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Article

FOOD CHEMICAL CONTAMINANTS

# A New Enrichment Method for Quantification of 5-Hydroxymethylfurfural by Indirect Flame Atomic Absorption Spectrometry in Honey and Jam Samples

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## Abstract

**Background:** Because of increasing amounts of 5-hydroxymethylfurfural (5-HMF) in processed foods and the complexity of the matrix, monitoring of trace 5-HMF requires accurate and reliable methods. Hence, an efficient sample pretreatment procedure is necessary for extraction and preconcentration of 5-HMF from the matrix.
**Objective:** In this study, a new and efficient sample preparation method utilizing ultrasound-assisted-cloud point extraction (UA-CPE), indirectly followed by flame atomic absorption spectrometry (FAAS), was introduced for the monitoring of trace amounts of 5-HMF in honey and jam samples.
**Method:** With this method, hydroxylamine was used as a derivatizing agent in the presence of Mn(II) and an anionic surfactant, SDS for extraction of 5-HMF at pH 9.0. For dispersing reagents in sample solution, low amounts of mixed surfactant, triton X-45 and SDS were mixed and fast-injected into the extraction media. A cloudy solution formed, and after reaction of 5-HMF with reagents, the cloudy solution was centrifuged. The extracted 5-HMF in the surfactant-rich phase was dissolved in acidic acetone and indirectly analyzed by FAAS.
**Results:** The method showed a detection limit of 1.27 μg/L in linear working range of 4–240 μg/L, good precision (2.3–6.5%), and recovery rates (93.5–97%) after preconcentration of 70-fold.
**Conclusions:** Within this study, an accurate and reliable method for the indirect quantification of 5-HMF in selected samples was successfully developed with a sensitivity improvement factor of 30.6.
**Highlights:** The figures of merit for the developed indirect method were appropriate. The applicability of the method for the analysis of 5-HMF in processed foods was excellent.

5-Hydroxymethylfurfural (5-HMF) is a heterocyclic compound that is released as a result of sugar degradation through the Maillard reaction when carbohydrate-rich foods are exposed to a thermal treatment (1, 2). 5-HMF content can vary greatly in different foods such as honey, jam, biscuit, milk, coffee, beer, and apple juice (3). In honey, 5-HMF is usually present as a result of the effect of normal honey acidity on reducing sugars at room temperature, however, its amount increases with improper storage and/or thermal treatment (4). Thermal processing of honey is usually carried out to reduce microbial degradation or change the tendency of honey to crystallize. Therefore, 5-HMF is considered as an indicator of quality deterioration resulting from uncontrolled overheating or improper storage conditions not only in honey, but also in a number of carbohydrate-containing foods. The World Health Organization (WHO) and European Union (EU) (5) international food control guidelines state that maximum allowable concentrations of 5-HMF in honey and apple juice are 40 and 50 mg/kg, respectively,

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with higher concentrations indicating deterioration and heat treatment. The interest in determining 5-HMF levels in foods is related to its toxicity. This compound and some derivatives (5-chloromethyl and 5-sulfuromethylfurfural) have been shown to have cytotoxic, genotoxic, mutagenic, and carcinogenic effects (6–8). Therefore, there is a great need for the development of a simple, sensitive, selective, and economical method for the continuous monitoring and determination of trace levels of 5-HMF in common foods such as honey.

Generally, the amount of 5-HMF in thermally processed fresh honey at temperatures higher than 135°C is either around the detection limit or lower than the detection limit for molecular absorption and emission techniques in the UV-visible region when used as a detection tool. In general, fresh honey contains less than 15 mg/kg depending on the pH of the product, its temperature, and its shelf life. Moreover, these techniques are not suitable for accurate and reliable determination of 5-HMF in food production without any pretreatment. For these reasons, a pre-separation and enrichment tool is required before determining with these techniques in order to get rid of the matrix and lower the detection limit. Some separation-enrichment methods have been reported for solid phase extraction (SPE) for 5-HMF, together with a suitable analytical technique such as UV, diode-array detection (DAD) and/or pulsed amperometric detection (PAD), MS, eletrochemical detection (Direct Current (DC), Pulse and Scan as operation mode) in GC, LC, capillary electrophoresis (CE) and micellar electrokinetic capillary chromatography (MEKC) to control the selectivity, and improve the sensitivity in terms of S/N ratio. However, these are based on tedious, time-consuming, and complex analytical processes such as solid phase microextraction with and without (SPME and HS-SPME), and vortex-assisted liquid-liquid microextraction (VA-LLME) (3, 9–22). The methods have limitations such as greater costs, more time consuming processes, and irreversible adsorption problem of substances in capillary columns, high operating pressure for effective separation, unsatisfactory enrichment factor, expert user requirement, large amounts of expensive organic materials and waste.

Unlike all of these methods, ultrasonic assisted-cloud extraction (UA-CPE), as a separation-enrichment tool, uses mainly diluted solutions, requiring low sample volume as well as relatively less consumption of toxic organic solvents that have a negative impact on the environment and human health. It attracts a lot of attention due to its "eco-friendly chemistry properties" (23). At the same time, UA-CPE provides a high recovery percentage and a large enrichment factor. It is also used successfully for enrichment of organic/inorganic species present in food, beverage, and environmental samples. Different methods are used to determine 5-HMF in different food matrices. Determination of trace levels of 5-HMF have been conducted by spectrometric methods (24, 25) and LC (26), GC (3), chromatographic techniques such as ion exclusion chromatography (IEC) (27), CE-DAD (28), and MEKC (15) using CE techniques. Analysis of 5-HMF in food production is usually carried out in routine analyses in the laboratory to determine critical limits. Contrary to large research laboratories, it is very difficult to provide the necessary financial support for such laboratories. Flame atomic absorption spectrometry (FAAS) is used directly or indirectly in organic-inorganic trace analysis in analytical chemistry, especially for use in narrow-budget research laboratories. It is a comparatively simple, fast, and inexpensive device that can be found in many research laboratories with its element selective detection power and versatility.

In this study, a new UA-CPE method is proposed for separating and enriching trace levels of 5-HMF from the sample matrix. For this purpose, to extract the chelated complex formed between Mn (II) and the pH-controlled oxime formed between hydroxylamine and 5-HMF at pH 9.0; SDS was used as both a counter-ion and sensitivity enhancer (as ion-pairing auxiliary ligand) in the extraction step. The formed ion-pair complex was then extracted into the micellar phase of Triton X-45 as extractant solvent. Since the micellar phase is very dense, the surfactant-rich phase was diluted with acidic acetone to 0.5 mL, and Mn, which is linearly related with the 5-HMF content, was indirectly monitored by FAAS. There are three special features in the center of the study: (1) the application of ultrasound energy shortens the extraction time and increases the extraction efficiency; (2) in the sense of new contribution to the literature, the facility of indirect monitoring of 5-HMF by FAAS being a selective detection tool; and (3) the use of pH-controlled ion-pair formation in the extraction process provides a significant improvement in both the sensitivity and selectivity of FAAS. In order to fully characterize the proposed UA-CPE process, the main parameters that affect ion-pair formation and extraction efficiency have been evaluated and optimized.

## Experimental

### Reagents and Solutions

All reagents were of analytical purity and were purchased from Sigma Aldrich (St. Louis, MO, USA). Triton X-45, Mn (II), hydroxylamine, and SDS, and diluting solvents such as ethanol, methanol, acetone, and tetrahydrofuran (THF) were provided from Merck (Darmstadt, Germany). The reagent's solutions [1.0% (v/v), 10 mg/L, $1.0 \times 10^{-3}$ mol/L, and $3.0 \times 10^{-3}$ mol/L, respectively] were prepared by dissolving, dilution in water, and vortex mixing (1200 rpm, 30 s) to produce a homogenous solution when necessary. Stock solution of 5-HMF (250 mg/L) was prepared by dissolving the appropriate amount in methanol and diluting with distilled water. Subsequently, working standard solutions were obtained by sequential dilution of the stock solution with aqueous methanol (9:1, v/v). Carrez I solution was prepared by dissolving 21.9 g zinc acetate dihydrate in water, adding 3 g glacial acetic acid, and diluting to 100 mL with water. Carrez II solution was prepared by dissolving 10.6 g potassium ferrocyanide in water and diluting to 100 mL. A 0.04 mol/L Britton-Robinson (B-R) buffer solution at pH 9.0 was prepared by mixing 0.04 mol/L levels of acetic acid, boric acid, and phosphoric acid, and diluting with HCl or NaOH (each one, 2.0 and/or 0.2 mol/L) using a pH meter to adjust the pH to 9.0. All solutions were kept in the refrigerator at 4°C before analysis. All test equipment used in the extraction step was washed with dilute nitric acid (2.0%, w/v) and rinsed three times before use with ultrapure water.

### Instrumentation

All devices used in this research were inexpensive and easy to use. The names and intended uses of these devices are listed below. It was used primarily with Shimadzu AAS-6300 PC FAAS (Kyoto, Japan) in the indirect appointment phase. Operating conditions for Mn: resonance wavelength 279.5 nm, lamp current 10 mA, slit 0.2 nm, 6.0 mL/min with sample uptake rate and 7 mm burner height, air/acetylene flow rate, and L/min 15/2. The ultrapure water used during the experiment was obtained from a Labconco (Kansas City, USA) water treatment system. The surfactant-rich phase formation was obtained and

facilitated using an ultrasonic bath (UCS-10 model, Seoul, South Korea) operating at 40 kHz, 300 W. A centrifuge (Universal 320 Hettich model, London, UK) was used to separate the surfactant-rich phase from the aqueous phase. The pH of the solutions was adjusted with a pH meter equipped with a glass electrode (JP Selecta, Barcelona, Spain).

### Samples and Sample Preparation for Analysis

All samples were obtained from local markets in Sivas, Turkey. Before the sample preparation step, semi-liquid and solid samples were thoroughly homogenized with a vortex mixer after mixing with some water.

The steps of the solving process under ultrasonic power are as follows (22):

(1) Transfer approximately 1.0 g of all samples to 100 mL beakers in a similar manner.
(2) Next, add 2.0 mL of 0.2 mol/L $HClO_4$ to all samples to minimize losses that can occur during protein removal when 5-HMF level is low, followed by the addition of Carrez-I (2.0 mL of 1.123 mol/L) and Carrez-II (2.0 mL of 0.288 mol/L).
(3) Bring the final volume of the mixture to 100 mL with water.
(4) Sonicate and dissolve the prepared mixture in an ultrasonic bath (300 W, 40 kHz) for 5 min at 40°C.
(5) Adjust the pH of the soluble mixture to 7.0 with dilute NaOH solution (0.15 mol/L).
(6) Centrifuge at 3500 rpm for 10 min.
(7) Filter the pretreated samples through a membrane filter (0.45 μm pore-size).

In addition, with and without dissolving under ultrasonic power to determine whether or not the acid concentration and heating between 20–60°C have an effect on both the formation and conversion of levulinic acid/formic acid and the polymerization of 5-HMF, the analysis of honey samples with spiked with 15, 25 and 50 μg/L levels was carried out, and the slopes of calibration curves (5, 10, 25, 50, 100, 150, 200, and 250 μg/L prepared from pure solvent and sample extracts) in terms of matrix effect were compared. A significant difference, except for a signal enhancement of +10.2%, was not observed between slopes of calibration curves from the results. As a result, the formation of 5-HMF and conversion to levulinic acid did not occur under dilute acid conditions at temperatures ≤40°C.

### Enrichment Method with UA-CPE

All extraction steps were carried out in 50 mL centrifuge tubes and parallel to the sample blank solution. First, add aliquots of the standard aqueous solution, containing 100 μg/L of 5-HMF in the optimization step or 35.0 mL sample solution in the enrichment step, to these tubes and adjust the pH of the solution to pH 9.0 with 1.5 mL of 0.04 mol/L B-R buffers. Sequentially, add 2.0 mL of $1.0 \times 10^{-3}$ mol/L $NH_2OH$, 2.0 mL of 10 mg/L Mn (II), 1.5 mL of $3.0 \times 10^{-3}$ mol/L SDS, 2.5 mL of 0.01 mol/L KCl, and 0.6 mL of 1.0% (w/v) Triton X-45 to the solution medium to form a hydrophobic ion-pair complex, and sonicate in an ultrasonic bath for 12 min at 40°C. Centrifuge the mixture for 8 min at 3250 rpm after the extractable complex formation. After centrifugation, collect the linearly related Mn-chelate complex with 5-HMF as a dense phase under the tube. Remove the aqueous phase by decantation and dilute the volume of the surfactant-rich phase containing the analyte (5-HMF) to 0.5 mL with acidic acetone (0.2 mol/L $HNO_3$), and then deliver the diluted phase with Mn to the nebulizer of FAAS for indirect analysis against the sample blank.

In order to ensure the reliability of the results obtained, the 5-HMF content of the samples was measured in a similar way for 40 min at 40°C under pre-treated samples with a modified spectrophotometric White method (29), which is also known as differential UV-photometry with and without bisulfite-reduction of 5-HMF, and evaluated comparatively. Analysis of the selected samples by the reference comparison method was carried out as follows.

Quantitatively transfer 1.0 g of the sample to a 50 mL flask, dissolve it in 25 mL of water, add 0.5 mL of Carrez-I and 0.5 mL of Carrez-II solutions, respectively, and dilute to 50 mL with water. Filter sample solutions through a 0.45 μm membrane filter. After discarding the first 10 mL of the filtrate, place 5 mL portions of the remaining solutions in two separate test tubes; add 5 mL of water or sample solution to one; to the second, add 5 mL of 0.2% (w/v) $NaHSO_3$ solution. Measure the absorbance of the solutions at 284 and 336 nm using a spectrophotometer. Determine their 5-HMF contents using the formula reported by the International Honey Commission (IHC) and recommended for the original White method (30):

$$5-\text{HMF (mg/kg)} = (A_{284} - A_{336}) \times 149.7 \times 5/W \quad (1)$$

where, W = the mass of the sample and the factor of 149.7 is a theoretical value associated with the molar absorption coefficient of 5-HMF at 284 nm. When performing regression analysis (for n: 6) for a range of 5-HMF solutions in the range of 0.1–5 mg/L under ultrasonic power, a good improvement in the regression data for the honey matrix was achieved according to the standard method as follows:

$$\text{Abs.} = 7.15 \times 10^{-3}\text{C (mg/kg)} + 0.0213,\ r^2:\ 0.9965 \quad (2)$$

The linear working range was 0.06–5 mg/kg, with detection and quantitation limits of 0.018 and 0.06 mg/kg, respectively. The difference was only 2.8%, when comparing the calibration curves for the possible matrix effect. Therefore, it can be concluded that this difference is not significant. Nevertheless, in the analysis of the samples, 250 μL of $1.0 \times 10^{-3}$ mol/L thiourea solution was added to the medium before analysis by FAAS to suppress the possible interference of similar aldehyde species.

In the optimization step and the analysis step of the samples, all studies were carried out in three and five replicates. Averages and their standard deviations were calculated for each study. Data processing and all statistical calculations (ANOVA analysis) were carried out using Microsoft excel® (Microsoft, version 2017). A 95% confidence level was adopted for all statistical calculations. The paired Student's *t*-test at the 95% confidence level was conducted to determine whether or not there is a significant difference between the 5-HMF levels obtained by using the two methods in sample extracts.

## Results and Discussion

### Optimization of Analytical Parameters

The efficiency of UA-CPE depends on various factors such as ultrasonic bath conditions, surfactant concentration, concentration of derivative and ion-pair forming reagents, pH, diluent type, and volume. All the above analytical variables have been extensively researched and optimized so that optimal conditions have been determined to obtain maximum extraction

efficiency, absorbance, and sensitivity for indirect measurement of Mn(II) linearly related with 5-HMF at 100 μg/L level by FAAS. In the optimization step, each point was followed with three replicates and their averages as error bars were taken into account.

### *The effect of pH and buffer concentration*

Extraction of 5-HMF by the UA-CPE method requires the formation of an ion-pair with sufficient hydrophobicity to be extracted into the small volume of the surfactant-rich phase. The pH plays a unique role in ion-pair formation, and later in UA-CPE yield. Therefore, pH was the first parameter to be evaluated for its effect of 5-HMF using the indirect determination of FAAS at the level of 100 μg/L. The effect of pH on extraction of extractable ion-pair complex was investigated in the range of 5.0–10.0 (Figure 1a). Quantitative extraction efficiency, which gives maximum sensitivity, was obtained at pH 9.0. At lower and higher pHs, the sensitivity gradually decreased. It is clear that a stable oxime based on condensation between a functional aldehyde group of 5-HMF and hydroxylamine at pH 9.0 formed for chelation with Mn(II) in the presence of SDS as both ion-pairing auxiliary ligand and stabilizer. Also, where the oximes as N, O donors are present in three main tautomer forms, the oxime (= N – OH), nitrone (= $N^+H - O^-$), and nitroso (– N = O), depending on pH, polarity, temperature, electrolyte, and solvent types of the microenvironment. This case is also assisted with the presence of an electron donor group like polar hydroxyl-methyl (acting such as a weak chelating benzyl alcohol on the furan ring) on the oxime moiety allowing to decrease the energy gap between the oxime and nitrone form, and stabilizes the latter, which facilitates nucleophilic addition reaction. Moreover, even if the nitrone form exhibits a higher reactivity than the oxime, especially in nucleophilic addition to unsaturated electrophile (5-HMF), it is implied in the literature (31) that the oxime form, in terms of chelation (herein, with $Mn^{2+}$ ions), is more reactive than the nitrone form at high pHs. For this reason, pH 9.0 is preferred and used for the next processes.

In addition, the effect of B-R buffer solution volume at the level of 0.04 mol/L at pH 9.0 (Figure 1b) was examined in the range of 0.05–2.5 mL to increase the sensitivity. The maximum absorption was achieved for a B-R buffer volume of 1.5 mL. The sensitivity was significantly lower at lower and higher buffer volumes.

### *Effect of concentrations of derivatizing and chelating agents [$NH_2OH$, Mn (II)]*

The effect of $1.0 \times 10^{-3}$ mol/L derivatizing reagent, $NH_2OH$ as nucleophile at pH 9.0 (Figure 2a) was examined in a 50 mL centrifuge tube in the range of 0.05–2.5 mL. It is clear that the sensitivity is linearly dependent up to a volume of 2.0 mL, where the basicity of $NH_2OH$ increased with increase in ethanol ratio as a polar solvent with pKa values of 5.93, and sequentially 5.82, 5.69, and 5.44 in water and water-ethanol mixtures (10, 30, and 50%, v/v) (32). An increase in the electron density at N-atom brings about the increase in the basicity of the substance as a result of interaction with hydrogen bonding between hydroxylamine and ethanol. In a similar way, it is believed that the oxime will be affected from polarity of solvent, pH, temperature of the environment, as well as buffer, electrolyte, and surfactant types and concentrations for chelation with $Mn^{2+}$ ions. The maximum absorbance was obtained in the presence of 2.0 mL of reagent. The absorbance gradually decreased at lower and higher volumes. Therefore, it is concluded that 2.0 mL of derivatizing reagent is sufficient for further studies.

Similarly, the effect of the Mn (II) of chelating metal ion at a concentration of 10 mg/L was examined in the range of 0.05–3.0 mL (Figure 2b), and the maximum absorbance was observed in 2.0 mL of Mn (II). At lower and higher chelating volumes, the sensitivity decreased sharply due to a concentration-dependent increase in the sample blank, in a manner that $Mn^{2+}$ ions,

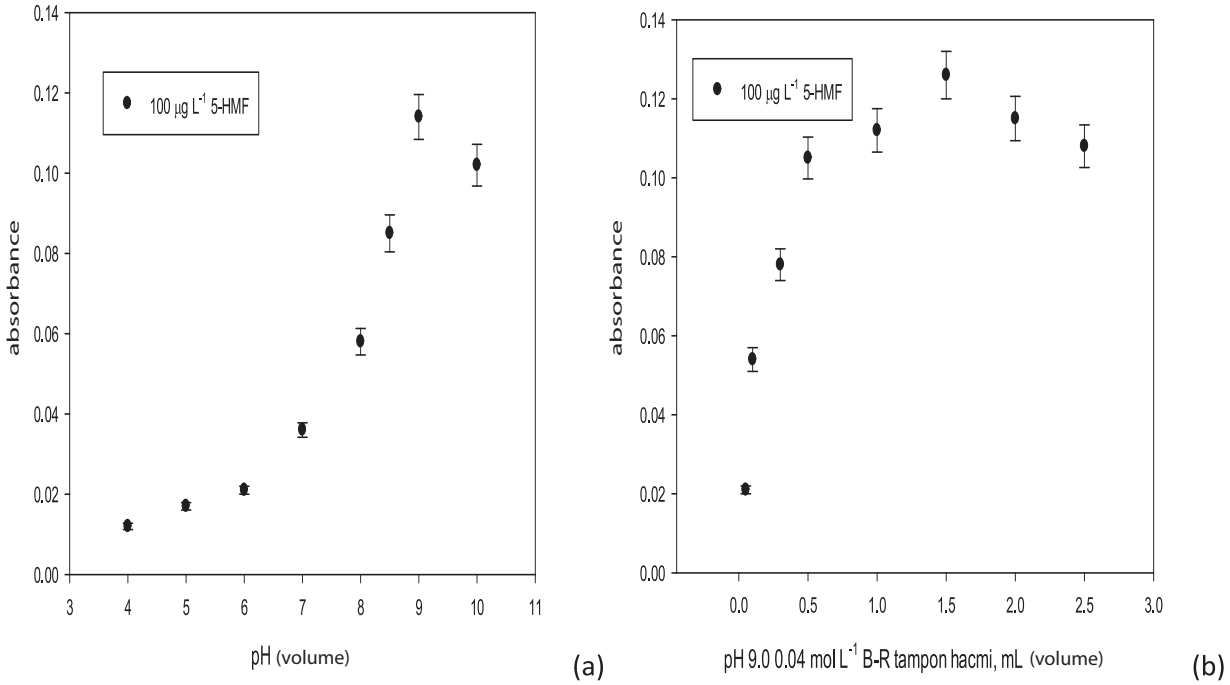


**Figure 1.** The effect of (a) pH and (b) 0.04 mol/L B-R buffer solution volume on the sensitivity.

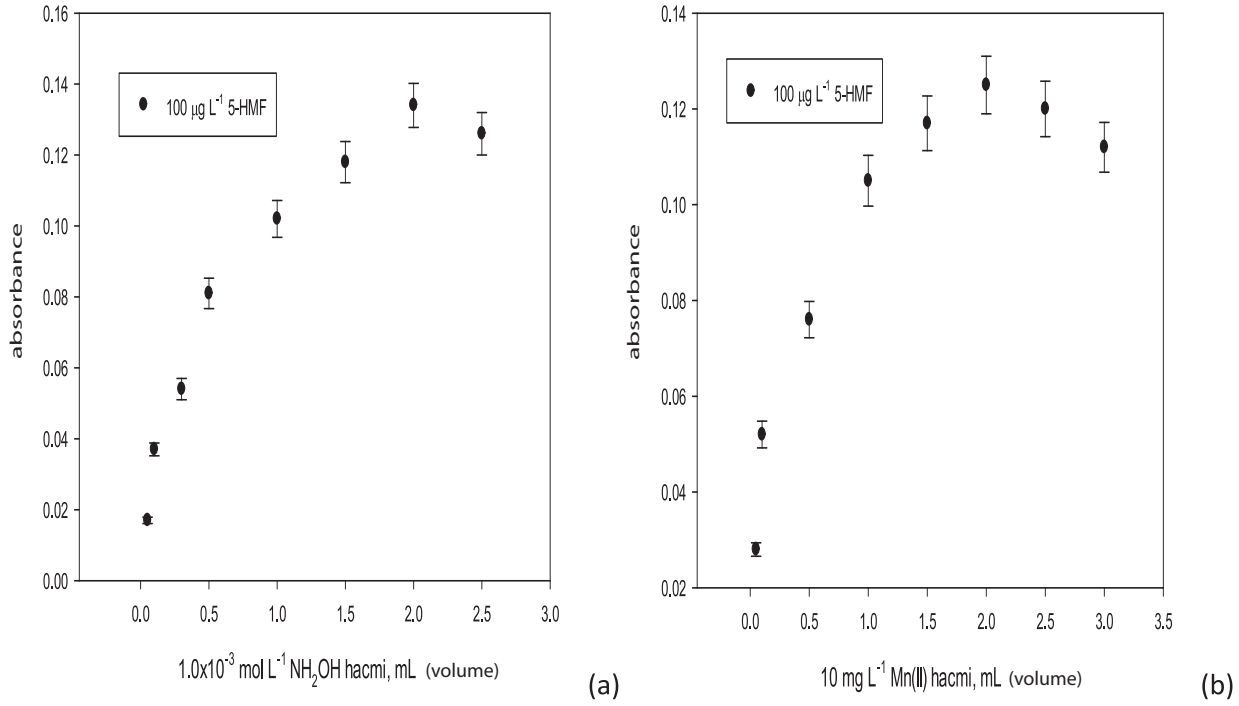


**Figure 2.** The effect of (a) $1.0 \times 10^{-3}$ mol/L $NH_2OH$ solution volume and (b) 10 mg/L Mn(II) solution volume on the sensitivity.

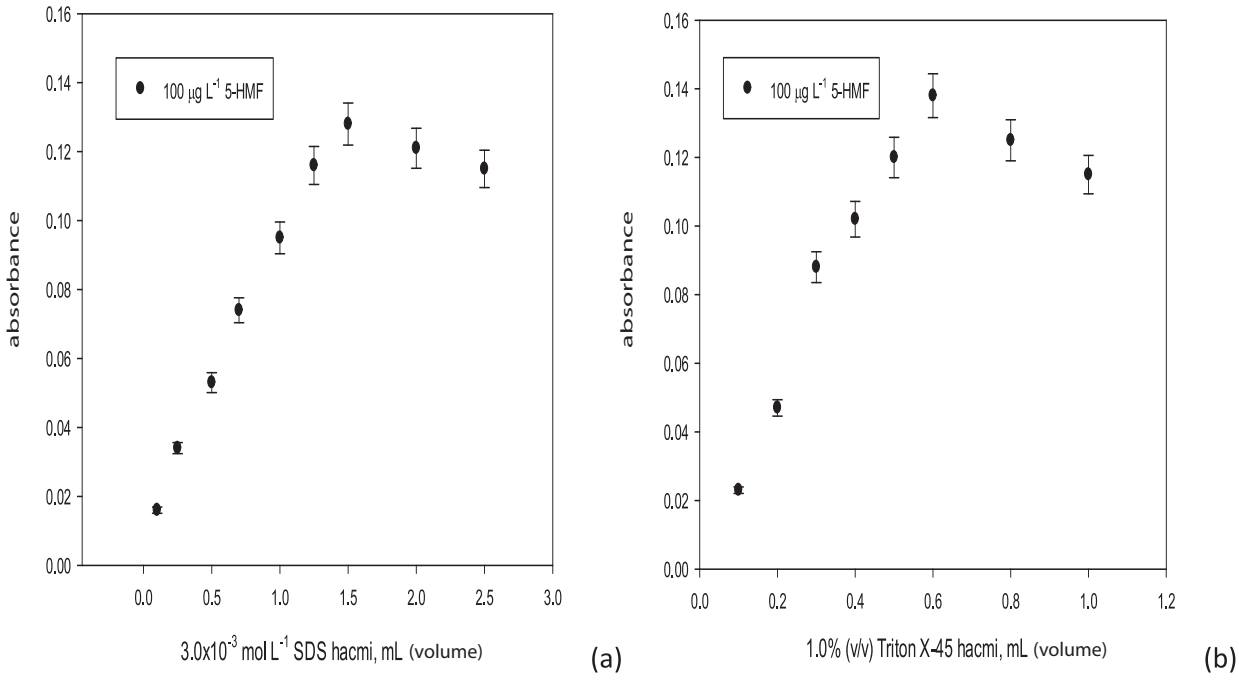


**Figure 3.** The effect of (a) ionic surfactant, $3.0 \times 10^{-3}$ mol/L SDS solution volume and (b) non-ionic surfactant, 1.0% (v/v) Triton X-45 solution volume on the sensitivity.

which act as highly weak, hard Lewis acid with a $pK_a$ of 10.6 (33), will form ion pairs in the presence of the SDS and complexing components of buffer at pH 9.0 where the hard-O, N donor atoms on oxime moiety participate in the coordination center of $Mn^{2+}$ ions. Therefore, it is concluded that 2.0 mL of Mn(II) for chelate formation is quantitatively sufficient for maximum extraction efficiency.

### *The effect of ionic (SDS) and non-ionic (Triton X-45) surfactant concentrations*

The effect of $3.0 \times 10^{-3}$ mol/L SDS on sensitivity was examined in the range of 0.1–2.5 mL (Figure 3a). With increasing surfactant volume up to 1.5 mL, the sensitivity increased and reached the maximum value. At volumes higher than 1.5 mL, it was observed that the sensitivity partially decreased, so as to lead to

an increase in the sample blank. Therefore, it was concluded that 1.5 mL of SDS is sufficient for maximum sensitivity.

Triton X-45 with physicochemical parameters of average oxyethylene unit of 4.5, clouding point of 35–45°C, critical micelle concentration (CMC) of 75 mg/L, and hydrophile-lipophile balance (HLB) of 9.8, is one of the most widely used nonionic surfactants in UA-CPE processes. Before analysis by FAAS, the centrifugation accelerates and facilitates phase separation for indirect detection of 5-HMF, due to its physiochemical properties such as high density of surfactant-rich phase, relatively low cloud point temperature, high purity availability, and low toxicity and cost (34, 35). The effect of 1.0% (v/v) Triton X-45 on the extraction efficiency of 5-HMF was examined in the range of 0.1–1.0 mL (Figure 3b). The absorbance of the complex increased linearly with an increasing volume of up to 0.6 mL and gradually decreased at higher volumes. This decrease is due to the increase in the volume and concentration of the micelle phase. In concentrations below this volume, the extraction efficiency of the complex was low as there were fewer surfactant molecules to quantitatively trap the complex. Therefore, it was concluded that 0.6 mL of surfactant is optimal for further studies.

*Electrolyte effect*

It is known that the ionic strength of solutions is one of the factors that affects the cloud point behavior of nonionic surfactants in separation-enrichment with UA-CPE. It has been observed that the presence of the electrolyte reduces the cloud point, causing more efficient and faster extraction. The lower cloud point is attributed to electrolytes that increase the dehydration of poly (oxyethylene) chains. According to Komaromy-Hiller et al. (36), this phenomenon is related to the orientation of ions towards the hydrophilic portions of micelles, which increases the interaction between micelles and consequently leads to the collapse of surfactant molecules for effective phase separation. In this study, the electrolyte effect conducted in the range of 0.5–5.0 mL for 0.01 mol/L KCl is provided in Figure 4, and

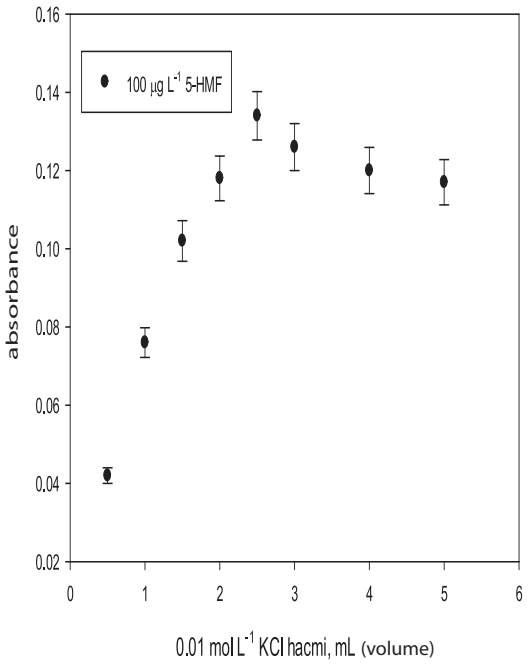


**Figure 4.** The effect of 0.01 mol/L KCl solution volume on the sensitivity.

demonstrates that the increased concentration of salt up to 2.5 mL has a significant effect on the extraction process (or the cloud point of Triton X-45) as a measure of sensitivity and at higher volumes, the sensitivity has been observed gradually to decrease.. Therefore, it was concluded that 2.5 mL 0.01 mol/L KCl was sufficient for a quantitative extraction.

*Effect of diluents*

In order to facilitate the detectability of the sample solution by FAAS, it was necessary to decrease the viscosity of the surfactant-rich phase. Different polarity solvents such as THF, acetone, acetonitrile, ethanol, methanol, and their acidic solutions (0.02 mol/L HCl) were tried in order to select the one producing the best results regarding sensitivity, reproducibility, and stability of the signal. The best result was obtained with acidic acetone. After phase separation, some acidic acetone was added to the surfactant-rich dense phase which was then submitted to nebulization of FAAS; where the micellar phase is diluted to about 0.5 mL for an enrichment factor of 70-fold from the enrichment of the optimal 35 mL sample (in the range of 5–50 mL). This amount of acidic acetone was chosen to ensure a sufficient volume of the sample solution for maximum sensitivity. For smaller volumes, the reproducibility of the signals was very poor, whereas for higher volumes, there was a gradual decrease in the signal due to excess dilution.

*Effect of equilibrium temperature and time*

The UA-CPE technique relies on the properties of many nonionic surfactants that become cloudy and form micelles when heated to cloud point temperature. The effect of the equilibrium temperature on the cloud point temperature was investigated in the range of 25–55°C. From the results, a temperature of 45°C was found to be sufficient to carry out quantitative extraction. Incubation time is also an important parameter to consider. From the results, it was observed that an optimal equilibrium time of 12 min was sufficient to obtain good extraction efficiency for the equilibrium time ranging from 1 to 20 min at 45°C.

*Effect of centrifugation rate and time*

The effect of centrifugation speed and duration on the sensitivity of the method for effective phase separation were investigated at different speeds (1000–4000 rpm) in 2–20 min intervals. From a series of repeated measurement results, a centrifugation time of 8 min at 3250 rpm was found to be sufficient for complete quantitative phase separation.

## Validation and Performance Features of the Method

For the validation process, the following parameters were determined by both direct calibration curve and matrix-matched calibration curve: linearity, recovery, intra-day/inter-day precision, LOD and LOQ, matrix effect and matrix dependent variations as established by the EU guidelines, and bioanalytical method validation (37, 38). Consequently, linearity and matrix effects (ME) were assessed by analyzing spiked samples at eight points in the range of 5–250 µg/kg (5, 15, 25, 50, 100, 150, 200, and 250 µg/kg) to cover an expected range of concentrations in samples. The limits of the method were obtained by the injection of analytical solutions in different concentrations prepared by dilution of the standard solution in the matrix blank extract which were obtained by the dilute acidic extraction procedure under sonication and confirmed experimentally. The LOD for 5-HMF has been defined as the lowest

**Table 1.** Analytical performance characteristics of the FAAS-combined UA-CPE method

| Analytical parameters | By direct calibration curve | By matrix-matched calibration curve[a] |
|---|---|---|
| Linear working range, μg/L | 4–240 | 5–200 |
| Slope, m | $(1.18 \pm 0.12)\times10^{-3}$ | $(1.30 \pm 0.13)\times10^{-3}$ |
| Intercept, b | $(1.24 \pm 0.05)\times10^{-2}$ | $(1.56 \pm 0.06)\times10^{-2}$ |
| Correlation coefficient, $r^2$ | 0.9961 | 0.9935 |
| LODs and LOQs, μg/L ($3\times s_{blank}/m$ and $10\times s_{blank}/m$, $n=10$) | 1.27, 4.24 | 1.38, 4.62 |
| Intra-day precision (RSD%, 25 and 100 μg/L, $n=5$ for same day) | 2.3–5.1 | 3.5–6.5 |
| Inter-day precision (RSD%, 25 and 100 μg/L, $n=3\times5$ for three consecutive days) | 3.5–6.5 | 4.1–7.5 |
| Accuracy (Recovery%, 25 and 100 μg/L, $n=5$) | 93.5–97.0 | 90.5–94.5 |
| Enrichment factor, EF | 70 | 70 |
| Sensitivity improvement factor, SIF | 30.6 | 33.6 |

[a] Calibration approach created after adding known volumes of the standard 5-HMF solution to the sample extracts, so that it falls within the range of 5–250 μg/L before enrichment with UA-CPE.

concentration that the analytical procedure can differentiate from the blank background noise while the LOQ is defined as the lowest validated spike level which meets the method performance acceptability criteria (mean recoveries were in the range 70–120%, with an RSD ≤20%). In this sense, under optimal conditions, the performance features of the method were comparably examined by two calibration curves. A direct calibration curve and a matrix-matched calibration curve, based on pure solvent and sample extracts, were highly linear between 4–240 and 5–200 μg/L, respectively, with correlation coefficients of 0.9961 and 0.9935. Table 1 summarizes the analytical features of the optimized method, including regression equation, linear working ranges, LODs and LOQs, enrichment factor, and repeatability/reproducibility within and between days. The LODs calculated from standard deviation of 10 replicate blank analysis and slope of calibration curves were 1.27 and 1.38 μg/L. The enrichment factor (35/0.5 = 70) was calculated as the ratio of the sample volume (35 to the surfactant-rich phase volume (0.5 mL) diluted with acidic acetone aspirated to the instrument prior to analysis by FAAS. After enrichment of the 35 mL sample, reasonable sensitivity improvement factors of 30.6 and 33.6 were obtained with direct calibration and matrix-matched calibration curves. The percentage recovery and precision data were between 90.5–97.0% and 2.3–7.5% (as RSDs), respectively, as a measure of accuracy and precision for the measurement of 25 and 100 μg/L 5-HMF for five replicates on the same day and three consecutive days. To estimate if the matrix substantially influences the sensitivity of the analyte, from the slopes of the calibration curves obtained for sample matrix ($b_{matrix}$) and the solvent ($b_{solvent}$), the matrix effect as % ME was calculated according to the formula below, and found to be +10.2% with a partial enhancement in analytical signal.

$$\%ME = (1 - b_{matrix}/b_{solvent}) \times 100 \quad (3)$$

This case shows that the method partly lacks specificity as other carbonyl compounds present or formed in the food during the process may also react with $NH_2OH$, so as to lead to overestimation of 5-HMF. On the other hand, assuming a matrix effect of ±20% is acceptable in this study, it can be said that a low matrix effect of +10.2% is quantitative in terms of reliability of further analysis results. Therefore, to avoid this matrix effect and ensure reliable results it is necessary to use matrix-matched calibration curves.

## The Matrix Effect

To evaluate the selectivity of the method, the effects of different ionic and non-ionic organic/inorganic species on determination of trace 5-HMF were investigated in UA-CPE. A known concentration of 5-HMF (100 μg/L) with different interfering concentrations was taken into account and the general extraction method was followed. From the results in Table 2, it can be said that the proposed indirect FAAS method is relatively selective for the determination of 5-HMF at trace levels. The effect of potential interfering organic species such as furfural, formaldehyde, and ascorbic acid can be largely controlled by the use of suitable masking reagents such as $Pb^{2+}$ and bisulfite prior to analysis. Moreover, in order to control the matrix effect in case of possible interference, besides diluting the sample solution, the matrix-matched calibration approach, which can be also used instead of the standard addition method to suppress the matrix effect, can be effectively used where the direct calibration approach is insufficient.

## Analytical Applicability of the Developed Method

In order to evaluate the analytical applicability of the proposed method, the intra-day and inter-day accuracy/precision analysis of two quality samples were carried out before and after spiking. Analysis results are given in detail in Table 3. The percentage recovery as a measure of accuracy and the the relative standard deviation amounts (RSDs) as a measure of precision were taken into consideration. From the intra-day and inter-day five replicate analysis results, an RSD of 4.0–5.7% and recoveries ranging from 89.3–95.0% were obtained. These results show that the method is quantitatively accurate and precise.

After concluding that the method is applicable to the analysis of real time samples, the developed method was analyzed and statistically checked in parallel with two different calibration approaches to take into account the possible sample matrix. At the same time, the accuracy and precision of both methods were evaluated comparatively (Table 4) by adding a standard of 25 μg/L after dilution of 250-fold. The results of the analysis by direct calibration ranged from 17.8 to 31.4 μg/L in the selected honey samples and ranged from 18.7 to 37.8 μg/L in jam samples. With the matrix-matched calibration curve, 5-HMF levels were in the range of 18.3–30.8 μg/L in honey samples while they were in the range of 18.1–38.1 μg/L in jam samples.

**Table 2.** Interference effect of possible matrix components on three replicate measurements of free 5-HMF at 100 μg/L level ($n = 3$)

| Interfering organic/inorganic species | Tolerance ratio, [Interferent]/[5-HMF] | Average recovery ± SD, %[a] |
|---|---|---|
| $NH_4^+$, $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ | 1500:1 | (97.0–99.5)±2.0 |
| $Sn^{2+}$, $Zn^{2+}$ | 1250:1 | (98.5–101.0)±2.0 |
| $Cl^-$, $Br^-$, $Pb^{2+}$ | 1000:1 | (98.0–101.5)±2.5 |
| $HPO_4^{2-}$, $SO_4^{2-}$, $HCO_3^-$ | 750:1 | (97.0–98.5)±2.5 |
| $Fe^{3+}$, $Co^{2+}$, $Cr^{3+}$ | 600:1 | (95.0–97.5)±2.0 |
| Bromobenzaldehyde, $Ni^{2+}$ | 500:1 | (97.0–102.5)±3.0 |
| 2-Chlorobenzaldehyde, phenol, 2-aminophenol | 350:1 | (93.5–97.0)±2.5 |
| $SO_3^{2-}$, $NO_3^-$, $F^-$, ethanol | 300:1 | (97.0–98.5)±3.0 |
| $Cd^{2+}$, $Ag^+$ | 250:1 | (97.0–98.5)±2.5 |
| 2-Nitrophenol, 4-nitrophenol | 200:1 | (94.0–96.5)±3.0 |
| $S_2O_3^{2-}$, $NO_2^-$ | 150:1 | (94.0–95.5)±2.5 |
| $Ni^{2+}$, $Cu^{2+}$ | 100:1 | (96.5–97.3)±2.5 |
| Benzaldehyde, 2,4-dinitrophenol | 80:1 | (100.5–102.5)±2.0 |
| $Fe^{2+}$, $VO^{2+}$, $VO_2^+$, $MoO_2^{2+}$ | 70:1 | (92.5–97.5)±2.5 |
| Ascorbic acid | 60:1 (200:1)[b] | 102.3 ± 2.0 |
| 2-Furfural, formaldehyde | 35,50:1 (100, 150)[c] | 103.5 ± 2.5 |

[a] The percent recoveries from three replicate measurements of binary mixtures and their relative standard deviations.
[b] Tolerance ratio obtained after improvement with pH 5.5 using 1.0 mL of 10 mg/L $Pb^{2+}$ as chelating metal ion.
[c] Before enrichment of 5-HMF from pre-treated samples, the tolerance rates obtained after pre-treatment of sample solutions with 1.5 mL of 10 mg/L bisulfite as a preservative around pH 5.0.

**Table 3.** Accuracy and precision studies for replicate measurement of free 5-HMF in two quality control samples with 15, 25, and 50 μg/L levels added to sample extracts diluted 1:250 times prior to analysis with FAAS

| Samples[a] | Sample volume, mL/dilution ratio | Spiking level, μg/L | Intra-day accuracy/precision (repeatability), $n = 5$ | | | Inter-day accuracy/precision (reproducibility), $n = 3 \times 5$ | | |
|---|---|---|---|---|---|---|---|---|
| | | | Found | Recovery, % | RSD, % | Found | Recovery, % | RSD, % |
| Geven flower honey[b] | 5/1:250 | – | 13.5 ± 0.7 | – | 5.1 | 12.5 ± 0.7 | – | 5.6 |
| | | 15 | 26.9 ± 1.3 | 89.3 | 4.8 | 26.1 ± 1.3 | 90.7 | 5.0 |
| | | 25 | 36.5 ± 1.7 | 92.0 | 4.6 | 35.5 ± 1.7 | 92.0 | 4.8 |
| | | 50 | 60.5 ± 2.5 | 94.0 | 4.1 | 60.0 ± 2.5 | 95.0 | 4.2 |
| Cherry jam | 5/1:250 | – | 23.5 ± 1.2 | – | 5.1 | 22.8 ± 1.3 | – | 5.7 |
| | | 15 | 37.1 ± 1.8 | 90.7 | 4.8 | 36.3 ± 2.0 | 90.0 | 5.5 |
| | | 25 | 46.3 ± 2.0 | 91.2 | 4.3 | 45.6 ± 2.3 | 91.2 | 5.0 |
| | | 50 | 70.0 ± 2.8 | 93.0 | 4.0 | 68.7 ± 2.8 | 91.8 | 4.1 |

[a] Two separate food samples that claimed to be completely organic and fresh.
[b] A perennial plant from legumes, locally known as "Geven" while systematically named gum genea, astragalus glycyphyllus, astragalus cicer, or leguminosae in plant biology. Their leaves are lined up across the branch in an elliptical way. Their blooms are are yellow, light yellow, white, and pink depending on their environment. They are thorny-perennial herbaceous plants with their length varying between 5 and 100 cm, depending on the type, and they are usually located at altitudes of 2000–2200 m, in Sivas, Turkey.

Samples of 1.0 g of honey and jam and 5.0 mL of the pre-processed and diluted sample solutions were analyzed before extraction. The 5-HMF contents of samples were in the range of 22.25–39.25 and 23.38–47.25 mg/kg, except for one jam sample. These are lower than the permitted limit value (40 mg/kg) prescribed by the EU for the formation of 5-HMF during processing in selected food matrices, and at a dose that does not pose a risk to human health.

## Comparison of the Method with Other Related Methods

The efficiency of the proposed method was evaluated by comparing the achieved analytical features with those of other extraction and detection techniques reported in the literature. As can be seen from Table 5, the method has a good intra-day and inter-day accuracy and precision in a linear working range of 60-fold. In addition, the detection limit and the pre-concentration factor of the method was generally better than those of other LC and CE techniques at different elution and detection modes, which are often used in separation and detection of 5-HMF in food and beverage matrices, but require a further separation/enrichment procedure such as vortex assisted-liquid-liquid microextraction (VALLME). In terms of operating parameters, the UA-CPE procedure was carried out using low-cost, simple devices and environmentally safe chemicals. The detection step was indirectly realized by using FAAS for Mn, linearly related to 5-HMF concentration, which is simple, easy to use, cost effective, and has fast measurement capabilities when compared to tedious, time-consuming, and complex chromatographic and electrophoretic techniques. Moreover, atomic spectrometric detection is available in many analytical research labs, and does not require an expert user to conduct 5-HMF analysis, unlike sensitive but more complex and expensive instrumental methods such as LC- or GC-MS.

**Table 4.** Determination of the 5-HMF levels in different honey samples, percent recoveries of the spiked samples, and comparison of the results with those obtained using the modified spectrophotometric White method (29)

| Samples | Sample volume, mL/ dilution ratio | By direct calibration | | | | By matrix-matched calibration | | | | Found by modified spectrophotometric White method, μg/L[b] | Experimental t- and F-values[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Added, μg/L 5-HMF | Found, μg/L 5-HMF[a] | RSD, % | Recovery, % | Added, μg/L 5-HMF | Found, μg/L 5-HMF[a] | RSD, % | Recovery, % | | |
| Filtered pine honey | 5/1:250 | – | 25.4 ± 1.0 | 3.9 | – | – | 24.7 ± 0.9 | 3.6 | – | 24.8 ± 1.0 | 1.16, 1.23 |
| | | 25 | 49.8 ± 1.6 | 3.2 | 97.6 | 25 | 49.3 ± 1.5 | 3.1 | 98.4 | – | – |
| Filtered flower honey | 5/1:250 | – | 28.5 ± 1.2 | 4.2 | – | – | 28.0 ± 1.0 | 3.6 | – | 27.8 ± 1.0 | 0.72, 1.44 |
| | | 25 | 53.0 ± 1.6 | 3.0 | 98.0 | 25 | 52.6 ± 1.5 | 2.9 | 98.4 | – | – |
| Honeycomp pine honey | 5/1:250 | – | 31.4 ± 1.2 | 3.8 | – | – | 30.8 ± 1.1 | 3.6 | – | 30.5 ± 1.0 | 0.66, 1.19 |
| | | 25 | 56.0 ± 1.8 | 3.2 | 98.4 | 25 | 55.3 ± 1.6 | 2.9 | 98.0 | – | – |
| Filtered honey (Zara, Sivas) | 5/1:250 | – | 27.3 ± 1.0 | 3.7 | – | – | 26.6 ± 0.9 | 3.4 | – | 26.8 ± 0.8 | 1.17, 1.23 |
| | | 25 | 51.6 ± 1.7 | 3.3 | 97.2 | 25 | 51.0 ± 1.6 | 3.1 | 97.6 | – | – |
| Honeycomp honey (Zara, Sivas) | 5/1:250 | – | 20.1 ± 0.8 | 4.0 | – | – | 19.4 ± 0.7 | 3.6 | – | 19.8 ± 0.8 | 1.61, 1.31 |
| | | 25 | 44.6 ± 1.4 | 3.1 | 98.0 | 25 | 43.5 ± 1.3 | 3.0 | 96.4 | – | – |
| Filtered honey (SEK brand) | 5/1:250 | – | 17.8 ± 0.7 | 3.9 | – | – | 18.3 ± 0.6 | 3.3 | – | 18.1 ± 0.7 | 1.21, 1.36 |
| | | 25 | 42.6 ± 1.4 | 3.3 | 99.2 | 25 | 43.5 ± 1.3 | 3.0 | 100.8 | – | – |
| Chestnut honey | 5/1:250 | – | 30.4 ± 1.1 | 3.6 | – | – | 29.7 ± 1.0 | 3.4 | – | 29.5 ± 1.0 | 1.05, 1.21 |
| | | 25 | 54.7 ± 1.6 | 2.7 | 97.2 | 25 | 54.3 ± 1.5 | 2.8 | 98.4 | – | – |
| Acacia honey | 5/1:250 | – | 28.2 ± 1.0 | 3.5 | – | – | 27.6 ± 0.9 | 3.3 | – | 28.0 ± 0.9 | 1.00, 1.23 |
| | | 25 | 52.8 ± 1.5 | 2.8 | 98.4 | 25 | 52.2 ± 1.4 | 2.7 | 98.4 | – | – |
| Citrus flower honey | 5/1:250 | – | 28.7 ± 1.1 | 3.8 | – | – | 28.1 ± 1.0 | 3.6 | – | 28.5 ± 1.0 | 0.90, 1.21 |
| | | 25 | 53.2 ± 1.5 | 2.8 | 98.0 | 25 | 52.7 ± 1.4 | 2.7 | 98.4 | – | – |
| Cherry jam | 5/1:250 | – | 38.7 ± 1.5 | 3.9 | – | – | 38.1 ± 1.5 | 3.9 | – | 38.2 ± 1.2 | 0.90, 1.21 |
| | | 25 | 61.2 ± 2.3 | 3.7 | 90.0 | 25 | 61.8.±2.3 | 3.7 | 94.8 | – | – |
| Cherry jam | 5/1:250 | – | 30.7 ± 1.3 | 4.2 | – | – | 28.7 ± 1.2 | 4.2 | – | 28.5 ± 1.0 | 0.90, 1.21 |
| | | 25 | 54.2 ± 1.8 | 3.3 | 94.0 | 25 | 51.7 ± 1.6 | 3.1 | 92.0 | – | – |
| Apricot jam | 5/1:250 | – | 18.7 ± 0.8 | 4.3 | – | – | 18.1 ± 0.8 | 4.4 | – | 18.5 ± 0.7 | 0.90, 1.21 |
| | | 25 | 43.0 ± 1.5 | 3.5 | 97.2 | 25 | 42.0 ± 1.4 | 3.6 | 95.6 | – | – |

[a] Average and standard deviation of five replicate measurements of 5-HMF with two calibration approaches after wet digestion under ultrasonic effect.
[b] The statistical t- and F-critical values at the 95% confidence level and 8 degrees of freedom are 2.31 and 6.39, respectively, to compare two average values of equal sample size for two independent samples t- and F test.
[c] The mean and standard deviation of the 5-HMF obtained using the modified spectrophotometric White method for samples similarly pretreated under ultrasonic power (300 W, 40 kHz) at 40°C for 5 min.

**Table 5.** Quantities of 5-HMF in foods analyzed by the current method, and comparison with other detection methods available in the literature

| Matrix type | Sample name | Detection method | Linear working range | Detection limit | Concn, μg/g | | Published data, mg/kg | References |
|---|---|---|---|---|---|---|---|---|
| | | | | | Average ($n = 3$) | RSD, % | | |
| Honey | Multiflorous A<br>Multiflorous B | RP-HPLC[a] | 0.13–100 mg/L | 0.04 mg/L | 38.3<br>4.6 | 3.0<br>10.0 | 0.8–138 | (3, 9) |
| Breakfast cereals | Honey rings<br>Cornflakes | LC | 10–150 μg/g | 0.03 μg/g | 24.7<br>46.8 | 3.0<br>4.0 | 4–193 | (11) |
| Beverages | Non-alcoholic<br>Alcoholic | HPLC/PAD | 0.1–100 mg/L | 3 μg/L, 280 nm | 8.5<br>6.3 | 4.0<br>7.0 | 4–22, 1–3 | (12) |
| Biscuit | Honey-biscuit | GC-MS | 25–700 μg/kg | 6 μg/kg | 7.0 | 5.0 | – | (13) |
| Jam | Plum | | | | 12.7 | 2.0 | | |
| Honey and vegetable oils | Honey | MEKC | 1–25 mg/L | 0.43 mg/L | 3–25 | 0.60–4.05 | 11–1145 | (14) |
| Foods | Honey, fruit juice | MEKC | 2.5–250 mg /g | 0.7 mg/kg | 40 | 3.0–10.2, 6.9–11.8 | 3.3–42.3,2.9–10.6 | (15) |
| Thermal processed foods | Honey | HS-SPME-HPLC | – | 7 μg/L, 280 nm | 200 μg/L | 1.4 | 66.1–179 | (16) |
| Honey | Different plant origin honey samples | RP-HPLC | 0.01–100 μg/L | 3 μg/L, 284 nm | | 0.2–1.47 | 5.90–155.8 | (17) |
| Beverages | Cola and fruit juices | SPE-AMTC-PAD[b] | 1–100 mg/L | 0.1 mg/L | 1.0 mg/L | 4.68 | 1.07–4.47 | (18) |
| Foods | Baby food, beer, and vinegar | SPME-GC-MS | 0.1–50 mg/L | 0.023 mg/L | 0.23 mg/L | 1.3–4.74 | 0.023–46.40, 0.023–27.87 | (19) |
| Beverages | Fruit juices | VALLME-HPLC-DAD | 1–5000 μg/L | 0.40–0.45 μg/L | 2–5000 μg/L | 1.5–3.2 | 0.71–28 | (20) |
| Foods | Fried products | HPLC-DAD-Full factorial CCD[c] | 0.8–56 mg/L | 7.6 μg/kg | – | 4.1–15.95 | 1.25 | (21) |
| Honey/alcoholic, non-alcoholic beverages | Different honey and alcoholic/non-alcoholic beverages | UA-CPE-Spectrophotometry | 6.5–275 μg/L | 1.96 μg/L | 25, 75 and 150 μg/L | 2.8–5.3, 3.8–5.6 | 0.903–1.555, 0.129–0.758 and 0.206–0.840 | (22) |
| Honey, jam | Different honey and jam samples | UA-CPE-FAAS | 4–240, 5–200 μg/L | 1.27, 1.38 μg/L | 25 and 100 μg/L | 2.3–6.5, 3.5–7.5 | 0.903–1.555, 0.129–0.758 and 0.206–0.840 | This study |

[a] RP = Reverse phase.
[b] AMTC = Amine trapped column chromatography.
[c] CCD = central composite design.

## Conclusions

A UA-CPE procedure was developed for extractive preconcentration of 5-HMF from honey and jam samples at pH 9.0 by using hydroxylamine and Mn(II) as derivatizing and chelating agents, with SDS and Triton X-45 used as mixed extracting surfactants. The developed protocol has been successfully employed for the indirect determination of trace 5-HMF in the selected honey and jam samples via FAAS. With respect to its achieved analytical features, the proposed method is simple, rapid, cost effective, and has a low LOD (1.27 μg/L), a wide in linear range (4–240 μg/L), a good recovery (≥90.5%), and is highly repeatable/reproducible (with RSD <7.5%). Given the importance of monitoring harmful substances like 5-HMF for food safety, this new method can benefit human health.

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## Conflict of Interest

The author declares that he has no conflict of interest.

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