF (5-hydroxymethylfurfural); Chemical parameters; Kinetics; Statistical analysis

1. Introduction

Honey is a semiliquid product (water: 15–18% approx.) which contains a complex mixture of carbohydrates, mainly glucose and fructose; other sugars are present as traces, depending on the floral origin. Moreover, organic acids, lactones, aminoacids, minerals, vitamins, enzymes, pollen, wax and pigments are present. Honey is produced either from many flowers or from single flower pollens. The single flower origin should assure a better quality of the product, when it guarantees a specific and well-defined flavour and aroma. Unifloral honeys, in fact, have highly characteristic aromas, indicating the presence of various components, mainly dependent on the original sources of nectar.

Recently the Codex Alimentarius Commission modified honey definition as ‘‘the natural sweet substance produced by all *honey-producing* bees from the nectar of plants or from secretions of living plants...’’, including not only *Apis Mellifera* (CAC, 2001); moreover, the standards proposed by the Codex Committee on Sugars (CCS) for honey (ALINORM, 01/25) were accepted. The norm (section 3 and annex) lists composition and quality factors; quality factors includ: the diastase activity of honey, usually not less of 8 Schade Units, and, in any case, not less 3 Schade Units, in honey with a low natural enzyme content, and the 5-hydroxymethylfurfuraldehyde (HMF) the content of which after processing and/or blending, shall not be more than 40 mg/kg, with the exception of honey coming from countries or regions with tropical ambient temperatures, where HMF content must not exceed 80 mg/kg.

Thus, the European Union, in order to simplify and up-date the legislation in some food sectors, and to follow the new standards of Codex on honey, published the EU Directive 2001/110/CE (L 10/47) where, in Annex II, honey description and chemical composition are listed. The EU Directive follows sections 2 and 3 of ALINORM 01/25, but with some differences. The point 3.2 of ALINORM stresses the effect of overheating on chemical composition changes and quality loss. The EU Directive, instead, stresses the loss and deactivation of natural honey's enzymes. The most important difference is in the HMF level; the European Union in addition to the two limits proposed by the Codex, is still continuing

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to propose a third limit of 15 mg/kg of HMF for honey with a diastase activity between 3 and 8 Schade units: no attention is paid to the other physical chemical characteristics of honey.

HMF levels and diastase testing, for measuring honey quality have been in use for over 75 years. A detailed description of the step-by-step process in using these tests, as well as a criticism of diastase as a quality index, has been reported by White (1994). Based on the assumption that an index for excessive storage and/or heat exposure should be: (1) easily measurable, (2) virtually absent in the fresh honey, (3) responsive in a predictable way to heating and storage, (4) independent of honey type or composition in its response, White (1994) proposed the HMF level as the only reliable heating/storage index in honey.

Honey processing frequently requires heating both to reduces viscosity, and to prevent crystallization or fermentation (Singh, Singh, Bawa, & Sekhon, 1988). Honey heating is usually carried out in two different ways: in air-ventilated chambers, at 45–50 °C for 4/7 days or by immersion of honey drums in hot water. Although, the second heating method is more efficient, the first is the most common. It is well known that heating of honey results in HMF, which is formed during acid-catalysed dehydration of hexoses (Belitz & Grosch, 1999). The presence in honey of simple sugars (glucose and fructose) and many acids is a favourable condition for the production of this substance.

Several factors influence the formation of HMF in honey: temperature and time of heating (Bath & Singh, 1999; Piro, Capolongo, Baggio, Guidetti, & Mutinelli, 1996; White, 1978); storage conditions; use of metallic containers (Cherchi, Porcu, Spariedda, & Tuberoso, 1997; Kubis & Ingr, 1998; Papoff, Campus, Floris, Prota, & Farris, 1995; Sancho, Muniategui, Huidobro, & Lozano, 1992; White, Kushnir, & Subers, 1964) and the chemical properties of honey, which are related to the floral source from which the honey has been extracted, these indicate pH, total acidity, mineral content (Anam & Dart, 1995; Bath & Singh, 1999; Hase, Suzuki, Odate, & Suzuki, 1973; Singh & Bath, 1997, 1998); however, no information on the correlation between chemical characteristics and HMF level of honey is available.

In this paper, the HMF levels during the heating process of four unifloral Sicilian honeys (Orange, Eucalyptus, Sulla, Chestnut) were determined; the kinetics of HMF formation were also investigated. The HMF content was correlated with the different chemical characteristics of the unifloral honeys analysed.

Whether the aim was to verify the most important factors, influencing HMF production, and the official limits are appropriate for all the studied honeys.

2. Materials and methods

2.1. Samples

Samples of 1 kg of each honey type: Orange (*Citrus aurantium* L.), Eucalyptus (*Eucalyptus camaldulensis* L.), Sulla (*Hedysarium coronarium* L.), Chestnut (*Castanea sativa* L.), were picked from stainless steel drums of 300 kg weight directly provided by local beekeepers (Zafferana Etnea, Catania, Italy). All samples were from the 2001 season and came from different areas of eastern Sicily. In fresh honeys, moisture, glucose, fructose, pH, free acids, lactones, total acidity, electrical conductivity, ash, diastase activity and HMF were determined.

2.2. Heating treatment

Samples (20 g of each honey) were transferred into two vials and heated in a thermostatic oven at 50, 70 and 100 °C. At definite time intervals, the vials were withdrawn, rapidly cooled and samples were analysed to determine the chemical characteristics and HMF. All experiments were conducted in duplicate.

2.3. Chemical analyses

Moisture was determined by measuring the refractive indices at 20 °C with a Carl Zeiss 16531 refractometer and the corresponding moisture content (%) was calculated according to AOAC method (1980).

Electrical conductivity was measured at 20 °C in a 20% (w/v) solution (dry matter basis) in deionised water (Louveaux, Pourtallier, & Vorwohl, 1973) by a Delta Ohm HD 8706 conductivity meter.

Ash was indirectly determined using the measured electrical conductivity and applying the following equation: $X_1 = (X_2 - 0.143)/1.743$ were: X_1 = ash value; X_2 = electrical conductivity in μS/cm at 20 °C (Piazza, Accorti, & Persano Oddo, 1991).

Free acids, lactones, total acidity and pH were measured using a Mettler Toledo MP 220 pH meter according to the Official Method (Repubblica Italiana, 1984).

Sugars (D-glucose and D-fructose) were determined by an enzymatic-spectrophotometric method, using a Boehringer Mannheim Enzymatic Bio Analysis, R-biopharm (Germany) kit.

Diastase determinations were conducted by an enzymatic-spectrophotometric method, using a Phadebas Amylase Test (Pharmacia & Upjohn Diagnostic AB) kit.

2.4. HMF analysis

Aliquots of honey samples were diluted to 50 ml with distilled water, filtered on 0.45 μm filter and injected into an HPLC (Varian 9012Q) equipped with a diode array detector (Varian, Star 330). The HPLC column was a Merck Lichrospher, RP-18, 5 μm, 125×4 mm, fitted with a guard cartridge packed with the same stationary phase (Merck, Milan). The HPLC conditions were the following: isocratic mobile phase, 90% water at 1% of acetic acid and 10% methanol; flow rate, 0.7 ml/min; injection volume, 20 μl. All the solvents were of HPLC grade (Merck, Milan). The wavelength range was 220–660 nm and the chromatograms were monitorated at 285 nm. HMF was identified by splitting the peak in honey with a standard HMF ($P > 98\%$ Sigma-Aldrich, Milan), and by comparison of the spectra of the HMF standard with that of one honey samples. The amount of HMF was determined using an external calibration curve, measuring the signal at $\lambda = 285$ nm.

2.5. Statistical analysis

Statgraphics plus software, version 5.0 was used to perform statistical analyses of the data obtained. One-way analysis of variance (ANOVA) was performed to examine the effects of heating, at different temperatures, on lactones, free acids, total acidity and pH; the *F*-test was used to estimate the statistically significant differences (*P*-value < 0.05). Moreover, a multiple linear regression model, to describe the relationship between formation of HMF at different temperatures and the independent variables, (time of heating, free acids or total acidity and pH) was elaborated. The model was statistically significant with *P*-value less than 0.05.

2.6. Kinetic measurements

Kinetic parameters, initial rate and K, were determined using the initial rate method (Frost & Pearson, 1953). Activation energies (E_a kcal mol^{-1}) were calculated from rate coefficients at different temperatures by applying the Arrhenius equation.

3. Results and discussion

Table 1 lists the chemical characteristics of the analysed honeys. Water contents were similar in Orange, Chestnut and Sulla honeys (from 17.5 to 18.5%) while Eucalyptus honey had the lowest value (15.2%). In terms of pH, electrical conductivity and ash content, Chestnut honey had the highest values, while Eucalyptus honey showed the highest concentration of free acids, lactones and total acidity. These data confirmed the declared floral origin of the honey samples.

The HMF concentration was low, both in Orange and Sulla honeys (5.95 and 1.23 mg/kg, respectively), and was not detectable in Eucalyptus or Chestnut honeys.

3.1. Heating treatment at 50 °C

Table 2 lists the HMF formation and the chemical parameters (free acidity, lactones, total acidity an pH), for the honey samples heated at 50 °C up to 144 h (6 days). The HMF level increased regularly after 60 h up to 144 h both in Orange and Sulla honeys and, at the end of heating process was 27.6 mg/kg and 14.3 mg/kg, respectively; from the beginning up to 60 h, HMF decreased probably due to HMF degradation, reactions (Wunderlin, Pesce, Amè, & Faye, 1998).

Eucalyptus honey had an HMF level lower than the detection limit, right up to 96 h; then, HMF concentration rapidly increased (20.5 mg/kg at 144 h). Chestnut honey had no detectable HMF content during the whole heating treatment.

All the chemical parameters (Table 2) showed the highest variation during the first 48 h of heating, changing at a slower rate there after. There are some exceptions; e.g., pH values remained almost constant in all samples, with the exception of Chestnut honey, which showed a decrease; lactones in Eucalyptus honey showed the highest starting value (9.5) and changed very little with heating.

3.2. Heating treatment at 70 °C

Table 3 shows the HMF formation and chemical parameters, for honey samples heated at 70 °C up to 96 h. Orange and Sulla honeys increased their HMF content from the beginning of the heating. In Chestnut and Eucalyptus honeys the HMF content was not detected up to 24 h; then the concentration rapidly increased in Eucalyptus honey, but slowly in Chestnut honey. After 96 h of heating, Chestnut honey showed the lowest HMF content (90.8 mg/kg), Eucalyptus honey showed the highest (513 mg/kg), while Orange and Sulla honeys showed intermediate concentrations, of 472 mg/kg and 331 mg/kg, respectively.

As well as heating at 50 °C, the highest variation of chemical parameters was from the beginning of treatment up to 4 h. The highest increase of free acidity occurred in Eucalyptus honey, passing from 22.5 to 29 mg/kg after 4 h, and to 32 mg/kg after 96 h, followed by Chestnut honey (22.5 mg/kg after 96 h). All samples, except Eucalyptus, showed an increase of lactones. The pH values of Eucalyptus, Orange and Sulla honeys remained almost constant with a tendency to a slight increase (0.2 pH units) while the pH of Chestnut decreased from 5.9 to 4.8 after 96 h.

3.3. Heating treatment at 100 °C

During heating at 100 °C, all honey samples showed considerable formation of HMF; after 4 h, the HMF concentration was 221 mg/kg in Orange honey, 219 mg/

Table 1
Characterisation of the different honey samples

Parameters	Orange	Eucalyptus	Chestnut	Sulla
Water (g %)	18.5±0.03	15.25±0.04	18.5±0.02	17.5±0.01
Glucose (g %)	31±0.7	31.5±0.7	25.1±0.8	32.2±1.0
Fructose (g %)	34±0.5	32.2±1.0	36.8±0.9	34.8±1.1
pH	3.4±0.03	3.7±0.03	5.9±0.01	3.4±0.02
Free acids (meq/kg)	22.5±0.7	23±0.7	9.7±0.3	15.5±0.3
Lactones (meq/kg)	2.5±0.7	9.5±0.7	1.7±0.3	2.5±0.3
Total acidity (meq/kg)	25±0.3	32.5±1.4	11.4±0.7	18±0.7
Electrical conductivity (μS)	193±0.4	413±0.8	1128±1.1	126±0.6
Ash (mg/kg)	0.03±0.02	0.1±0.02	0.6±0.05	0.008±0.03
Diastase activity (Schade)	7.6±0.2	33.9±0.3	27.3±0.9	10.8±0.2
HMF (mg/kg)	5.95±0.04	–	–	1.23±0.1

kg in Eucalyptus honey, 146 mg/kg in Sulla honey, but only 51.8 mg/kg in Chestnut honey. This trend was maintained up to 60 h when the HMF concentration reached the highest values in all honey.

The variation of chemical parameters at this temperature is reported in Table 4. Free acidity increased in all samples, but most in Chestnut (18-fold the starting value) and in Eucalyptus (4-fold the starting value) honeys; in Orange and Sulla honeys the increase of free acidity did not exceed 2.5-fold the starting value. Lactones showed similar behaviour to that observed at lower temperatures. The greatest decrease of pH values was in Chestnut honey, passing from 5.92 to 3.55 after 60 h. All other samples had a slight increase up to 12 h, than a decrease to values from 3.2 to 3.55.

3.4. Kinetic measurements

Table 5 lists the initial rates, pseudo first order rate and the activation energies of the HMF formation in honey samples. Kinetic parameters showed different trends according to the heating temperature.

At 50 °C, Chestnut honey showed no HMF formation; Orange honey showed the highest initial rate, followed by Eucalyptus and Sulla honeys.

At 70 °C, these trends were confirmed for Orange, Eucalyptus and Sulla honeys while Chestnut honey showed an initial rate one order lower than all others; the pseudo first order rate also confirms the lower reactivity of Chestnut honey at 70 °C.

At 100 °C, the reaction of HMF formation was speeded up in all types of honey. Chestnut honey showed the highest initial rate and pseudo first order rate constant, followed by Orange and Eucalyptus and, finally, by Sulla honeys.

The calculation of the activation energies emphasised that Orange, Eucalyptus and Sulla honeys had similar E_a values. Chestnut honey had the highest E_a, confirming its reduced reactivity to HMF formation, although E_a in this case was an underestimated value, calculated using the two points at the highest temperatures (70 and 100 °C).

3.5. Statistic analysis

The multiple linear regression analysis (Table 6) clearly showed that HMF levels in honey samples, heated at 100 and 70 °C, were significant correlated only with time of heating. In the Chestnut sample, the HMF was also correlated with pH, although the linear model exclusively formulated with hours of heating had a R^2 of 94.09%. Sulla honey, also, showed a correlation with free acidity, even though the most important factor remained the time of heating. The statistical models formulated were significant for all honey samples, with the extreme values in Orange and Sulla honeys, (R^2 of 95.78 and 99.8%), respectively. Intermediate values were in Eucalyptus and Chestnut, with R^2 values of 97.16 and 97.07%, respectively.

At 70 °C, time of heating was the most important factor for HMF level in the different honey samples. The correspondent statistical models (Table 6) showed R^2 (correlation coefficient) values of 95.06, 94.47 and 96.16% for Orange, Eucalyptus and Sulla honeys, respectively. On the other hand, the statistical model elaborated for Chestnut honey had no statistical significance. In fact, all the used independent variables (time, free acidity and pH) were not correlated with the HMF formation; as consequence, it was not possible to produce any predictive model. In Sulla honey, HMF, besides being correlated with time, was also correlated with pH; the corresponding model explains the 97.7% of HMF formed during heating at 70 °C.

At the lowest heating temperature (50 °C), some differences among samples were seen (Table 6). At this temperature the chemical composition of each honey is important for HMF levels. The formulated model for Eucalyptus was statistically significant, but it explained only 78.95% of HMF variability. Time of heating was still the most important factor, but the corresponding model, coming from the exclusion of free acidity, had no statistical significance (R^2 60.38). In Sulla honey, HMF was correlated with time of heating and total acidity, the corresponding model explained 89.36% of

Table 2
Variation of chemical characteristics, during the heating at 50 °C, for the analysed honeys (all data expressed as meq/kg except for HMF mg/kg and pH)

Hours heating	Free acids	Lactones	Total acidity	pH	HMF	Free acids	Lactones	Total acidity	pH	HMF
Eucalyptus						*Chestnut*				
Initial value	22.5a±0.71	9.5c d±0.71	32.0a±3.10	3.66a±0.03	nd	9.7a±0.35	1.7a±0.35	11.4a±0.71	5.92d±0.01	nd
48	33.8c±0.35	8.3a b±0.35	42.1bc±0.10	3.74b±0.04	nd	12.7bc±0.35	9.5bc±0.71	22.2b±1.06	5.22b±0.02	nd
60	32.5bc±0.71	7.8a±0.35	40.3bc±1.06	3.70ab±0.02	nd	13.7de±0.35	9.3b±0.35	23.0b c±.0.10	5.16a±0.02	nd
72	35.5d±0.71	8.8bc±0.35	44.3de±1.06	3.74b±0.02	nd	13.7d e±0.35	10.7d±0.35	24.4c d e±0.71	5.13a±0.03	nd
84	33.5c±0.71	9.3c±0.35	42.8cd±1.06	3.74b±0.02	nd	12.2b±0.35	11.2de±0.35	23.4bcd±0.71	5.22b±0.03	nd
96	31.7b±0.35	10.3d±0.35	42.0bc±0.10	3.75b±0.01	nd	13.7d e±0.35	10.3bc d±0.35	24.0c d±.0.10	5.12a±0.02	nd
108	31.5b±0.71	11.3e±0.35	42.8cd±0.35	3.73b±0.04	12.0±0.47	13.2cd±0.35	11.3de±0.35	24.5cde±0.71	5.35c±0.03	nd
120	31.7b±0.35	12.3f±0.35	44.0de±0.10	3.75b±0.02	14.2±0.70	13.5cde±0.71	12.2e±0.35	25.7e±1.06	5.36c±0.03	nd
144	33.7c±0.35	11.8ef±0.35	45.5e±0.71	3.71ab±0.02	20.5±0.25	14.2e±0.35	10.5c d±0.71	24.7d e±1.06	5.13a±0.03	nd
Orange						*Sulla*				
Initial value	22.3a±0.35	2.5a±0.71	24.8a±0.35	3.43a±0.03	6.0±0.04	15.3a±0.35	2.7a±0.35	18.0a±0.71	3.43a±0.02	1.3±0.11
48	26.5cd±0.71	11.5d±0.71	38.0c±1.41	3.51bc±0.01	3.2±0.50	16.5ab±0.71	8.7c±0.35	25.2b±1.06	3.48b±0.01	nd
60	26.5cd±0.71	8.8b±0.35	35.3b±1.06	3.52bc±0.01	5.8±0.13	16.5ab±0.71	7.2b±0.35	23.7b±0.35	3.47ab±0.01	0.2±0.10
72	26.5cd±0.71	7.8b±0.35	34.3b±1.06	3.53bc±0.01	7.7±0.12	16.5ab±0.71	11.5d±0.71	28.0c±1.41	3.48b±0.02	2.6±0.15
84	26.8d±0.35	8.8b±0.35	35.6b±0.71	3.50b±0.01	11.2±0.24	17.5b±0.71	11.5d±0.71	29.0c±0.41	3.49b±0.01	3.7±0.25
96	24.8b±0.35	8.8b±0.35	33.6b±0.71	3.56d±0.01	13.1±0.22	17.5b±0.71	10.5d±0.71	28.0c±1.41	3.48b±0.01	4.4±0.28
108	25b±0.01	10.3c±0.35	35.3b±0.35	3.56d±0.01	16.8±0.12	17.5b±0.71	10.5d±0.71	28.0c±1.41	3.55d±0.04	7.5±0.14
120	25.5bc±0.71	12.5de±0.71	38.0c±0.41	3.53cd±0.01	19.2±0.11	16.5ab±0.71	8.2bc±0.35	24.7b±0.35	3.50bc±0.01	9.2±0.08
144	25.3b±0.35	12.8e±0.35	38.1c±0.71	3.52bc±0.01	27.6±4.16	16.5a b±0.71	8.5b c±0.71	25.0b±0.10	3.54cd±0.02	14.3±1.38

Table 3
Variation of chemical characteristics, during the heating at 70 °C, for the analysed honeys (all data expressed as meq/kg except for HMF mg/kg and pH)

Hours heating	Free acids	Lactones	Total acidity	pH	HMF	Free acids	Lactones	Total acidity	pH	HMF
Eucalyptus						*Chestnut*				
Initial value	22.5a ± 0.71	9.5b c d ± 0.71	32.0a ± 1.41	3.66a ± 0.03	nd	9.8b ± 0.35	1.8a ± 0.35	11.6a ± 0.71	5.90e ± 0.01	nd
4	29.0bc ± 1.41	8.0ab ± 0.01	37.0bc ± 1.41	3.78bc ± 0.01	nd	8.0a ± 0.01	6.5bc ± 0.71	14.5ab ± 0.71	5.80d ± 0.06	nd
8	28.5b ± 0.71	7.5a ± 0.71	36.0b ± 0.01	3.77b ± 0.01	nd	9.0a b ± 1.41	6.0b ± 0.01	15.0b ± 1.41	5.80d ± 0.01	nd
12	28.0b ± 1.41	9.0a bcd ± 1.41	37.0bc ± 0.01	3.79bc ± 0.02	nd	10.0b ± 0.01	7.5bc ± 2.12	17.5bc ± 2.12	5.70d ± 0.01	nd
24	29.0bc ± 0.01	9.0abcd ± 0.01	38.0c ± 0.01	3.79bc ± 0.01	nd	12.0c ± 0.01	8.5bcd ± 2.12	20.5c ± 2.12	5.40c ± 0.08	nd
48	30.0bcd ± 1.41	8.5abc ± 0.71	38.5c ± 0.71	3.82cd ± 0.01	290 ± 5.63	16.8d ± 0.35	8.0bcd ± 0.01	24.8d ± 0.35	5.00b ± 0.01	33.2 ± 4.28
60	31.5cde ± 0.71	10.0cd ± 0.01	41.5d ± 0.71	3.87de ± 0.01	286 ± 17.61	16.5d ± 0.71	8.5bcd ± 0.71	25.0d ± 1.41	5.10b ± 0.01	18.4 ± 1,44
72	33.0e ± 1.41	13.5e ± 0.71	46.5e ± 0.71	3.87e ± 0.04	365 ± 40.0	19.0e ± 0.01	9.0cde ± 1.41	28.0d ± 1.41	5.00b ± 0.07	42.0 ± 9.78
84	33.0e ± 1.41	9.5bcd ± 0.71	42.5d ± 0.71	3.86de ± 0.04	387 ± 51.57	20.8f ± 0.35	10.5de ± 0.71	31.3e ± 0.35	5.00b ± 0.10	56.0 ± 3.53
96	32.0de ± 1.41	10.5d ± 0.71	42.5d ± 0.71	3.92f ± 0.01	513 ± 6.70	22.5g ± 0.71	11.5e ± 0.71	34.0e ± 1.41	4.80a ± 0.02	90.8 ± 1.18
Orange						*Sulla*				
Initial value	22.0a ± 0.35	2.5a ± 0.71	24.8a ± 0.35	3.43a ± 0.03	6.0 ± 0.04	15.3a ± 0.35	2.8a ± 0.35	18.1a ± 0.71	3.43a ± 0.02	1.3 ± 0.11
4	24.5b ± 0.71	9.5bc ± 0.71	34.0b ± 0.01	3.60b ± 0.01	6.9 ± 0,18	17.5b ± 0.71	10.0d ± 0.20	27.5b ± 0.71	3.46ab ± 0.05	2.4 ± 0.64
8	25.0b ± 0.01	10.5cd ± 0.71	35.5bc ± 0.71	3.60b ± 0.02	12.6 ± 0.18	17.0ab ± 0.22	8.0bc ± 1.41	25.0b ± 1.41	3.48abc ± 0.06	6.8 ± 0.30
12	24.0ab ± 0.01	11.0d ± 0.01	35.0bc ± 0.02	3.58b ± 0.02	20.9 ± 0.33	18.5b ± 2.12	7.0b ± 0.10	25.5b ± 2.12	3.49abc ± 0.04	12.8 ± 1.08
24	24.0ab ± 1.41	10.5cd ± 0.71	34.5bc ± 0.71	3.56b ± 0.02	47.5 ± 0.78	17.5b ± 0.71	8.0bc ± 1.41	25.5b ± 2.12	3.47ab ± 0.06	32.2 ± 3.47
48	26.0bc ± 0.01	9.8bc ± 0.35	35.8cd ± 0.35	3.63b ± 0.01	149 ± 6.04	17.5b ± 0.71	8.0b c ± 1.41	25.5b ± 2.12	3.57d ± 0.03	96.8 ± 3.59
60	29.0d ± 1.41	10.0cd ± 0.01	39.0e ± 1.41	3.42a ± 0.09	208 ± 15.25	18.0b ± 0.10	9.5cd ± 0.71	27.5b ± 0.71	3.56cd ± 0.02	142.7 ± 4.87
72	28.5d ± 0.71	8.8b ± 0.35	37.3de ± 0.35	3.45a ± 0.05	291 ± 11.42	17.8b ± 0.35	10.0d ± 0.01	27.8b ± 0.35	3.59d ± 0.12	189.8 ± 6.25
84	28.5d ± 2.12	10.5cd ± 0.71	39.0de ± 1.41	3.62b ± 0.04	372 ± 25.77	18.5b ± 0.71	8.5b c d ± 0.71	27.0b ± 1.41	3.60d ± 0.01	222.83 ± 20.28
96	27.5cd ± 0.71	10.0cd ± 0.01	37.5de ± 0.71	3.64b ± 0.06	472 ± 7.81	18.0b ± 1.41	7.0b ± 0.01	25.0b ± 1.41	3.52bcd ± 0.01	331.4 ± 17.35

Table 4
Variation of chemical characteristics, during the heating at 100 °C, for the analysed honeys (All data expressed as meq/kg except for HMF mg/kg and pH)

Hours heating	Free acids	Lactones	Total acidity	pH	HMF	Free acids	Lactones	Total acidity	pH	HMF
Eucalyptus						*Chestnut*				
Initial value	22.5a±0.71	9.5b±0.71	32.0a±1.41	3.66c±0.03	nd	9.8a±0.35	1.75a±0.35	11.5a±0.71	5.92f±0.01	nd
4	26.5ab±0.71	6.5a±0.71	33.0a±1.41	3.97d±0.16	219±13.71	20.0a±2.83	10.0b±1.41	30.0b±1.41	5.04e±0.18	51.8±4.38
8	32.5bc±0.71	9.5b±0.71	42.0b±0.01	4.03d±0.16	752±45.34	33.5b±3.54	11.5b±2.12	45.0b±1.41	4.60d±0.06	281±1.94
12	37.5c±0.71	10.0b±1.41	47.5b±0.71	3.91d±0.10	1517±70.3	47.0c±0.01	14.5bc±2.12	61.5c±2.12	4.36e±0.05	672±59.8
24	49.0d±1.41	16.0cd±1.41	65.0c±0.01	3.66c±0.08	4153±93.0	83.5d±7.78	16.5c±2.12	100d±9.90	4.02b±0.06	2417±21.7
36	63.5e±3.54	15.5c±0.71	79.0d±4.24	3.52bc±0.06	7085±257	119.0e±1.41	17.0c±2.83	136e±4.24	3.87b±0.01	3998±375.6
48	77.5f±6.36	17.5cd±2.12	95.0e±4.24	3.37ab±0.01	9962±1680	147.0f±9.90	19.0c±2.83	166f±12.7	3.69a±0.03	6673±90.3
60	87.0g±4.24	18.5d±0.71	105.5f±4.95	3.27a±0.02	13651±316.1	181.0g±7.07	17.5c±2.12	199g±9.19	3.55a±0.02	10657±837
Orange						*Sulla*				
Initial value	22.3ab±0.35	2.5a±0.71	24.8a±0.35	3.43abc±0.03	6.0±0.04	15.3bc±0.35	2.8a±0.35	18.1a±0.71	3.43ab±0.02	1.3±0.11
4	20.5a±0.71	8.5b±0.71	29.0ab±1.41	3.59cde±0.06	221±20.2	11.0a±1.41	9.5b±0.71	20.5ab±0.71	3.71c±0.16	146±4.86
8	24.0ab±1.41	9.5b±0.71	33.5bc±2.12	3.80e±0.23	829±33	13.0ab±1.41	8.5b±0.71	21.5b±2.12	3.67bc±0.10	608±16.6
12	24.5b±2.12	10.5bc±2.12	35.0c±4.24	3.67de±0.13	1534±73.3	14.0b±1.41	8.5b±0.71	22.5bc±0.71	3.68bc±0.16	1242±24.5
24	29.0c±2.83	13.5d±0.71	42.5d±2.12	3.54bcd±0.01	4138±200	17.0c±1.41	9.0b±1.41	26.0c±2.83	3.51abc±0.12	3347±139
36	37.0d±1.41	12.5cd±0.71	49.5e±0.71	3.44abcd±0.07	6672±255	22.5d±0.71	13.0c±1.41	35.5d±2.12	3.40a±0.11	5952±144
48	44.0e±1.41	13.5d±2.12	57.5f±0.71	3.31ab±0.06	8144±1880	26.0e±1.41	10.5b±0.71	36.5d±0.71	3.32a±0.06	7881±229
60	55.5f±2.12	11.0bcd±1.41	66.5g±0.71	3.23a±0.01	13271±1982	36.5f±0.71	13.0c±1.41	49.5e±0.71	3.30a±0.08	10991±397

HMF variability. In this case, moreover, exclusively correlating HMF with time, the corresponding model lost statistical significance (R^2 73.33%). Orange honey was differentiated from all others. HMF level, resulting at the same time, correlated with three independent variables: time, pH and free acidity. The formulated model explained 96.47% of HMF variability, with a P-value <0.01, and all terms were statistically significant and all must therefore be considered. In fact, the corresponding model, HMF/Time, explained only 76.02% of HMF variability, confirming that HMF level in orange honey depends on time of heating, pH and free acidity.

At high temperature (100 °C) no difference, related to HMF formation, can be measured among honeys of different origin. In this case, the driving force, as confirmed by multiple linear regression analysis, is time of heating. On the other hand, a strong increase of free acidity in Chestnut honey, at this temperature, was observed. During the heating at 70 °C, the most important factor for HMF formation is, also, the time. But, at this temperature, some differences among honeys are seen. Orange and Eucalyptus, after 96 h, had 513 and 472 mg/kg of HMF, respectively; Sulla had 330 mg/kg; while Chestnut honey, had only 91 mg/kg.

At lower temperature (50 °C), these differences were much more evident; all samples developed different amounts of HMF. First, the sample with the highest pH value, Chestnut honey, did not form any HMF even after 144 h (6 days) of heating. In other honeys, experimental data as well as statistical treatment showed that the time of heating, as well as free and total acidity and pH values, might play important roles in HMF formation.

Use of HMF level as the only index of time and/or temperature abuse in honey, as proposed by White (1994), could work, but, there are considerations about the present limits in honey standards.

Table 5
Kinetic parameters for HMF formation

Sample	T (°C)	Initial rate (mol kg^{-1} s^{-1})	Pseudo first order rate coefficients (s^{-1})	Ea (kcal mol^{-1})
Orange	100	6.06×10^{-7}	2.05×10^{-7}	32.5
	70	1.66×10^{-8}	5.63×10^{-9}	
	50	6.78×10^{-10}	2.29×10^{-10}	
Sulla	100	4.63×10^{-7}	1.47×10^{-7}	33.4
	70	1.30×10^{-8}	4.14×10^{-9}	
	50	4.27×10^{-10}	1.36×10^{-10}	
Eucalyptus	100	6.05×10^{-7}	1.92×10^{-7}	33.7
	70	1.36×10^{-8}	4.34×10^{-9}	
	50	5.28×10^{-10}	1.68×10^{-10}	
Chestnut	100	7.56×10^{-7}	2.92×10^{-7}	43.6
	70	4.47×10^{-9}	1.73×10^{-9}	
	50	–		

Table 6
Multiple linear regression analysis

Honey	Parameter	Estimate	Standard Error	T Statistic	P-Value
Dependent variable: HMF Temperature 100 °C					
Eucalyptus	Constant	7496.76	5820.56	1.287	0.2220
	Time	342.13	90.58	3.776	0.0026
	Free acidity	−123.99	81.03	−1.530	0.1519
	pH	−1385.24	1395.73	−0.992	0.3406
Chestnut	Constant	−8414.99	2987.35	−2.817	0.0155
	Time	336.85	121.67	2.768	0.0170
	Free acidity	−38.42	44.04	−0.872	0.4002
	pH	1516.09	537.62	2.820	0.0155
Orange	Constant	−1364.43	6227.37	−0.219	0.8303
	Time	114.24	46.16	2.474	0.0292
	Free acidity	116.68	82.85	1.408	0.1844
	pH	−341.66	1570.07	−0.217	0.8314
Sulla	Constant	−421.80	1863.37	−0.226	0.8247
	Time	148.68	7.39	20.126	0.0000
	Free acidity	87.42	22.04	3.965	0.0019
	pH	−311.75	473.77	−0.658	0.5230
Dependent variable: HMF temperature 70 °C					
Eucalyptus	Constant	68.95	1422.11	0.048	0.9619
	Time	5.98	0.62	9.693	0.0000
	Free acidity	−9.26	7.77	−1.191	0.2511
	pH	35.84	417.69	0.085	0.9327
Chestnut	Constant	−220.10	144.77	−1.520	0.1479
	Time	0.45	0.51	0.879	0.3921
	Free acidity	4.59	3.21	1.430	0.1719
	pH	29.88	23.83	1.254	0.2279
Orange	Constant	−45.32	425.27	−0.106	0.9165
	Time	5.34	0.46	11.559	0.0000
	Free acidity	−10.69	6.69	−1.597	0.1298
	pH	74.19	98.99	0.749	0.4645
Sulla	Constant	1083.84	351.14	3.087	0.0071
	Time	3.61	0.19	19.031	0.0000
	Free acidity	−3.85	4.23	−0.910	0.3761
	pH	−301.51	102.60	−2.938	0.0096
Dependent variable: HMF temperature 50 °C					
Eucalyptus	Constant	210.59	134.88	1.516	0.1408
	Time	0.21	0.03	7.140	0.0000
	Free acidity	−0.79	0.38	−2.046	0.0600
	pH	−53.02	37.94	−1.397	0.1840
Orange	Constant	309.94	52.18	5.939	0.0000
	Time	0.23	0.01	17.320	0.0000
	Free acidity	−1.66	0.30	−5.503	0.0001
	pH	−77.98	15.62	−4.993	0.0002
Sulla	Constant	−48.34	58.53	−0.826	0.4227
	Time	0.12	0.02	1.012	0.0000
	Total acidity	−0.66	0.14	−4.549	0.0005
	pH	17.34	17.13	6.753	0.3286

The statement, that the amount of HMF is independent of honey type and composition, could sometimes be wrong. Different compositions and, moreover, different pH values, could lead to different HMF levels, particularly at low heating temperatures. The limit of 40 mg/kg of HMF in Chestnut honey seems too high. On the other hand, the HMF limit imposed by the EU for honeys with low diastase activity (3–8 Schade units) seems to be very restrictive for some types of honey, e.g., citrus. In fact, looking at data shown in this paper, the Chestnut honey could be heated at 50 °C for 1 week without any measurable amount of HMF, while orange honey after 4 days at the same temperature has already exceed the legal limit (15 mg/kg).

It might be more useful to relate the HMF limit to the pH of honey. For instance, for honeys with a pH <4, a limit of 40 mg/kg, including citrus honey, but for honeys with a pH >4 a lower limit (20–25 mg/kg). Moreover, it should be put on evidence that HMF is a *thermal index* rather than a quality index. Other variables, e.g., aroma profile (Verzera, Zappalà, Campisi, & Bonaccorsi, 2001) or sensory analysis, should be introduced in evaluating honey quality.

4. Conclusions

This study has substantiated that:

- the HMF concentration in honey is also related to honey composition (pH, acidity) at heating temperatures below 50 °C;
- HMF as a quality index of chestnut honey is inappropriate or, in any case, too high;
- 15 mg/kg of HMF for Orange honey is too low; 40 mg/kg as a limit would be much more appropriated; and
- the HMF limit in honey should be related to the initial honey pH.

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