DEVELOPMENT & VALIDATION OF 5-HMF CONTENT BY UV SPECTROPHOTOMETER IN HONEY & FRUIT JUICES AVAILABLE IN NAVI-MUMBAI

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Abstract: *5- Hydroxy Methyl Furfural(5-HMF) is a compound present in several processed foods and various marketed products. The presence of 5-HMF is an indicator of quality deterioration in various marketed processed foods. Biologically, 5-HMF has pros and cons on human health so its presence is still a topic of debate. Aim of the work is to determine and quantify the 5-HMF present in such processed marketed foods in our local markets. Marketed Honey products and Fruit Juices samples easily available in Navi-Mumbai were chosen for determination and Quantification of 5-HMF content using simple and easily available UV-Visible Spectrophotometer in a UG level College Laboratory. By literature study and survey of previous developments on the topic, a favorable & convenient method was decided for the determination of 5-HMF content. Standard and chemical reagents were ordered from the manufacturer for reference and various validation studies. Maximal wavelength was obtained from Standard UV spectrum and absorbances were recorded of standard dilutions. Similarly, sample solutions of marketed honey products were prepared by a suitable solvent system i.e. Acetonitrile: HPLC graded water in 50:50 proportion. For Fruit juices, the supernatants were collected by centrifuging and preparing a sample solution by using HPLC graded water as the solvent. The absorbance of each sample was recorded and the concentration of 5-HMF was determined. Validation of the Analytical method was decided and by studying the ICH guidelines Q2 (R1) the parameters were decided to be done. Performing various analytical method validation parameters, the analytical method was validated and was found to be linear, robust, satisfactorily precise, and poorly specific in the presence of impurities, degradants, and adulterants. Forced degradation studies revealed that on extra humid conditions the degradation of 5-HMF is favored. The project study focuses on the easy, simple, convenient, and cheaper spectrophotometric method for determination 5-HMF in the marketed product. It was concluded that the above method is cheaper, less sophisticated, and easily available for UG level student in their college laboratory.*

INTRODUCTION

Food products are given thermal treatments to possess desirable sensory properties or texture features, assure microbiological safety, and eliminate enzymatic activities (1). 5-Hydroxymethylfurfural (HMF) is a six-carbon heterocyclic aldehydic compound produced while the thermal treatments of carbohydrate-containing foods as a result of **Maillard** reaction (the non-enzymatic browning reaction) and Caramelization. Its chemical formula is 5(hydroxymethyl)-2-furancarboxaldehyde ($C_6H_6O_3$) (2), and it is used in the synthesis of certain organic compounds (3) and Novolac resins (4). It is also used as an intermediate substance in the synthesis of some crown ethers (5), and the production processes of some polymers, surfactants, solvents, pharmaceutics, and plant protection agents (6).

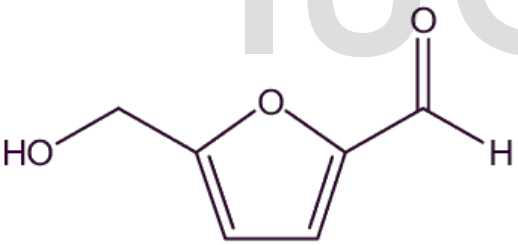

Fig.1. The structural formula of 5-Hydroxymethyl-2-furfuraldehyde

The following factors influence the formation of HMF in foods:
(a) carbohydrate content
(b) physicochemical properties (pH, total acidity)
(c) thermal treatment
(d) water activity
(e) long-term storage, and
(f) use of metallic containers.

HMF has been used to evaluate the sensorial properties of food products. The changes in the colour, flavour, and taste of food products during processing and storage are related to the HMF content. It is recognised as an indicator of improper processing and storage conditions. Therefore, HMF generally is known as an indicator of quality deterioration as a result of excessive heating or storage in a wide range of foods and food products (7, 8).

In general, monosaccharides (i.e. fructose or glucose) are the basic substrates for HMF production. Moreover, disaccharides and most polysaccharides are hydrolyzed and broken down into simple sugars, which, subsequently, act as a precursor for HMF formation. The process also may be strongly enhanced under acidic conditions in the absence of amino groups (9). In acidic medium, HMF is formed by the decomposition of hexoses during the heating after a slow enolization and a fast β-elimination of three water molecules (10). Therefore, foods containing simple sugars and acids, namely honey, jam, cereal products, and fruit and vegetable products favour the formation of HMF.

Although HMF is a by-product of thermal processing, its impact on human health is still a contentious topic. There is much debate over its toxicity, genotoxicity, mutagenicity, and carcinogenicity (1). Some authors believe that HMF is a natural component of traditional foods, having no risk to human health (11), while others believe that HMF can act as a neurotoxin and consequent to accumulation in the body and combination with proteins eventually lead to lesions in muscles and the viscera (12). However, in more recent extensive studies, HMF has been proved to have a wide range of positive effects, such as antioxidative, anti-allergic, anti-inflammatory, anti-hypoxic, anti-sickling, anti-hyperuricemic effects (13).

Durling et al. reported significant HMF-induced DNA damage after 3 hours of exposure to 100 Mm HMF. They reported that HMF can break DNA and damage it; however, the damage was only observed at high concentrations (14). Moreover, HMF can be metabolized to 5-sulfooxy methyl furfural (SMF), a reactive intermediate that binds to DNA and causes mutagenic effects. Both HMF and SMF are weak intestinal carcinogens in mice (15). From a safety perspective, HMF is produced in large quantities, and the levels can exceed 1g/kg in several food items; however, honey is the only food for which a legal limit on HMF concentrations has been set (16). The Codex Alimentarius (17) has established that after processing and/or blending, the HMF content of honey must not exceed 80 mg kg-1. The European Union (EU) recommends a lower limit of 40 mg kg-1 with the following exceptions: 80 mg kg-1 is allowed for honey that originates from countries or regions with tropical temperatures (18). The legal limit of 40 mg kg-1 had already been issued for honey by the Institute of Standards and Industrial Research of Iran (ISIRI) (19). Given the fact that HMF is usually a recognized parameter indicating the freshness and quality of foods, some researchers have determined the amount of HMF in some kinds of food including bread (20), coffee (21), honey (22, 23, 24, 25), fruit juice (22, 25), raisins (26), milk (27), instant coffee (28), biscuits, jam, and breakfast cereal (22).

The quantitative analysis of 5-HMF in clinical research and therapeutics is of great importance as in foods (29). Various methods have been defined for the measurement of 5-HMF levels, including colorimetric, spectroscopic, chromatographic, polarographic, and two spectrophotometric methods; **White's** method and **Winkler's** method (30,31). HPLC method and spectrophotometric methods were recently tested by the International Honey Commission (IHC) (32). The first used before the spectrophotometric methods were optical and chemical methods (29). The basis of the **White's** method is based on the measurement of UV absorbance of clarified aqueous honey solutions with and without bisulfite. In the other spectrophotometric **Winkler** method, the UV absorbance of honey solutions with barbituric acid and p-toluidine is measured. Although these two methods are fast, their sensitivity and specificity are not sufficient. Also, the use of carcinogen p-toluidine in **Winkler'**s method is a disadvantage. The disadvantage of the HPLC method is that it is more expensive, but it provides advantages in terms of both labour and time (32,33). In the HPLC method is according to **Jeuring** and **Kuppers**: firstly, honey is dissolved in water. 5-HMF is determined on a reversed-phase HPLC column with water and methanol as an isocratic mobile phase after 983millipore filtration (33). Borate is used as a supporting electrolyte in the electrochemical method. The basis of the method is a single and sharp reduction signal against the silver or silver chloride (34). **Yuan et al.** have used the ion exchange liquid chromatography with photodiode array detection technique (35). Another method used in the 5-HMF analysis is the automated flow injection method which provides a detection range of 5-40 ppm (36). Caffeine is used as an internal standard in micellar electro-kinetic capillary chromatography, which is used in the 5-HMF analysis. This technique allows rapid quantification of the sample, especially in honey without prior pre-treatment (36). The real-time coupled with time of flight mass spectrometry is another method used in the 5-HMF analysis (37).

Bogdanov et al reported that the comparison of official methods has been investigated widely only for samples with high HMF content (38). **Ciriaci et al** reported that in recent years a substantial reduction of HMF content in honey has been observed, revealing how beekeepers have developed remarkable skills in adopting processing techniques that both comply with product characteristics and pay greater

attention to the early stages in the production and conservation of honey to guarantee better product quality. Both from a legal and quality point of view, it is important that there be a concordance of results for the determination of HMF in honey obtained with different official methods, and in all possible ranges, above all at high HMF levels. Nevertheless, following the drastic reduction of HMF levels in fresh honey, there needs now to be a specific study concerning honey samples with low HMF concentrations of about 1 mg/kg (39)(40). **Zappala et al** analyzed only 4 samples with very low HMF content, so no conclusion can be drawn about the comparison between the White and the HPLC methods (41). **Surh and Tannenbaum 1994**; **Lee et al. 1995; Sommer et al.** recent results on the metabolic activation of HMF show genotoxic potential in vitro of this compound due to its transformation by human sulfotransferases to 5-sulfoxymethyl-2-furfural (SMF), which was shown to be mutagenic. **Archer et al; Bruce et al; Zhang et al** Also, initiation/promotion studies indicate that HMF may act as an initiator and promoter of colon cancer in rats (43). **Cordella et al.** improved the method for the analysis and evaluation of the quality of honey. They proposed the determination of high-performance chromatography profiles of sugars with anion exchange**. Khalil et al**. investigated the correlation between the physicochemical properties of honey and HMF formation and found that there was a strong correlation between free acids and total acidity, while there was only a moderate correlation between Ph and lactones (44).

Reyes-Salas et al. reported an electrochemical approach for HMF detection. In this method, a single and sharp reduction signal was created at −1100 Mv versus argentum or argentum chloride, while borate was used as a supporting electrolyte (45). **Yuan and Chen et al.** reported another method is the ion exchange liquid chromatography–photodiode array detection technique (46). Another method involves automated fow injection, as reported by **Iglesia et al.**, which is based on the operating principle of the **Winkler** method and provides a detection range of 5–40 ppm (47). **Rizelio et al.** reported Micellar electro-kinetic capillary chromatography (MEKC) is another rapid method that uses caffeine as a standard. The technique is suitable for the rapid quantification of HMF particularly in honey samples, without requiring sample pretreatment (48). **Rajchl et al** reported a unique and efficient rapid screening technique is direct analysis in real-time (DART) coupled with time-of-flight mass spectrometry (TOF-MS), which has been reported to yield a chromatogram with high resolution (49). **Toker et al**. recommended central composite face-centered design (CCFD) of response surface methodology (RSM) as a useful experimental technique for this purpose (50). According to **Hunter et al.**, this versatile and effective systematic tool can be used to determine the optimal levels of the contributing factors for the parameters concerned (51). The mathematical expression derived by this methodology can be employed to develop predictive models upon setting the levels of the various influencing factors to reach the optimum HMF concentration in food. **Bertelli et al**. published an effective method for the detection of honey adulterated using sugar syrups. It involves one-dimensional (1D) and two-dimensional (2D) nuclear resonance (NMR) coupled with multivariate statistical analyses (52). **Almeida-Muradiana et al.** used Fourier transform infrared attenuated total reflectance spectroscopy (FT-IR ATR) to analyze various honey samples from north-eastern Brazil (53).

Based on the information from the literature review, we conclude that the differences between the methods cause very low levels of changes in the 5-HMF results. On the other hand, the use of incorrect or inadequate procedures in the 5-HMF determination leads to inaccurate results. Spectrophotometry may provide relatively cheaper options compared to the capital-intensive chromatographic methods.

Hence, the purpose of this study was to develop and validate a simple, fast, and specific UV-spectroscopic method.

MATERIALS & METHODS

1.Materials

1.1. Chemical Reagents:

5-Hydroxy Methyl Furfural Standard, HPLC Grade Water, Acetonitrile, was purchased from a registered vendor. Laboratory reagents such as HCL (0.1N & 1N), NaOH (0.1M & 1M) and prepared 3% H_2O_2 solution.

1.2. Instruments:

Analytical balance, Sonicator, Centrifuge, Hot Air Oven, Freezer, UV-Visible Spectrophotometer easily available in a UG-level student laboratory.

1.3. Samples:

Various brands of Marketed Honey Products and Fruit Juices available in local markets of Navi Mumbai. Marketed Brands of Honey such as, Baidyanath Honey, Phondaghat Pharmacy Honey, Patanjali Honey, Diet Honey, Lion Honey, Dabur Honey and Uttarakhand Honey. Marketed Brands of Fruit Juices and variable flavours such as, Alo Fruit juice (Mango), Alo Fruit juice (Anaar), Tropicana Fruit Juice (Guava), Tropicana Fruit Juice (Litchi), Nestea (Lemon), Appy (Apple), Mulmina Fruit Juice (mango), Frooti (Mango).

2. Methods

2.1. Preparation of Standard solutions: -

1. Several concentrations of 5-HMF were prepared from the working standard solution (2µg/ml, 4 µg/ml, 6µg/ml, 8µg/ml, 10µg/ml, 12µg/ml, 14µg/ml, 16µg/ml, 20µg/ml) to obtain the calibration curve.
2. 50mg of 5-HMF standard was diluted with Acetonitrile: HPLC graded water (50:50) up to 50ml of volumetric flask.
3. 5ml was pipetted out from the above solution and diluted up to 50ml
4. From the above solution, 1ml solution was taken and diluted up to 10ml using Acetonitrile: HPLC graded water (50:50)
5. The absorbance of the resulting solution was recorded at 284nm and 336nm.

2.2. Preparation of Honey samples: -

1. 1ml of the honey sample was diluted up to 10 ml using Acetonitrile: HPLC graded water (50:50).
2. The absorbance of the resulting solution was recorded at 284nm and 336nm.

2.3. Preparation of Fruit Juice samples: -

1. 5ml was pipetted after shaking the fruit juice sample properly and centrifuged in 20ml tubes at 5000rpm for 5 minutes.
2. The supernatants were collected and diluted (1 in 50 dilutions) with HPLC graded water.
3. The absorbance of the resulting solution was recorded at 284nm and 336nm.

3. Method Validation

3.1. Linearity:

- A linear relationship is evaluated across the range of the analytical procedure.
- Evaluated by visual inspection of a plot of signals as a function of analyte concentration or content.
- For the establishment of linearity, a minimum of 5 concentration is recommended. (known that it is a straight line).

3.2. Robustness:

1) By changing the composition of the solvent system: -

Several concentrations of 5-HMF were prepared from the working standard solution from 2µg/ml to 20µg/ml to obtain the calibration curve.

a) Composition 1 - Acetonitrile: HPLC graded water (60:40)

1. 50mg of 5-HMF standard was diluted with Acetonitrile: HPLC graded water **(60:40)** to 50ml volumetric flask.
2. 5ml was pipetted out from the above solution and diluted to 50ml.
3. From the above solution, 1 ml solution was taken and diluted to 10ml using Acetonitrile: HPLC graded water (50:50).
4. The absorbance of the resulting solution was recorded at 284nm and 336nm.

b) Composition 2 - Acetonitrile: HPLC graded water (40:60)

1. 50mg of 5-HMF standard was diluted with Acetonitrile: HPLC graded water **(40:60)** to 50ml volumetric flask.
2. 5ml was pipetted out from the above solution and diluted to 50ml.
3. From the above solution, 1 ml solution was taken and diluted to 10ml using Acetonitrile: HPLC graded water **(40:60).**
4. The absorbance of the resulting solution was recorded at 284nm and 336nm

2) By changing the Wavelength: -

1. 50mg of 5-HMF std was diluted with Acetonitrile: HPLC graded water (50:50) to 50ml volumetric flask.
2. 5ml was pipetted out from the above solution and diluted to 50ml.
3. From the above solution, 1 ml solution was taken and diluted to 10ml with Acetonitrile: HPLC graded water (50:50).
4. The absorbance of the resulting solution was recorded at **280nm** and **290nm**.

3.3. Precision:

Inter-Day: -

1. 10µg/mL of Standard HMF concentration was prepared using Acetonitrile: HPLC graded water (50:50).

2. Similarly, sample solutions of Honey & Fruit Juices were prepared using a proper solvent.
3. Absorbances of standard, as well as all the samples, were recorded on Day 1st, Day 3rd & Day 5th.

3.4. Specificity:

1) Procedure for Honey sample: -

1. 1ml of the honey sample was diluted up to 10 ml using Acetonitrile: HPLC graded water (50:50).
2. From that solution, 1ml was taken + 1 ml of 0.1N HCL solution.
3. It was diluted with Acetonitrile: HPLC graded water (50:50).
4. It was then placed at 50ºC for 24 hours.
5. The absorbance of the resulting solution was recorded at 284nm and 336nm.

The same procedure by adding 1N HCL, 0.1N NaOH, 1N NaOH, and 3% H_2O_2 in place of 0.1N HCL.

2) Procedure for Fruit juices: -

1. 5ml was taken from shaking the fruit juices sample properly and centrifuged in 20ml tubes at 5000rpm for 5min.
2. The supernatant was collected and diluted (1 in 50 Dilution) with HPLC graded water.
3. 1ml from the above solution was makeup up to 10ml with HPLC graded water.
4. 1ml was taken from the above solution and mix with **1ml of 0.1N NaOH** solution and then makeup with HPLC graded water up to 10ml.
5. It was then placed at 50ºC for 24 hours.
6. After 24 hours the absorbance of the resulting solution was taken at 284nm and 336nm.

The same procedure was repeated adding 1N NaOH, 1N HCL, 0.1N HCL, 3% H_2O_2 in place of 0.1N HCL.

RESULT & DISCUSSION

The results from part **2.1.** were revealed and percentage of drug in standard dilutions were determined by recording absorbance of the resultant solution at maximal wavelength i.e. 284nm.

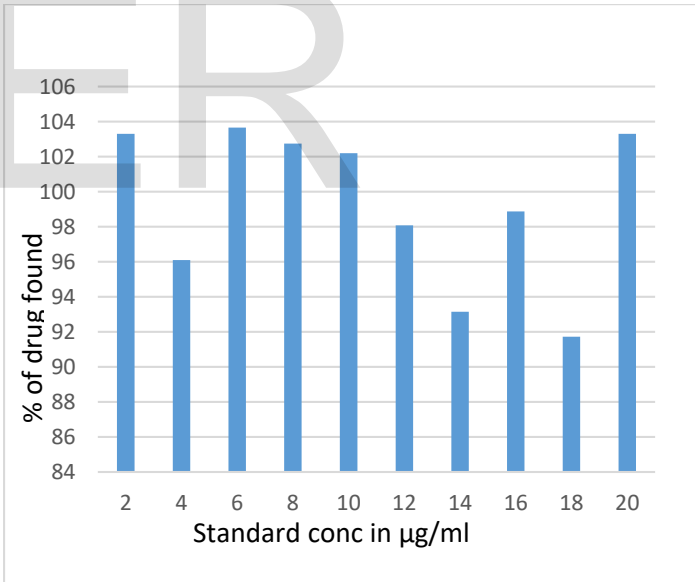

Fig.2. 5-HMF standard dilution results

For convenience in the work, we labelled samples in the following manner:

Table.1. Codes for Honey samples

Code	Name of the sample
F1	Alo Fruit Juice (Mango)
F2	Alo Fruit Juice (Anaar)
F3	Tropicana Juice (Guava)
F4	NesTea (Lemon)
F5	Tropicana Juice (Litchi)
F6	Frooti Juice (Mango)
F7	Appy Fruit Juice (Apple)
F8	Mulmina Juice (Mango)

Table.2. Codes for Fruit Juice samples

The results from part **2.2.** were revealed and amount of 5-HMF content in Marketed Honey Products were determined.

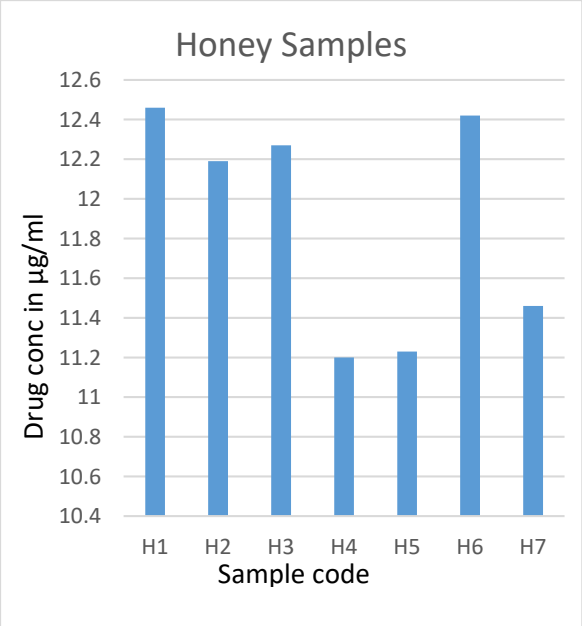

Fig.3. Drug content in Honey samples

From the above results, we found out that 5-HMF concentration in marketed Honey products were approximately in a range of **11µg/ml – 12.5µg/ml**.

The results from part **2.3.** were revealed and amount of 5-HMF content in Marketed Fruit Juice samples were determined.

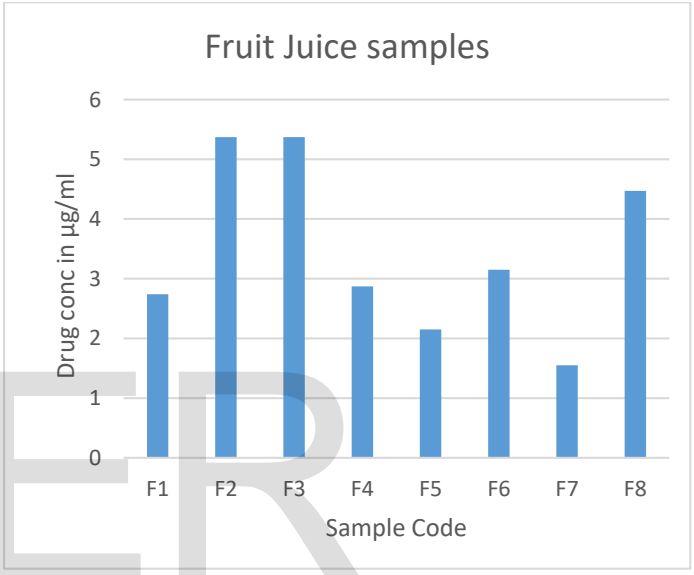

Fig.4. Drug content in Fruit Juice samples

Code	Name of the sample
H1	Baidyanath Honey
H2	Phondaghat Pharmacy Honey
H3	Patanjali Honey
H4	Dabur Honey
H5	Diet Honey
H6	Lion Honey
H7	Uttarakhand Honey

From the above results, we found out that 5-HMF concentration in marketed Fruit Juice

products were approximately in a range of **1.5µg/ml – 5.5µg/ml**.

From part **2.2.** & **2.3.** we found out that concentration of 5-HMF in marketed Honey Product brands was higher in comparison with marketed Fruit juice brands available in Navi Mumbai.

Method Validation Parameters

1) Linearity:

The results from part **3.1.** were revealed and Linear relationship of the analytical method was determined by plotting a drug concentration versus absorbance curve and applying linear regression function.

The results obtained from the analysis of standard 5-HMF at various concentrations with equation **y=0.664x+0.2171** and regression value **R^2 = 0.996** which is a good and acceptable value as per ICH guidelines.

2) LOD & LOQ:

a) Limit of Detection (LOD)

The lowest amount of analyte in a sample which can be detected (not necessarily quantitated as an exact value). Mathematically, it is given by

LOD = 3.3 × (S.D of Intercept/slope)

LOD = 1.99788µg

b) Limit of Quantification (LOQ)

The lowest amount of analyte in a sample can be quantitatively determined with suitable accuracy and precision. Mathematically, it is given by

LOQ = 10 × (S.D of Intercept/slope)

LOQ = 6.05421µg

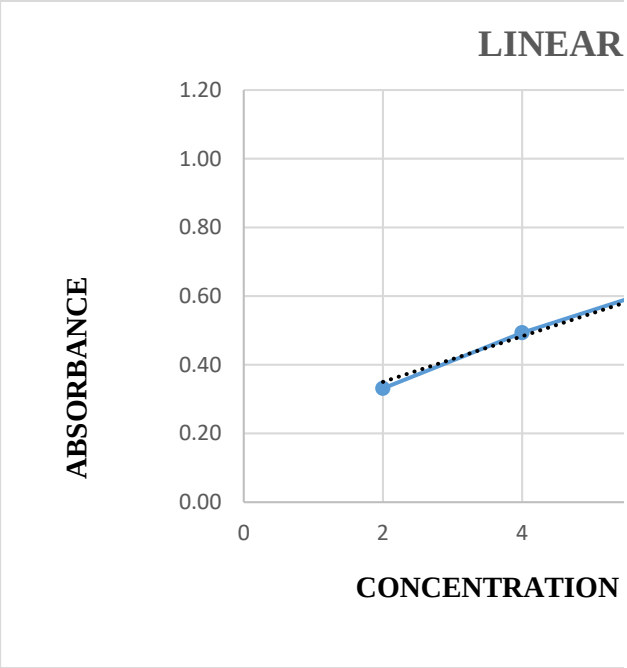

Fig.5. Linearity Graph

For further validation parameters the results were calculated in % RSD and mean %RSD for each parameter was determined and found out to be,

Sr.	Validation Parameter	Mean %RSD
3)	Robustness	
	Wavelength varied	2.99%
	Composition varied	1.92%
4)	Precision	12.33%
5)	Specificity	31.94%

Table.3. %RSD for validation parameters

As per ICH guidelines, acceptable criteria for validation parameters is %RSD < 5%. Therefore, we found that Precision and Specificity parameter do not comply for acceptance criteria. While, Linearity and Robustness complies for acceptance criteria.

CONCLUSION

A simple, convenient, and cheaper spectrophotometric method has been developed and validated to quantify 5-HMF levels in marketed honey product brands and fruit juices brands using simple lab techniques and UV-Visible Spectrophotometer easily available in UG-level students Laboratory. In the work we carried out, we found the presence of 5-HMF in marketed Honey products as well as Fruit Juice brands in variable amount. The presence of 5-HMF in various food products can be detected by this method approximately.

As per ICH guidelines, the analytical method used for determination of 5-HMF content was validated and was found showing Linearity, Robust, satisfactorily precise, and poorly specific in the presence of any impurities or degradants. The 5-HMF levels were not dependent on the duration of the storage of the products.

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