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Study of immobilisation of aniline for an optical detection of Hydroxyl-Methyl-Furfural by spectrophotometric technique

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Abstract:
The current study proposed an optical sensing material based on the immobilised aniline in filter paper coated with chitosan for the detection of hydroxymethylfurfural (HMF) based on reflectance spectrophotometry. The reflectance intensities of the reagent were measured at a wavelength range of 250 - 500 nm before and after the reaction with the HMF. The sensing material was found to have an optimum response at pH 3.0 with immobilised aniline of 8.0×10^{-3} M. The relative standard deviation (RSD) of reproducibility and photostability were found 1.96 and 1.40 %, respectively. The results showed no significant deference between the HMF method and the high performance liquid chromatography standard method, with a correlation coefficient (R^2) and a slope value of 0.99 and 0.96, respectively. By comparing the results obtained using the designed sensor with that obtained with established standard HLPC method, it was found that the HMF content in honey samples was in great agreement.
Keyword: Optical sensor, aniline, chitosanhydroxymethylfurfural, spectrophotometric technique.

الملخص:

في هذه الدراسة تم تعيين الهيدروكسي ميثيل فور فيورال في العسل بواسطة تثبيت الانيلين مع الكيتوسان باستخدام جهاز المطياف الضوئي. كثافة الادمصاص للانيلين درست عند

طول موجي 250–500 نانومتر قبل وبعد التفاعل مع هيدروكسي ميثيل فور فيورال. أفضل النتائج وجدت عند الاس الهيدروجيني (3) لهيدروكسي ميثيل فور فيورال وتركيز الانيلين عند 8×10^{-3} M. أوضحت النتائج أنه لا يوجد فارق كبير بين طريقة الهيدروكسي ميثيل فورفيورال وتقنية الكرماتوقرافيا السائلة عالية الأداء. وقد وجد ان الانحراف المعياري لمدى ثبات تكرار استقراريه العينة واستقرارها الضوئي كان 1.96 و 1.40 % على التوالي.

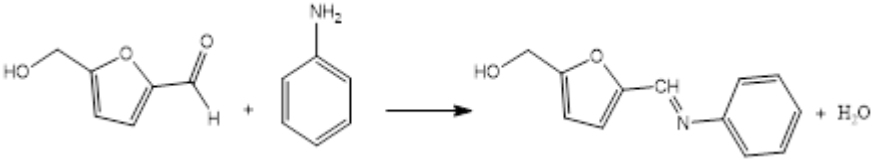
1.Introduction

HMF is an acyclic aldehyde produced as a result of sugar degradation [1]. The presence of simple sugars (glucose and fructose) and various acids in honey is said to be a favorable condition for HMF production. HMF is formed in honey by heating monosaccharides under acid condition, and it can be used in the quality control of honey [2]. HMF is usually absent in fresh and untreated food, however: its concentration is also reported to increase as a result of heating processes [3]. But its concentration is also reported to increase as a result of heating processes [4]. Therefore, HMF is a recognized parameter that is related to the freshness and quality of such food. A number of factors affect HMF formation in honey during storage.

The Codex Alimentarius has established that the HMF content of honey after processing and/or blending must not be higher than 80 mg/kg [5]. However, the European Union recommends a lower limit of 40 mg/kg, with the following exceptions; 80 mg/kg is allowed for honey that originates from countries or regions with tropical temperatures; whereas only 15 mg/kg is allowed for honey with low enzymatic levels [6]. The International Honey Commission (1999) recommends thet there are three reported methods for the determination of HMF [7]. These methods include two spectrophotometric methods that are widely used in routine analysis, namely determination based on protocols by Winkler, White and a chromatographic technique using high performance liquid chromatography (HPLC). High HMF concentrations have

been reported to cause mutagenic activities that can be dangerous for humans if consumed [8]. Both spectrophotometer methods are fast; however, these methods have low specificity and sensitivity (in particular, systematically positive). Aniline is used as a reagent for biochemical reactions that produce peroxides or phenols. In volatile organic compounds (VOC) culture, HMF is one of the volatile organic compounds (VOC) for honey; HMF is used as an indicator for adulteration and overheating during honey processing and is also used as a quality indicator [9] .

In this article, the performance of the immobilised aniline in filter paper coated with chitosan as a reagent phase in the development of optical reflectance sensor for HMF determination has been demonstrated. The determination of HMF using colour reagent aniline was achieved; aniline for determination HMF, which interacts with the nitrogen group for producing reactions that are confirmed through azomethine group colour products. Figure 1 shows the reaction mechanism between HMF and aniline.



(HMF) aniline (E)-(5-((phenylimino) methyl) furan-2-yl) methanol

Figure 1. The reaction mechanism between HMF and aniline

2. Material and Method

2.1 Materials

The current study used these materials: chitosan (Aldrich; Mw 1861.50), aniline (E. Merck); HMF (Sigma); phosphate buffer (reinst), acetic acid (CH_3COOH) 36%, ethanol 95% (Systerm). All these chemicals were applied without being further purified

2.2 Preparation of stock solution

Preparation of chitosan with 2% (w/v) concentration was measured by dissolving 2 g of the chitosan powder in 100 ml of acetic acid (0.1 M). This was followed by stirring the viscous chitosan solution overnight at room temperature.

The required amount of 0.0698 gm aniline was dissolved in deionised water for the purpose of preparing 25 mL of an aniline standard stock solution with concentration of 3.0×10^{-2} M. Dilutions of the stock solution of 3.0×10^{-2} M aniline, was done for the preparation of aniline solution in the concentration range of 1.0 – 10.0×10^{-3} M [10]. The standard HMF solution of 3.0×10^{-2} M was prepared by dissolving 0.094 gm of HMF in 25 ml deionised water. Eight serial dilutions in deionised water were made from standard HMF solution to prepare HMF solution in the concentration range of $1.0 – 8.0 \times 10^{-3}$ M.

2.3 Preparation of Sensing Material

The sensor design used in this study was as in previous work with slight modifications [11]. For preparation of sensing material, 0.3 ml of the mixture of aniline in chitosan solution was pipetted into the petri dish. This coating process method was used to immobilise aniline in filter paper coated with chitosan. The filter paper was used as a immobilisation surface because it has a low cost, does not need complex pre-treatment, easy to handle, prevents coagulation of colloids, can be used in different solvent media, and allowing detection of non-polar molecules [12]. Next, the filter paper strip soaked with aniline in chitosan was dried by placing the filter paper in the refrigerator overnight

2.4 Instrumentation and Measurement Procedure

The study used a reflectance spectrophotometer (model Perkin Elmer, US). For the purpose of measuring all reflections in spectrophotometric studies. Filter paper that contained the phosphate buffer solution (pH 3.0), aniline (3×10^{-3} M), and HMF (3×10^{-3} M) was used for immersing aniline the in filter paper coating with immobilised a chitosan. Moreover, the reflections studies were recorded between the wavelenght 250 - 500 nm.

2.5 Optimisation the Response of Sensing Material

All the characterisations of the chemical reactions between the immobilised aniline and the HMF were carried out using optical sensing material reflectance spectrophotometer. The reflectance measurement of the analyte was captured in the reflectance visible wavelength from 250 to 500 nm.

The constructed HMF sensor was evaluated in triplicates with respect to its photostability; affect the pH, dynamic linear concentration range, reproducibility, and interference studies. The photostability of the sensor was studied by exposing the reagents to the light source continuously for 6 hrs, and the reflectance measurements were taken every 30 min. The impact of pH was examined by varying the pH of the reaction medium. The dynamic linear response range of the HMF concentration of the sensor was determined by placing the optimized in a series of HMF solutions from 1.0×10^{-3} to 6.0×10^{-6} M at pH 3.0. The method of reproducibility was performed by employing repeating 7 independent experiments, using the same concentration of 3.0×10^{-3} M for HMF and aniline at the almost same pH.

An evaluation of the developed method was made of possible interferences from the major compounds (glucose, fructose) commonly presence in honey. The interference was evaluated based on the comparison of the reflections data between the target and interference compounds. Then, solutions containing 3×10^{-3} M of HMF were also determined. Finally, solutions containing 3×10^{-3} M of HMF and one of the major compounds at concentrations equal to that of HMF were also determined by using the sensing material. All samples were analysed using reflectance visible double-beam spectrophotometer at the wavelength range from 200 to 500 nm.

The reflectometric HMF sensor was then validated and compared with the standard acidimetric HPLC method for determination HMF in honey samples. The samples of honey were collected from the farmer in Green Mountain in Libya. The honey was buffered at the optimised pH by using, phosphate buffer solution. The honey

sample was then deionised into a solution. Next, a series of known HMF concentrations within the range of 0.0 to 6.0 × 10^{-3} M compared with another standard HPLC method. Finally, the HMF concentrations in the deionised were determined with standard HPLC method. For HMF determination using reflectometric HMF sensor, honey samples were directly analysed without pre-treatment steps.

An evaluation of the developed method was made of possible Five grams of honey samples were diluted up to 50 ml with deionised water, and immediately injected in an HPLC. The HPLC column was a Merck Lichrospher, RP- 18.5 ml, 125 mm, fitted with a guard cartridge packed with the same stationary phase. The HPLC conditions were the following: isocratic mobile phase, 90% water and 10% methanol; flow rate, 1.0 ml/min; injection volume, 10µl. All the solvents were HPLC grade. The wavelength range was 220–500 nm and the chromatograms were monitored at 280 nm. HMF was identified by splitting the peak in honey with a standard HMF and by comparison the spectrum of HMF standard with that of honey samples. The amount of HMF was determined using an external calibration curve, measuring the signal at 280 nm.

3. Results and Discussion

Figure 2. Shows the reflectance spectra of aniline before and after reaction with HMF. Reaction with HMF caused an increase in the reflectance intensity due to the change in colour of the reagent phase from yellow to dark yellow after reaction with HMF. All reflectance measurements in this study were carried out at this wavelength whereby the effect of the diffuse reflection resulted into a higher reflectance signal. This indicates that the light struck on the substrate surface was the reason behind the occurrence of this diffuse reflection especially when the light penetrated the measured medium and reflected at the surface after partial reflectance and multiple scattering within the medium happened. These are assigned to π -π* and n - π*, transition, respectively. The reflectance spectra of 3 ×10^{-3} M of the Schiff bases showed similar reflectance spectra of the light, which were shifted to higher wavelengths. Moreover,

there was an increase or appearance of the peak due to n - π* transition, thus confirming the azomethine group. Based on the reflection, the maximum reflectance difference between the two spectra before and after reaction with HMF was at 370 nm, which was used for subsequent quantitative studies.

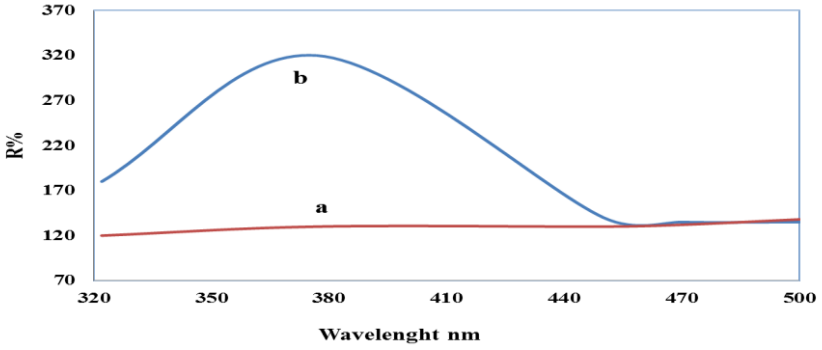


Figure 2. Reflectance spectra of the immobilised the aniline in filter paper coated with chitosan (a) after reaction with HMF (b) at pH 3.0 and the same concentrations of 3 x 10^{-3} M.

To optimise the sensor response, an experiment at different pH solutions of HMF was carried out and the result is shown between the immobilised aniline, with the HMF reflectance signals at the range of 250 - 500 nm in Fig. 3. As shown, the greatest relative reflectance occurred at pH 3.0; therefore this pH was selected as the optimum response and was used for subsequent experiments. Additionally, pH is an important factor during the storage of honey, because it is related to the stability and shelf life of the product [4]. At lower pH, the sensor was found to produce a low reflectance signal because the medium is too acidic. higher pH, the reaction of HMF and aniline to produce a secondary compound which is due to the reduction of the azomethine group caused the reflectance signal to decrease. The response of the HMF sensor was found to peak at pH 3.0 .

The number of protons involved in the rate determining step is found to be two. A shift of the peak potential towards more negative value

with increase in pH indicates the existence of a protonation reaction coupled with the azomethine group reduction process.

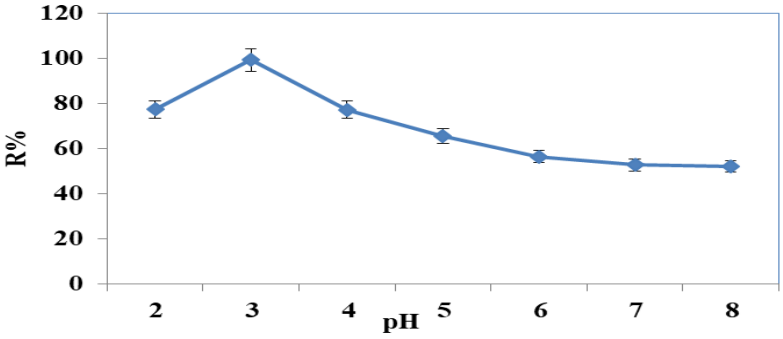


Figure 3. The effect of pH on the immobilised the aniline upon reaction with $3x10^{-3}$ M HMF

Based on the results obtained from all the techniques, was found to a give a single well defined peak in acidic solutions (pH 3). Increase of pH 3.0 leads to decrease of the peak current. In the acidic medium the peak of the compound is due to the reduction of > C = N group in two electron process, as reported by Reddy et a1 reported the same of pH 3.0 with extensively studied electrochemical behaveour of azomethine group containing pesticides [13]. Typical cyclic azomethine group are shown in Figure 4, the reduction mechanism is as follows.

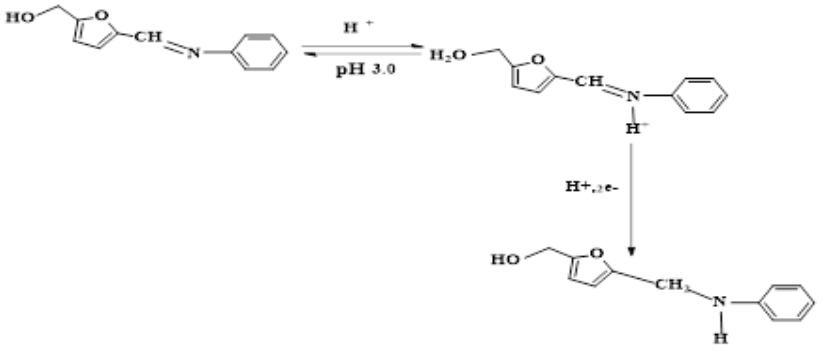


Figure4: The mechanism of the pH effect on the azomethine group

A study on the effect of reagent concentration used during immobilisation of aniline in filter paper and coating with chitosan to the sensing response at the same HMF concentration (3×10^{-3} M) of the wavelength range 250-500 nm was also observed. It is apparent from Figure 5 that the reflectance intensity increases with increasing aniline concentration (8×10^{-3} M) used during immobilisation of the reagent.

The optimum aniline loading was observed at a concentration of 8×10^{-3} M, which was later, used for all subsequent studies. A similar trend of response was also obtained in a flow injection method for Co (II) determination based on immobilised 2-(4-pyridylazo) resorcinol in chitosan membrane as reported by Yusof and Ahmad [14].

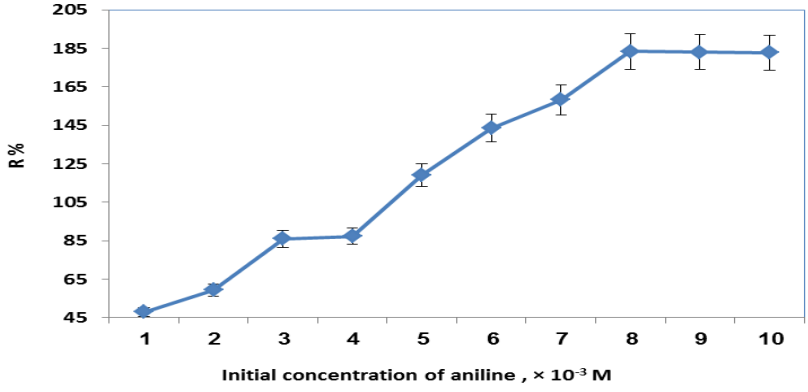


Figure 5. The effect of initial concentration aniline during immobilised of the reagent in chitosan and the concentration of HMF was fixed at 3×10^{-3} M.

A study on photostability of the sensing material was manually to detect the possibility of photoleaching or photodecomposition of the reagent phase when it was continuously exposed to a light source for a long period of time (Figure 6). In this study, it was demonstrated that the reagent phase is stable and no photodecomposition occurred. The leaching of the immobilised

reagent was evaluated by monitoring the sensing response continuously for 6 hrs when the sensor was immersed in a solution. Sundari et al. 2006 have reported similar photodecomposition time [15]. The study indicated that for this duration of time, the sensor response is quite stable with R.S.D. of 1.4 %.

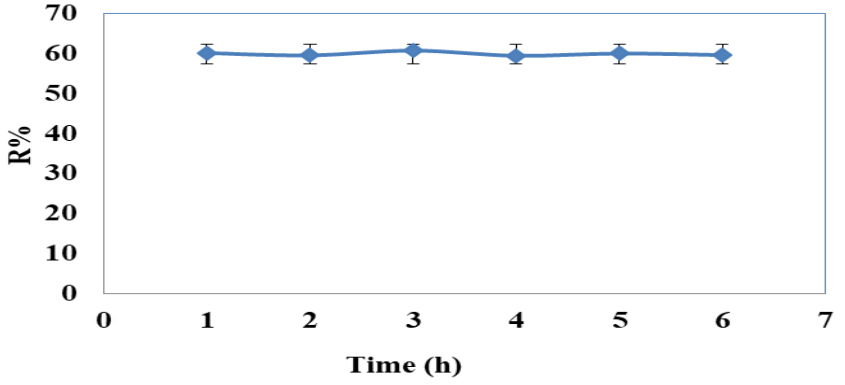


Figure 6. The photostability of the immobilised aniline during a study period of (6 hrs)

Figure 7 shows the results concerning the response curve of the sensor towards HMF in the concentration range of 1×10^{-1} M to 8×10^{-3} M. Based on these results, in the initial study, there was a drastic increase of the reflectance signal with the increasing HMF concentrations. However, at higher HMF concentrations, this response slowly leveled off and even became saturated at 6×10^{-3} M HMF. Such result indicates that the more reactions between the sensor and HMF in the adjacent phase that was fostered by higher HMF concentrations resulted into a higher signal. However, finally, this reflectance signal plateaud due to the complete accupation of all available sites by the HMF. The inset is evident of obtaining the calibration curve under optimised conditions. Moreover, the relative reflectance had a linear correlation (R^2 = 0.9859) with HMF concentration in the concentration range of 1- 6×10^{-3} M.

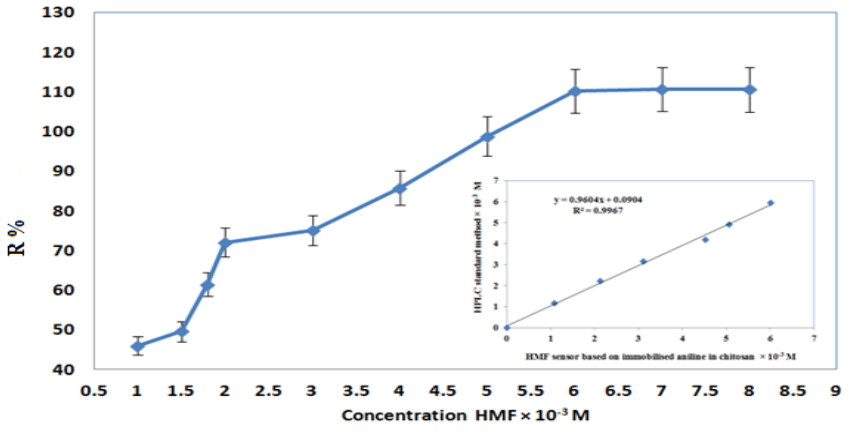


Figure 7. The response curve of the method at different concentration of HMF. Inset is the linear dynamic range of the HMF solution.

The interference from several compounds during HMF determination was also examined in this work. Table 1 summarises the degree of interference measured for several compounds of HMF: compounds. The tolerance ratio of each compounds was taken as the largest amount yielding an error below ±5% [16]. From Table 1, Glucose and Fructose exhibited error values lower than 5%, and therefore these compounds did not interfere during HMF detection. In solution studies, however, these compounds were found to give less error compared when the reagent is immobilised. Further examination of Table 1 showed that error values of higher than 5% were recorded for compounds studied. Since HMF is more mobile in solution phase, this gives more opportunity for compounds to interfere. On the other hand, in its immobilised form, the more rigid compound structure of HMF with aniline will diminish the chance for interaction between HMF and the compounds that in turn yield less interference. Several theoretical concepts have been attempted to elucidate the study of interference compounds in solution system and its immobilised [17]. Castoldi et al, 2016 reported that glucose and fructose are the most dominant compounds identified in the honey sample [2].

Table 1. The degree of interference of HMF compounds:

Compounds	% Interference (Reagent Immobilised)	% Interference (Reagent Not Immobilised)
Glucose	-0.89	-89.69
Fructose	-0.48	-85.84

% **Interference** = $(x - y)/y \times 100\%$, where x and y are the reflectance reading in the presence and absence of the compounds, respectively

Figure 8 shows the analytical performance of the sensing material compared with HMF established method, HPLC standard method, for HMF concentration range of 1 – 6 x 10^{-3} M. The results showed great match between the HMF method and the HPLC standard method, with the correlation coefficient (R^2) 0.99 and slope results value was 0.98 .

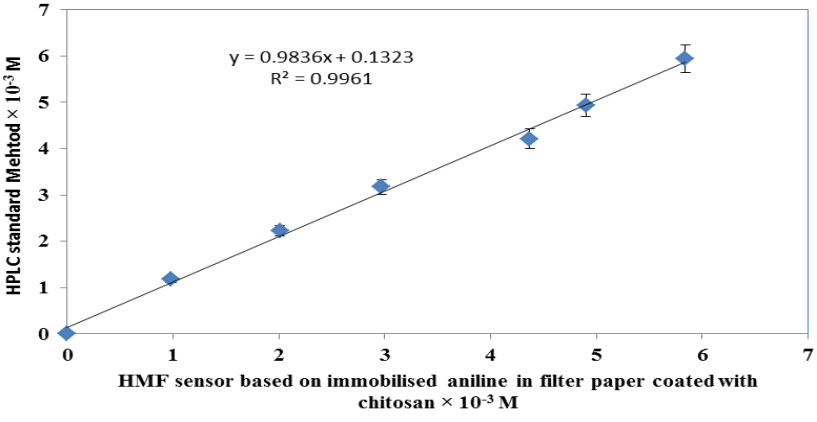


Figure 8. Comparison between the HMF concentrations measured by chitosan / aniline sensor and HPLC standard in this work

To further examine the method performance especially for real sample analysis, an experiment has also been carried out using the method developed in this study to determine the concentration of HMF in honey sample .Comparing the results obtained using the sensor with HPLC standard method, it was found that the HMF content in honey sample as in the Table 2. The Statistical analysis

for the comparison of the two means taken from the standard HPLC method and the developed HMF method was also assessed. Statistical analysis was conducted using the technique that was first described by Miller [18,19].

Table2. Comparison between absorption HMF method and standard HPLC method in the determination of $t_3 = 3.18$ ($P = 0.05$).

Concentrations x10^{-3} Mol L^{-1}	**Developed method x10^{-3} M mean±SD (n=3)**	**HPLC method x10^{-3} M mean±SD (n=3)**	**t-value**
1	0.98±0.09	0.99±0.01	0.08
2	2.01±0.01	2.18±0.06	0.03
3	2.97±0.02	3.32±0.04	0.02
4	4.37±0.05	4.23±0.08	0.02
5	4.90±0.02	4.84±0.09	0.01
6	5.84±0.05	5.35±0.09	0.06
Real Sample	1.78±0.06	2.25±0.01	0.02

Table 2 shows that the critical value is greater than the calculated values of (t). This signifies that there is no significant difference between the two methods at the 3% level.

4. Conclusion

The spectrophotometric method of aniline was successfully use at deferent the concentration of HMF as an analyte. This study show that HMF determination based on aniline is rapid, simple, with reasonable reagent. This method has a good effective for the quantitative determination of HMF. The method measurement was carried out of optimum pH response at 3.00 and immobilised aniline of 8.0×10^{-3} M. The reproducibility and photostability studies show a good RSD value of 1.96 % and 1.40 %, respectively.

The results obtained for HMF determination in unknown honey samples using the reflectance method were in a good agreement with those obtained from HPLC standrad. The interference study

has revealed no influence due to presence of glucose and fructose. The results showed insignificant between the HMF method and the HPLC standard method, for the determination of HMF concentration in the range of 1 - 6 x 10^{-3} M with good correlation coefficient (R^2) and slope of 0.9961 and 0.9838, respectivly. Therefore, a sensor based on immobilisation of aniline in chitosan has been successfully employed for the quantitative determination of HMF.

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