https://doi.org/10.31871/WJRR.18.6.17
World Journal of Research and Review (WJRR)
ISSN: 2455-3956, Volume-18, Issue-6, June 2024 Pages 01-04

A Simple Method for Quantification of 5-hydroxymethylfurfural in Honey of Different Botanical Origins

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***Abstract*—5-hydroxymethylfurfural (HMF) is a compound resulting from the dehydration of fructose in an acidic environment in the presence of water, where heat accelerates this reaction. Honey, due to its high fructose content or adulteration with inverted sugar syrups, may contain HMF, indicating prolonged storage, improper heating, or processing. This can affect its commercialization, especially with the increase in international honey trade by Brazil. The Ministry of Agriculture has established a limit of 60 mg kg^{-1} of HMF in honey as a quality control measure. Determining HMF in honey involves analytical methods such as direct injection, mass spectrometry, or colorimetric methods, which can be complex and costly. The proposed method uses extraction based on the QuEChERS method and liquid chromatography with UV detection, avoiding heating and acidification during sample preparation.**

***Index Terms*—honey, HMF, method validation.**

I. INTRODUCTION

5-hydroxymethylfurfural (HMF) is a compound formed by the dehydration reaction of fructose in an acidic environment in the presence of water, with heat being an important factor to accelerate this reaction [1]. Honey is one of the foods where the presence of HMF can be identified, either due to the high fructose content in its composition (38%) or due to commonly performed adulterations through the addition of inverted sugar syrups. The presence of HMF is also indicative of prolonged storage time, improper heating, and processing of the food [2-5].

In recent years, Brazil has been standing out in the international market with an increase in honey trading. In 2020 alone, Brazil exported 45.7 thousand tons of natural honey, with a return of US$ 98.560 million [6]. The nutritional composition of honey depends on many factors such as: nectar origin; the species of bee that produced it; type of flower; soil; climatic conditions, and the presence of contaminants such as HMF can generate negative economic impacts on its commercialization.

As a measure of honey quality control, the Ministry of Agriculture, Livestock (MAPA) through the Department of Inspection of Animal Origin Products (DIPOA) has established a maximum acceptable limit of HMF in honey of 60 mg kg^{-1} [7].

The determination of HMF in honey has different analytical method approaches. In the literature, methods such as direct injection, making only a dilution of honey in water, which depending on the concentrations, can present many interferents in the chromatogram; methods that use mass spectrometry for quantification, using expensive inputs and not so accessible equipment; and colorimetric methods such as Wrinkle and White methods, which have many steps and toxic reagents [2,4,8-9].

The objective of this work was to present a simple, fast, and effective method for the quantification of HMF in honey, in which the extraction was based on QuEChERS and liquid chromatography coupled to a UV detector. This is a method that does not involve heating and acidification, thus avoiding the formation of HMF during sample preparation.

II. MATERIAL AND METHODS

A. Standard and reagents

The HMF standard was acquired from Sigma-Aldrich, Brazil, with 99% purity. Methanol (99.9%) of HPLC grade and acetic acid (99.8%) P.A. grade were obtained from J.T. Baker. Acetonitrile (99%) and sodium sulfate (99.0%), both P.A. grade, were obtained from Merck. Since sodium sulfate is a non-anhydrous reagent, it was subjected to drying at 120°C for 48 hours to remove any possible incorporated moisture. After treatment, it was stored in desiccators with silica gel. Deionized water was purified using the Barnstead Nanopure ultrapure water system.

Standard solutions were prepared by weighing HMF on an analytical balance to achieve a concentration of 1000 µg mL^{-1} and dissolved in water. Storage was carried out in a freezer at an approximate temperature of -18°C.

B. Equipment and chromatographic conditions

The chromatographic conditions were based on the methods described by [8-10].

The instrument used for quantification of HMF was a high-performance liquid chromatograph with UV-vis absorbance detector from Shimadzu, equipped with a quaternary pump (LC-20AD), an autosampler (SIL-20A), a column oven (CTO-20A), and a detector (SPD-10Avp) connected to a computer running LC Solution software for control and data acquisition.

The mobile phase consisted of deionized water with 1% (v/v) acetic acid and HPLC-grade methanol in a ratio of 90:10 (v/v), respectively, with a flow rate of 1.0 mL min-1. The

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injection volume was 20 µL. An analytical column Agilent Zorbax SB C-18 (150mm x 4.6 mm, particle size 3.5 µm) was used for separation. The column oven temperature was set to 35°C. The wavelength for detection of HMF was 285 nm. All statistical analyses were performed using Statistica 7.0 software.

The detection of sugars was conducted using an HPLC connected to an UV detector (Waters, USA) equipped with an analytical column (300 mm × 7.8 mm, 5 µm; Rezex Roa-Organic Acid H+, Phenomenex, USA). The column temperature was set at 55 °C. The mobile phase consisted of 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.6 mL min^{-1}. HMF was detected at 285 nm. The injection volume was 20 µL. A calibration curve was constructed by injecting standard solutions at five concentrations (0.66–4.98 µg mL^{-1}). The curve had a correlation coefficient (R) greater than 0.99.

C. Samples

Fifteen honey samples were collected from apiaries and markets, which were divided into 3 classes according to the honey's origin: orange blossom, eucalyptus, and wildflowers. Of the 15 samples, 13 are from São Paulo, 1 from the southern region of Minas Gerais, and 1 from the interior of Rio Grande do Sul. The samples were stored in a refrigerator at a temperature of 4 ± 3°C.

In order to evaluate whether the honey samples met the standards of Identity and Quality according to Brazilian legislation, the pH of the samples, presence of colorants, and sugar profile were assessed [7].

For pH determination, 10g of sample were weighed into a 250 mL beaker, 100 mL of deionized water were added, the mixture was homogenized, and the pH was measured.

To determine the presence of colorants, 1g of honey was weighed and dissolved in 10 mL of deionized water. After homogenization, approximately 2 mL of a 5% sulfuric acid solution were added.

The sugar analysis were done using a 150 mg honey diluted in 10 mL of 5 mmol L^{-1} H_2SO_4 (J.T.Baker, USA) under vigorous manual stirring, filtered through a polyvinylidene difluoride membrane filter (0.20 µm; Millipore, USA), and immediately analyzed by HPLC-UV.

D. Sample preparation to HMF analysis

The developed method is based on the modification of the QuEChERS method proposed by [2,11].

For the extraction procedure, 10.0 g of honey was weighed into a 50 mL polypropylene centrifuge tube, followed by the addition of 10 mL of water. The mixture was homogenized on a vortex until all the honey was completely dissolved. Then, 10 mL of acetonitrile was added and homogenized for 1 min on a vortex. Afterward, 6 g of sodium sulfate was added to the mixture and homogenized again for another 1 min. The samples were then centrifuged at 8000 RPM for 10 min. An aliquot of 1 mL of the supernatant was transferred to a glass test tube and subjected to evaporation with continuous airflow at room temperature. After complete drying, 1 mL of water was added and homogenized. The extract was then filtered through a 0.45 µm hydrophilic PVDF membrane and directed for chromatographic analysis.

E. In house validation

Internal validation was performed according to the criteria and recommendations of Guideline SANTÉ 11312/2021 [12]. The parameters considered were: linearity, limit of detection (LOD), limit of quantification (LOQ), selectivity, recovery, and precision.

Selectivity was verified by comparing signals at the retention time of HMF from a representative honey sample and analytical standard.

The LOD of the method was calculated considering 3 times the baseline noise amplitude. The LOQ was determined as the level at which the analyte could be detected accurately and quantified.

Linearity was determined from an analytical curve with five points prepared by successive dilutions in the range of 1-10 µg mL^{-1}. The presence of outliers was evaluated by the Hubber test and homoscedasticity by the Cochran test. Residual analysis was performed with a normal distribution of calibration points.

The precision and accuracy of the method were tested through recovery studies at two different concentration levels corresponding to 30 and 60 µg kg^{-1} in five repetitions each. Recovery data were calculated by comparison between the added value and the concentration obtained after analysis, and the results were expressed as percentage recovery. Precision results were expressed by the coefficient of variation (CV%).

The validated method was applied to the samples in triplicate, and all statistical analyses were performed using Statistica 7.0 software.

III. RESULTS AND DISCUSSION

A. Optimization of the chromatographic method

Reverse-phase C18 chromatographic columns were tested to evaluate retention time, symmetry, and peak broadening. The tested columns were Phenomenex (4.6 mm x 250 mm, 5 µm), Waters Symmetry (3.9 mm x 150 mm, 5 µm), and Agilent Zorbax (4.6 mm x 150 mm, 3.5 µm). Initially, chromatographic conditions of the method were tested, comparing different mobile phase compositions such as water:acetonitrile and water:methanol in ratios from 90:10 (v/v) to 50:50 (v/v). The 90:10 ratio in the water:methanol composition showed better peak symmetry. The Agilent Zorbax analytical column was chosen for optimizing the chromatographic conditions of the method. Method optimization was performed using a 2^3 experimental design. Tests were conducted with variations in temperature, flow, and different columns. The Agilent Zorbax C-18 column showed better separation performance, with lower retention time and peak symmetry.

B. Sample Preparation

Ten (10) grams of honey sample were weighed and diluted in 10 mL of water. In the partitioning step, the QuEChERS method uses magnesium sulfate, which heats the sample to approximately 40°C. This sample heating effect was not desired, as it catalyzes the formation of HMF in honey. Therefore, besides the extraction solvent, the quantities of partition salts interfering with HMF extraction were also evaluated. Sodium sulfate, sodium chloride, and the type of solvent (acetonitrile, acetone, and ethyl acetate) were assessed. It was concluded that acetonitrile would be the solvent used, 6 g of sodium sulfate would be used, and that sodium chloride could be discarded as it had no effect on

recovery. Additionally, the addition of an ambient temperature evaporation step for sample concentration using nitrogen gas and compressed air was tested. The recovery of HMF was verified, and it was found that the use of gas was indifferent, so compressed air was chosen. As shown in Fig. 1, there was a considerable reduction in interferents after the evaporation step.

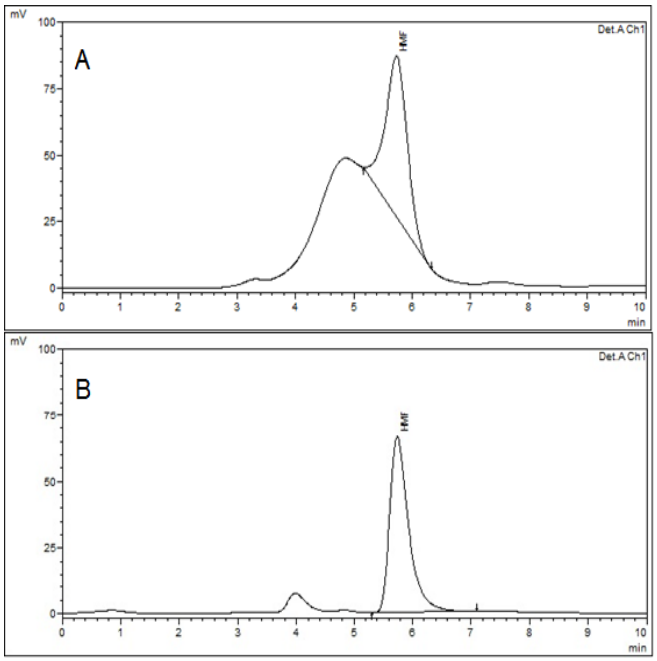

Fig 1. Comparative chromatograms of the sample cleaning step (a) without evaporation and (b) with evaporation.

C. *Effect of pH correction in the sample*

It is known that the formation of HMF occurs through the isomerization of fructose in an acidic medium, and that temperature increases the conversion rate. Therefore, an initial attempt to alkalize the medium was made, as described by [2], with the addition of 150 µL of NH_4OH. However, recoveries below the acceptable range were observed. The effects of pH alteration in the sample were then studied through tests with acetic acid and ammonium hydroxide. The test was performed on fortified samples in triplicate, and three extractions were performed: samples alkalized with 150 µL of NH_4OH, acidified with 150 µL of acetic acid, and without pH adjustment. Recoveries for alkalized samples were around 65%, for samples without pH adjustment, it was 85%, and for acidified samples, it was the same as samples without adjustment. The reduction in recovery is probably due to the degradation of HMF in a basic medium [13-14].

D. *Method validation*

The obtained values for LOD and LOQ were, respectively, 0.05 µg mL^{-1} and 30 mg kg^{-1}. The achieved LOQ value was due to the absence of honey samples free from the presence of HMF. Considering that the tolerable limit of HMF residue in honey is 60 mg kg^{-1}, the method has the necessary sensitivity to quantify at relevant levels for inspection. The method proved to be linear, with a correlation coefficient of 1.00, and curves prepared with water. Accuracy and precision were evaluated on two different days, through fortification in quintuplicate at levels of 30 mg kg^{-1} and 60 mg kg^{-1}. The evaluated parameters were recovery and precision. Intermediate precision was evaluated by comparing the results obtained on different days. The data obtained are presented in Table I. All method parameters are in accordance with those recommended by SANTE (2021) [12].

Table I. Recovery means among fortifications and coefficients of variation.

Level (mg kg^{-1})	Recovery (%)	CV (%)
30	83	1.32
60	83	0.78

E. *Characterization of honey samples and quantification of HMF*

The pH value of honey can be influenced by the pH of nectar, soil, or the association of plants for honey composition. Bee mandibular substances added to the nectar during transportation to the hive can also alter the pH of honey. According to Brazilian legislation, the pH of honey should range between 3.3 and 4.6. The obtained results varied between pH = 4.04 (orange blossom, São Paulo state) to as high as 4.74 (eucalyptus blossom, São Paulo state). Sample honey 11, with pH 4.74, does not meet the identity standard required by legislation.

The indicative test for the presence of coloring substances in honey, whether natural or artificial, did not indicate any color change. If there are coloring substances added to the honey, the color gradually shifts from violet to pink.

The analysis of the sugar profiles of the honeys was performed by liquid chromatography with a refractive index detector, quantifying the concentrations of sucrose, glucose, and fructose present in each sample. Figure 2 shows a chromatogram of the sugar analysis.

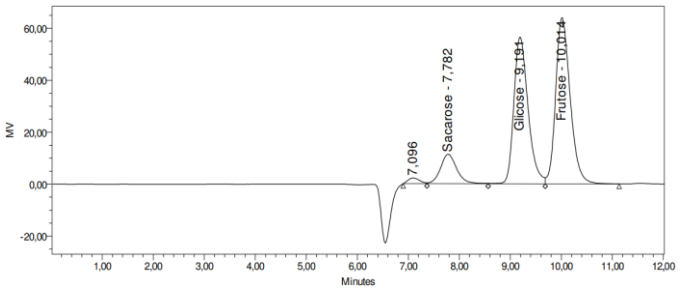

Fig 2. Chromatogram demonstrating the sugar profile in honey.

Honey is a concentrated solution of sugars with a predominance of glucose and fructose. It also contains a complex mixture of other carbohydrates, enzymes, amino acids, organic acids, minerals, aromatic substances, pigments, and pollen grains, and may contain beeswax from the extraction process [7]. Regarding reducing sugars, glucose and fructose, except for one sample (# 13), are below the minimum established of 65g/100g of honey. As for sucrose, legislation sets a maximum limit of 6g/100g of honey. Only samples #9 and #17 met the criteria.

For the quantification of HMF, the validated method was applied to the 18 samples. The analyses were conducted in duplicate accompanied by quality control (control sample and fortifications at validated levels). All samples showed contamination by HMF, ranging from 3.26 to 148.05 mg kg^{-1} as presented in Table II.

Table II. Results of honey sample characterization and HMF quantification.

#	Honey	pH	HMF (mg kg^{-1})	Sucrose (g kg^{-1})	Glucose (g kg^{-1})	Fructose (g kg^{-1})
01	Orange	4.04	13.74	87.56	327.86	393.86
02	Orange	4.20	17.31	101.21	329.43	383.52
03	Orange	4.16	11.73	82.44	290.15	387.83
04	Orange	4.18	13.00	104.47	303.62	359.46
07	Eucalyptus	4.25	148.05	111.18	310.38	375.88
08	Eucalyptus	4.36	79.69	73.04	325.58	376.93
09	Eucalyptus	4.56	4.42	56.22	355.26	390.74
10	Eucalyptus	4.48	8.94	86.95	288.20	410.69
11	Eucalyptus	4.74	4.28	87.58	286.97	403.81
13	Wildflower	4.36	3.26	193.54	184.98	138.68
14	Wildflower	4.50	8.48	81.40	301.92	359.96
15	Wildflower	4.42	6.76	76.52	317.39	350.04
16	Wildflower	4.18	45.31	90.83	325.85	357.46
17	Wildflower	4.32	30.36	55.74	362.08	352.06
18	Wildflower	4.30	61.11	90.34	339.78	363.32

The honey samples #7, # 8, and #18 showed contamination above the maximum permitted limit for HMF (60 mg kg^{-1}).

IV. Conclusion

The method developed, validated, and proposed in this work meets the criteria established by the SANTE guideline. The method offers a fast and cost-effective sample preparation, enabling its applicability in the laboratory. Both the characterization of the samples and the HMF analyses indicate that honeys from different botanical origins do not have standardization regarding quality and identity standards.

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