Determination of Hydroxymethylfurfural (HMF) in Honey Based on 4-aminoantipyrine Immobilised in sol-gel/chitosan Using Reflectance Spectrophotometric Technique

Mashri Ahmad Yahia [a], Musa Ahmad [b]

[a] Chemistry Department, Faculty of Science, Sebha University, Libya

[b] School of Chemical Science and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

Email: Mshre71@yahoo.com

Abstract

: The current study proposed an optical sensing material based on 4-aminoantipyrine (4AAP) immobilised in filter paper coated with sol-gel/chitosan for the detection of hydroxymethylfurfural (HMF) by reflectance spectrophotometry method. 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 sensor showed optimum activity at pH 6. The optimum chitosan concentrations and enzyme loading were found to be at 2% (w/v) and 0.08 mg, respectively. A linear response of the sensor was obtained in the hydroxymethylfurfural concentration range of 0.0004 to 0.1 M. The reproducibility of the sensor was good, with an observed relative standard deviation of 0.9% (n = 8).

Keyword: Optical sensor; 4-aminoantipyrine; sol-gel/chitosan; hydroxymethylfurfural; reflectance spectrophotometric technique; pH.

1.-Introduction

Honey is defined as a natural substance with high nutritional value due to the complex composition. Essentially, it consists of sugar and water with important secondary components such as minerals, organic acids, enzymes, proteins, and vitamins (Abd Rashid et al.,2020). The composition and nutritional properties of honey very dependent on the flora, climate, and soil (Ajlouni & Sujirapinyokul 2010).

In general, there are some features characterising honey, which are sweetness and stickiness. Besides, it's being as a naturally complex liquid food. The natural honey produced by bees is made from the nectar of flowers. Studies have shown that such bee honey is nutritionally and medically beneficial or useful since it contains some biological active components such as flavonoids, ascorbic acid, and a-tocopherol in addition to other useful elements such as various amino acids, organic acids as well as readily obtainable sugars (Turhan et al., 2008). As stipulated by the international food regulations, honey represents itself as a purely natural product to which no other types of substances or elements are allowed to be added (Al Naggar, et al., 2021). It was found that the addition of water to honey leads to reduced in the activity with increasing water content (Abdellah, et al., 2020).

HMF is practically absent in fresh honey, but concentrations increase with the temperature and time of heating, as well as due to improper storage and adulteration (Ajlouni & Sujirapinyokul,

2010: Miotto 2011). At low heating temperatures, the composition and pH can also influence the formation of HMF in honey (Miotto, 2011). HMF is formed in honey by heating monosaccharides under acid conditions (Castoldi et al., 2016) and the measurement of HMF concentration can be used in the quality control of honey (Amariei et al.,2020).

The reaction between HMF and 4-AAP to produce Schiff-based compound has been characterised by using UV-Visible spectrophotometer. Schiff bases are compounds containing the azomethine group (-HC=N-), which is formed through condensation reaction between ketones or aldehydes with primary amines. Formation of Schiff base generally takes place under acid or base catalysis or with heat. Figure. 1 shows the reaction between HMF and 4-AAP.

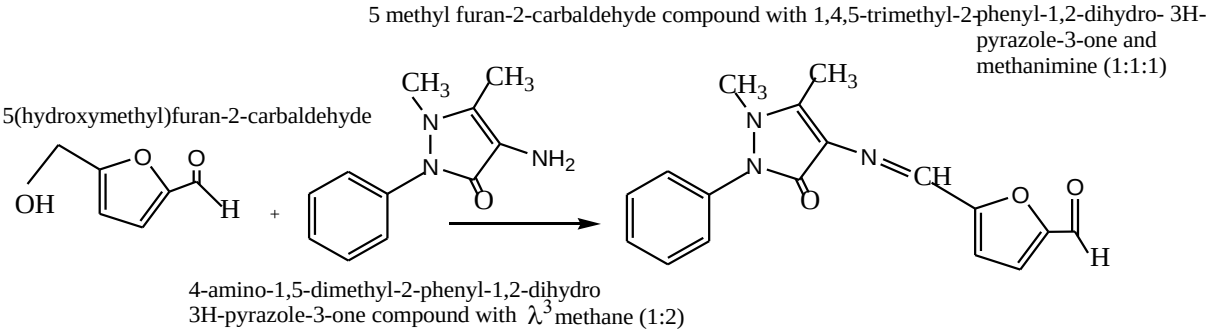


Fig.. 1 Structure of possible reaction between 4-AAP and HMF

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 Solutions preparation

The standard hydroxymethylfurfural (HMF) solution of 3×10^{-2} M was prepared by dissolving 0.094 g of HMF in 25ml deionised water. Eight serial dilutions in deionised water were prepared from standard HMF solution to the measure of HMF solution with concentration in the range of (1–8) $\times10^{-3}$ M.

Preparation of the 4-aminoantipyrine (4-AAP) standard stock solution of 3×10^{-3} M was achieved by dissolving 0.152g of 4-AAP in 25mL deionised water. Serial dilutiond for the preparation of (1–8) $\times$ 10^{-3} M of (4-AAP) prepared by appropriate dilution of the stock solution in deionised water.

The preparation of pH amounting to 3.403 g anhydrous KH_2PO_4 and 4.355 g anhydrous K_2HPO_4 was done by dissolving separately in 250 mL of freshly deionised water and then the two solutions were mixed with different volumes.

2.3. Materials preparation

2.3.1 Sol-gel membrane

Preparation of the homogeneous stock solution of sol–gel was conducted by mixing 40 mL of TEOS, 20 mL of 0.1 M HCl, 80 mL of ethanol, and 4.2 mL of triton-x 100 together. Before using this stock, a solution of sol–gel was maintained at room temperature overnight in order to ensure the completion of the condensation and hydrolysis (Mohammad et al., 2007).

2.3.2 Chitosan membrane:

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 (Abdullah et al., 2006).

2.4 Preparation of Sensing Material:

The sensor design used in this study was as in previous work with slight modifications (R. Tiwari et al., 2007). Sol–gel has been widely used for sensor development during the past decade, driven by the unique characteristics of its simple preparation that can be done at room temperature and it is suitable for the immobilisation of various reagents (Jeronimo et al., 2007 Kim,et al.,2013). On the other hand, chitosan is also transparent, has good adhesion to surface of filter paper, and it is the immobilisation of some reagent. In this study, sol-gel/chitosan was chosen for the immobilisation of 4-AAP because it is non-toxic, biocompatible, and biodegradable (Dash et al., 2011).

The use of sol-gel/chitosan has been previously reported by Jaafar et al. (2006), based on their work on optical biosensor using stacked films for the detection of phenolic compounds. It has been mentioned that the use of hybrid sol-gel / chitosan yielded enough hydrophobic and porosity properties to allow the MBTH reagent to be retained and the leaching problem could be improved (Jaafar et al., 2006).

2.4.1 4-Aminoantipyrine hybrid in sol-gel / chitosan:

Preparation of immobilised 4-aminoantypirine in gel/chitosan was started by mixing 2 mL of sol-gel with 2 mL chitosan in a sample bottle. Then the sol-gel/chitosan solution was stirred until a homogenous solution was obtained. This was followed by the addition of 0.7 mL of saturated 4-aminoantypirine and phosphate buffer (pH 6.0) to the homogenous solution of the sol-gel/chitosan and stirred continuously by using magnetic stirrer until homogenous solution was achieved. After 25 minutes, the solution became colourless and was ready to use. Then 0.4 ml of the mixture of 4-aminoantypirine in sol-gel/chitosan solution was pipetted into a petri dish. A paper filter strip was then immersed into the petri dish. Next, the paper filter strip was

soaked in a mixture of sol-gel/chitosan with 4-aminoantypirine and dried by placing it in the refrigerator overnight.

2.4.2 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 6.0), 4-aminoantypirine (6×10^{-3} M), and HMF (4×10^{-4} M) was used for immersing 4-aminoantypirine the in filter paper coating with immobilised a chitosan. Moreover, the reflections studies were recorded between the wavelength 250 - 500 nm.

3. Optimisation the Response of Sensing Material :

All the characterisations of the chemical reactions between the immobilised 4-AAP 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 6.0. 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 4×10^{-4} M of HMF were also determined. Finally, solutions containing 4×10^{-4} 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.

4. Results and Discussion

The reaction between immobilised 4-AAP in hybrid sol-gel/chitosan with HMF produced a Schiff base which gave maximum reflectance at 350 nm (Figure 2.) due to the changes in colour of the immobilised 4-AAP from colourless to yellow. Figure 2. Shows the reflectance spectra of 4-AAP 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 colourless to yellow after reaction with HMF. All reflectance measurements in this study were carried out at a 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 $\times 10^{-3}$ 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 380 nm, which was used for subsequent quantitative studies.

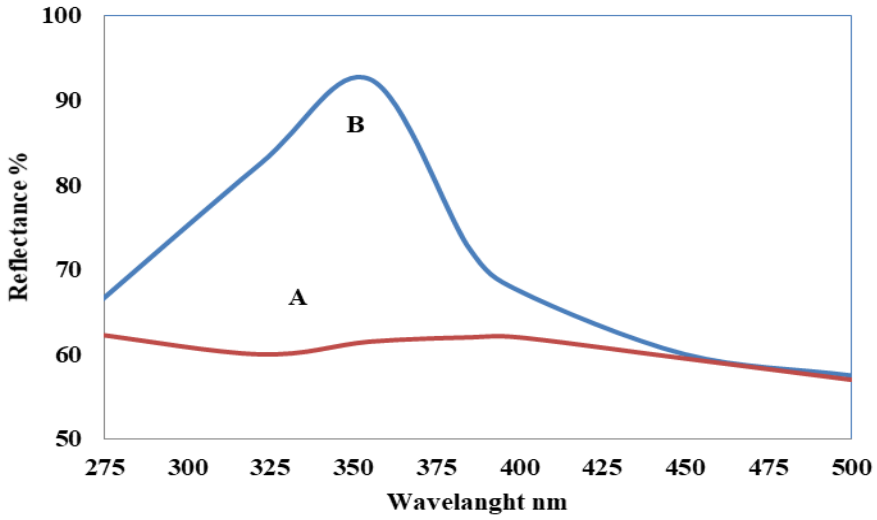


Fig. 2. Reflectance spectra of the immobilised 4-AAP alone in hybrid sol-gel/chitosan (A) and after reaction with HMF (B)

4.1 Optimisation of pH:

The sensing material response to the variation of pH is determined using phosphate buffers in the range of 2 to 8 as shown in Figure 3. The optimum response was obtained at pH 6.0. The higher reflectance signal was observed at acidic region and reaches optimum at pH of 6.0. The optimum pH is found to be the same as in solution on pH 3.0 because there is no effect on the functional group in the 4-AAP during immobilisation (Musa et al,.1998).The same trend of results is also reported by Musa et al. (1998) where in their work the same optimum pH of pH 6.0 was observed both in solution and immobilised form.

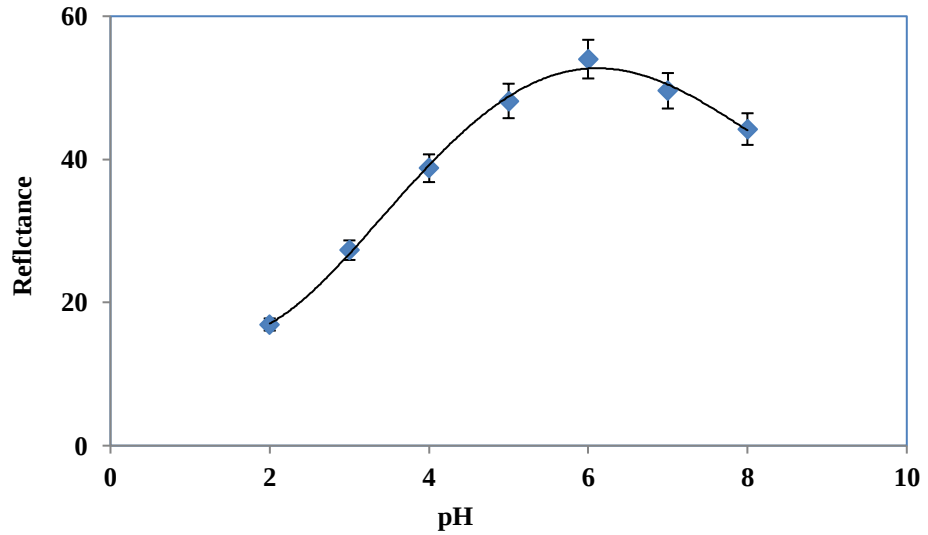


Fig. 3. The effect of the variation in the pH of phosphate buffer on the sensing response.

4.2 The effect of 4-AAP concentration:

The effect of different concentration of immobilised 4-AAP upon reaction with HMF has been investigated by measuring the reflectance signal using UV-Vis spectrophotometer at fixed HMF concentration of 3 x 10^{-4} M. The reflectance increased with the increasing of 4-AAP concentration at the initial stage and the response slowly decreased when 4-AAP concentration reach 5 × 10^{-4} M. The use of higher concentration of 4-AAP allowed more reactions to occur, thus higher reflectance signal was observed. The reflectance signal ultimately became plateau as almost all immobilisation sites have been fully occupied by the HMF compound. The phenomena has been explained by Abdullah et al.(2006) in their work on the quinone produced from the enzymatic oxidation of phenol was reacted with 3-methyl-2-benzothiazolinone hydrazone (MBTH) to produce a maroon colour adduct.

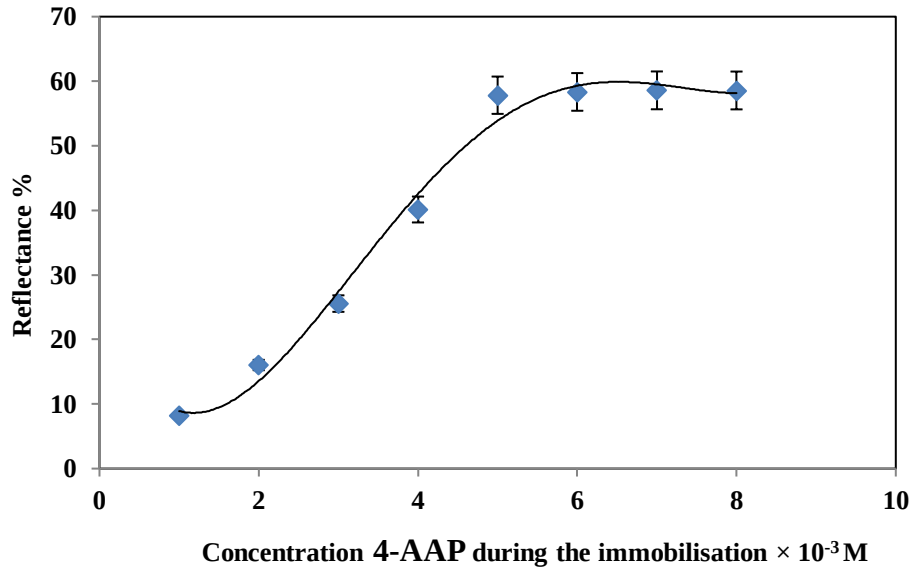


Fig. 4. The effect of immobilised 4-AAP concentration on the response upon reaction with HMF concentration was fixed at 3 × 10^{-4} M.

4.3 Photostability study of the sensing material:

Reflectance spectrophotometric method was employed to study the effect of light on the immobilised 4-AAP where the immobilised reagent was constantly exposed to the light source for 6 hrs (Figure 4.). This process aimed to determent the occurrence of photoleaching or photodecomposition at the Ambien temperature and light conditions at reagent concentration of 3 ×10^{-4} M. The obtained RSD value of the experimented is 0.9%, which indicate that the immobilised reagent was stable against light at ambient conditions.

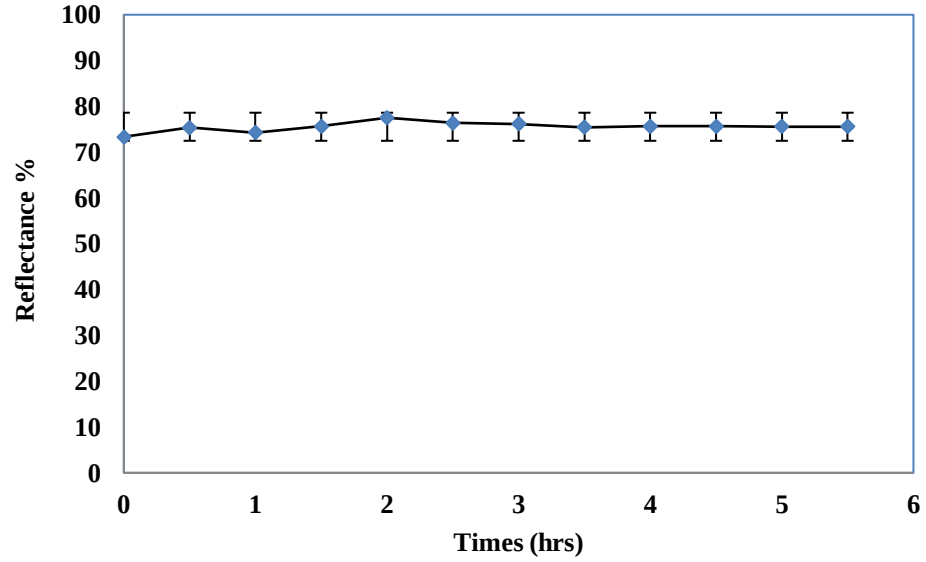


Fig. 5. The photostability of the immobilised 4-AAP against time of 6 hrs.

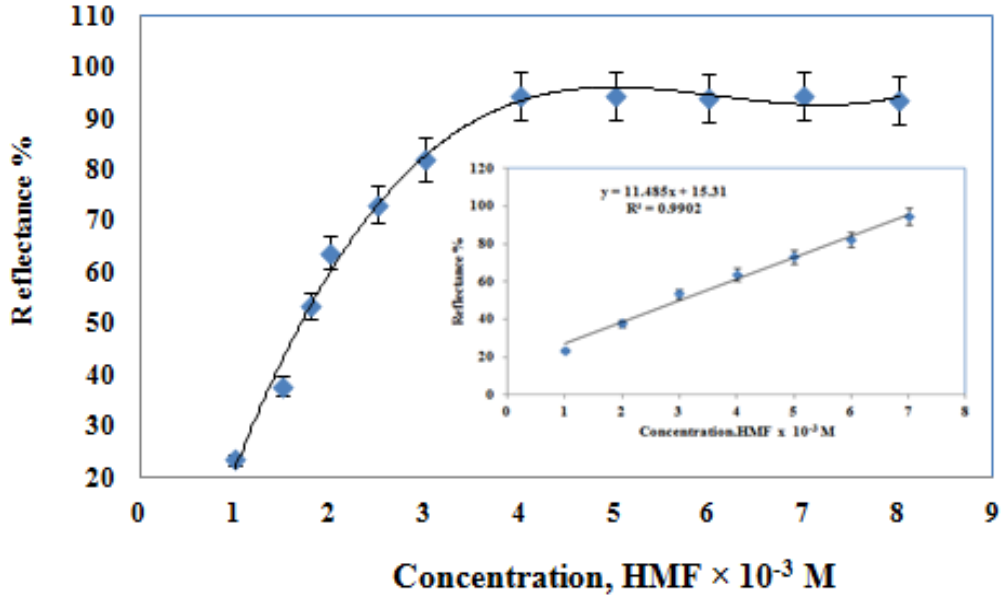


Fig. 6. The response curve of the different concentration of HMF (inset is the linear dynamic range of the (HMF sensor)

4.5 The dynamic range of HMF concentration :

Figure 6. Shows the increasing of reflectance signal with the increasing of HMF concentration, until the HMF concentration reach 4×10^{-4} M where the response become plateau. It is clear that the higher HMF concentration facilitated the occurrence of more reactions between the immobilised 4AAP and analyte molecules. Therefore, a higher signal was observed. Ultimately, the spectrophotometer signal reached a plateau after a full occupation of almost all sites by the analyte. The same trend at results was also reported by Tan *et al.*, (2012) based on

a facile single-step immobilisation of cobalt (II) ion onto high capacity Dowex HCR-W2 microspheres . A linear relationship between reflectance and HMF concentration at range of 1 - 4 × 10^{-4} M, was achieved with a linear correlation of R^2 = 0.99.

The interference from several compounds during HMF determination was also examined in this work. Based on the interference studies conducted for glucose and fructose that might be presence in honey samples, it can be noted that the interfering compounds did not show any significant interference since the percentage of interference are observed to be well below ± 5% (Table 1). The percentage interference was calculated by the equation shown below Table1. The same method has been used and reported by Ahmad and Narayanaswamy (2002).

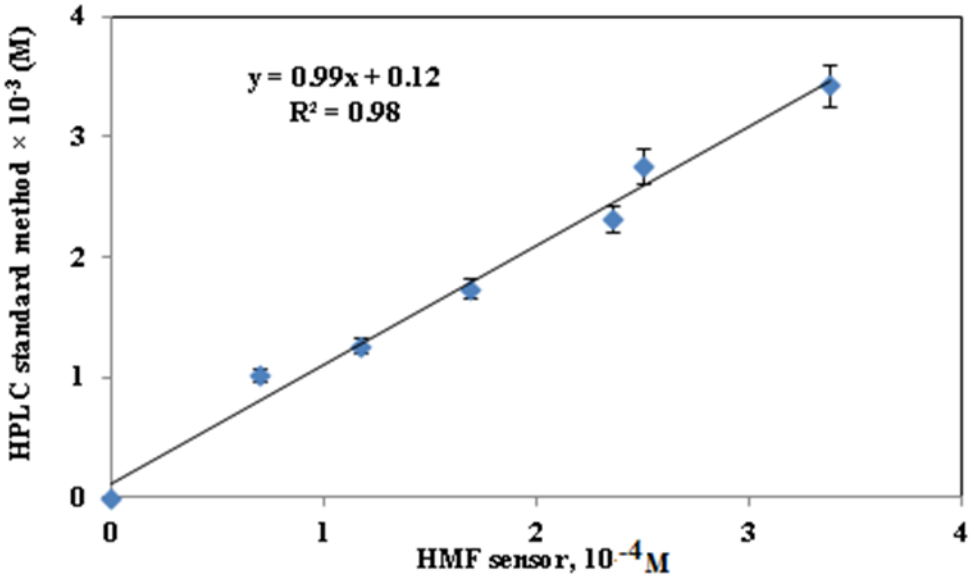
Table1. Degree of interference of some compounds (glucose and fructose) of using HFM sensor based on immobilised 4AAP at the same concentration 3 x 10^{-4} M

Interfering compounds	(x) mean±SD (n=3)	(y) mean±SD (n=3)	Percentage of interference
Glucose	157.58±0.07	157.52±0.10	0.04%
Fructose	157.11±0.08		- 0.26%

% interference = (a − b)/b × 100%, where a and b are the reflectance reading in the presence and absence of the interfering respectively.

4.6 validation study

Sensing material developed in this study has been applied to determine the HMF concentration, and the results have been validated by comparison to the HPLC standard method. As shown in Figure 7, the results have revealed that a great agreement between the sensing material developed in this study and the HPLC standard method with a good coefficient R_2 and slope value at 0.98 and 0.99 respectively. To further examine the method performance especially for real sample analysis, an experiment has also been carried out using both methods which has been developed in this study and standard method, to determine the concentration of HMF in honey sample. The results obtained are shown in the Table 2. The comparison of the two means taken from the standard HPLC method and the developed sensing material revealed that there was no significant difference between both methods. Statistical analysis was conducted using the t-test value technique. The statistical analysis results showed that the difference between the two methods is insignificant with percentage error of less than 5% level. Also f-value has been calculated and the value obtained no significant (less than 39) and two methods give similar results.



Fi.e 7. The validation study of the HMF method with HPLC standard method for determining HMF concentration

Table 2 .Comparison between the developed method based on immobilised 4-AAP for the determination of HMF in honey.

Concentrations × 10^{-3} Mol L^{-1}	Developed sensing material × 10^{-3} M mean±SD (n=3)	HPLC method × 10^{-3} M mean±SD (n=3)	t-value	f-value
1	0.09±0.05	0.09±0.02	0.35	1
2	0.19±0.09	0.17±0.01	0.01	0.80
3	0.32±0.10	0.28±0.09	0.01	0.76
4	0.41±0.12	0.43±0.05	0.03	1.09
5	0.47±0.21	0.48±0.07	0.02	1.04
6	0.6±0.23	0.59±0.06	0.01	0.96
Honey real sample	0.13±0.02	0.22±0.01	0.03	2.86

Note: The critical value, t3 = 3.18 (P=0.05) f2,2 = 39 (P=0.05)

5. Conclusion

An optical sensing material based on immobilised of 4-AAP in chitosan/sol-gel has been successfully developed for the detection of HM. The response of the sensing material was

dependent on the concentration of 4-AAP used for immobilisation, pH and HMF concentration. A linear relationship was obtained at HMF concentration in the range of 1- 4 × 10^{-4} M. The interference study has revealed no significant interference due to presence of glucose and fructose in honey. The results showed a great agreement between the sensing material and the HPLC standard methods, with correlation coefficient (R_2) and slope value at 0.98 and 0.99 respectively. The developed sensor has a good potential use in quantitative determination of HMF in industrial of honey with LOD 0.12 and LOQ 0.36**.**

REFERENCES

Ahmed, J., Prabhu, S., Raghavan, G. & Ngadi, M. 2007. Physico-chemical, rheological, calorimetric and dielectric behavior of selected Indian honey. Journal of Food Engineering, 79, 1207-1213.

Abd Rashid, N., Mohammed, S. N. F., Syed Abd Halim, S. A., Ghafar, N. A., & Abdul Jalil, N. A. (2022). Therapeutic potential of honey and propolis on ocular disease. Pharmaceuticals, 15(11), 1419. https://doi.org/10.3390/ph15111419

Abdellah, F., Makhloufi, C., Boukraa, L., Hammoudi, S. M., Amel, S. A. F. A., Dellel, N., Benamara, A., Benhadiri, M., Marouf, N., & Benaraba, R. (2020). Physico-chemical properties and antibacterial and antioxidant activity of two varieties of honey from Algerian steppe. Journal of Apitherapy and Nature, 3(2), 59–74. https://doi.org/ 10.35206/jan.774052

Castoldi, K., Milani, M. I., Rossini, E. L., Pezza, L. & Pezza, H. R. 2016. Flow injection analysis of 5-(hydroxymethyl)-2-furaldehyde in honey by a modified Winkler method. Analytical Sciences, 32. 413-417.

Al Naggar, Y., Giesy, J. P., Abdel-Daim, M. M., Ansari, M. J., Al-Kahtani, S. N., & Yahya, G. (2021). Fighting against the second wave of COVID-19: Can honeybee products help protect against the pandemic? Saudi Journal of Biological Sciences, 28 (3), 1519–1527. https://doi.org/10.1016/j.sjbs.2020.12.031

Anand, S., Deighton, M., Livanos, G., Morrison, P. D., Pang, E. C., & Mantri, N. (2019). Antimicrobial activity of Agastache honey and characterization of its bioactive compounds in comparison with important commercial honeys. Frontiers in Microbiology, 10, 263. https://doi.org/10.3389/fmicb.2019.00263

Ajlouni, S. & Sujirapinyokul, P. 2010. Hydroxymethylfurfuraldehyde and amylase contents in Australian honey. Food Chemistry, 119. 1000-1005.

Turhan, I., Tetik, N., Karhan, M., Gurel, F. & Tavukcuoglu, H. R. 2008. Quality of honeys influenced by thermal treatment. LWT-Food Science and Technology, 41. 1396-1399.

Amariei, S., Norocel, L., & Scripcˇa, L. A. (2020). An innovative method for preventing honey crystallization. Innovative Food Science & Emerging Technologies, 66, Article 102481. https://doi.org/10.1016/j.ifset.2020.102481

Diacu, E. & Tantaveanu, E. F. 2007. Determination of moisture content and its correlation with other parameters in honey quality control. Revista De Chimie-Bucharest-Original Edition, 58. 1310.

Abdellah, F., Makhloufi, C., Boukraa, L., Hammoudi, S. M., Amel, S. A. F. A., Dellel, N., Benamara, A., Benhadiri, M., Marouf, N., & Benaraba, R. (2020). Physico-chemical properties and antibacterial and antioxidant activity of two varieties of honey from Algerian steppe. Journal of Apitherapy and Nature, 3(2), 59–74. https://doi.org/ 10.35206/jan.774052

Carvalho, C. A., Sodré, G. S., Fonseca, A. A., Alves, R. M., Souza, B. A. & Clarton, L. 2009. Physicochemical characteristics and sensory profile of honey samples from stingless bees, Anais da Academia Brasileira de Ciências, 81. 143-149.

Dash, M., Chiellini, F., Ottenbrite, R. & Chiellini, E. 2011. Chitosan—A versatile semi-synthetic polymer in biomedical applications. Progress in Polymer Science, 36,. 981-1014.

Kim, M.A.; Lee, W.Y.2003. Amperometric phenol biosensor based on sol-gel silicate/nafion composite film. Analytical. Chimecal. Actuators, 479, 143-150

Jaafar, A. Musa, A. Nadarajah, K. Lee, Y.H. Hamidah, S.2006. Immobilisation of tyrosinase in chitosan film for an optical detection of phenol. Sensors and Actuators B:Chemical, 114(2), 604-60

Mohammad, R., Ahmad, M. & Daud, J. M. 2007. Potensi kurkumin sebagai penunjuk pH semula jadi untuk pembangunan sensor optik pH. Malaysian Journal of Analytical Sciences, 11. 351-360.

Abdullah, J., Ahmad, M., Heng, L. Y., Karuppiah, N. & Sidek, H. 2006. Stacked films immobilisation of MBTH in nafion/sol-gel silicate and horseradish peroxidase in chitosan for the determination of phenolic compounds. Analytical and Bioanalytical Chemistry, 386. 1285-1292.

Ahmad, M. & Narayanaswamy, R. 2002. Optical fibre Al (III) sensor based on solid surface fluorescence measurement. Sensors and Actuators B: Chemical, 81, 259-266.

R. Tiwari, S. Buse, C. Herstatt, 2007, Innovation via global route: Proposing a reference model for chances and challenges of global innovation processes, Proceedings of the Second International Conference on Management of Globally Distributed work, Indian Institute of Management, Bangalore, pp. 451-465.