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Research Article

Quantification of a Carcinogenic and Cytotoxic compound: HMF(5-hydroxymethyl-2-furfural) using HPLC(High-Performance Liquid Chromatography)

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ABSTRACT

In this research work, Extraction of HMF (5-hydroxymethylfurfural) from honey was done and the amount was estimated by using the reference standard of HMF on HPLC. Honey samples were received for testing from two companies and the reference standard was obtained from Sigma-Aldrich. Honey is a natural product which is produced by honey bees (Apis mellifera). The composition of honey changes depending on its floral, geographical and entomological sources. HMF concentration in fresh honey is mostly absent or is present in very low amounts, but its concentration increases during processing, upon storage for longer periods of time and upon heating/storing at higher temperatures. HMF contributes to sometimes fatal effects (mutagenic, chromosomal aberrations, genotoxic, organotoxic, carcinogenic, cytotoxicity towards mucous membranes, the skin, the eyes and the upper respiratory tract). Extraction of HMF from honey was carried out by making Carrez I and Carrez II solutions. An accurate and specific Reversed-Phase HPLC method was developed due to the urgent requirement of an analytical method to estimate the quantity of HMF in honey. A mobile phase with a combination of Acetonitrile and Water with 0.2% Formic Acid was used with a flow rate of 0.6 ml/minute and the separation was done on Phenomenex Non-Polar C18 Column with dimensions of 150 mm X 4.6 mm X 3 µm at a wavelength of 282 nm. Analysis was carried out with a run time of 15 minutes. The Calibration curve method was adopted for the estimation of HMF in honey with R2 value of 0.999. These results were repeated two times in the laboratory; hence Repeatability Precision was performed to validate the results. The retention time of the HMF reference standard was 6.53 minutes and retention time of the samples (Sample 1 and Sample 2) was 6.63 minutes and the retention time variation of ± 0.1 minutes was observed which is acceptable and was within limits. The amount of HMF in honey in the samples were found to be within the limit of 40 mg/kg as per FSSAI. The amount of HMF present in Sample 1 and

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and Sample 2 was found to be 2.20 mg/kg and 5.28 mg/kg respectively. HMF was extracted from honey and a new analytical HPLC method was developed. This new HPLC method developed would be economical, reproducible, and repeatable. The amount of HMF present in Honey samples was found to be 3.80 mg/kg and 6.50 mg/kg. The results were found to be within the limits (40 mg/kg) as specified by FSSAI regulatory guidelines.

INTRODUCTION

In this research work, Extraction of HMF (5-hydroxymethylfurfural) from honey was done and the amount was estimated by using the reference standard of HMF on HPLC. Natural products have been used for ages as dietary supplements. Natural products like Honey are nutritional and beneficial for our body. Honey is a natural medicinal product which is produced by honey bees (Apis mellifera) after they collect the nectar from flowers. Honey is a natural sweetener that is added to food to give flavor of sweetness [1]. Honey has many secondary metabolites like Flavonoids, Alkaloids, Terpenoids, Phenolics, etc which can heal various diseases related to gastrointestinal and respiratory tracts [2, 3, 4]. Honey is used in the treatment of Cancer. Honey is useful for the skin as it gives an anti-oxidant effect, promotes the development of new tissue, cures wounds, acne, eczema, dandruff, tinea and psoriasis as it is anti-inflammatory, anti-fungal and anti-bacterial properties [2, 4, 5]. Honey contains amino acids and reducing sugars like glucose and fructose in majority and other sugars like trehalose, melibiose, turanose and raffinose in low amounts [1, 2, 3, 4, 6, 7]. An organic compound 5-hydroxymethyl-2-furfural (HMF) is produced from dehydration of reducing sugars in honey in acidic environments (amino acids in honey) when heated. This is called as Maillard reaction which is a non-enzymatic browning reaction. [1, 3, 7, 8, 9]. This adulteration [3] leads to toxic effects. HMF has carcinogenic, mutagenic, genotoxic, chromosomal aberrations, organotoxic and cytotoxic on mucous membranes, the skin, the eyes and the upper respiratory tract [1, 7, 8]. HMF is a heterocyclic compound containing 6 carbon atoms comprising of a furan ring on which two functional groups i.e., formyl and hydroxymethyl groups are attached at the second and fifth positions, respectively (Figure 1). HMF is a white-colored compound that is highly soluble in water [1, 8]. HMF concentration indicates honey's freshness and quality. The concentration of HMF is negligible in fresh honey but increases during its processing or heating, upon storage for longer periods or storing in higher temperatures [1, 7, 10]. The FSSAI (The Food Safety and Standards Authority of India) also marks honey's quality as degraded if HMF in honey exceeds the limit of 40 mg/kg. According to Codex Alimentarius, HMF in Honey must not exceed the limit of 40 mg/kg after processing and blending, except for honey obtained from tropical climatic region [10]. Due to this adulteration and processing of honey, there is an urgent need to develop an analytical method to quantify the amount of HMF present in honey as this leads to serious health issues and can lead to the death of an individual if the quality of honey (marketed and sold to the public) is not checked properly [1, 3, 7, 8, 10]. In this research work, a new analytical method to quantify the amount of HMF in honey was developed on HPLC (High-Performance Liquid Chromatography) by using the Calibration curve method as there are very few articles on HPLC analysis of HMF in honey were reported before, but they were not reproducible in the laboratory and were uneconomical [10 - 21], few articles reported for LC-MS/MS analysis of HMF in pharmaceutical products [22, 23, 24], few articles reported for detection of adulterants using NMR [25]. And there is no official method in FSSAI too for the quantification of HMF in honey. Hence, this new analytical method was developed. First, the Extraction of HMF from honey was carried out and then it was quantified on HPLC using the reference standard for HMF with 99%

purity, obtained from Sigma-Aldrich. These results were repeated twice in the same lab on the same instrument to validate the results for the Repeatability Precision Validation parameter.

MATERIALS AND METHODS

Chemicals and Solvents:

5-hydroxymethyl-2-furfural (HMF) with a purity of 99.8% and molecular formula C6H6O3 obtained from Sigma-Aldrich (Merck), Bangalore, India. Acetonitrile with a purity of 99.93% and Formic acid with a purity of 99.0% were obtained from Merck (India) Ltd., Mumbai, Maharashtra. Milli-Q Water was used from Milli-Q Direct Water Purification System - Milli-Q - Type 1 Ultrapure Water procured from Merck, India. All other reagents and chemicals used were of AR Grade commercial quality.

Instruments and Chromatographic conditions:

The HPLC system used was Shimadzu LC-20 with a UV-visible DAD detector, binary pumps, Column compartment with oven, Autosampler and other parts of the system. A 0.45 µm PVDF HPLC Membrane Syringe filter was used. Chromatographic separation (Reverse phase) was carried out on a C18 Phenomenex Column with dimensions of 150 mm X 4.6 mm X 3 µm in Isocratic mode. The mobile phase comprising of Water with 0.2% Formic Acid and Acetonitrile with a ratio of 90:10 (v/v) at a flow rate of 0.6 ml/minute. Column temperature was kept at 30°C. The injection volume was 10 µl. The Wavelength (λmax) on which the peaks were observed was 282 nm. The run time for the analysis was 15 minutes. The chromatographic conditions are given in Table 1 and Table 2.

Experimental procedures:

Preparation of Standard stock solution:

The Standard stock solution of the HMF reference standard was prepared with a 2 mg/ml concentration in methanol.

Preparation of Calibration points:

The Calibration points were prepared by ½ serial dilutions of Standard stock solution to prepare ten calibration points i.e., 0.012 ppm, 0.024 ppm, 0.049 ppm, 0.098 ppm, 0.195 ppm, 0.391 ppm, 0.780 ppm, 1.563 ppm, 3.125 ppm, 6.25 ppm. These were used to plot on the Calibration curve of HMF Reference Standard.

Preparation of Sample:

10 g of honey was weighed in a 50 ml volumetric flask, dissolved in 50 ml of Milli-Q Ultrapure water and mixed well. From the above solution, 25 ml of the solution was pipetted out in a 50 ml volumetric flask in which 0.6 ml of Carrez-I and 0.6 ml of Carrez-II solutions were added and stirred well, then the volume was made up to the mark with water. The solution obtained was milky in appearance. The contents of the flask were filtered through a Whatmann Filter paper, where the first 10 ml of the filtrate was discarded. Then this filtrate was filtered through a 0.45 µm PVDF HPLC Membrane Syringe filter and transferred to the HPLC glass vials for the chromatographic analysis.

Carrez-I solution:

21 g of Zinc acetate dehydrate was dissolved in 10-15 ml of water and 3 g glacial acetic acid was added and diluted to 100 ml with water.

Carrez-II solution:

10 g of Potassium ferrocyanide was dissolved in 10-15 ml of water and then diluted with 100 ml of water.

RESULTS

Analytical Method Development:

Different Mobile phase compositions and different buffers were tested before finalizing the best mobile phase for the analysis of HMF in honey. For obtaining consistent best results, a mobile phase of Water with 0.2% Formic Acid and Acetonitrile with a ratio of 90:10 (v/v) was chosen with a flow rate of 0.6 ml/minute. We observed sharp peak shapes (Gaussian peak shape) with this column chosen. The Calibration plot showed a

linear calibration curve with a correlation coefficient, R2 value of 0.999 (Figure 2). The retention time of the HMF reference standard was 6.53 minutes and retention time of the samples (Sample 1 and Sample 2) was 6.63 minutes and the retention time variation of ± 0.1 minutes was observed which is acceptable and was within limits. The initial four peaks observed in chromatogram of Samples of honey received for testing are the other impurities present in the honey samples as the two peaks of these four peaks are not observed in HMF Reference Standard's chromatogram. The results were validated for Repeatability Precision validation parameter and the analysis was repeated twice.

- The amount of HMF in honey in the samples were found to be within the limit of 40 mg/kg as per FSSAI.
- The amount of HMF present in Sample 1 and Sample 2 was found to be 3.80 mg/kg and 6.50 mg/kg respectively.
- The chromatogram of HMF Reference Standard is given in Figure 3. The chromatograms of Sample 1 and Sample 2 is given in Figure 4 and Figure 5 respectively.
- The results of HMF in Samples of honey received for testing are given in Table 3.

DISCUSSION

This analytical method developed for the first time was well analysed by testing different mobile phases, flow rates, chromatographic column. This method was repeated to see whether it gives consistent results. And it gave consistent results mentioned in Table 3. Few other samples of honey were analysed by this method, apart from those mentioned above, and the amount of HMF present in these samples of honey exceeded the limit of 40 mg/kg. And these results were repeated twice to validate the method and same results(values) were obtained even after repetition. This showed adulteration in honey and these samples were found to be unsafe for consumption. These samples which exceeded the limit as per FSSAI (Food Safety and Standards Authority of India), were reported to the Government of India, New Delhi, for further action. Therefore, it can be said this research helped the Government of India to address this serious issue and take action against those companies whose samples exceeded the HMF limit. Thus, this method was found to be suitable for analysing the amount of HMF present in honey samples as it was economical, robust and precise(reproducible) as it gave consistent results on repetition of the analysis. HMF was extracted from honey samples received for testing. A new HPLC (High-Performance Liquid Chromatography) method for quantifying the amount of HMF (5-hydroxymethyl-2-furfural) was developed for the first time. This method is economical, reproducible and repeatable. This method will help detect adulteration in honey as HMF is harmful for humans. This investigation is essential for the health of public. The results were found to be within the limits specified by FSSAI for the maximum amount of HMF to be present in honey i.e., within 40 mg/kg.

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Table 1. Chromatographic Conditions Of Hmf Analysis In Honey

Parameters	Chromatographic Conditions
Mobile Phase	Acetonitrile and Water with 0.2% Formic acid
Chomatographic Column (Stationary phase)	Phenomenex C18; 150mm x 4.6 mm x 3 µm
Flow rate	0.6 ml/minute
Column temperature	30°C
Run time	15 minutes
Injection volume	10 µl
Wavelength	282 nm
Elution Mode	Isocratic

Table 2. Mobile Phase Composition Of Hplc Analysis In Isocratic Mode

Run time (minutes)	Mobile phase A (Water with 0.2% Formic acid)	Mobile phase B (Acetonitrile)
15	90	10

Here the mobile phase composition is Water with 0.2% Formic acid:Acetonitrile (90:10)

Table 3. Results Of Hmf In Samples Of Honey

SR. No	Sample Code	Results of HMF (5-hydroxymethyl-2-furfural)
1.	Sample 1	3.80
2.	Sample 2	6.50

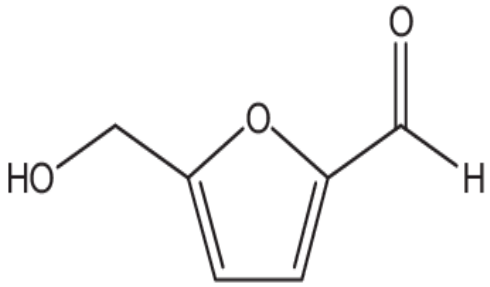
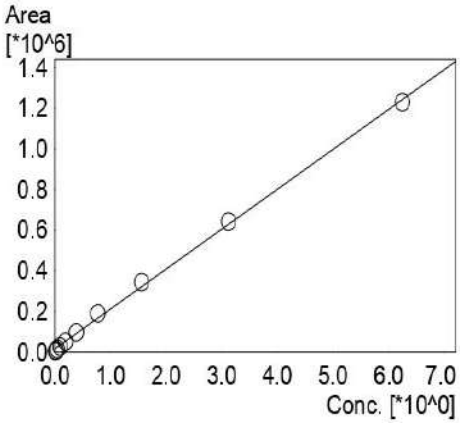


Fig. 1. Structure of HMF (5-hydroxymethyl-2-furfural) [1]

<Calibration Curve>

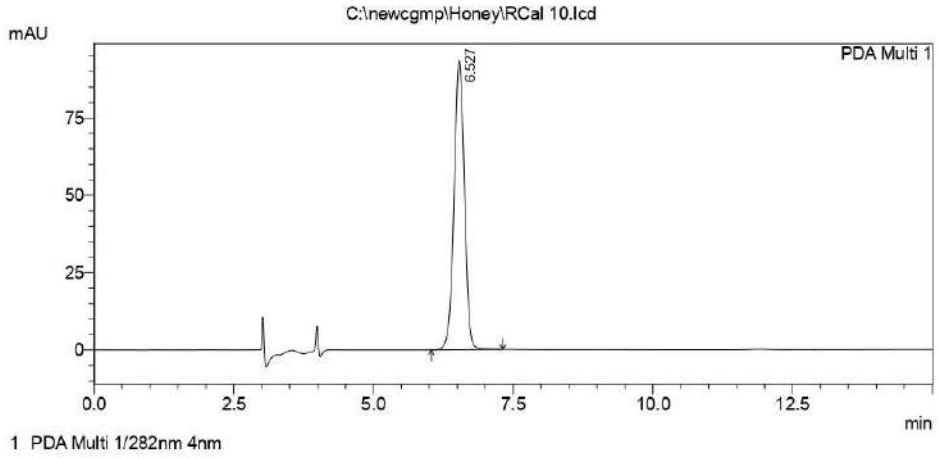
ID# : 1
Name : HMF
Quantitative Method : External Standard
Function : f(x)=196488*x+15316.9
Rr1=0.9993390 Rr2=0.9986784
MeanRF:261523 RFSD:49982.6 RFRSD:19.1121
FitType : Linear
ZeroThrough : Not Through
WeightedRegression : None
Detector Name : PDA



#	Conc (Ratio)	Area	Area
1	0.012	4348.1	4348
2	0.024	7556.9	7557
3	0.049	14676.3	14676
4	0.098	27067.8	27068
5	0.195	50607.1	50607
6	0.391	96724.4	96724
7	0.780	190727.8	190728
8	1.563	344108.4	344108
9	3.125	642169.7	642170
10	6.250	1228846.8	1228847

Fig. 2. Calibration Curve of HMF(5-hydroxymethyl-2-furfural) in Honey.

The calibration curve of HMF was plotted with concentrations ranging from 0.012 ppm to 6.25 ppm and was found to be linear with a R2 value of 0.9986



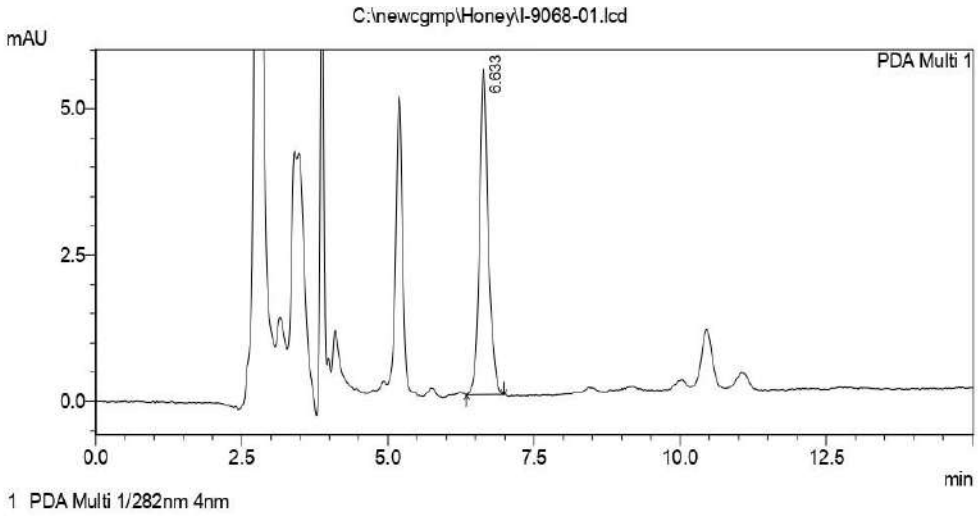
Quantitative Results

PDA

ID#	Name	Ret. Time	Area	Height	Conc.
1	HMF	6.527	1228847	93457	6.176

Fig. 3. Chromatogram of HMF Reference Standard (Reference Standard 10)

The chromatogram depicting the Reference standard of HMF with the highest concentration i.e., 6.25 ppm at a retention time of 6.527 minutes



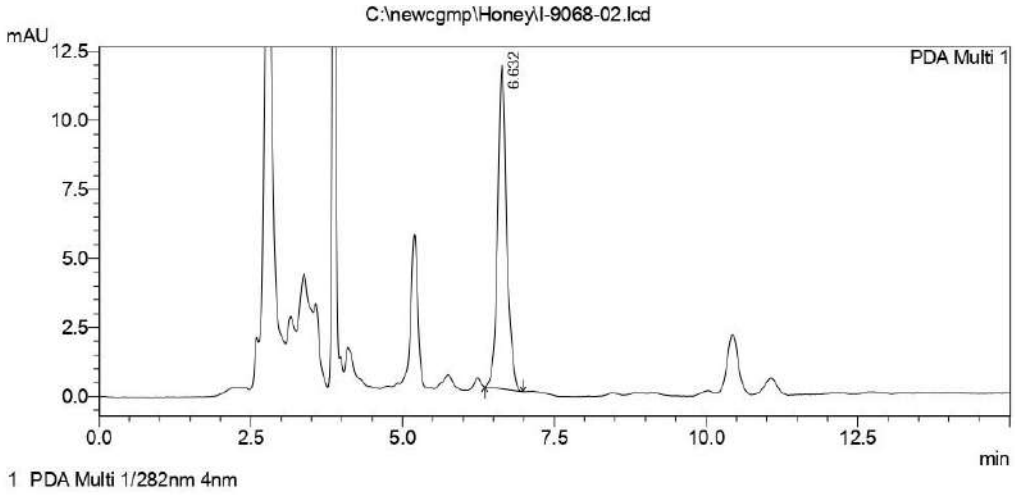
Quantitative Results

PDA

ID#	Name	Ret. Time	Area	Height	Conc.
1	HMF	6.633	58465	5562	0.220

Fig. 4. Chromatogram of Sample 1 of Honey

The figure shows the chromatogram of HMF in honey sample 1 with a retention time of 6.633 minutes



Quantitative Results

PDA

ID#	Name	Ret. Time	Area	Height	Conc.
1	HMF	6.632	119217	11716	0.529

Fig. 5. Chromatogram of Sample 2 of Honey

The figure shows the chromatogram of HMF in honey sample 2 with a retention time of 6.632 minutes