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Electronic Supplementary Information (ESI)

Detection of Azadirachtin from Neem Kernels using a Paper-based Sandwich Sensor

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S.1 Comparison of color intensities

The blank and the GMF dots containing samples were analysed using ImageJ software using relative red intensity (RRI), relative green intensity (RGI), relative blue intensity (RBI) and the normalized gray intensity (NGI). The values were calculated using the equations:

$$\text{RRI} = \text{Red intensity of the blank} - \text{Red intensity of the sample} \quad \text{(S1)}$$

$$\text{RGI} = \text{Green intensity of the blank} - \text{Green intensity of the sample} \quad \text{(S2)}$$

$$\text{RBI} = \text{Blue intensity of the blank} - \text{Blue intensity of the sample} \quad \text{(S3)}$$

$$\text{NGI} = 255 - [(0.2123 \times \text{Red intensity}) + (0.7152 \times \text{Green intensity}) + (0.072 \times \text{Blue intensity})] \quad \text{(S4)}$$

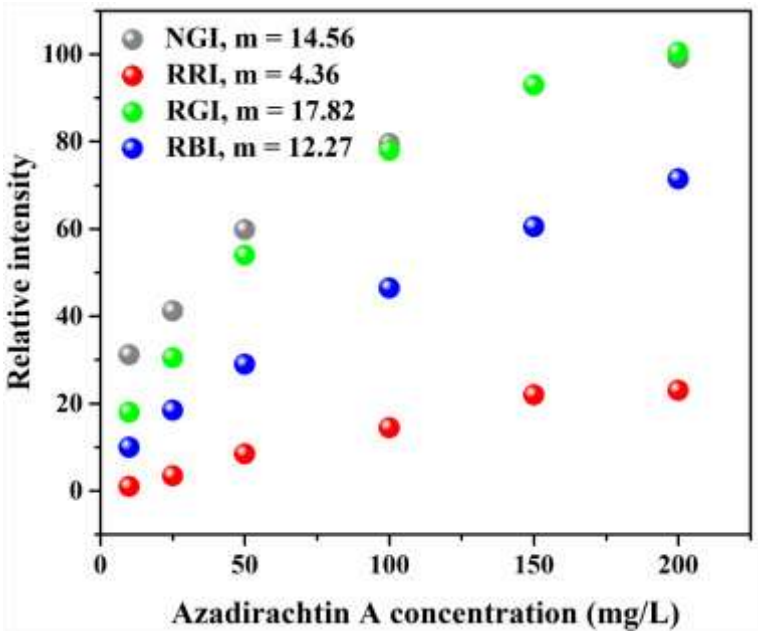

Figure S1: The variation in relative red intensity (RRI), relative green intensity (RGI), relative blue intensity (RBI) and normalized gray intensity (NGI) with concentration.

In Figure S1, the slope (m) for each of the relative intensities is also specified. Of all the relative intensities, RGI shows the highest slope or sensitivity. Hence, all measurements were performed using RGI values.

S.2 Acidified vanillin assay – Drop test

Since the method involves concentrated sulfuric acid, it was necessary to have an acid-resistant base for the assay. Parafilm promised the best results as the acid drops did not denature the film. The hydrophobicity of the parafilm caused the drops to remain as such even after several days, posing difficulty in handling and image analysis (Figure S2). The GMF paper was acid resistant, hydrophilic, and an ideal choice for paper-based assay. This helped in the handling and image analysis of these dots.

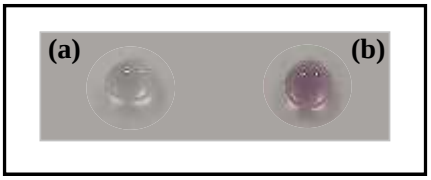

Figure S2: Acidified vanillin assay as a drop test: (a) the blank drop and (b) the drop with Aza. The drops remained as such on the parafilm sheet, making image analysis difficult and unsafe.

S.3 Glass Microfiber Filter paper

The GMF paper has two sides – a smooth, spongy surface, while the other has patches (Figure S3). The patchy surface was preferred as the reaction surface, as the reagent flow was smooth and rapid.

Figure S3: The two sides of the GMF dots – (a) the smooth, spongy surface can be seen, where the flow of reagents is not even and (b) patchy surface enables the uniform and easy flow of reagents.

S.4 Optimization of size of the paper dots

Experiments were performed with two sizes of paper substrates (addressed as paper dots) – 3mm and 5mm. Two paper dots of each size were arranged side by side to compare the sample response with their respective blank. The vanillin solution, H_2SO_4, and Aza concentrations were 100 mg/mL, 36 N, and 200 mg/L, respectively. The effect of paper dot size during colorimetry is shown in Figure S4.

The volumes of vanillin, acid, and Aza were taken to be 4, 2, and 7 µL, respectively, for the 3mm dots. As shown in Figure S4(a), we observed these dots were overloaded with reagents. The wet dots make image analysis difficult as the excess reagents spread during scanning during image analysis. This creates imprecise boundaries, leading to erroneous RGI values during image analysis. Hence, it is necessary to have dried dots. Reducing the reagent volume could be an option but decreasing reagent volume lower than 2 µL was not attempted as precise control of such low volumes is difficult. Thus, 5 mm is chosen as the size of the GMF dots. This was able to accommodate a total of 19 µL of reagents. The vanillin, acid, and Aza volume was 6, 4, and 9 µL, respectively. The dots were relatively dry in this case, as shown in Figure S4(b). Scanning of these larger dots yielded better images with good boundaries. Paper dots larger than 5 mm were not tried since this would involve the use of higher volumes of acid, giving rise to safety concerns.

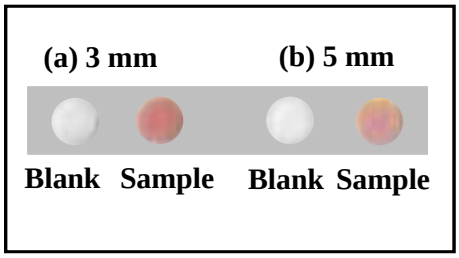

Figure S4: Images of the blank and sample obtained using 3 mm and 5 mm GMF dots.

S.5 Optimization of sequence of addition of reagents

The assay involves the addition of three reagents: vanillin, acid, and Aza. Understanding the reaction mechanism of these reagents for colour development is essential. Six different combinations of addition sequence of reagents were analysed, namely (a) vanillin solution-methanol/Aza-acid, (b) vanillin solution-acid-methanol/Aza, (c) acid-methanol/Aza-vanillin solution, (d) methanol/Aza-vanillin solution-acid, (e) acid-vanillin solution-methanol/Aza and (f) methanol/Aza-acid-vanillin solution. The objective here was to find the most appropriate order of addition of reagents. The assay was conducted using the standard volumes and concentrations of reagents on 5 mm GMF dots, and the images obtained are shown in Figure S5. It was found that the order of addition of reagents played a significant role in determining the uniformity and intensity of the colour on the GMF dots. As seen in Figure S5 (a), (d), and (f), the reagents did not spread out uniformly, and thus, a ring pattern was formed at the periphery of the GMF dots. In addition, it is also clear from these images that for uniform coloration, it is necessary to have an acidified vanillin base on which Aza can act. For quantitative detection using a colorimetric assay, having a uniform colour distribution over the surface is desirable. Moreover, adding an Aza sample as the last reagent is favourable for a sensor application. Based on this requirement, either vanillin-acid-Aza (case b) or acid-vanillin-Aza (case e) can be used. In the present study, we have used case (b) as the preferred order, i.e., vanillin solution followed by acid followed by methanol/Aza.

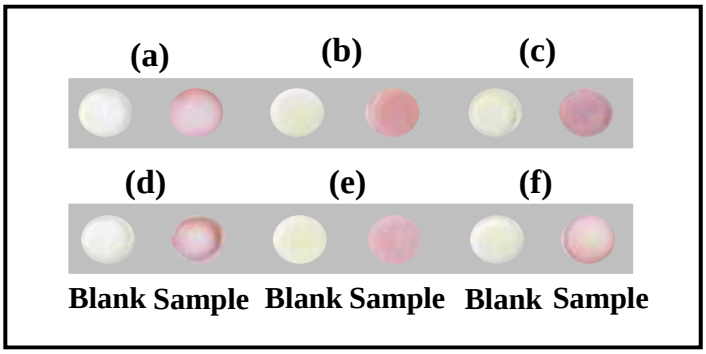

Figure S5: Effect of order of addition of reagents. (a) vanillin solution followed by Aza/methanol followed by acid (b) Vanillin-Acid-Aza/methanol, (c) Acid-methanol/Aza-vanillin solution. (d) Aza/methanol-vanillin solution-acid e) Acid-vanillin solution-methanol/Aza (f) Aza/methanol-acid-vanillin solution.

S.6 Acidified vanillin assay at different acid strength

Acidified vanillin assay gives the magenta color in the presence of Aza at high concentration of sulfuric acid. At any other concentration, the assay does not yield color. Figure S6 indicates the acidified vanillin assay at three different acid concentrations-18M, 9M and 4.5M. The first set of images show the acid in presence of vanillin alone, where yellow color formation happens only at 18M acid. The same result is observed in the presence of Aza. Hence, 18M was chosen as the standard acid concentration.

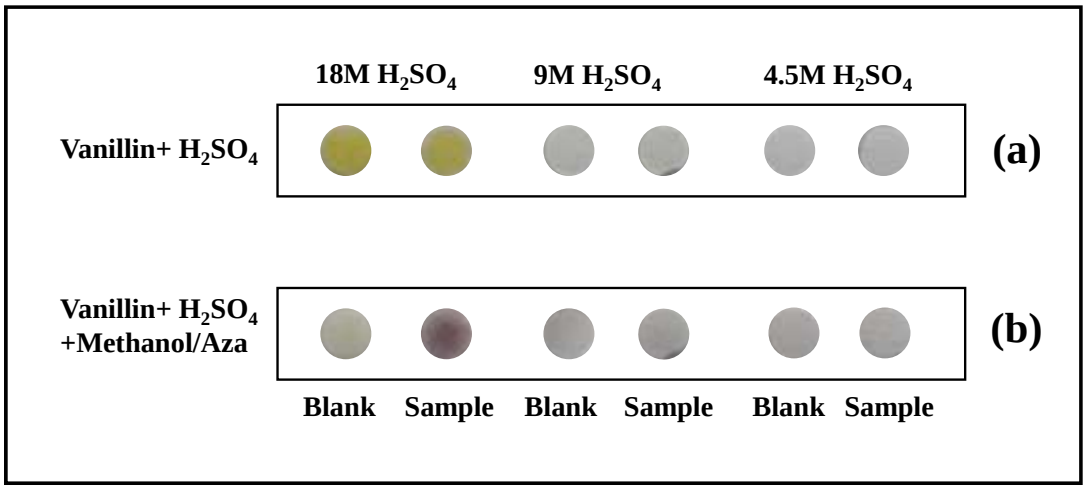

Figure S6: Acidified vanillin assay at different concentrations of acid: 18M, 9M and 4.5M acid. (a) shows the GMF dots acidified with sulfuric acid (to maintain uniformity in figures, the dots are shown in duplicates) and (b) shows acidified vanillin treated GMF dots in presence of methanol/Aza.

S.7 Repeatability and reliability

All the assays was performed in triplicates by the same person and the average of the three measurements are reported in Table S1. The standard deviation and % relative standard deviation (%RSD) are also indicated. The % relative standard deviation was calculated according to the equation:

$$\%\ Relative\ standard\ deviation = \frac{Standard\ deviation}{Average\ RGI\ value} \times 100\ \%$$

Table S1: Repeatability of the assay

Sample No	% Aza from colorimetry	Average RGI values	Standard deviation	% Relative Standard Deviation
1	0.21	9	0.04	0.44
2	0.32	15	0.00	0
3	0.39	19	0.02	0.11
4	0.46	22.67	0.01	0.044

Reliability indicates the precision of the assay when performed at different conditions, like different days and by different people. The assay, performed on different days, is averaged, and reported in Table S2:

Table S2: Reliability of the assay

Sample No	% Aza from colorimetry	Average RGI values	Standard deviation	% Relative Standard Deviation
1	0.24	10.67	0.06	0.56
2	0.31	14.33	0.04	0.28
3	0.39	19	0.05	0.26
4	0.42	20.67	0.04	0.20

The % RSD was in the range of 0-0.4% for intra-day and 0.2-0.6% for inter-day repeats. The % RSD below 2 % signifies high precision of the proposed analytical system.

S.8 Estimation of % Aza content in neem seed kernels

The % content of Aza in the seed kernels were calculated using the slope of the calibration curve, Y = 1.64X – 2.49. The detailed calculation is shown in Table S3.

Table S3: Estimation of % Aza content from neem seed kernels using slope value from calibration plot.

S.N.	RGI value (From Colorimetry)	Conc. from slope (Y = 1.64X – 2.49)	Final Conc. (Conc. × 30)/1000
1	8.43	6.66	0.19
2	14.67	10.47	0.31
3	19	13.11	0.39
4	21.6	14.69	0.44