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Modification of White Method for Quantitative Evaluation of 5-hydroxymethylfurfural in Honey

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Abstract *The White method for determination of 5-hydroxymethylfurfural (5-HMF) content in honey was successfully modified using Perchloric acid ($HClO_4$) as replacement for zinc acetate ($Zn(CH_3CO_2)_2{\cdot}2H_2O$) and potassium ferrocyanide ($K_4Fe(CN)_6{\cdot}3H_2O$) to serve as Deproteinizing agent, and Sodium bisulphite ($NaHSO_3$) was replaced with sodium pyrosulphite ($Na_2S_2O_5$) for the chromophore removal of 5-HMF at 284 nm. The proposed method was validated by evaluation of parameters such as linearity, precisions (reproducibility and intermediate), accuracy, and limit of detection (LOD), limit of quantification (LOQ), ruggedness and robustness. The correlation coefficients for the calibration curves were 0.9994 and 0.9923. The method is in agreement with Beers-Lamberts law at the concentration range of 5, 10, 15, 20 and 25 mg/kg. The values of reproducibility and intermediate precision in honey samples were 2.65, 2.67, 3.03, 4.73, and 1.90 % respectively. The recoveries for the analyses were between 81.4 % and 104.6 %, LOD and LOQ were 0.12 and 0.36 mg/kg at 284 nm and 0.06 and 0.17 mg/kg at 336 nm respectively. The ruggedness of the method was 1.23 and 1.00 %, and the robustness were 0.64 and 0.42 %. The results obtained suggest that Perchloric acid and sodium pyrosulphite can successfully replace zinc acetate, potassium ferrocyanide and Sodium bisulphite which are scarce and expensive reagents. The Modified method is suitable for routine determination of 5-HMF in honey samples*.

Keywords: *5-hydroxymethylfurfural, honey, perchloric acid, sodium pyrosulphite, carre*

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1.0 Introduction

The Codex Alimentarius regulation for honey (1987), define honey as the natural sweet substance produce by *Apis Mellifera* bees from the nectar of plants or extra floral secretion that bees transform and store. Honey starts out as nectar that bees collect from flowers. Basically, nectar is sugar-rich liquid produced by plants in glands called nectaries and is use to attract pollinating insects and birds. It's a sugary fluid which includes the aromatic oils that give flowers their scent, as well as other trace substances (Abraham, 2011). Hydroxy- methylfurf - ural (5-hydroxymethylfurfural) is a six-carbon heterocyclic organic compound containing both aldehyde and alcohol (hydroxymethyl) functional groups (Pereira *et al.,* 2011). The ring of the structure is centered on furan moieties, whereas the two functional groups, that is, formyl and hydroxyl-methyl groups, are linked at the second and fifth positions, respectively.

5-hydroxymethylfurfural is a solid, yellow substance that has a low melting point but is highly soluble in water and its chemical formula is 5(Hydroxymethyl)-2-fuancarboxaldehyde ($C_6H_6O_3$) (Ames, 1992). The presence of simple sugars such as glucose and fructose and various acids has been reported to be favorable for honey production (Zappala *et al.,* 2008).

Fig. 1: Chemical structure of HMF

roxymethylfurfural has attracted several research interests because of its carcinogenic potential for humans. Some studies have shown that this metabolite can be converted in *vivo* to 5-sulfooxymethylfurfural (SMF), a genotoxic compound (Surh *et al.,* 1994). In addition, at high concentrations, HMF is cytotoxic, causing irritation to eyes, upper respiratory tract, skin and mucous membranes (Bruce *et al.,* 1993). For this reason, the Codex Alimentarius and the European Commission have set 40 mg/kg as a maximum concentration of HMF in honey except those from tropical countries and honeys with low enzyme levels, in which the HMF limit is set at 80 and 15 mg/Kg respectively (Codex, 1987; European Commission, 2001). Three methods for determination of hydroxymethyl - furfural are described and validated by the IHC (Bogdanov *et al.,* 1997). However, only two of them are recommended for use: the HPLC and the white method. Winkler method is not recommended for determination of HMF because one of the reagents (p-toluidine) is carcinogenic (Stefan *et al.,* 2004). Both spectrophotometer methods are fast but not very specific and sensitive. In particular, systematic positive interference and the use of p-toluidine (a recognized carcinogenic compound), are some of the reason that also made the Winkler method to be discarded (Winkler *et al.,* 1995). On the other hand, the RP-HPLC method is more accurate and sensitive than spectrophotometric methods but quite slow and very expensive. According to Anklam (1998) the suitability of the analytical methods for 5-HMF is unsatisfactory and requires further investigation. Therefore, this study is aimed at modifying the white method for determination of hydroxymethylfurfural in honey using perchloric acid and sodium pyrosulphite as replacement for the scarce and expensive carrez reagents and sodium bisulphite.

2.0 Materials and Methods

2.1 Reagents and solutions

Analytical grade: methanol, ethanol, sodium pyrosulphite ($Na_2S_2O_5$), Carrez solution II: 30 g zinc acetate (Zn $(CH_3CO_2)2{\cdot}2H_2O$), Carrez solution I: 15 g potassium ferrocyanide (K_4Fe $(CN)_6{\cdot}3H_2O$), sodium bisulphite ($NaHSO_3$), and perchloric acid ($HCLO_4$) were obtained from Steve Moore chemical store Zaria, Kaduna State. 5-Hydroxymethylfurfural was obtained from sigma-Aldrich (Santa Ana, CA, USA) and the Stock solutions of 5-HMF (1000 mg L^{-1}) was prepared in MeOH-water solution (50:50, v/v) at a 1000 mg/L concentration and store at 4 ^{0}C until analysis. Five solutions of different concentrations (5, 10, 15, 20 and 25 mg/L) were prepared for calibration curves.

2.2 Honey samples

For the purpose of this study, five (n=5) honey samples were obtained across the Kachia Local Government Area in Kaduna State, and was stored at ambient temperature (4 ^{0}C), in the dark, until the experiment.

2.3 Determination of 5-hydroxymethylfurfural content by the two methods

2.3.1 White method:

Five gram of honey sample was weighed into a 50 mL beaker. The sample was dissolved in approximately 25 mL of distilled water and transferred quantitatively into a 50 mL volumetric flask. 0.5 mL of Carrez solution I was added and mixed. 0.5 mL of Carrez solution II was also added and mixed. The solution was diluted to volume with distilled water (a drop of ethanol was added to suppress foam) and followed by filtration. 5.0 mL of the solution was pipette into each of the two test tubes. 5.0 mL of water was added to one of the test tubes and were agitated for them to be well mixed. 5.0 mL of sodium bisulphite solution (0.2%) was also mixed with the second test tube to obtain a reference solution. The absorbance of the sample solution against the reference solution at 284 and 336 nm (in 10 cm quartz cells) was determined (A.O.A.C, 1990).

2.3.2 Modified method

Five gram of honey sample was weighed into a 50 mL beaker. The sample was dissolved in approximately 25 mL of water and transferred quantitatively into a 50 mL volumetric flask. 2 mL of ice-cold perchloric acid was added and mixed. The solution was placed on ice for 5 minutes, followed by filtration. 0.02 mL of ice-cold neutralization solution was also added to the sample solution and mixed to neutralize the sample and precipitate excess PCA. 5.0 mL of the solution was pipetted into each of the two test tubes. 5.0 mL of water was added to one of the test tubes and mixed well. Again, 5.0 mL of sodium pyrosulphite solution (0.2%) was added to the second test tube and mixed well (the reference solution) in which the 284 nm chromophore of HMF was removed. The absorbance of the solution against the reference solution at 284 and 336nm (in 10 cm quartz cells) was determined. The quantitative value of HMF was determined using the proposed formula for the method (Bogdanov *et al.,* 1997).

The hydroxymethylfurfural content of honey was calculated using the following equation:

$$HMF(mg/kg) = \frac{A284-A336) \times 74.87}{W} \quad (1)$$

where A284 and A336 are absorbance reading at 284 and 336 nm. 74.87 is the correction factor. The correction factor was calculated from the following equation

$$\text{Factor} = \frac{126 \text{ x } 100 \text{ x } 1000 \text{ x } 100}{16830 \text{ x } 1000} = 74.8 \quad (2)$$

where 126 = Molecular Weight of HMF, 16830 = Molar absorptivity of HMF at 284 nm (IHC, 2002).

2.4 Method validation

The modified method was validated according to ICH (2005) guide lines for validation of analytical procedures in order to determine the linearity, precision, accuracy, percentage recovery, limit of detection, limit of quantification, ruggedness and robustness.

2.5 Statistical analysis

All analyses were carried out in triplicates and the data was presented as means ± standard deviations. Linear regression analysis and paired sample t-test were used to compare the quantified variables in the samples of honey.

3.0 Results and Discussion

3.1 Calibration curve

Calibration curves for determination of 5-hydroxymethylfurfural in honey obeys Beers-Lamberts law within the range of 5-25 mg/kg. Correlation coefficient is a statistical measure that calculates the strength of the relationship between the relative movements of two variables (ICH, 2005). The coefficients of determination (R^2) were 0.9994 (Fig. 2) for absorbance at 284 nm and 0.9923 for the absorbance at 336 nm (Fig. 3). It was observed that the absorbance at 336 nm had the least R^2 value. There was direct relationship and positive correlation between the absorbance and the concentrations (Table 1). In comparison, the method has a better calibration curves compared to the known classical method. Hameed *et al*. (2019) reported also reported R^2 value of 0.98 when he used the classical method.

Table 1. Calibration parameters for 5-hydroxymethylfurfural at 284 and 336 nm

Parameter	284 nm	336 nm
R^2	0.9994	0.9923
Intercept (a)	- 0.0345	0.303
Slope (b)	0.1534	0.002
LOD (mg/kg)	0.12	0.06
LOQ (mg/kg)	0.36	0.17

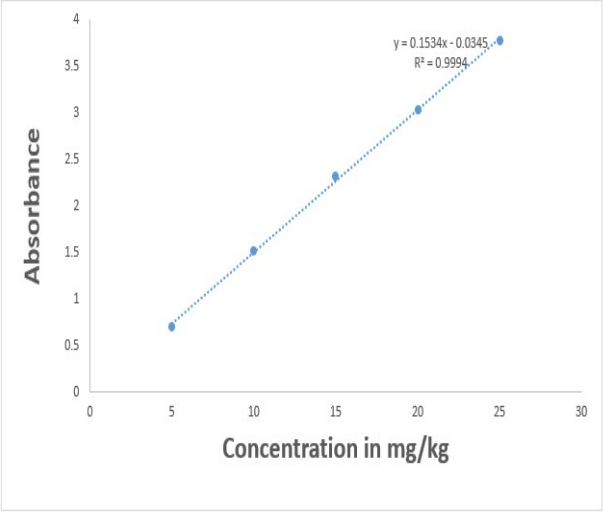

Fig. 2: Calibration curve of 5–hydroxymethylfurfural at 284 nm

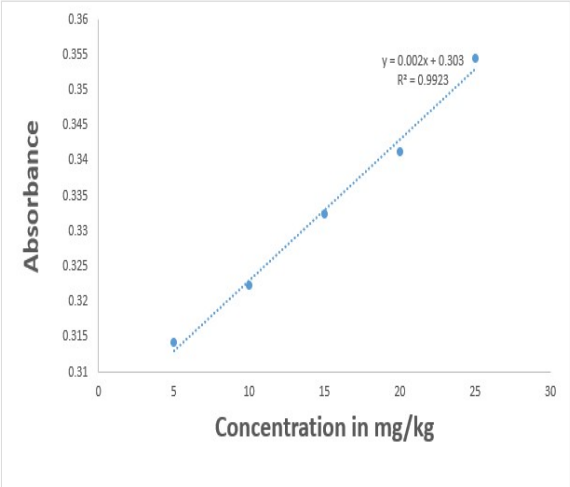

Fig. 3: Calibration curve for the determination of 5-hydroxymethylfurfural at 336 nm

Table 2. The comparison of results obtained by the two methods

Sample	White method (mg/kg)	Modified method (mg/kg)
S1	43.26±0.01	45.93±1.06
S2	52.64±0.14	56.00±0.01
S3	28.13±0.01	32.63±0.01
S4	26.51±0.01	24.17±4.14
S5	54.02±0.00	39.94±0.06
Pearson's correlation		0.8183
P-value		0.3743

3.2 The comparison of results obtained by the two methods

Comparison of both methods was carried out on different types of honey samples (sample S1, S2, S3, S4 and S5) (Table 3). The White method gave result in the ranged of 28.13 to 54.02 mg/kg while the modified method gave results that ranged from 24.17 to 56.00 mg/kg. However significant was found in the range obtained for sample S5 which was 54.02 mg/kg by White method and 39.94 mg/kg, but there was no significant difference between results obtained for the two methods in sample S1, S2, S3 and S4. The Pearson's correlation (0.8183) indicated positive (+) correlation between the two methods. The p-value (0.374) calculated for the reference and proposed method was greater than the alpha level chosen (0.05). The t-test indicated no significant difference between the results obtained for the mean concentration of HMF by Modified method and reference method (Table 2).

3.3 Precision

The precision of the procedures was determined by repeatability (intra-day) and intermediate precision (inter-day). One sample solution containing the target level of analytes was prepared. Eight replicates were made from sample solution and analyzed according to the final method procedure. The precision of the method was checked using the International Conference Harmonization (2005). The precision (repeatability) for the method was 2.65 % while intermediate precision was found to be 2.67, 3.03, 4.73 and 1.90 % respectively for Operator 1-Day 1, Operator 2-Day1, Operator 1-Day 2 and Operator 2-Day 2 respectively (Table 4). The relative standard deviation (RSD %) were within the acceptable limit of <15 RSD %, which shows good precision for the method. Viviane (2012) and Hameed *et al*. (2019) reported the precisions of 5.41 and 12.5 % respectively. In comparison, these values are higher than the one of the proposed methods, which confirmed that the proposed method is précised.

3.4 Accuracy

The accuracy of the assay method was evaluated with the recovery of the standards from excipients. Percentage recovery values ranged from 81.4 to 104.6 % for the spiking solution at three different concentrations (1, 2 and 3 mg/kg). The relative standard deviations were found to be 2.65, 0.48 and 0.22 % respectively (Table 5). This is within the ranged of 1-5 % and indicate that the method is accurate (Varvey, 2000). Maryam and Farzaneh (2015) reported % RSD of 6.1, 4.4 and 7.4 % for honey samples which were relatively higher than the ones reported by this method. Hameed *et al.,* (2019) reported the mean recovery values range from 73 to 89 % for HMF. This shows that the proposed method has a very good accuracy and specificity (Varvey, 2000).

3.5 Limit of detection (LOD) and limit of quantification

Limit of detection is the lowest quantity of analytes an analytical method can detect but not

necessary quantify. The LOD values were 0.12 and 0.06 mg/kg, which point to good sensitivity of the method compared to 0.02 and 0.0.15 mg/kg reported by Maryam (2012) and Hameed *et al.,* (2019). LOQ as the smallest quantity of analyte an analytical method can detect and quantify. The LOQ of the two absorbance were 0.36 and 0.17 mg/kg which also implies good sensitivity of the method compare to 0.06 and 0.07 mg/kg reported by Maryam (2012) and Hameed *et al*., (2019). The LOD and LOQ were found to have small values indicating the sensitivity of the method (Table 1).

3.4.2 Ruggedness and robustness

The ruggedness of the method was carried out by two different analyst and the respective absorbance and concentrations of HMF were recorded. The relative standard deviation determined by Analyst 1 and 2 were 1.23 and 1.00 % indicating that the method is rugged compared to ICH (2005) guideline (Table 6 and 7). The robustness analysis was carried out to determine the effluence of small but deliberate variation in the wavelength. The relative standard deviation (RSD %) at wavelength 284 and 336 nm was 0.37 % and 0.24 % at wavelength 283 and 335 nm (Table 8 and 9).

Table 3. Comparison of the reagents used between Modified method and some of the reported methods

Methods	Reagents	Comments
Modified	• Sodium pyrosulphite ($Na_2S_2O_5$) • Perchloric acid ($HCLO_4$)	Stable, sensitive and not expensive.
White	• Sodium bisulphite ($NaHSO_3$) • Carrez solution I: potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$) • Carrez solution II: zinc acetate ($Zn(CH_3CO_2)2 \cdot 2H_2O$)	Expensive and not readily available
Winkler	• p-toluidine (C_7H_9N) • Barbituric acid ($C_4H_4N_2O_3$)	Carcinogenic, less sensitive and not readily available
HPLC	• Carrez solution I: 15 g potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3H_2O$) • Carrez solution II: 30 g zinc acetate ($Zn(CH_3CO_2)2 \cdot 2H_2O$)	Expensive and not readily available

Table 4. Results obtained from repeatability studied for evaluation of HMF

Parameter	HMF (mg/kg)	RSD %
Precision-Repeatability	56.12	2.65
Precision-intermediate: Operator 1, Instrument 1, Day 1.	55.76	2.67
Operator 2, Instrument 2, Day 2.	54.82	3.03
Operator 1, Instrument 1, Day 2.	56.85	4.73
Operator 2, Instrument 2, Day 2.	56.36	1.90

Table 5. Results obtained from accuracy studied for evaluation of HMF in honey

Levels	Concentration added (mg/kg)	Concentration found (mg/kg)	Recovery %	RSD %
50 %	1	58.74±1.15	81.4	2.65
100 %	2	58.91±0.0.28	84.6	0.48
150 %	3	59.90±0.13	104.6	0.22

Table 6. Result showing Ruggedness by Analyst 1.

Analysis 1			
Concentration (mg/kg)	**Absorbance (336)**	**Absorbance (284)**	**Statistical analysis**
56.82	0.3162	4.2358	
57.77	0.3168	4.1761	Mean = 57.29 mg/kg
57.39	0.3169	4.1511	SD = 0.71
56.35	0.3170	4.0819	RSD = 1.23 %
58.10	0.3165	4.1981	

Table 7. Result showing Ruggedness by Analyst 2.

Analysis 2			
Concentration (mg/kg)	**Absorbance (336)**	**Absorbance (284)**	**Statistical analysis**
56.66	0.3150	4.0991	
57.51	0.3150	4.1558	Mean = 57.03 mg/kg
56.21	0.3159	4.0698	SD = 0.57
57.43	0.3170	4.1522	RSD = 1.00 %
57.33	0.3144	4.1434	

Table 8. Result showing Robustness at 336 and 284 nm

Concentration (mg/kg)	Absorbance (336 nm)	Absorbance (284 nm)	Statistical analysis
57.62	0.3044	4.1538	
57.61	0.3038	4.1510	Mean = 57.32 mg/kg
56.91	0.3039	4.1042	SD = 0.37
56.94	0.3031	4.1056	RSD = 0.64 %
57.52	0.3038	4.1452	

Table 9. Result showing Robustness at 337 and 283 nm

Concentration (mg/kg)	Absorbance (337)	Absorbance (283)	Statistical analysis
57.50	0.3018	4.1416	
57.18	0.3005	4.1190	Mean = 57.33 mg/kg
57.59	0.3021	4.1483	SD = 0.24
57.40	0.3051	4.1382	RSD 0.42 %
57.00	0.3072	4.1138	

4.0 Conclusions

The White method was successfully modified for evaluation of 5-HMF in honey and was validated. Satisfactory results were obtained in relation to linearity, precision (repeatability and intermediate precision), accuracy, ruggedness, and robustness, limit of detection and limit of quantification which show that the proposed method was suitable for 5-HMF evaluation in honey. Comparison of the proposed and the standard method was also carried out on five honey samples. From the results obtained, it can be concluded that White method for determination of 5-HMF in honey was successfully modified and validated. There was no statistically significant difference ($P<0.05$) between the means concentrations of 5-HMF determined by the Modified and Winkler methods. Similarly, there was no significant difference between the standard deviations calculated for both methods. Hence the two methods gave accurate, precise and satisfactory results for the concentration of 5-HMF in honey and is recommended as a simple, cheap, precise, accurate, rugged, robust and easy applicable method for determination of 5-HMF content in honey. Hence, the modified method can be applied as an alternative (or complementary) analytical technique to the recommended White method for total estimation of 5-HMF content in honey.

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