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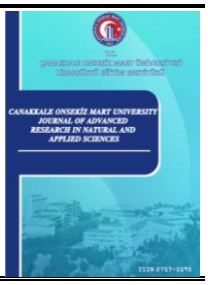

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Single Laboratory Validation of Four Methods for Quantification of HMF in Honey

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Research Article

Abstract − Hydroxymethylfurfural or 5-(hydroxymethyl)-2-furaldehyde (HMF) has been absent or found in honey naturally at very low amount. It is formed in honey mainly by heating process and improper storage conditions. HMF has been reported to have cytotoxic, carcinogenic, and mutagenic effects and thus regulatory agencies in many countries impose restrictions on its maximum levels in honey. Validated methods have been required for effective and specific detection and quantification of HMF in food samples. In this study, a single laboratory validation study was conducted on four quantification methods: direct spectral analysis, High Performance Liquid Chromatography (HPLC) analysis, Seliwanoff and Winkler methods. All methods showed linearity with the lowest R^2 value of 0.992. Two method performance parameters, accuracy, and precision were satisfied by each four methods with recovery values at 98.2%, 100.2%, 102.5% and 103.3% and *RSDr* (relative standard deviation) % values at 6.97%, 6.19%, 2.87% and 0.90% for spectral analysis, Seliwanoff, HPLC and Winkler methods, respectively. Based on the measurement uncertainties of four quantification methods, honey samples spiked with HMF at the final concentration of 0.004mg/0.1g were reported as 0.004 mg/0.1g ± 0.00025 mg/0.1g by spectral analysis, 0.0036 mg/0.1g ± 0.000691 mg/0.1g by Seliwanoff method, 0.004 mg/0.1g ± 0.00045 mg/0.1g by HPLC and 0.0039 mg/0.1g ± 0.00022 mg/0.1g by Winkler methods (k=2, confidence level of 95%). The validated methods can quantify HMF in honey with a target concentration of 0.004 mg/0.1g, specifically and accurately.

Keywords − *HMF, honey, method validation, Seliwanoff, uncertainty*

1. Introduction

An organic compound, 5-(hydroxymethyl)-2-furaldehyde (HMF), is a water soluble furanic compound produced by the dehydration reaction of sugar molecules, especially fructose and sucrose under acidic conditions. It is formed in sugar containing foods by a non-enzymatic browning reaction or dehydration of hexoses as a result of heating or improper and longer storage [1,2]. Since it is not found in fresh and untreated foods, the presence of HMF in food indicates excessive heat-treatment, spoilage, and possible adulteration of food products with other sugars or syrups. Fresh honey generally does not contain HMF while its concentration increases during heating process or storage [3]. Thus, the presence of HMF in honey is an indicative of honey quality. Previous studies have indicated the negative effects of HMF in humans and animals [4-7]. It has been reported that administration of HMF at certain concentrations lead to skin lesions or tumors in rats [8-10]. Lee et al. [11] presented the mutagenicity of HMF via sulfonation of its allylic hydroxyl functional group in rat. Monien et al. [12] showed the hepatocarcinogenic activity of HMF due to its reaction with DNA. Due to these cytotoxic, carcinogenic, and mutagenic effects of HMF, Codex Alimetarius Alinorm 01/25 [13] establish a

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maximum HMF content as 80mg/kg in honey. European Union Council Directive 2001/110/EC [14] also set the limit of 40 mg/kg HMF in honey (80 mg/kg of HMF for honey produced in tropical regions). According to the Turkish Food Codex Honey Communique 2020/7 [15], the maximum amount of HMF in honey is 40 mg/kg while originating from tropical regions is 80 mg/kg. Since HMF is a quality marker indicating improper storage, heating treatment or other adulterations in honey, quantitative analysis methods have been playing an important role for ensuring food safety. There have been different methods proposed for the quantification of HMF in literature [16-21]; however, according to the International Honey Commission (IHC) [22] and High Performance Liquid Chromatography (HPLC) [23], Winkler [24] and White [25] methods have been applicable for HMF determination in honey. HPLC methods have been validated [26] thus far and also comparative studies have been conducted with Winkler and White methods [27] to provide reliable and accurate results.

In this study, two new spectral methods were used for the quantification of HMF in honey in addition to HPLC and Winkler methods approved by IHC. Direct use of spectrophotometer at 284 nm (without any reactive reagents) provided an easy way for determination of HMF without any interference present in honey. Another spectral method, Seliwanoff method, depended on the measurement of the absorbance of colored products formed from the reaction between resorcinol and HMF. Winkler method, Seliwanof method, direct spectral measurement and HPLC analysis were compared based on validation parameters. Their responses to honey samples spiked with HMF and honey samples after thermal process were evaluated. These four methods were validated by a single laboratory validation study and all method performance parameters were found in the range of acceptable limits. Moreover, measurement uncertainties were calculated for all methods at the maximum acceptable limit of HMF allowed for honey. Thus far, this is the first study that compares the performances of four different methods for determination of HMF in honey.

2. Materials and Methods

2.1. Reaction Reagents and Solutions

The chemicals used in this study (HMF (analytical standard, ≥98.0%), furfural (≥98.0%), resorcinol (99.0%), *p*-toluidine (99.0%), barbituric acid (99.0%), fructose (≥98.0%), galactose (≥98.0%), sucrose (≥98.0%), glucose (≥98.0%),HCl, acetic acid (≥99.0%), 2-propanol (≥99.0%), and methanol (HPLC grade, ≥99.0%) were purchased from Sigma-Aldrich (St Louis, MO, USA).

HMF stock solution was prepared by dissolving 0.01 g of HMF in 1 mL of distilled water. HMF intermediate standard solutions were prepared by diluting HMF stock solution with distilled water to have final concentration of 0.001mg/g, 0.002mg/g, 0.003mg/g, 0.004mg/g, 0.005mg/g and 0.006mg/g. Both stock and intermediate standard solutions were kept at 4°C and in dark till use. Fructose solutions at 0.57 mg/mL, 5.7 mg/mL and 57 mg/mL concentrations were prepared separately in 0.1%, 0.5%and 1.0% HCl solution and heated at 80°C for 4, 7, 9, 11, and 13 min. After heat treatment, fructose solutions were measured by UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at 284 nm. The yield (Y_{HMF}) of dehydration reaction was calculated by (2.1). Galactose, glucose, and sucrose were used as negative control in selectivity analysis.

$$Y_{HMF}(\%) = \frac{\text{moles of HMF produced}}{\text{moles of initial fructose}} \times 100 \quad (2.1)$$

Seliwanoff test reagent for HMF detection, resorcinol, was prepared in 15% HCl solution to have final concentration of 5 mg/mL. Winkler test reagent, p-toluidine, was prepared in acetic acid (1 g/mL) and diluted to 100 mL with 2-propanol. Barbituric acid solution at 0.5% was used for sample solution. The reagent

solutions were kept in the dark till use.

2.2. Samples

Honey samples used in this study were taken from local markets in Turkey. They were made from flower nectar and had a Brix° value of 75. Honey samples were prepared by suspending 2.0 g of honey in 10 mL of water. For quantification of HMF, honey samples were spiked with known amount of HMF (0.001 mg/g, 0.002 mg/g, 0.003 mg/g, 0.004 mg/g, 0.005 mg/g and 0.006 mg/g) and analyzed by four methods. The honey samples without addition of HMF were used as negative controls. Moreover, honey samples were heated at 50°C, 70°C and 90°C for 10, 30, 60, 90, 120, 720 and 1440 min and further analyzed by four methods to quantify HMF in heat-treated samples.

2.3. Methods

2.3.1. Spectral Analysis

HMF intermediate standard solutions at concentrations of 0.001 mg/g, 0.002 mg/g, 0.003 mg/g, 0.004 mg/g, 0.005 mg/g and 0.006 mg/g were analyzed by UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at 284 nm. All analysis was performed in triplicate and absorbance values of all standard solutions were given in mean ± standard deviations. Based on data, spectral calibration curve was constructed, and the equation of spectral calibration curve was used in further quantification experiments of HMF.

2.3.2. HPLC Analysis

HMF intermediate standard solutions and dehydration products at concentrations of 0.001 mg/g, 0.002 mg/g, 0.003 mg/g, 0.004 mg/g, 0.005 mg/g and 0.006 mg/g were analyzed by HPLC (Agilent 1260 Infinity, Agilent Technologies, USA) configured with 1260 Infinity II Quaternary pump, standard autosampler and degasser using a method proposed by Elmastaş et al. [28]. C18 column (250 mm x 4.6 mm, 5 µm particles) was used with DAD detector for quantification of HMF at 285 nm. Samples were injected through the column at the flow rate of 1.0 mL/min at 25°C with mobile phase of methanol: water (10:90, v:v). Honey samples were homogenized in water and filtered through a 0.45 µm filter before HPLC analysis. Each sample was analyzed in triplicate.

2.3.3. Winkler Method

Winkler method is another spectrophotometric method used for HMF analysis in honey samples. The determination of HMF by Winkler protocols depends on the measurement of absorbance values of test samples and reference solutions at 550 nm [24]. For test samples, 500 µL of both standard HMF and dehydration products were mixed separately with 500 µL of *p*-toluidine solution and 100 µL of barbituric acid solution, while, for reference samples, 100 µL of water was added instead of barbituric acid to *p*-toluidine: sample (1:1) mixture. The absorbance of sample solutions was measured against reference solutions at 550 nm. The calibration curves were constructed both for standard HMF solutions and dehydration products. For both spiked and heated honey samples, 10 g of each sample was suspended in 20 mL of water and then the same procedure explained above was applied. The amount of HMF in honey samples were obtained from the equation of calibration curve.

2.3.4. Seliwanoff Method

The third spectrophotometric method for determination of HMF was based on Seliwanoff analysis. The reaction parameters were optimized by evaluating the effects of resorcinol concentration and heating time. The resorcinol solution at final concentrations of 2.5 mg/mL, 3.75 mg/mL and 5 mg/mL were mixed with dehydration products (1:1 volume ratio) and heated at 100°C separately for 3 and 5 min. The absorbance of heated solutions was measured at 520 nm. At optimum conditions previously determined (2.5 mg/mL resorcinol solution and 5 min-heating), different concentrations of HMF intermediate standard solutions and dehydration products were tested by this assay and calibration curves were constructed based on their absorbance values at 520 nm against HMF concentrations. The optimized Seliwanoff method was assayed on honey samples spiked with HMF at different concentrations and heated honey samples for the quantification of HMF in samples. Basically, 500 µL of honey samples homogenized in water was added to the equal volume of resorcinol solution and heated at 100°C for 5 min. The absorbance at 520 nm was recorded for each sample. HMF in spiked and heated honey samples were quantified by regression equations of calibration curves.

2.4. Method Validation

Single laboratory validation of four methods for the quantification of HMF in honey was performed according to the International Organization for Standardization (ISO) 5725-2 [29]. Method validation parameters (selectivity, linearity, LOD, LOQ, precision and accuracy) were evaluated for all four methods.

The selectivity of methods to HMF was evaluated by testing other sugar samples at optimum conditions of each method. Galactose, sucrose, fructose, and glucose solutions at concentration of 0.004mg/g were analyzed spectrophotometrically at 284 nm, at 550 nm with *p*-toluidine and barbituric acid, at 520 nm with resorcinol reagent, and at 285 nm by chromatographic method. The calibration curves obtained by plotting absorbance of samples at 284nm, 520 nm and 550 nm and peak areas of samples at 285 nm against HMF concentrations gave information about the linearity of these methods. The equations described by $y = ax + b$ were evaluated in the concentration range in the study and R^2 values indicated the sensitivity of the measurements. LOD and LOQ values of all four methods were calculated based on the standard deviations of blank samples. LOD was represented as three times the standard deviation of blank whereas LOQ was represented as ten times the standard deviation of blank. The repeatability was the measure of the precision of four methods in this study. The HMF solution at the concentration of 0.004 mg/0.1g was prepared as three replicates and analyzed by four methods in triplicate. The standard deviation (*Sr*) and relative standard deviation (*RSDr*) were calculated based on the response of four methods. The accepted precision limit was the repeatability value lower than 20% in this study. The accuracy referred as the closeness of results to the true value was evaluated by recovery parameter. The HMF solution at the concentration of 0.004 mg/0.1g was prepared as three replicates and analyzed by four methods in triplicate. The percentage recovery (%) was calculated for each sample analyzed by each method. The accepted accuracy limit was the recovery values of 70-120% for all methods.

2.5. Measurement Uncertainty

All validation data was used for the calculation of measurement uncertainty of four methods. The uncertainties from calibration curve (U_{calib}), repeatability (U_{RSr}) and accuracy (U_{Rec}) were selected as main sources of uncertainty in these methods and their equations were given below (2.2-2.5).

$$U_{calib} = \frac{s}{b_1}\sqrt{\left(\left(\frac{1}{p}\right) + \left(\frac{1}{m}\right) + \left(\frac{(c_0 - c')^2}{s_{xx}}\right)\right)} \quad (2.2)$$

$$U(RSD)_r = \sqrt{\frac{RSD^2}{n}} \quad (2.3)$$

$$U(Rec) = Rec\sqrt{\left(\frac{s_{obs}^2}{n \times c_{obs}^2}\right)} \quad (2.4)$$

$$U = \sqrt{U_{calib}^2 + U_{Rec}^2 + U_{RSD_r}^2} \quad (2.5)$$

where *s* represented the standard deviation of residuals of calibration curve; b_1 represented the slope of the calibration curve; *p* was the number of measurements; *m* was the number of standards used for calibration curve; *co* was calculated concentration from calibration curve; *c*' was the mean of concentrations of standard; *n* was the number of repetitions in repeatability calculations; *Rec* was recovery value and *U* was the combined uncertainty. The uncertainty results were given for four methods as extended uncertainty by multiplying the combined uncertainty with coverage factor, *k* ($k = 2$, for a confidence level of 95%).

3. Results and Discussion

In this study, the four methods for HMF quantification were validated by single laboratory validation study and all method performance parameters and measurement uncertainties were evaluated for all methods [30].

3.1. Fructose Dehydration to HMF

HMF, a cyclic aldehyde, is formed in honey at lower pH by the degradation of reducing sugar through the Maillard reaction as a result of heat treatment or long storage of honey. The presence of HMF or the increase in its amount due to heating or improper storage is used as an indicator of honey quality [1,3,31]. The HMF formation in honey depends on certain factors such as temperature, pH [32], moisture content [33], water activity and mineral content of honey [25], however, the main factor affecting the rate of HMF formation is the ratio of fructose to glucose [34,35]. Due to lower reactivity of glucose at lower pH and its slower enolization which is the rate-limiting step for 5-HMF formation, the dehydration of fructose yields more HMF than glucose [31]. Therefore, fructose solutions prepared in 0.1%, 0.5%, and 1.0% HCL solution at different initial concentrations were used for HMF formation after heating at 80°C. Based on higher yield (Y_{HMF}) of dehydration reaction, the optimum conditions for HMF production from fructose was set as 57 mg/mL of initial fructose in 0.5% HCL solution. HMF formed in solution was used as a sample in method validation studies together with standard HMF solutions in subsequent experiments. This solution was used to represent a heat-treated or long-term stored honey sample to test the four methods for the determination of HMF.

3.2. Spectral Analysis

The spectral analysis of HMF was performed by a UV-Vis spectrophotometer at 284 nm. Both standard HMF solutions and HMF solutions formed via fructose dehydration were used for the construction of calibration curves (Figure 1A & B). The calibration curve of standard HMF solutions gave the equation of $y = 139.4x - 0.133$ (R^2= 0.993) (Figure 1A) which was found to follow Beer's law in the concentrations range of 0.001-1 mg/L. Turner et al. [36] stated that the molecular absorption coefficient of HMF was calculated as 16.830 liters $mole^{-1}$ cm^{-1} (or 16.830 M^{-1} cm^{-1}). When converted to a coefficient based on molecular weight, it became 133.57 L g^{-1} cm^{-1}. In another study, direct spectrophotometric method was used for the determination

of HMF, and it was reported that the molar absorption coefficient was 16070 M^{-1} cm^{-1} at 284 nm [37]. If converted into a coefficient based on molecular weight it was found as 127.54 L g^{-1} cm^{-1}. The slope of the calibration curve in this study was 139.4 and the extinction coefficient converted to gram of HMF was close to other values described previously in literature so that calibration curve constructed with the use of standard HMF solution at 284 nm was applicable for the quantification of HMF.

HMF solutions formed by the dehydration of fructose (57 mg/mL in 0.5% HCL at 80°C) contained HMF at the concentration of 0.002, 0.003, 0.004, 0.005 and 0.006 mg/0,1g after heating for 4, 7, 9, 11 and 13 min, respectively. The calibration curve generated by plotting the concentration of HMF versus their absorbance at 284nm was an equation of $y = 149.2x - 0.207$ (R^2= 0.992) (Figure 1B). According to "Harmonized Methods of the International Honey Commission" [38], the theoretical factor based on molar extinction coefficient was 149.7 to quantify HMF in honey spectrophotometrically. When compared with the slope of the calibration curve constructed by fructose dehydration's products, it was observed that they were not significantly different from each other. Based on these results, it was concluded that these two calibration curves can be used for direct spectral determination of HMF in aqueous solution and honey.

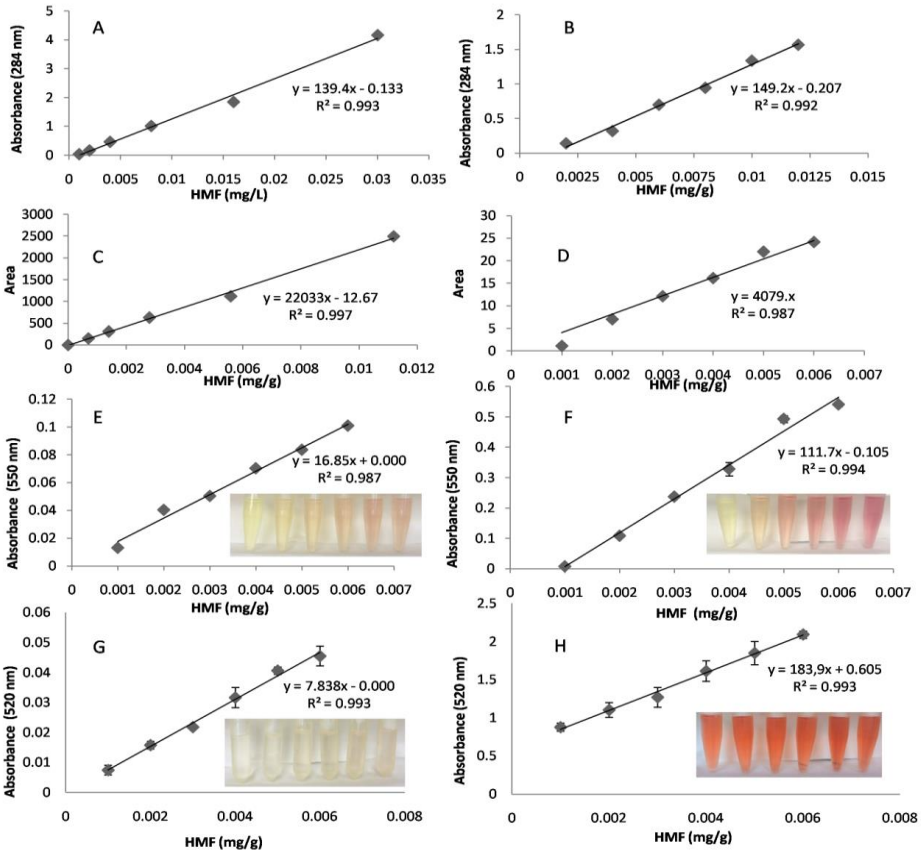

Figure 1. Correlations between HMF concentration and method response obtained by A& B) direct spectrophotometer; C&D) HPLC; E&F) Winkler method and G&H) Seliwanoff method in standard HMF solutions and fructose dehydrated products (n=3) (inlet images were digital images of products formed by reaction between HMF and Winkler/Seliwanoff reactive)

3.3. HPLC Analysis

Generally, analytical method has been preferred for HMF quantification due to its higher sensitivity and the lack of toxic compounds like *p*-toluidine used in Winkler method. Moreover, it was found that the presence of interferences in honey affected the accuracy of spectrophotometric methods [23,27,39]. Therefore, the

accuracy and sensitivity of spectral methods used in this study was compared with the results from HPLC analysis. The chromatographic determination was carried out with both standard HMF solutions and HMF solutions produced via fructose dehydration. As shown in Figure 1C and D, the calibration curves constructed with standard solutions and dehydration products in the range of 0.001-0.006 mg/0.1g gave the standard equations of $y = 22033x - 12.67$ (R^2=0.997) and $y = 4079x$ (R^2=0.987), respectively.

3.4. Winkler Method

Winkler method is one of the methods recommended by the International Honey Commission for the quantification of HMF [22]. The method principle depends on the formation of colored product after reaction between HMF, barbituric acid and *p*-toluidine and quantitative determination by measuring its absorbance at 550 nm [24]. Although Winkler method is not preferred because of the use of carcinogen p-toluidine, it provides advantages in terms of analysis cost and time as compared to HPLC method [40].

In this study, in order to determine the concentration of HMF in honey, two calibration curves were constructed by plotting the absorbance values of reaction products at 550 nm versus the concentration of standard HMF solutions or HMF in dehydrated fructose solutions. As shown in Figure 1E and F, the equations of calibration were $y = 16.58x + 0.0001$ (R^2=0.987) and $y = 111.7x - 0.105$ (R^2=0.994) and the slopes of the equation represented the extinction coefficients of colored products under the experimental conditions in this study. According to R^2 values, the calibration curve obtained from fructose dehydration products more accurately represented the experimental responses. Since it better represented the honey sample than standard HMF solutions, it was concluded that the calibration curve constructed by dehydration products could be used for HMF analysis in honey samples by Winkler method.

In addition to absorbance measurement, visual analysis of colored products formed in the presence of toluidine and barbituric acid was performed. The formation of red color was more obvious in dehydration products with increasing HMF content (inlet image in Figure 1E) than standard HMF solutions (inlet image in Figure 1F). As seen in digital images of red colored products and their intensities, Winkler reaction gave more clear result dehydration products with increasing HMF content.

3.5. Seliwanoff Method

The last method tested for HMF quantification in this study was Seliwanoff method. The principle of Seliwanoff method is the condensation reaction of HMF produced by three dehydration reactions between monosaccharide and resorcinol to produce a colored product, xanthenoid. Since ketoses give Seliwanoff reaction faster than aldoses, this test is mainly used for the discrimination of aldoses and ketoses. Under acidic conditions fructose is dehydrated and this dehydrated product reacts with resorcinol in a condensation reaction to give a cheery red colored product. In this study, therefore, the quantification of HMF was performed both by spectrophotometric measurements and color analysis of colored reaction product.

Before construction of calibration curves and testing real samples, resorcinol concentration and heating time were optimized with HMF solutions produced via fructose dehydration. Reaction solutions containing 0.001, 0.002, 0.003, 0.004, 0.005 and 0.006 mg/0,1g of dehydrated products and resorcinol solutions at 2.5 mg/mL, 3.75 mg/mL and 5 mg/mL were heated for 3 min and, the absorbance values of colored products were in the range of 0.1-0.25 (Figure S1A). With the increases in HMF concentration, the absorbances of colored product increased for all concentrations of resorcinol. As seen from graph and observed from the color intensities of HMF solutions at 0.004 mg/0.1g (inlet image in Figure S1A), the resorcinol solution at 5 mg/mL gave highest

absorbance value with more intense pinkish color. When heating was applied for 5 min, absorbance values increased to approximately five times the 3-minute reaction results and color intensities of reaction products became more intense (Figure S1B). Seliwanoff reaction between HMF solutions and 2.5 mg/mL of resorcinol gave higher absorbance values with higher slope as compared with other resorcinol solutions. As a result, optimum parameters for Seliwanoff method were determined as 2.5 mg/mL and 5 min for resorcinol concentration and heating time, respectively.

For HMF quantification, calibration curves for both standard HMF solutions and HMF solutions produced via fructose dehydration were constructed following optimization studies. As seen in Figure 1G and 1H, linear relationships between HMF concentration and absorbance value of colored reaction product were represented by regression equation of $y = 7.838x + 0.0001$ (R^2=0.993) and 183.9x + 0.605 (R^2=0.993) after reaction with standard HMF solutions and dehydrated product, respectively. When standard HMF solutions were analyzed by Seliwanoff test, the absorbance values of each HMF concentration were considerably below than the values of corresponding solutions having fructose dehydration products. In addition, the correlation coefficient of ≥ 0.99 indicated relatively strong relationship between HMF concentrations in dehydrated products and absorbance values, thus, it was concluded that the calibration curve of dehydrated products can be used for spectral determination of HMF in honey by Seliwanoff method. Beside absorbance values, digital images of both standard samples and dehydrated products (inlet image in Figure 1G and 1H, respectively) were revealed the accuracy of use of the calibration curve of dehydrated products for HMF quantification. The clear red color formation was observed by fructose dehydrated products after reaction with Seliwanoff reagent.

3.6. Method Validation and Measurement Uncertainty

As a healthy sweetener, honey, contains fructose and glucose as main sugars and other mono-, di-, tri-, oligo- and polysaccharides [41,42] and sum of fructose, glucose and sucrose content is both quality and ripeness parameter of honey [14]. Therefore, the specificity of these four instrumental methods was evaluated with testing fructose, glucose, sucrose, and galactose under same test conditions as used for HMF.

Specificity of HPLC method is evaluated by the peak regions of interferences [44]. In this study, retention time was estimated at 9.31 min for the chromatogram of HMF standard solution. The peaks of interferences (glucose, fructose, and sucrose) were not observed in the region of HMF peak (data not shown). Moreover, the absence of interference peaks at the resolution lower than 1.5 times of peak of HMF supported the specificity of HPLC method for separation of HMF than other sugars [44-46]. Direct spectral analysis of HMF and possible interferences showed the specificity of the method by giving significantly different absorbance value for HMF than other analytes (Figure 2A). The specificity analysis of Winkler method (Figure 2B) indicated that significantly higher absorbance value was obtained for HMF as compared to the values of other sugars. The reagents of Winkler reaction formed a red colored product only if there was HMF in the solution; otherwise, the color remained similar as the initial color of reagents. In Winkler method, the possible reaction mechanisms are explained by the opening of furan ring in sugar such as HMF and fructose and forming a product with barbituric acid and p-toluidine that can absorb light in visible region [47]. Therefore, it is expected for fructose and HMF derived from furan to react with Winkler method's reactive. In this study, Winkler method was found highly specific for HMF detection and quantification under specified reaction conditions. As mentioned, the basic principle of Seliwanoff Test is the reaction between ketose dehydrated in the presence of acidic solution and resorcinol to form xanthenoid with pink color. Thus, it is expected that fructose and sucrose give positive results while galactose and glucose require more reaction time to give positive results. As shown in Figure 2C, the Seliwanoff reaction gave positive result only for HMF solution under optimized

condition. The red colored product was generated by the reaction between HMF and resorcinol reactive. Therefore, it could be concluded that with resorcinol amount and reaction time optimized in this study, Seliwanoff method was highly specific for HMF detection and quantification.

The validation of four methods for HMF analysis used in this study was carried out by determining LOD, LOQ, accuracy (recovery) and precision (repeatability) parameters and measurement uncertainties of methods based on EURACHEM Guide and ISO standard [48]. Table 1 summarized the method performance parameters at maximum limit of HMF determined by codex and uncertainty budgets of four methods. According to the Codex Alimentarius Commission Manual [49], it has been reported that the acceptable recovery values for 10 mg/kg and 100 mg/kg of analytes are in the range of 80 to 110% and 90 to 107%, respectively. Although the lowest recovery in sample with the content of 0.004mg/0.1g HMF was observed with direct spectral analysis (98%), all four methods met the acceptable criteria for the recovery.

The Codex Alimentarius Commission Manual [49] has also given the precision requirements based on Horwitz equation ($2*C^{-0,1505}$). At mass fraction of 10 mg/kg, the calculated *RSDr* % value is *RSDr* %≤ 22, while it is *RSDr* %≤ 16 for mass fraction of 100 mg/kg. In this study, *RSDr* % values were found as 6.97%, 6.19%, 2.87% and 0.90% for spectral analysis, Seliwanoff, HPLC and Winkler methods, respectively. Therefore, it was concluded that all four methods satisfied the precision requirements at the HMF concentration of 0.004mg/0.1g. When LOD and LOQ values were compared, it was shown that the analytical method had lowest LOD and LOQ values as expected. The highest values of LOD and LOQ were 0.0004 mg/0.1 g and 0.0013 mg/0.1 g for Winkler method.

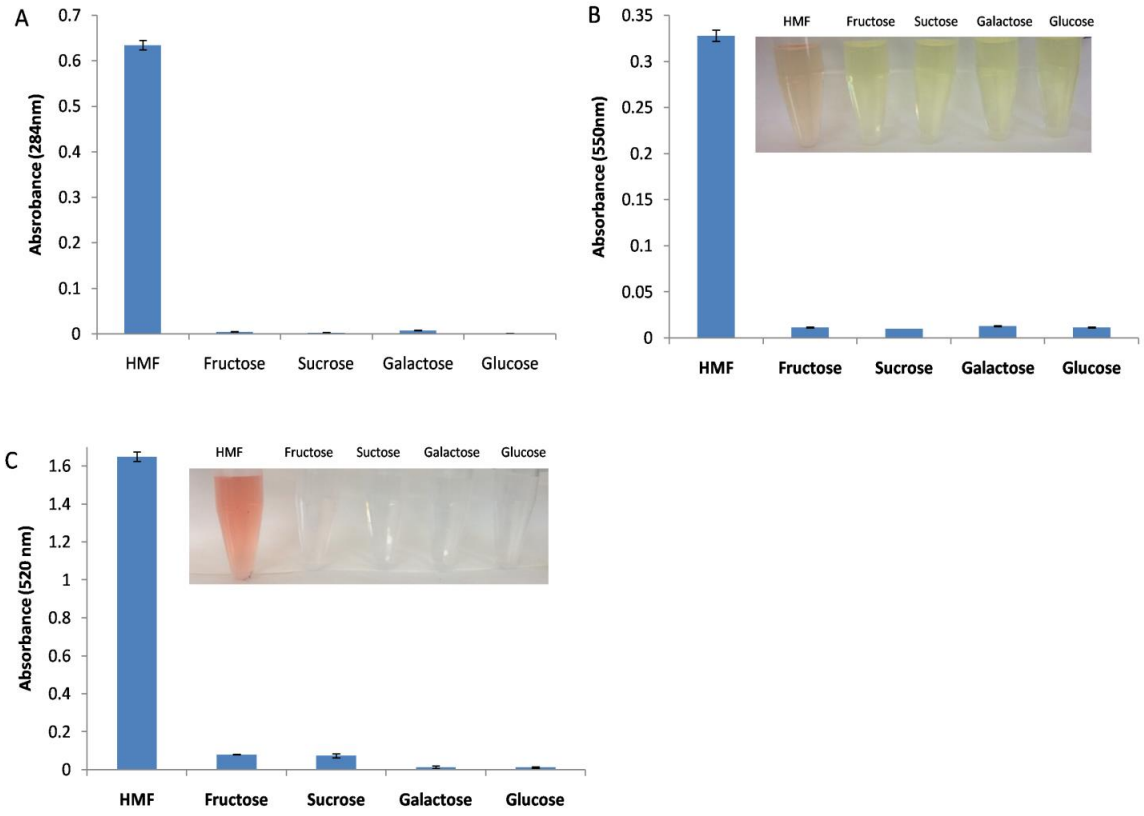

Figure 2. Specificity analysis of spectral methods for HMF; A) Direct measurement of interferences (fructose, sucrose, galactose, and glucose) by spectrophotometer at 284 nm, B) measurement by Winkler method at 550 nm, C) measurement by Seliwanoff method at 520 nm (inlet images were digital images of products formed by reaction between analytes and Winkler/Seliwanoff reactive) (n=3)

Table 1. Method performance criteria and uncertainty budgets of four methods used for the HMF determination in honey

Methods	Recovery (%)	Relative Standard deviations of repeatability (*RSDr*, %)	LOD (mg/0.1g)	LOQ (mg/0.1g)	Uncertainty of calibration curve	Uncertainty of repeatability	Uncertainty of recovery	Expanded uncertainty**, *U*
Spectral Analysis	98.2	6.97	0.0011	0.0035	0.01249	0.02793	0.02845	0.00025
HPLC Analysis	102.5	2.87	0.0004	0.0013	0.04768	0.01171	0.16971	0.00045
Winkler Method	103.3	0.90	0.0012	0.0038	0.02803	0.00369	0.00381	0.00022
Seliwanoff Method	100.2	6.19	0.0007	0.0023	0.07868	0.02525	0.02531	0.00069

*Data represented the means of three measurements. P-values less than 0.5 were considered statistically significant.
**with coverage factor of 2, confidence level of 95%.

Three main components contributing to uncertainty budgets were determined as uncertainty of calibration curve, recovery, and repeatability for these methods. Measurement uncertainty budgets of these methods calculated from validation data were also shown in Table 1. HMF solution at the final concentration of 0.004 mg/0.1g, therefore, were reported as 0.004 mg/0.1g ± 0.00025 mg/0.1g, 0.0036 mg/0.1g ± 0.000691 mg/0.1g, 0.004 mg/0.1g ±0.00045 mg/0.1g and 0.0039 mg/0.1g ± 0.00022 mg/0.1g (k=2, confidence level of 95%) by spectral analysis, Seliwanoff method, HPLC and Winkler method, respectively. The use of calibration curve affected directly both the results of quantification and uncertainty of measurement. Since all four methods in this study analyzed the amount of HMF based on calibration curves of fructose dehydrated products, the main component of uncertainty budgets is expected to be the uncertainty of calibration curve. However, the most effective component in the uncertainty sources was the calibration curve for only Seliwanoff method. Uncertainty associated with recovery contributed more for uncertainty budgets of spectral analysis, HPLC and Winkler method which could be explained by the difference of percent recovery values from 100% (or recover values from unity).

The aim of this study was the validation of four methods for HMF determination in honey and providing their comparisons based on validation and uncertainty parameters. Previously, Zappalà et al. [27] presented a study to compare three methods for the determination of HMF in honey. It was concluded that the results of HPLC and White methods were approximately similar while it was higher when analyzed by Winkler method, and HPLC method was preferable to quantify HMF in honey due to the toxicity of Winkler reactive reagent and UV interferences in spectral analysis. A recent study described an in-house validation study based on Seliwanoff test to determine HMF in honey [50]. The proposed method had precision and accuracy in the range of 2.52–5.14% and 95.83% to 96.65%, respectively and showed a linear relationship with Winkler method and HPLC.

In this study, four methods were validated and defined for HMF quantification. Spectral analysis was used for the measurement of HMF amount directly without using any reagents. Seliwanoff method was used with resorcinol as reactive reagent which does not show any known toxicity. Besides Seliwanoff method and spectral analysis, Winkler and chromatographic method were carried out for quantitative determination of HMF after validation. Based on method performance parameters of validation study, all four methods were found to be applicable for the quantification of HMF. The measurement uncertainty of quantification of HMF by four methods based on the validation data of spiked samples was described by their linearity, recovery, and repeatability in this study. To our knowledge, this is the first study for defining direct spectral method for HMF quantification and validation of four methods to determine HMF.

3.7. Determination of HMF in Real Samples

Honey samples spiked with HMF at the final concentrations of 0.001, 0.002, 0.003, 0.004, 0.005 and 0.006 mg/0,1g were analyzed separately by four methods in this study. HPLC method was selected as reference method and spectral methods were compared with reference method. The correction factors of 0.41, 0.82 and 1.25 were used in the calculation of HMF concentrations to balance the effects of the honey matrix on the results of Spectral analysis, Seliwanoff and Winkler methods, respectively. Figure S2 represented the graph of the HMF concentrations calculated from the calibration curves of spectral analysis, Seliwanoff and Winkler methods against the HMF concentrations calculated based on the HPLC calibration curve. The comparison of the responses of three spectral methods with a chromatographic method gave the slopes of linear regression at 1.086, 1.059 and 1.062 for spectral analysis, Seliwanoff and Winkler methods, respectively. The R^2 values were ≥0.986 which indicated the relatively strong relationship between the results of these methods at this concentration range. Moreover, the calculated concentrations of spiked honey samples and the recovery values (%) of each method were given in Table 2. The recoveries of each method at each spiked HMF concentration were in the acceptable range of 70-120%. All these results indicated the applicability of four methods for the quantification of HMF in honey with accepted method performance criteria for recovery.

Table 2. Concentrations of HMF spiked to honey and the recoveries of four methods

	Spectral analysis		Seliwanoff method		HPLC analysis		Winkler method	
HMF spiked (mg/0.1g)	HMF (mg/0.1g)	Recovery %	HMF (mg/0.1g)	Recovery %	HMF (mg/0.1g)	Recovery %	HMF (mg/0.1g)	Recovery %
0.001	0.0011	108.7	0.0010	101.6	0.0012	118	0.0010	100
0.002	0.0023	116.2	0.0020	100.5	0.0021	105	0.0020	100.7
0.003	0.0034	114.8	0.0035	115	0.0033	110	0.0030	100.8
0.004	0.0039	98.2	0.0040	100.2	0.0041	102.5	0.0041	103.3
0.005	0.0050	98.9	0.0049	98.3	0.0047	94.6	0.0050	100.6
0.006	0.0061	102.4	0.0055	92.3	0.0056	93.3	0.0055	92.3

HMF is also used as heating index of honey and its concentration has been changed by temperature and heating time. Karabournioti and Zervalaki [51] showed that incubation of honey samples at 35°C, 45°C, 55°C and 65°C for 24 hours changed the initial HMF concentration from 2.25 mg/kg to 3.45, 3.75, 4.35 and 19.00 mg/kg, respectively. They concluded that temperature higher than 55°C resulted in a significant increase in HMF concentration regardless of the exposure time, but still lower than that recommended by international standards and codex. Another study reported that heating floral honey at 100°C for 75 and 90 minutes yielded HMF concentrations of 55.41 and 73.78 mg/kg [52]. Therefore, besides spiked honey samples, heated honey samples were analyzed in this study in order to evaluate the effectiveness of these methods for the quantification of HMF in real samples. The initial concentration of HMF in honey was lower than the detection limits of spectral methods. Three different temperatures were set and HMF concentrations were determined at certain time intervals by spectral methods. Table S1 summarized HMF concentrations produced upon heat treatment at 50°C, 70°C and 90°C. Figure S3 also showed the effect of heating temperature and time on HMF content of honey samples. Heating process at 50°C did not yield a HMF concentration higher than 40 mg/kg which is the maximum limit for HMF allowed in honey by Codex and EU directive [13,53]. However, even after 10 min of heat treatment at 70°C and 90°C, the amounts of HMF exceeded this maximum limit in honey. Moreover, these concentrations calculated by the calibration curves of each spectral method was not significantly different from each other which mean that spectral analysis, Seliwanoff method and Winkler method could be applicable for detection of HMF in heated honey.

4. Conclusion

In this study, three spectral methods and HPLC were used to evaluate HMF concentration in honey. These four methods were validated by single laboratory validation study. The measurement uncertainties were calculated for all methods at the maximum acceptable limit of HMF allowed for honey. The method validation and uncertainty results prove that these methods can be successfully used to determine HMF content in honey.

Author Contributions

All the authors equally contributed to this work. This paper is derived from the first author's master's thesis supervised by the second author. They all read and approved the final version of the paper.

Conflicts of Interest

All the authors declare no conflict of interest.

Supplementary Material

https://dergipark.org.tr/en/download/journal-file/30476

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