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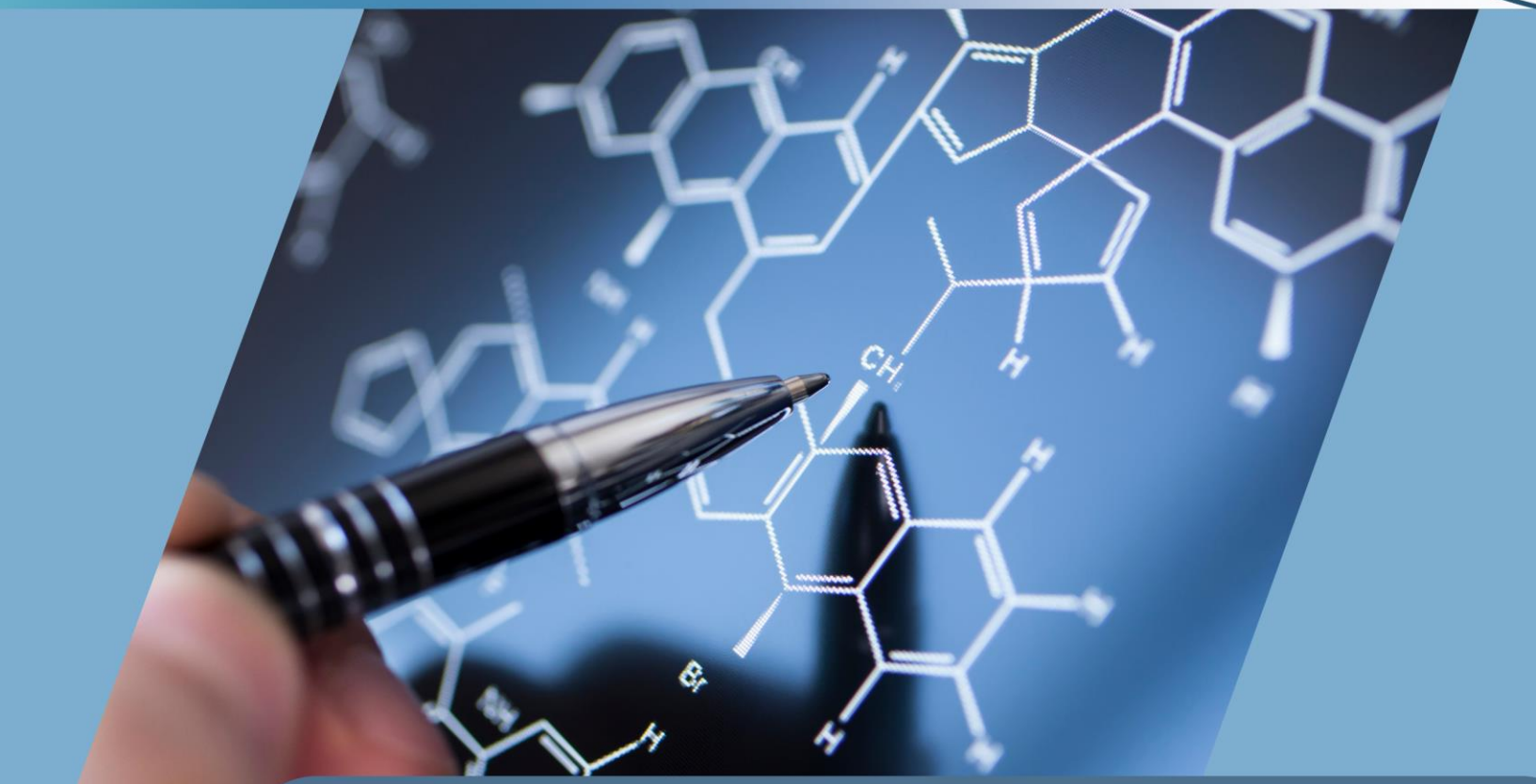

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Spectrophotometric Determination of 5-Hydroxymethylfurfural in Honey Samples from Al-Marj City in Libya using White Method

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A B S T R A C T

5-hydroxymethylfurfural (HMF) content was determined as a parameter for honey quality for three Sidr honey samples from Al-Marj city in Libya. In this study, the spectrophotometric quantification of HMF in honey was performed using White method which is one of the most accurate and recommended official methods. The honey samples were obtained from three different farms that distribute honey to most of the retail centers across the city. The aim of this study was to determine HMF concentration in samples from this geographical region to provide some data for future studies. HMF can be considered one of the most important parameters for honey testing and its concentration can predict if heat was used in any stage of honey production. All samples showed normal HMF concentrations indicating good honey quality, good storage conditions, and no possible adulteration nor heating. The preparation of a full report for the honey quality in this region is recommended.

1. Introduction

Honey is a natural sweet material that is created by honeybees. Bees collect nectar from flowers, and for further processing, bees combine it with substances of their own and finally store it in the honeycomb to ripen (Codex Alimentarius Commission). Honey testing is essential for the assessment of its quality and authenticity worldwide during consumption and purchase using several methods; these methods are utilized to determine the chemical composition of honey as well as its biological activity (Żak & Wilczyńska, 2023). Diastase number and 5-hydroxymethylfurfural are the most frequent parameters tested for honey quality (Sakač & Sak-Bosnar, 2012; White, 1994).

5-(hydroxymethyl-)furan-2-carbaldehyde, or 5-Hydroxymethylfurfural (HMF) is a heterocyclic aldehyde formed by dehydration reactions of reducing sugars, mainly fructose in acidic medium. Its concentration is usually expressed in milligrams per kilogram, and it is naturally present even in fresh honey but in low concentrations. HMF is formed as a result of non-enzymatic dehydration of glycans (caramelization) during storage or as a Maillard reaction byproduct during thermal honey treatment (Capuano & Fogliano 2011; Silva et al., 2016; Veríssimo et al., 2017).

HMF could also be found in other foodstuff including pastries, beverages, caramel solution, and coffee. In general, HMF could be used as a parameter and a special marker compound for quality for a wide range of processed fruits and food products (Capuano & Fogliano 2011; Rada-Mendoza et al., 2002; Rada-Mendoza et al., 2004; Cristina 2010). Inappropriate storage conditions including the type of storing containers and moisture contribute to HMF formation. High HMF content in honey may also indicate the

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addition of syrups or another kind of sugars. Therefore, The HMF content is regarded as a measure of the freshness of honey as well as proof of its quality, which can be impacted by heat treatment, storage conditions, or sugar factors including storage conditions, heat-treatment or adulteration with sugars (Tosun 2013 & Veríssimo et al., 2017).

HMF is quantified in honey due to its potential harmful effects on human health since it is carcinogenic substance (Abraham et al., 2011; Janzowski et al., 2000; Teixidó et al., 2006). HMF can be rapidly absorbed from the gastrointestinal tract in our bodies, and at high concentrations HMF is cytotoxic with LD_{50} of 3.1 g/kg body weight in rats and with a dietary intake of 1.6 mg per person a day (Capuano & Fogliano 2011, Veríssimo et al., 2017). According to the Codex Alimentarius Commission, honey HMF content after processing should not exceed 40 mg/kg, and for honey coming from regions with tropical temperatures 80 mg/kg is the limit.

There are three methods to quantify HMF in honey using UV or UV-VIS detectors recommended by The International Honey Commission (IHC); two of them are conventional spectrophotometric techniques (White and Winckler methods), and the third is using high performance liquid chromatography (HPLC method) (Bogdanov, 2009; Zappala et al., 2005). The UV-VIS spectrophotometric determination of HMF in honey by White is based on reacting HMF with the bisulfite anion (HSO_3^-) (Teixidó et al., 2011; White, 1979). The White method gave more accurate HMF values when compared to Winckler method and gave almost identical HMF concentrations to the ones determined by HPLC. The White method also uses low-cost reagents compared to HPLC and is considered a greener alternative to Winckler's (Bogdanov, 2009).

The current study aimed to investigate whether heat was utilized in any stage of honey production in Al-Marj, Libya. Heat could lower the quality of honey by producing Maillard by-products such as HMF or affecting enzymes that are naturally present in honey. In acidic solutions like honey, heat could elevate the level of HMF. In this study, HMF concentration was quantified spectrophotometrically using the conventional, and most recommended official method developed by White. Other honey-quality parameters such as the enzyme levels (diastase and invertase) were not the focus of the study since if heat was used, the enzymes would be affected. The purpose of this study was to quantify HMF in honey samples from this geographical region to provide some data for future studies. The importance of this data is to highlight cities for honey export.

2. Materials and Methods

The current study used White's method to determine the concentration of HMF in three honey samples obtained from different random farms across Al-Marj city, Libya; these Sidr honey samples were collected and analyzed before the flood that happened in the region in 2023 (UNICEF, 2023). The determination of HMF content in this study was based on the determination of UV absorbance of HMF of "White honey sample" against a reference sample at 284 nm where HMF should absorb. The interference of other components present in honey at this wavelength was avoided by subtracting the difference between the absorbances of a clear aqueous honey solution (i.e., after the addition of deproteinating agents) and the same solution after the addition of bisulfite. HMF content is then calculated after subtraction of the background absorbance at 336 nm (White, 1979; Bogdanov, 2009).

2.1. Preparing the Reagents

For White method, solutions of 15% potassium ferrocyanide (Carrez solution I), 30% zinc acetate (Carrez solution II), and 0.2% sodium bisulfite were prepared in volumetric flasks and renewed daily. Carrez solution I was prepared by dissolving 15.0 g of potassium hexacyanoferrate(II), $K_4Fe(CN)_6 \cdot 3H_2O$ in water and made the volume up to 100. mL. Carrez solution II was then prepared by diluting 30.0 g of zinc acetate dihydrate, $Zn(CH_3.COO)_2.2H_2O$ and then made up to 100. mL. Finally, Sodium bisulfite solution 0.20 g/100.0 g was prepared by dissolving 0.20 g of solid sodium hydrogen sulfite $NaHSO_3$ in water and then diluted to 100 mL (White 1979; Bogdanov, 2009).

2.2. Equipment

Double-beam spectrophotometer operating in a wavelength range including 284 and 336 nm is needed to measure the absorbance of the sample solution since the absorbance of HMF is from 250-330 (the maximum is 284). In this study, spectrophotometer (Cecil Aquarius CE 7400) was used, and the cuvette was a 1-cm quartz cell. Vortex mixer was used to homogenize the honey samples.

2.3. Sample preparation and procedures

For each sample, five grams of honey were weighed into a beaker and dissolved in about 20 mL distilled water, and then transferred quantitatively into a 50 mL volumetric flask. The solution afterward was homogenized and 0.5 mL of Carrez solution I and 0.5 mL of Carrez solution II were added. Finally, the flask was filled to the mark with distilled water. The solution was filtered through general-purpose filter paper so the precipitated proteins in honey have no contribution to the UV-VIS absorbance leaving only HMF absorbance; 5.0 mL of the filtrate was then pipetted in each of two test tubes. 5.0 mL of water was added to one of the test tubes (the sample solution), and 5.0 mL of 0.2% sodium bisulfite solution was added to the other (the reference solution or White solution). The absorbance reading was measured using the spectrophotometer at 284 nm (for the maximum HMF absorbance) and then at 336 nm. According to White, about 94% of the HMF absorbance band at 284 nm was reduced by the presence of the bisulfite indicating the reaction between the two compounds (White, 1979 & Bogdanov, 2009).

3. Results and Discussion

Following the White method, the UV-VIS absorbance of a clarified honey solution (sample) was determined against a reference solution of the same honey sample after the destruction of the 284 nm chromophore of HMF by its reaction with the bisulfite. This way the background absorbance of honey was not included leaving only HMF absorbance in the sample. The difference between sample absorbance (without bisulfite) and the reference (with bisulfite) indicated the absorbance of HMF. Thus, the difference absorbance eliminated the contribution of other honey constituents. The HMF was then quantified using its literature absorptivity value. The absorbance of the bisulfite is negligible at 336 nm, and it is about 0.014 at 284 nm. According to White, the absorbance values then were placed in the official formula developed by White (see Equation 1 below). Where: A_{284} and A_{336} are the absorbance values at 284 nm and 336 nm respectively, and m is the sample mass of 5.0 g. The factor 149.7 was calculated by (126/16830) x (1000/10) x (1000/5). Where: 16830 is HMF molar absorptivity at wavelength of 284 nm, 1000 is the conversion factor from grams of sample to milligrams, and 5 is the honey mass (White, 1979).

$$HMF(mg/kg) = (A_{284} - A_{336})\, x149.7\, x\, \frac{5}{m} \quad \textbf{(1)}$$

Table 1 below shows the absorbance values for the three honey samples with their corresponding HMF concentration that were calculated using Equation 1. For honey samples 1, 2, and 3, the HMF concentrations were 26.65, 10.93, and 28.00 mg/kg respectively. The three samples collected randomly from different nearby farms from Al-Marj city in Libya were good-quality honey samples and fulfilled the European standards for honey. Maillard reaction products such as HMF depend directly on the processing temperature (White, 1979; Cristina et al, 2010). This concludes that the three honey samples were not exposed to any kind of heat treatment during processing. Since HMF levels were normal, heat was not used, and other parameters of honey quality such as diastase number and invertase levels would not be a concern for this study.

Table (1) Absorbance values for honey samples with the corresponding HMF values.

Honey sample	Absorbance at 284	Absorbance at 336	HMF content in mg/kg
1	**0.248**	**0.07**	**26.65**
2	**0.223**	**0.15**	**10.93**
3	**0.327**	**0.04**	**28.00**

4. Recommendation and Future Work

HPLC is used today for measuring HMF levels in food products. However, it is considered an expensive technique and that is the reason why the White method is still on top of conventional spectrophotometric methods for HMF quantification. Development of a safe, quick and reliable technique for measuring HMF content is still needed. Using more environmental-friendly chelating agents to deproteinize honey, as a replacement for Carrez solutions, is a possibility for a better quantification of HMF in honey without producing chemical waste. The chemical waste produced by White method limits its utilization in educational settings including graduation projects and as a biochemistry laboratory experiment. The replacement of Carrez solutions would make White method one of the recommended biochemistry laboratory experiments for science major and premedical college students since it uses a real, inartificial sample (honey). That would have an impact on students' learning experience and would better prepare them for their career. The modification of White's method

recommended by Okibe et al., can only be used in industry but not for educational purposes since Carrez solutions were replaced with corrosive, strong acid, the perchloric acid (Okibe, et al., 2020; Perchloric acid. SDS, 2023). For a greener quantification of HMF, the development of a more benign method is still needed since the White method utilizes reagents that could be replaced. In addition, including more parameters in the future to generate a full, intensive honey-quality report should be considered; these parameters include ash content, moisture, acidity, conductivity, and enzyme levels.

5. Conclusion

HMF levels were normal for honey samples that were obtained from the main distributors from Al-Marj city in Libya using the official White method. The significance of this research was providing insights into this geographical region and testing the main indicator for overheating honey. The focus was whether heat was used in any stage of honey production in these farms. HMF was the main heat-byproduct that naturally present in honey, but its health issues emerge at high concentrations. If honey was heated in any stage, other parameters would be affected as well including diastase number and invertase levels. Since the main heat-byproduct is HMF, the current study focused on the determination of its concentrations to provide data regarding this particular region to support further scientific research.

Conflict of Interest: The author declares that there are no conflicts of interest.

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