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Spectrophotometric Assessment of 5-HMF in Thermally Treated Honey: Analytical Performance Comparison of Classical and Modified Methods

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Abstract: To ensure high-quality honey, and to delay crystallization or fermentation, honey is subjected to heat treatment. The heating process, as well as the duration and storage conditions, can lead to an increased level of 5-HMF (5-hydroxymethylfurfural), which exhibits numerous adverse effects on human health. The objective of the present study was to evaluate the evolution of 5-HMF levels in different varieties of honey from Romania, thermally treated at different temperatures between 45 and 75 °C. Both classical spectrophotometric methods, such as White and Winkler methods, and two modified simpler methods were used. The methods were compared based on their analytical reactions and analytical parameters such as linearity, LOD and LOQ, sensitivity, accuracy, and precision. The best-performing method was the one that employed thiobarbituric acid. The level of 5-HMF in the control samples and samples treated at 45 °C are below the limits accepted by the legislation (40 mg/kg). At higher temperatures, such as 55–65 °C, the values in some honey species, especially polyfloral species, already exceed the accepted threshold (57.5 ± 0.7 mg/kg; 98.8 ± 4.6 mg/kg) whereas at 75 °C, the 5-HMF level is very high (142.8 ± 8.7 mg/kg; 453.8 ± 51.3 mg/kg). The results obtained indicate that 5-HMF level increases gradually with temperature and is variety dependent.

Keywords: 5-HMF; honey; heat treatment; spectrophotometry; thiobarbituric acid; analytical performance; limit of detection

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1. Introduction

Honey is one of the most well-known and widely used natural foods, with a very long history, being used since antiquity due to its nutritional and therapeutic values. It is produced by honeybees (*Apis mellifera* L.) from flower nectar or honeydew, which are stored and transformed over time in the crop, mixed with bee saliva to mature. Bees use nectar as the raw material for honey production, which in turn affects the composition of the final product [1].

Among the disaccharides in honey, sucrose is the most encountered, and it is also an important indicator of honey quality. A high sucrose content may indicate that syrups or cane sugar have been added to the honey during harvesting or preparation [2,3]. The composition of honey is quite variable and primarily depends on the botanical origin, the flower source, as well as other factors, such as temperature, season, environmental factors, the physiological state of the bees, honey handling and processing, and storage conditions [4]. The main phenolic compounds in honey, such as phenolic acids and their derivatives, flavonoids, confer honey its antioxidant and antibacterial properties. As for

volatile compounds in honey, the most significant are organic acids, alcohol, hydrocarbons, ketones, terpenes, furan or pyran derivatives, aromatic compounds, and aldehydes [5].

To ensure high-quality honey, maintain its freshness, extend its shelf life, delay crystallization or fermentation, and reduce its viscosity, it is usually processed by heating or sterilization. This process can also lead to the formation of undesirable tastes and aromas, resulting in the loss of nutritional value and sensory quality of the food. Additionally, compounds that are not normally present in freshly harvested honey may develop during the heating, storage, or preservation processes, revealing mutagenic, carcinogenic, and cytotoxic effects, such as heterocyclic amines, nitrosamines, and aromatic hydrocarbons. These compounds are called neoformed contaminants. Among such compounds, 5-hydroxymethylfurfural (5-HMF) has drawn the attention of the scientific community. It has been shown that 5-HMF can be carcinogenic, hepatotoxic, nephrotoxic, and irritating to the eyes and respiratory tract if present above permissible limits [6–8].

Considering all of these aspects, reducing the 5-HMF content in honey and, subsequently, the risks it poses to consumers, have become important trends in assuring the safety of honey, as well as that of other foods where 5-HMF may appear [6,7]. The 5-HMF remains constant during the transient phase in honey, while an increase in 5-HMF content occurs in the isothermal phase subjected for a given period, indicating that heat treatment during the transient phase is less harmful to honey, but high temperatures for longer periods should be avoided [9]. The formation of 5-HMF is interdependently related to chemical characteristics such as pH, lactone content, mineral content, free acid content, and total acidity, which in turn are linked to the floral source of the collected honey samples [10]. To date, the establishment of limits for the intake of 5-HMF in foods has only been focused on a few products, so it is very important to develop analytical monitoring techniques for 5-HMF in foods that can lead to its quantification for sustainable food safety and security [11].

The Codex Alimentarius Commission has set the maximum admissible concentration of 5-HMF in honey after processing to be less than 80 mg/kg, as opposed to the European Union (2002) recommendation, which sets an even lower limit, of 40 mg/kg: 80 mg/kg for honey originating from tropical countries or regions, while only a limit of 15 mg/kg is allowed for honey with low enzymatic levels [12].

Risk assessment for 5-HMF needs to be continuously improved, as it is a newly formed contaminant, present in a wide variety of foods, and consumed by humans on a daily basis. Especially in the case of foods with therapeutic or medicinal roles, monitoring, detection, and determination of these compounds, are crucial as they can be potentially harmful, unhealthy, and toxic to the human body. The International Honey Commission (IHC) recommends three methods for determining 5-HMF. These methods include two spectrophotometric methods, widely used in the routine analysis: the White spectrophotometric method (which is based on comparing the absorption at 284 nm and 336 nm, and using sodium bisulfite to eliminate background interference due to different aldehydes that may be present in honey, depending on its floral origin, or products that occur during storage and conditioning) [13,14] and the Winkler spectrophotometric method (based on measuring the absorbance of a red complex formed with *p*-toluidine, at 550 nm, or with the less toxic *p*-aminobenzoic acid, at 384 nm), as well as a chromatographic method, namely, high-performance liquid chromatography (HPLC) [15,16]. The classical White method involves measuring the UV absorbance of honey solutions, with and without the presence of bisulfite, while the classical Winkler method involves measuring the absorbance of honey solutions in the visible range, with *p*-toluidine and barbituric acid reagent. Regarding the HPLC method, honey is simply dissolved in water and, after filtration, 5-HMF is determined on an HPLC column with reversed phase elution with water and methanol as the

mobile phase. The HPLC method has the advantage of separating 5-HMF from other components, thus avoiding interference during determination.

A more recent spectrophotometric method for the determination of 5-HMF has been proposed, namely the qualitative Seliwanoff test, a quantitative determination through the reaction of 5-HMF with the Seliwanoff reagent, resulting in the formation of a red color [17]. A modified Winkler method has also been described, using flow injection analysis (FIA), which is more environmentally friendly, using *p*-aminobenzoic acid instead of *p*-toluidine reagent. The method is an efficient and environmentally friendly technique for analyzing 5-HMF in honey [15]. However, several studies have shown that the White and Winkler methods, which are also spectrophotometric methods, are rapid but have low specificity and sensitivity, and these spectral methods may be subjected to interferences from other compounds present in complex matrices [13]. Chromatographic methods are often used for quantifying 5-HMF in honey, providing higher precision results, being simpler, less time-consuming methods of determination, with higher sensitivities [18,19]. Such methods also include HPTLC methods [20,21].

The aim of this study was to modify and verify the spectrophotometric methods for 5-HMF determination, then compare the results, and finally determine 5-HMF in a real matrix, in the form of untreated and heat-treated honey.

Therefore, by monitoring the 5-HMF level at different temperatures, the optimal heat-treatment temperature for each honey type can be determined, as well as the temperature at which the 5-HMF level may exceed the accepted limits.

2. Materials and Methods

2.1. Chemicals

5-(Hydroxymethyl)furfural (98%) was purchased from Acros Organics and stored at 2–8 °C. Reagents such as *p*-toluidine, *p*-amino-benzoic acid, and thiobarbituric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and barbituric acid, 99% was acquired from ThermoScientific (Waltham, MA, USA). The salts for Carrez I solution ($K_4[Fe(CN)_6]$) and Carrez II solution ($Zn(CH_3COO)_2$) were obtained from Reactivul București (Bucharest, Romania). The sodium sulfite solution was prepared from the heptahydrate salt obtained from Reactivul București (Bucharest, Romania). Buffer solutions used in the Winkler method (optimal pH = 2.55) were prepared from a universal, Britton-Robinson buffer solution, (0.1 M boric acid; 0.1 M phosphoric acid, and 0.1 M acetic acid), starting from pH = 1.70, followed by the addition of NaOH solution (20%). pH adjustment for the White method (optimal pH = 4.5) was conducted with a buffer solution of acetic acid/sodium acetate, 0.1 M, purchased from VWR. Ultrapure water was used for preparing the solutions.

2.2. Instrumentation

For spectroscopic measurements, a nanospectrophotometer (NP80, Implen GmbH, Munich, Germany), was used. Sample solutions were measured in plastic microcuvettes. Additionally, for high-throughput measurements, a plate reader (Spark 10M, Tecan Group Ltd., Männedorf, Switzerland), was employed. In this case, the samples were measured in Eppendorf microplates with 96 wells. For sample preparation, an analytical balance, micropipettes, Eppendorf tubes, centrifuge tubes, a Vortex mixer, a centrifuge, and an oven for heat treatment were used. A pH meter was used for pH adjustment and optimization.

2.3. Preparation of Standard Solutions and Analytical Reagents

For the stock solution of 5-(hydroxymethyl)furfural, 14.35 mg of HMF was weighed and dissolved in deionized water in a 25 mL volumetric flask, resulting in a stock solution of 11.37 mM. Successive dilutions of tenfold (1.14 mM) and one hundred-fold (0.11 mM) from

the stock solution were prepared daily for analysis. For the Winkler reagents, *p*-toluidine was prepared by weighing 1.0 g, dissolving it in 5 mL of DMSO, followed by the addition of 1 mL of glacial acetic acid, and bringing the solution to volume with 10 mL of DMSO, resulting in a stock solution of 933 mM. The *p*-amino-benzoic acid solution was prepared in the same manner, resulting in a stock solution of 933 mM. The barbituric acid solution was prepared by weighing 0.2500 g, which was dissolved in 50 mL of deionized water, resulting in a stock solution of 39 mM. The reagent used in the modified Winkler method, thiobarbituric acid, was prepared by weighing 0.2812 g, which was dissolved in 50 mL of deionized water, resulting in a stock solution of 39 mM. For the White method, the sodium sulfite solution (0.8% *w*/*v*) was freshly prepared daily by weighing 0.4 g, which was dissolved in deionized water to a final volume of 50 mL. For preparing the Carrez I solution ($K_4[Fe(CN)_6]$), 7.5 g of potassium ferrocyanide was weighed and dissolved in 50 mL of deionized water, and for the Carrez II solution ($Zn(CH_3COO)_2$), 10 g of zinc acetate was weighed and dissolved in 33.3 mL of deionized water.

2.4. Honey Samples, Sample Preparation, and Heat Treatment

A total of 15 honey samples were purchased from beekeepers in 3 different regions of Romania. The honey samples were collected in the summer of 2022, except for one honey sample, which was collected in the summer of 2021. Immediately after collection, the samples were transferred to sterile storage containers and kept at 2–4 °C to prevent 5-HMF formation. The honey variety, sampling location, and collection date, as well as the sample codes, are listed in Table 1. For the heat-treatment experiment, honey samples were divided into five groups corresponding to the temperature at which they were treated, in 15 mL polypropylene tubes. These groups included the control group (kept at 4 °C), and groups treated at 45 °C, 55 °C, 65 °C, and 75 °C. A known mass of 2 g—with a 4 decimal precision—of each type of honey was weighed and subjected to heat treatment in an oven for 24 h. After heat treatment, the samples needed to be prepared for analysis. For this, untreated and heat-treated honey types were dissolved in 5 mL of deionized water. Then, 250 µL of Carrez I solution was added, and the samples were vortexed, followed by the addition of 250 µL of Carrez II solution. The volume was adjusted to 10 mL, and the samples were thoroughly mixed. The samples were centrifuged at 1500 rpm for 4 min to prepare them for analysis.

Table 1. Types of honey, their geographical and floral origin, harvest dates, and sample code.

Sample Number	Variety of Honey	Sampling Location (Location-County)	Harvest Date	Sample Code
1.	Acacia	Sanislău, Satu-Mare	June 2022	S1
2.	Acacia	Dioșod, Sălaj	June 2022	S2
3.	Acacia	Salva, Bistrița-Năsăud	May 2022	S3
4.	Acacia	Deja, Sălaj	May 2022	S4
5.	Honeydew	Panic, Sălaj	June 2022	M1
6.	Honeydew	Verveghiu, Sălaj	June, 2022	M2
7.	Honeydew	Romuli, Bistrița-Năsăud	August 2022	M3
8.	Honeydew	Dioșod, Sălaj	July 2022	M4
9.	Polyfloral	Dioșod, Sălaj	July 2022	P1
10.	Polyfloral	Panic, Sălaj	July 2022	P2
11.	Polyfloral	Romuli, Bistrița-Năsăud	August 2021	P3
12.	Rapeseed	Deja, Sălaj	April 2022	R1
13.	Rapeseed	Dioșod, Sălaj	May 2022	R2
14.	Sunflower	Deja, Sălaj	July 2022	FS1
15.	Sunflower	Dioșod, Sălaj	August 2022	FS2

2.5. Initial Optimization of the Assays

For optimizing the pH in the White method, a spectrophotometric test was conducted in plastic cuvettes. Different buffer solutions were added in decreasing pH order: pH = 9.05; 8.03; 7.04; 6.03; 5.01; 4.03; 3.53; 2.05. Then, 20 μL of HMF (1.14 mM) was added, followed by 80 μL of bisulfite solution, and the absorbance was measured at 284 nm. A reference solution was also prepared by adding 230 μL of deionized water and 20 μL of HMF (1.14 mM). For the Winkler method, preliminary tests were conducted first to observe the absorption wavelength of barbituric acid and thiobarbituric acid. Spectrophotometric tests were performed similarly. Initially, 900 μL of water, 100 μL of HMF (1.14 mM), 100 μL of *p*-toluidine reagent, and 20 μL of barbituric acid were added to a plastic cuvette, and absorbance was measured at 550 nm and 400 nm. Another test was conducted with only barbituric acid, adding 40 μL of HMF (0.11 mM), 80 μL of water, 20 μL of HCl (1 M), and 40 μL of barbituric acid, and measuring the absorbance at 395 nm. Thiobarbituric acid was tested similarly, with absorbance values measured at 360 nm and 426 nm. To optimize the pH, a test was performed in an Eppendorf microplate reader with 96 wells. Buffer solutions were added (pH = 1.70; 2.12; 2.55; 3.06; 3.52; 4.11; 4.54; 5.02; 5.68; 6.19; 6.86; 7.20), followed by 80 μL of HMF (0.11 mM) and 80 μL of barbituric acid or thiobarbituric acid. In the last wells, remaining buffer solutions were added (pH = 7.76; 8.44; 9.36; 10.78), along with 20 μL of HMF (1.14 mM) and 170 μL of bisulfite solution. After optimizing the pH, the pH values of the honey samples were measured three times, after dissolving them in 5 mL of deionized water.

2.6. Construction of the Calibration Curves for the Employed Methods

For the analysis of honey samples, five spectrophotometric methods were tested. The White method, described in the literature, utilizes Carrez I and II solutions, and bisulfite solution for analysis. Sodium acetate buffer, with an optimal pH of 4.5, was used in this case, and absorbance values were read at 284 nm. The classic Winkler method, also found in the literature, uses a *p*-toluidine solution. The bands appeared at 550 nm, explaining the red coloration. The only disadvantage of this method is the use of *p*-toluidine reagent, which is toxic and carcinogenic. Therefore, a modified Winkler method was proposed, using *p*-aminobenzoic acid reagent instead of *p*-toluidine, with absorbance measured at 384 nm. No significant absorption was observed at 550 nm in this case. Two other simpler methods were proposed. The first method uses only barbituric acid (BA method), while the second method uses only thiobarbituric acid (TBA method). The reaction product obtained after treating 5-HMF with barbituric acid absorbs at 395 nm, while after treatment with thiobarbituric acid, the product absorbs at two different wavelengths, 360 nm, and 426 nm. Yellow coloration was observed during the reaction for both methods. The reaction to thiobarbituric acid is preferred because the compound formed with HMF is much more intensely colored than the one formed with barbituric acid, which is less sensitive. Our preliminary tests indicate that in the method with barbituric acid, there was a 30-min incubation time after adding the acid, while in the case of thiobarbituric acid, there was a 15-min incubation time. Before working with real samples, calibration was performed for each method.

2.7. Analytical Procedure for Honey Samples Analysis

For the analysis of real honey samples, the White method and the simpler proposed method with thiobarbituric acid, as well as the classic Winkler method, were used. An Eppendorf microplate with 96 wells was used for measurements. In the case of the White method, 100 μL of each sample (both control and heat-treated samples) was taken, to which 125 μL of buffer (pH = 4.5) and 74 μL of bisulfite solution (0.8%) were added.

Two sets of measurements were made for each sample. The first set with the reference solution, water, and the second set with the sulfite solution. Absorbance was measured at 284 nm. For the proposed method with thiobarbituric acid (both control and heat-treated samples), 60 µL of the sample, 200 µL of HCl (1 M), and 80 µL of thiobarbituric acid were added. Before adding thiobarbituric acid, there was an incubation time of 15 min. Two sets of measurements were also made in this case. Absorbance was measured at 426 nm. The applied workflow steps are presented in Figure 1. To determine accuracy, a spiking experiment was conducted, which involved adding a known amount of 5-HMF to a honey sample and then checking if the added quantity was recovered in the sample. Accuracy is assessed by calculating the recovery rate (expressed as a percentage). For this purpose, three untreated honey samples with different concentrations of HMF were selected. Specifically, Sample 1 (P2-Polyfloral-Panic) with the highest measured concentration of 5.3 µg/mL, Sample 4 (FS1-Sunflower-Deja) with an average concentration of 2.7 µg/mL, and Sample 12 (R1-Rapeseed-Deja) with the lowest measured concentration of 0.71 µg/mL were chosen. Standard additions were made at three different concentrations: 0.5, 2, and 5 µg/mL. The stock solution of HMF had a concentration of 1435 µg/mL. First, a series of samples without addition (reference samples) and standard samples were prepared by adding 1 mL of water to three tubes, followed by the additions, i.e., the calculated concentrations of HMF. In the first tube, diluted HMF (1.14 mM) was added because the concentrations were too low, and errors may occur, so the sample must be diluted. After preparing the spiked solutions, the White, Winkler, and TBA methods were applied to determine the accuracy.

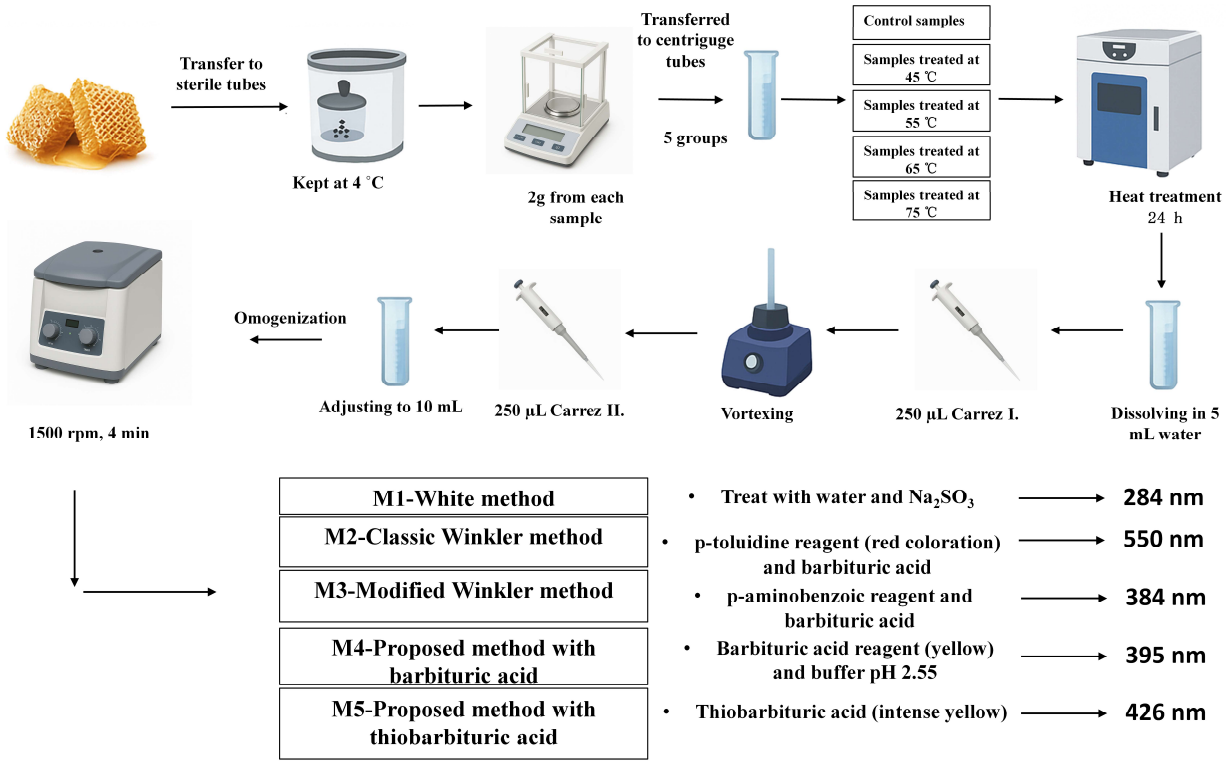


Figure 1. Experimental steps for 5-HMF assessment in honey using the five employed spectrophotometric methods.

For measurements, Eppendorf microtiter plates with 96 wells were used. Moreover, 100 µL of spiked samples (from Standard I, Addition I, Addition II, Addition III), 125 µL of buffer (pH = 4.5), and 75 µL of bisulfite solution were added for one set of measurements, while for the other set, water was added instead of sulfite. The samples were measured in duplicate. This experiment was also applied in the case of the proposed method with

thiobarbituric acid. Notably, 60 µL of spiked sample, 200 µL of HCl (1 M), and 80 µL of thiobarbituric acid were added. The incubation time before measurement was 15 min. The samples were measured in duplicate. The experiment was also applied in the case of the classic Winkler method, where 74 µL from spiked samples, 188 µL of Winkler reagent, and 38 µL of barbituric acid were added. For the reference, Standard I, II, and III, and water were added in this case.

2.8. Statistical Analysis

To validate an analytical method, certain parameters and characteristics of method performance need to be evaluated, such as linearity, limit of detection (LOD), limit of quantification (LOQ), sensitivity, precision, and accuracy. To determine linearity, a calibration curve needs to be constructed. Linear correlation analysis at a confidence level of 95% can be used for determination. The limit of detection and limit of quantification are calculated from the calibration curve. In this process, the equations (3·SD)/m and (10·SD)/m are used, where m represents the slope of the curve, and SD is the standard deviation of the residuals from the calibration curve. The sensitivity of a measurement process characterizes the change in the analytical response produced by the analytical instrument, reported to the variation in concentration of the component in the final sample. It can be determined from the calibration curve, representing the slope of the line. Precision is evaluated by repeatability, which involves preparing three samples from each method and analyzing them on the same day. The relative standard deviation (RSD%) is calculated to estimate repeatability. For accuracy evaluation, recovery tests must be performed, and the recovery degree is calculated. The spiking method is used, where a known amount of 5-HMF is added to a sample to assess if it can be detected in that sample. To evaluate the accuracy, the recovery degree (%) is calculated [22,23].

3. Results and Discussion

3.1. Investigation of the Performances of the White Method for 5-HMF Determination

The first method used in this study is the classical White method, which is based on the reaction with sodium sulfite, whose absorbance is measured at 284 nm. In this method, two series of measurements are made for each honey sample, one based on dissolving the sample in a reference solution, which is water, and the other after mixing with sodium sulfite solution. Before using the spectrophotometric methods, pH optimization is necessary to obtain the ideal range for measurements and to assess whether the pH range influences the measurements. The percentage of the consumed HMF as a function of pH is presented in Figure 2, alongside the distribution diagram of the bisulfite species. In the graph presented in Figure 2, the black dots represent the percentage of 5-HMF consumed by sulfite, which overlaps very well with the red dots, calculated theoretically with Curtiplot, representing the degree of ionization of sulfurous acid, αHSO_3^-, which is the fraction of the monoprotonated species, HSO_3^-. Thus, there are three species, SO_3^{2-}, HSO_3^-, and H_2SO_3, one completely deprotonated, one monoprotonated, and one completely protonated. The distribution curve of the monoprotonated species overlaps very well with the percentage of 5-HMF consumed, indicating that this species reacts with 5-HMF. The data also indicate that the optimal pH for the White method is within the 3.5–5.0 pH interval.

As can be observed in Figure 3, the UV molecular absorption spectra of 5-HMF in the aqueous solution, before the addition of sulfite, present a band centered on 284 nm. When sulfite is added, which reacts with 5-HMF, the absorbance decreases for each species, indicating that a reaction occurs between the two compounds. The calibration curve has a good coefficient of determination, close to the value of 1, $R^2 = 0.9986$, indicating that the line fits very well with the experimental data.

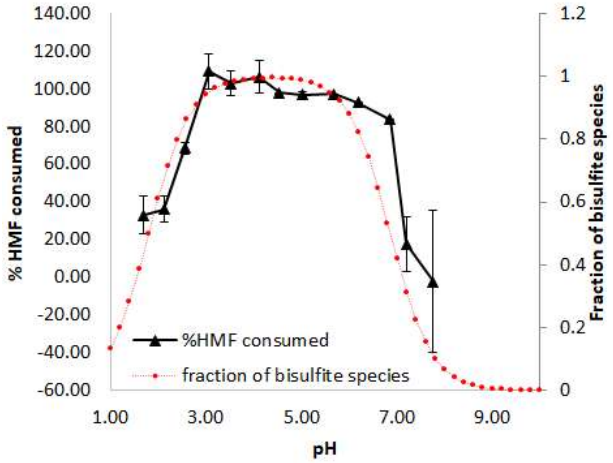


Figure 2. Percentage of 5-HMF consumed as a function of pH superimposed on the distribution diagram of the bisulfite species.

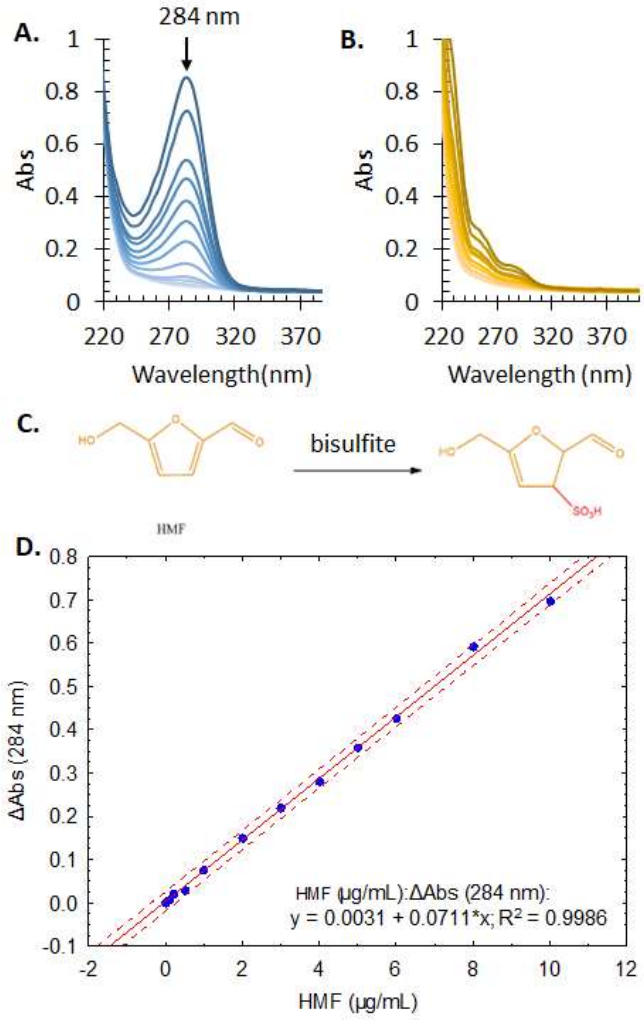


Figure 3. White method for 5-HMF determination in honey. UV molecular absorption spectra of the 5-HMF standards before (**A**) and after (**B**) reaction with bisulfite. (**C**) Reaction of 5-HMF with bisulfite anion. (**D**) Calibration curve for the White method, ΔAbs at 284 nm (before–after) as function of 5-HMF concentration. The signed * in the equation of the regression line stands for multiplication signed. The dashed red line represents the confidence bands for regression line (red continuous line) at the probability of 95% and the blue circle are the measured mean values.

3.2. Investigation of the Performances of the Classic Winkler Method for 5-HMF Determination

Since in the White method measurements are performed in the UV region, at 284 nm, there may be compounds that can hinder the sensibility of the method, especially in honey samples, which are rich in various aromatic compounds. Instead of the White method, the classical Winkler method is more often used. This method uses *p*-toluidine and barbituric acid as analytical reagents. In this case, as well, two sets of measurements are made: one with the reference solution, water, and *p*-toluidine, and the other with the barbituric acid solution and *p*-toluidine. The absorbance is measured at 550 nm, because of the appearance of a red coloration. The UV-vis spectra of the colored compound alongside the reaction and calibration curve are presented in Figure 4. From these data, one can observe the variation in absorbance, i.e., the increase in absorbance of 5-HMF in the presence of barbituric acid and *p*-toluidine, which is caused by the appearance of the red coloration resulting from the reaction of 5-HMF with *p*-toluidine. The absorbance is measured at 550 nm, and the coefficient of determination in this case is slightly lower than that of the White method, $R^2 = 0.9976$, but still acceptable. A nonlinearity behavior is also observed in the 0–10 µg/mL working domain.

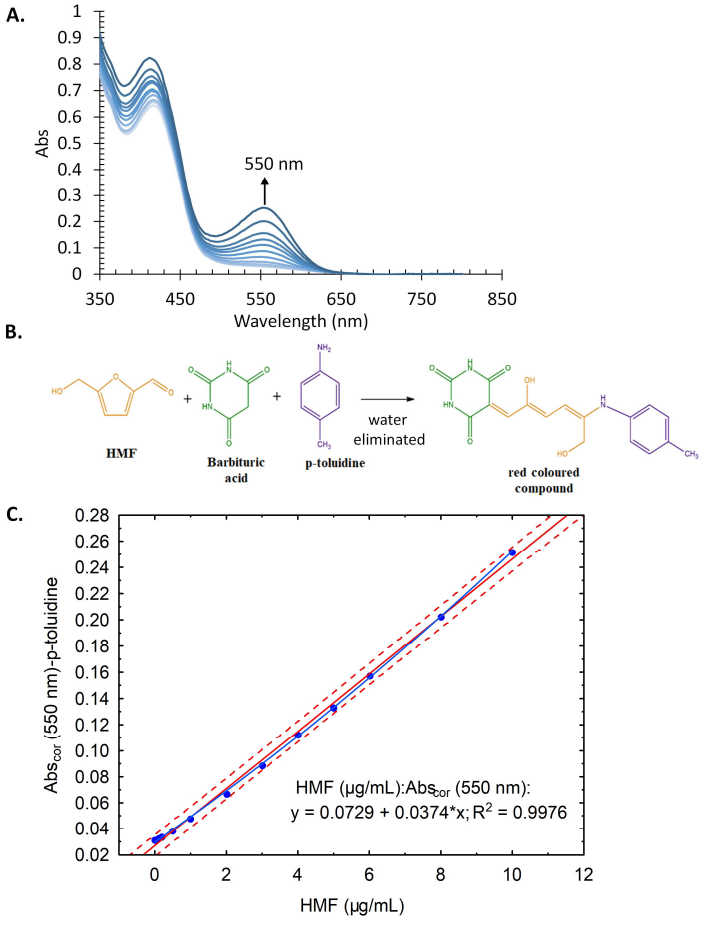


Figure 4. Classical Winkler method for 5-HMF determination in honey. (**A**) UV-Vis molecular absorption spectra of the red compound (550 nm) obtained during the reaction (**B**) of 5-HMF standards with barbituric acid and *p*-toluidine. (**C**) Calibration curve for the classical Winkler method, Abs at 550 nm as function of 5-HMF concentration. A linear fitting curve (red) and a nonlinearity (blue curve) are indicated. The signed * in the equation of the regression line stands for multiplication signed. The dashed red line represents the confidence bands for regression line (red continuous line) at the probability of 95% and the blue circle are the measured mean values.

3.3. Investigation of the Performance of the Modified Winkler Method for 5-HMF Determination

Since the classical Winkler method uses *p*-toluidine, which is a highly toxic, carcinogenic agent for the human body, a modified Winkler method has been proposed as presented in the introductory section. In this modified method, *p*-amino-benzoic acid is used instead of *p*-toluidine, which is a much more environmentally friendly, non-toxic alternative, and, barbituric acid is still present. The absorbance is observed in this case at 384 nm, and nothing appears at 550 nm.

The UV molecular absorption spectra, the analytical reaction, and the calibration curve data are presented in Figure 5. The absorbance is measured at 384 nm, and the coefficient of determination in this case is almost the same as that of the classical Winkler method, $R^2 = 0.9975$. The modified Winkler method is not very efficient, as the blank, the reagent itself presents high absorbance values, and the wavelengths are in the ultraviolet range, affecting the sensitivity and the linearity. The reproducibility of the method is also limited.

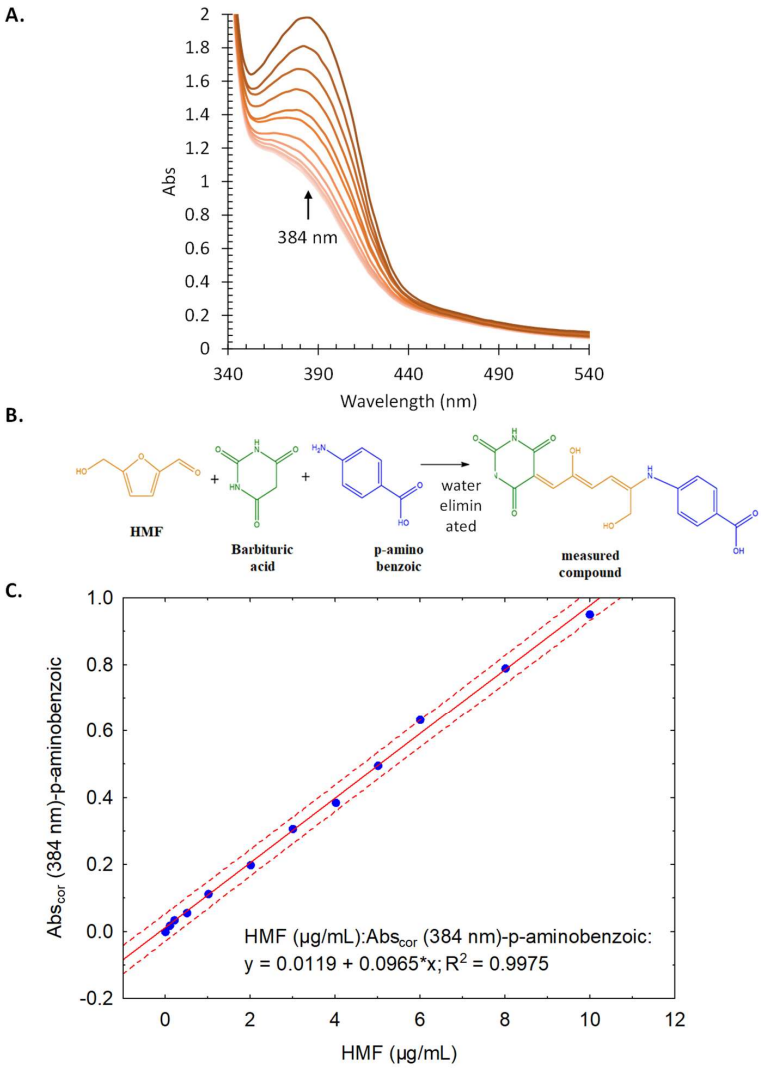


Figure 5. Modified Winkler method for 5-HMF determination in honey. (**A**) UV molecular absorption spectra of the detected compound (384 nm) obtained by the reaction (**B**) of 5-HMF standards with barbituric acid and *p*-aminobenzoic acid. (**C**) Calibration curve for the modified Winkler method, corrected absorbance at 384 nm (Abs after blank signal subtraction) as a function of 5-HMF concentration. The signed * in the equation of the regression line stands for multiplication signed. The dashed red line represents the confidence bands for regression line (red continuous line) at the probability of 95% and the blue circle are the measured mean values.

3.4. Investigation of the Performances of the Simplified Methods for 5-HMF Determination

A simplified method has been proposed here, which uses only the reagent, barbituric acid (BA method). In this case, the absorbance is measured at 395 nm, which is caused by the appearance of a yellow coloration resulting from the reaction of 5-HMF with barbituric acid. The reaction is depicted in Figure 6.

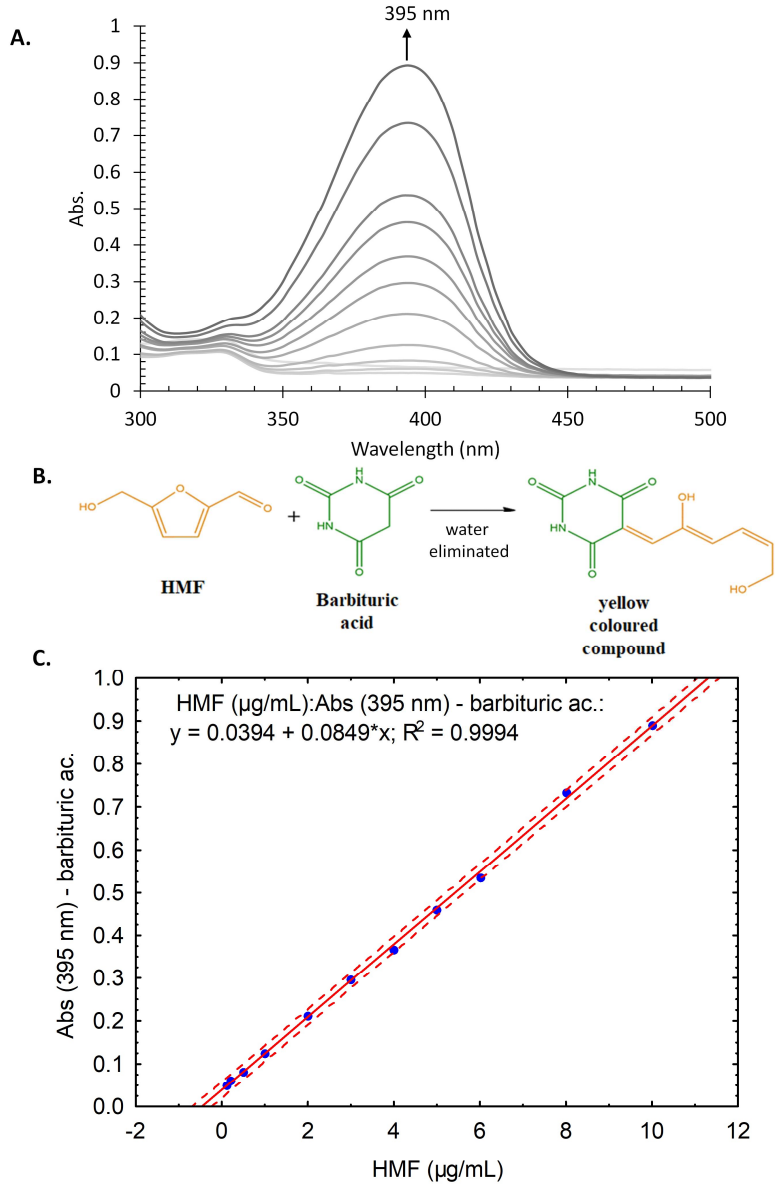


Figure 6. Simplified method for 5-HMF determination in honey. (**A**) UV molecular absorption spectra of the detected compound (395 nm) obtained by the reaction (**B**) of 5-HMF standards with barbituric acid only. (**C**) Calibration curve for the simplified method, absorbance at 395 nm as a function of 5-HMF concentration. The signed * in the equation of the regression line stands for multiplication signed. The dashed red line represents the confidence bands for regression line (red continuous line) at the probability of 95% and the blue circle are the measured mean values.

For this method, the coefficient of determination has the highest value obtained among all of the used methods, $R^2 = 0.9994$, indicating that the newly proposed method works very well for detecting 5-HMF. One problem associated with the use of barbituric acid in this context is that it can react with other types of aldehydes as well and may give a reaction to 5-HMF. However, these types of compounds are expected to be present in real samples

in much lower concentrations than 5-HMF. Another newly proposed method makes use of thiobarbituric acid, as it has been suggested (TBA method). In this case, the absorbance is measured at 426 nm, which is caused by intense yellow coloration. The color of the complex between 5-HMF and thiobarbituric acid is much more pronounced than the one with barbituric acid, as indicated in Figure 7.

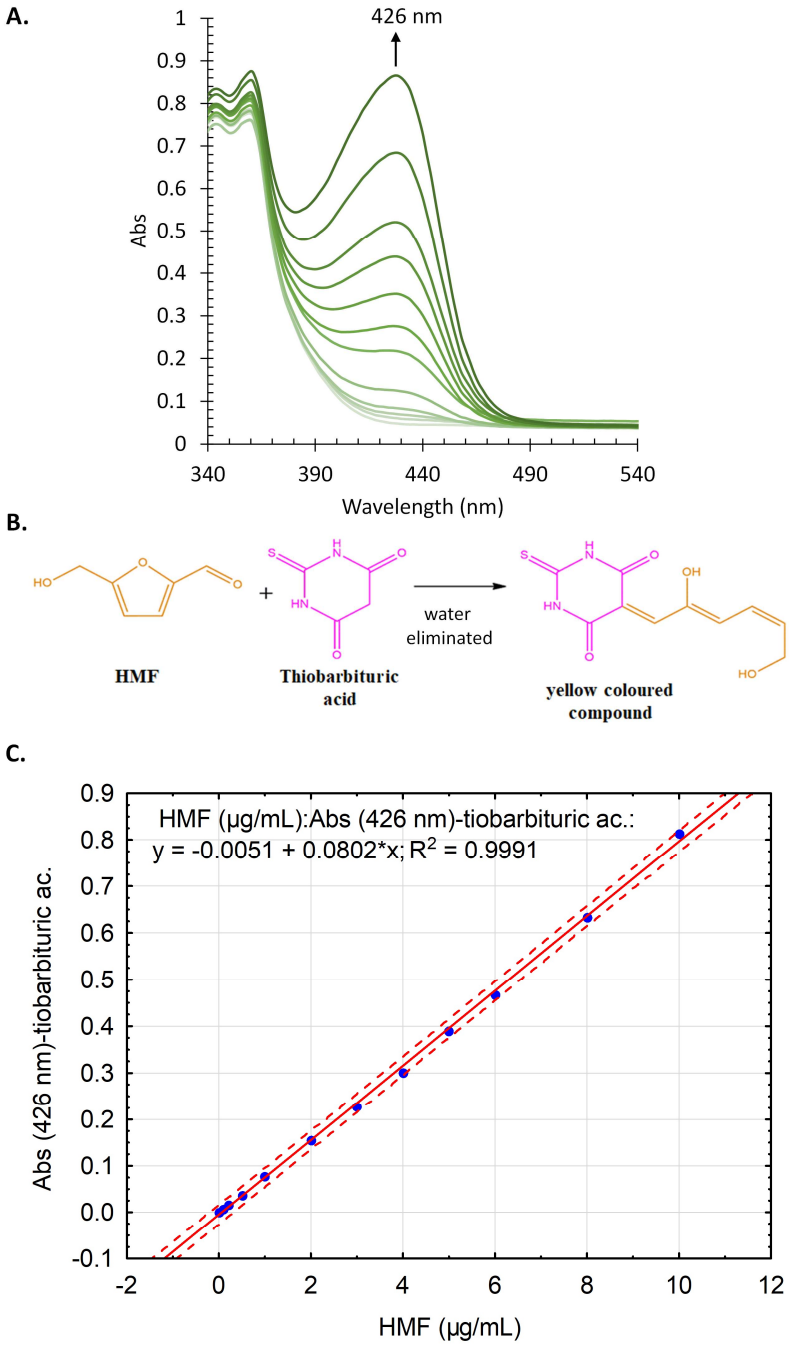


Figure 7. Simplified method for 5-HMF determination in honey. (**A**) UV molecular absorption spectra of the detected compound (426 nm) obtained by the reaction (**B**) of 5-HMF standards with thiobarbituric acid only. (**C**) Calibration curve for the simplified method, absorbance at 426 nm as a function of 5-HMF concentration. The signed * in the equation of the regression line stands for multiplication signed. The dashed red line represents the confidence bands for regression line (red continuous line) at the probability of 95% and the blue circle are the measured mean values.

As can be observed in Figure 7, the variation in absorbance, (i.e., the increase in absorbance) in the presence of thiobarbituric acid due to the reaction with 5-HMF, the former complex absorbs at a higher wavelength, 426 nm than in the case of the BA method. This could be a significant advantage since the interference of other present compounds is less pronounced as the working wavelength increases. The coefficient of determination also has a better value in this case, very close to 1, slightly lower than in the case of the method with barbituric acid, with a value of $R^2 = 0.9991$. Thus, the proposed method with thiobarbituric acid works very well for detecting 5-HMF.

3.5. Linearity, Detectability, and Accuracy Performances of the Employed Methods

The calibration curves have produced very good coefficient of determination (R^2) values, around 0.998–0.999, which are closely related to the linearity of the method. In our case, the best values were obtained with the methods proposed with barbituric acid ($R^2 = 0.9994$) and thiobarbituric acid ($R^2 = 0.9991$), but even with the methods known from the literature, the obtained values are acceptable and suitable. The calibration curve showed linearity in the concentration range of 0.5–10.5 μg/mL in the case of the method with barbituric acid, and 0–10 μg/mL in the case of the method with thiobarbituric acid. Therefore, it can be concluded that the proposed methods using barbituric acid and thiobarbituric acid are the most suitable for determining 5-HMF in honey samples, especially the method with thiobarbituric acid, where the color of the complex from the reaction with 5-HMF is more pronounced.

Within this study, the analytical parameters of the methods were determined, namely: limit of detection (LOD), limit of quantification (LOQ), precision, linearity, sensibility, and accuracy. Regarding LOD, LOQ, and sensitivity, they are summarized in Table 2. The sensibility of the method is given by the slope of the calibration curve. The best value was obtained in the case of the modified Winkler method with *p*-aminobenzoic acid (0.0965 mL/μg), but also in the cases of methods with barbituric acid (0.0849 mL/μg) and thiobarbituric acid (0.0802 mL/μg), the sensitivity is very good.

Table 2. Analytical performance characteristics of the employed analytical methods.

Method	Wavelength (nm)	R^2	Calibration Sensitivity (mL × μg^{-1})	LOD (0–1 μg/mL) In Solution (μg/mL)	LOQ (0–1 μg/mL) In Solid [1] (mg/kg)
White	284	0.9986	0.0711	0.24	6.74
Winkler-classic	550	0.9976	0.0374	0.11	3.03
Winkler-modified	384	0.9975	0.0965	0.17	4.83
BA method	395	0.9994	0.0849	0.06	1.80
TBA method	426	0.9991	0.0802	0.02	0.53

[1] Calculated when applying the exact protocol indicated in Section 2, for 2 g sample and 10 mL final volume.

Regarding LOD and LOQ, in the range of 0–1 μg/mL, higher values are presented in the case of the White method, both in μg/mL (which is the concentration in solution), and in mg/kg (in solid form). The best result appears in the case of the method with thiobarbituric acid, in the concentration range of 0–1 μg/mL, with LOD = 0.53 mg/kg and LOQ = 1.58 mg/kg, indicating that the method is very sensitive. Thus, it can be inferred that the proposed methods, alongside the classical ones, are very sensitive, and precise, with good repeatability for conducting measurements, especially the proposed method with thiobarbituric acid, which is advantageous also due to its environmental friendliness compared to, for example, the classical Winkler method, which uses a carcinogenic reagent.

Based on these previously described data, only the TBA method was chosen to be presented further. The accuracy of this method was evaluated based on the recovery of 5-HMF in honey, using the standard addition method for samples P2, FS1, and R1. In sample P2, the recovery rate is between 106 and 114%, in sample FS1 it is between 99 and 105%, and in sample R1 it is between 95 and 100%, indicating good accuracy and the absence of matrix effects. Accuracy can be affected by the sample processing procedure, matrix effects, and analyte concentration. The recovery rate should be between 80 and 120%, and all results fall within this range, indicating that the analytical methods operate with remarkable accuracy. The results are summarized in Table 3.

Table 3. Accuracy testing in real honey samples by spiking, using the TBA method.

Sample	Added (µg/mL)	Found (µg/mL)	Recovery Rate (%)
Sample P2	0.00	1.49	-
	0.50	2.06	114.47
	2.00	3.62	106.58
	5.00	7.06	111.45
Sample FS1	0.00	0.26	-
	0.50	0.77	102.63
	2.00	2.24	99.01
	5.00	5.56	105.92
Sample R1	0.00	0.09	-
	0.50	0.58	97.79
	2.00	2.00	95.5
	5.00	5.09	100.04

3.6. Determination of 5-HMF in Real Honey Samples Using the TBA Method

Table 4 presents the results obtained from the TBA method, which is the most efficient of the tested methods, for each honey species, both before and after thermal treatment at different temperatures.

Table 4. Content of 5-HMF (mg/kg) in analyzed honey samples using the TBA method.

	Untreated Honey Samples		Samples Treated at 45 °C		Samples Treated at 55 °C		Samples Treated at 65 °C		Samples Treated at 75 °C	
Sample Codes	HMF ± CI [1]	RSD [2] (%)	HMF ± CI	RSD (%)	HMF ± CI	RSD (%)	HMF ± CI	RSD (%)	HMF ± CI	RSD (%)
S1	6.4 ± 0.8	8.2	7.2 ± 0.7	6.0	11.8 ± 0.5	2.8	27.6 ± 0.1	0.2	142.8 ± 8.7	3.8
S2	5.3 ± 0.2	2.8	6.5 ± 0.7	6.8	9.0 ± 0.4	2.9	24.1 ± 0.5	1.4	172.1 ± 0.3	0.1
S3	7.9 ± 0.4	3.6	8.7 ± 0.1	0.6	13.6 ± 0.2	0.9	26.6 ± 3.2	7.6	201.3 ± 3.3	1.0
S4	10.7 ± 1.1	6.4	11.5 ± 1.3	7.0	15.5 ± 0.5	2.0	35.2 ± 1.0	1.9	197.4 ± 8.4	2.7
M1	15.4 ± 1.2	4.8	17.4 ± 0.3	1.0	25.1 ± 0.4	0.9	49.3 ± 0.2	0.2	236.8 ± 0.1	0.0
M2	6.8 ± 0.6	5.1	9.0 ± 0.1	0.9	13.6 ± 0.2	0.2	36.1 ± 2.0	3.5	256.4 ± 3.1	0.8
M3	11.1 ± 0.1	0.1	12.2 ± 0.7	3.5	17.7 ± 0.4	1.5	38.4 ± 4.2	7.0	163.4 ± 25.2	9.7
M4	8.5 ± 0.3	1.9	7.4 ± 0.7	5.7	11.8 ± 0.2	1.5	27.0 ± 2.1	4.9	195.0 ± 3.5	1.1
P1	8.4 ± 0.4	3.3	10.4 ± 0.5	2.9	14.2 ± 0.0	0.2	30.6 ± 2.7	5.6	169.8 ± 3.1	1.1
P2	25.2 ± 1.4	3.6	34.5 ± 2.7	5.0	57.5 ± 0.7	0.7	98.8 ± 4.6	2.9	453.8 ± 51.3	7.1
P3	13.9 ± 0.6	2.8	20.2 ± 0.6	1.8	22.9 ± 0.3	1.0	46.8 ± 4.5	6.0	304.2 ± 10.1	2.1
R1	3.4 ± 0.1	1.7	3.3 ± 0.6	10.8	6.6 ± 0.8	7.9	21.2 ± 1.6	4.8	163.7 ± 2.1	0.8
R2	4.3 ± 0.7	9.7	5.3 ± 0.2	2.4	8.7 ± 0.2	1.8	26.2 ± 1.4	3.5	217.6 ± 4.7	1.4
FS1	13.5 ± 0.9	4.2	16.4 ± 0.6	2.5	21.4 ± 1.0	2.9	77.1 ± 6.1	5.0	370.6 ± 26.1	4.4
FS2	11.0 ± 1.0	5.5	12.0 ± 1.2	6.1	15.4 ± 1.5	6.0	33.9 ± 4.1	7.6	373.7 ± 21.3	3.6

[1] CI—confidence interval at 95% probability, $n = 3$. [2] RSD—relative standard deviation, $n = 3$.

It can be observed that in the case of control samples, the values are below those established by the European Union or the Codex Alimentarius Commission. A more significant value appears in the case of sample P2 (25.2 ± 1.4 mg/kg), as well as in the cases of samples M1 (15.4 ± 1.2) and P3 (13.9 ± 0.6), but not significantly exceeding the accepted range. After the first thermal treatment at 45 °C, an increase in the quantity of 5-HMF in different samples can be observed. Samples M1, P2, and P3 still present higher values. In the case of sample P2, the value (34.5 ± 2.7) almost reaches the maximum permissible quantity of 40 mg/kg. Moving further to a temperature of 55 °C, a more significant increase can be observed. Sample M1 presents a quantity of 25.1 ± 0.4 mg/kg, sample P3 has a value of 22.9 ± 0.3 mg/kg, and sample P2 has a value of 57.5 ± 0.7 mg/kg, which already exceeds the accepted threshold in the literature. At a temperature of 65 °C, the increase is even more pronounced, with several samples exceeding the permissible limit, such as S4 (35.2 ± 1.0 mg/kg), M1 (49.3 ± 0.2 mg/kg), P2 (98.8 ± 4.6 mg/kg), which shows a very sharp increase, FS1 (77.1 ± 6.1 mg/kg), and sample P1 (30.6 ± 2.7 mg/kg), which presents a slower increase compared to other polyfloral species, such as acacia samples like S2 (24.1 ± 0.5 mg/kg), none of which have yet reached the threshold.

At a thermal treatment of 75 °C, the increases are very significant for each sample, exceeding the accepted threshold. The increases are much more pronounced for samples P2 (453.8 ± 51.3 mg/kg), FS2 (373.7 ± 21.3 mg/kg), FS1 (370.6 ± 26.1 mg/kg), and P3 (304.2 ± 10.1 mg/kg). The increases are less significant for acacia species, such as S1 (142.8 ± 8.7 mg/kg), compared to other honey species, especially when compared to polyfloral or honeydew varieties.

The high content of 5-HMF in polyfloral species can be explained by the fact that these species are composed of nectar from various types of flowers, with higher sugar content and a much higher degree of crystallization. Therefore, thermal treatment for these honey types is almost inevitable to prevent crystallization. In the case of honeydew samples, which are composed of plant or insect secretions, the sugar content is lower, and crystallization is rarely observed even after prolonged storage. Hence, the 5-HMF content is lower compared to polyfloral species. For unifloral species such as acacia, rapeseed, and sunflower, the increase in 5-HMF content is much slower because these samples are composed of nectar from the same flower. Therefore, crystallization in these cases is not as remarkable as in polyfloral species.

The relative standard deviation (RSD%), which is related to precision, was calculated for all honey samples both before and after thermal treatment. The obtained results show an acceptable RSD% for each sample, whether control or thermally treated, with values below 10%.

3.7. Investigations of the Relationship Between pH and 5-HMF Content

pH conditions may also be an important factor during honey storage, as it is closely related to the stability and the validity period of the honey product. The pH of each honey sample was measured three times, and the mean of these values, standard deviation, and relative standard deviation (RSD%) were calculated and presented in Figure 8. The average pH for different honey species ranged between 3.72–4.86.

The calculated RSD% values refer to the deviation of the set of scattered numbers, in this case, the pH, around the mean value. It can also be observed from the graph that the greatest variability, dispersion around the mean value, occurs in the polyfloral honey samples. In the case of honeydew species, there is also a greater difference compared to other honey species such as acacia, rapeseed, and sunflower, where the values are much closer to one another, not so different, and the variability is not as evident as in the polyfloral species. Looking at the calculated RSD% values, it is obvious that the highest values occur

in polyfloral species: specifically, the polyfloral honey from Romuli has a value of 2.40, and the polyfloral honey from Dioșod has a value of 1.46. Similarly, higher values compared to other species are also observed in the case of honeydew species, namely, the honeydew honey from Verveghiu has a value of 1.01, while the honeydew honey from Romuli has a value of 1.65. The lowest values are found in rapeseed species, with rapeseed honey from Deja having a value of 0.24, and in acacia (0.48) and sunflower species (0.48) with lower values. Therefore, it can be concluded that pH variation is most significant in polyfloral species and honeydew species.

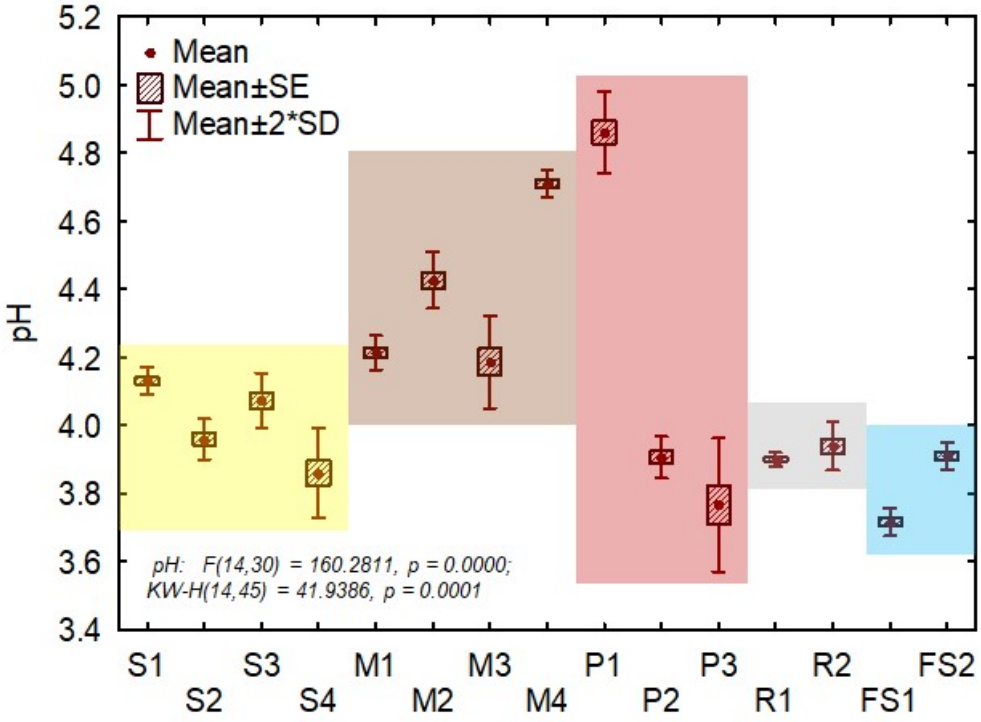


Figure 8. pH variation in the analyzed honey samples. The honey samples are color grouped according to the type of honey (see Table 1). The signed * indicated in the "2*SD" stands for multiplication sign.

When it comes to the correlation between 5-HMF content, and pH, although the literature specifies a dependency, no statistically significant correlation was observed in this study between 5-HMF concentration and the average pH value of honey samples, as the free acidity in honey is insufficient, and does not influence the concentration. The correlation coefficient presents a value of $r < 0.4$ and $p > 0.05$, indicating no significance, both in the case of control samples and thermally treated samples. It is obvious that the concentration of 5-HMF is significantly higher when the pH of honey is below 4, thus in more acidic conditions.

The formation of 5-HMF in honey is in line with 5-HMF in acid and fructose reaction systems. Therefore, it can be concluded that 5-HMF is transformed by the dehydration of fructose and glucose in honey. The formation of 5-HMF mainly occurs at low pH, and usually, no 5-HMF formation occurs at pH 7. Glucose is mainly formed at high pH values, and no glucose is detected at pH 3 or lower. Lactic acid is formed mainly at high pH values, and the formation of acetic acid can be observed at all initial pH levels. However, selectivity increases with increasing pH value. Levulinic acid is observed only at low pH. Therefore, pH value matters when discussing the quantity of 5-HMF in honey, especially in very acidic conditions, in a pH range of 2.2–3, but pH can also influence the texture, stability, and shelf life of honey.

4. Conclusions

To conclude, well-established methods such as White, Winkler as well as other simplified versions were tested for their precision, sensitivity, accuracy, and suitable linearity for 5-HMF determination. The chemistry behind all of these methods, as well as their analytical performances, are investigated in this study. The best performances in terms of linearity, LOD, and LOQ were found for the simplified thiobarbituric acid method. As expected, the study reveals that the level of 5-HMF increases with the applied temperature for the heat treatment of the honey, reaching very high values for polyfloral and sunflower types, but reduced values for the rapeseed type. No significant correlation was found between the pH of the honey and the generated 5-HMF content after heat exposure.

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