Front. Chem. Sci. Eng.
https://doi.org/10.1007/s11705-019-1790-7
COMMUNICATION

A simple, azulene-based colorimetric probe for the detection of nitrite in water

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Electronic Supplementary Material

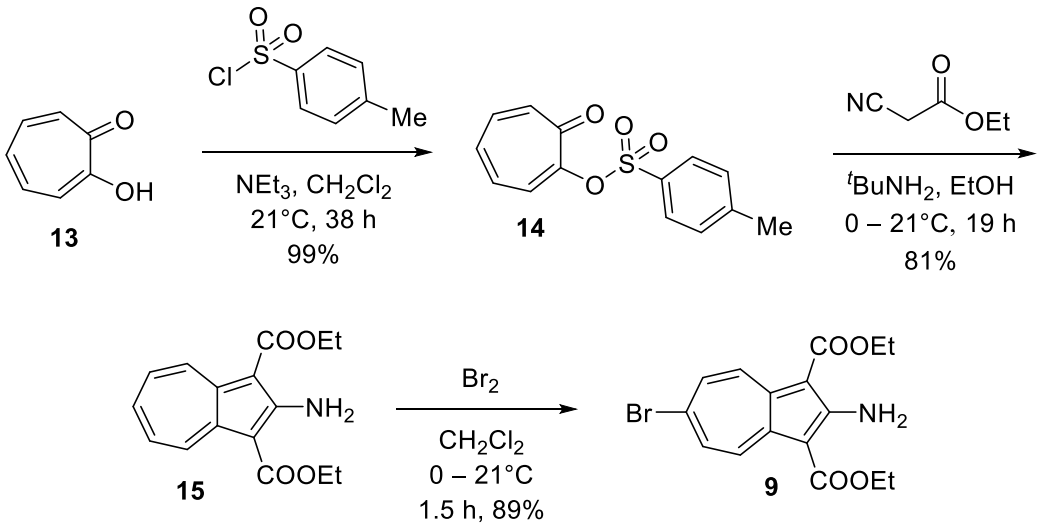
Reagents, General Procedure and Equipment

Solvents and reagents used were reagent grade, purchased from Fisher Scientific, Sigma-Aldrich and Fluorochem. All chemicals and solvents purchased were used without further purification.

Unless stated otherwise, ambient conditions were used for each reaction. Inert conditions were achieved using anhydrous solvents and by allowing the reaction to proceed under an atmosphere of nitrogen. Anhydrous solvents were dried using an Innovative Technology PS-400-7 Solvent Purification System.

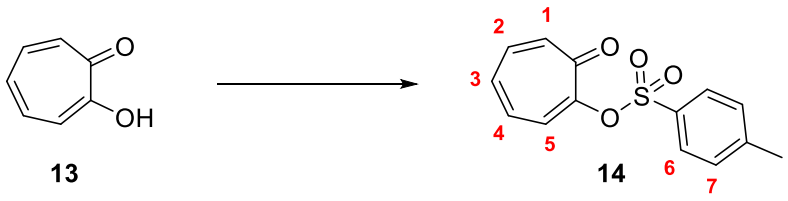
Mass spectra were recorded on a microTOF mass spectrometer, with electrospray ionisation (ESI) used as the ionisation method. All ^{1}H and ^{13}C NMR spectra were obtained from a 500 MHz Agilent ProPulse, for which proton decoupling was active for 13C NMR. All chemical shift values (δ) are reported in ppm relative to tetramethylsilane (internal standard), referenced to the residual solvent peak of the deuterated solvent used. Multiplicity of the recorded peaks are listed as s for singlet, d for doublet, t for triplet, q for quartet, or m for multiplet. Coupling constants (*J* / Hz) are given where calculable. All UV/Vis experiments were performed on a Shimadzu UV-1800 UV spectrometer, for which a quartz cuvette of 1 cm path length was used.

Overall Synthetic Scheme



Scheme S1. Synthesis of nitrite probe 9.

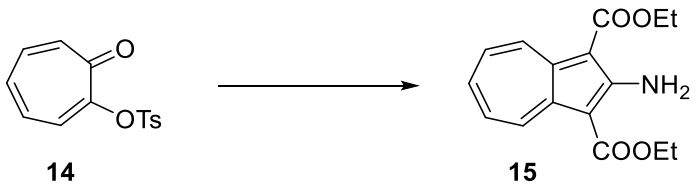
7-Oxocyclohepta-1,3,5-trien-1-yl 4-methylbenzenesulfonate (14)



Under an atmosphere of nitrogen, tropolone **13** (5.00 g, 40.94 mmol, 1.0 eqv) and tosyl chloride (7.81 g, 40.94 mmol, 1.0 eqv) were dissolved in anhydrous CH_2Cl_2 (60 mL), into which NEt_3 (5.71 mL, 40.94 mmol, 1.0 eqv) was added dropwise. The solution was further diluted in CH_2Cl_2 (60 mL) to prevent a suspension from forming, and left to stir for 38 hours, forming a yellow slurry. The reaction was quenched with ice, extracted with CH_2Cl_2 (3 × 100 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure affording 7-Oxocyclohepta-1,3,5-trien-1-yl 4-methylbenzenesulfonate **14** as a crystalline yellow solid (11.30 g, 99 %) without any further purification required.

δ_H (500 MHz, $CDCl_3$) 7.91 (2H, d, *J* 8.3 Hz, H^6), 7.46 (1H, d, *J* 9.4 Hz, H^1), 7.33 (2H, d, *J* 8.1 Hz, H^7), 7.21, (1H, ddd, *J* 12.3, 7.9, 1.1 Hz, H^4), 7.15 (1H, d, *J* 12.3 Hz, H^5), 7.08 (1H, ddd, *J* 11.6, 7.9, 1.1 Hz, H^3), 6.98 (1H, ddt, *J* 10.9, 9.4, 1.3 Hz, H^2), 2.45 (3H, s, CH_3). δ_C (125 MHz, $CDCl_3$) 179.5 (C=O), 155.3 (O-C), 145.6 (SC), 141.3 (C^5), 136.4 (C^4), 134.8 (C^3), 133.6 ($CH_3\underline{C}$), 130.9 (C^2), 130.0 (C^1), 129.7 (C^7), 128.7 (C^6), 21.9 (CH_3). Analytical data in agreement with those previously reported.[1]

Diethyl 2-aminoazulene-1,3-dicarboxylate (15)

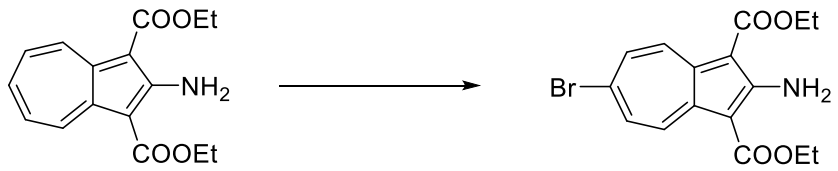


14 **15**

7-Oxocyclohepta-1,3,5-trien-1-yl 4-methylbenzenesulfonate **14** (4.50 g, 16.29 mmol, 1.0 eqv) and ethyl cyanoacetate (3.82 mL, 35.83 mmol, 2.2 eqv) were dissolved in ethanol (80 mL) and cooled to 0 °C, into which *t*-butylamine (4.28 mL, 40.72 mmol, 2.5 eqv) was added dropwise. The solution was left to stir for 19 hours, during which the system warmed to room temperature and the product, an orange precipitate, had formed. The precipitate was filtered and the filtrate concentrated to ~30 mL under reduced pressure, cooled to 0 °C, into which water (100 mL) was added to further precipitate the product. The precipitate was filtered, washed with water and dried under vacuum to afford diethyl 2-aminoazulene-1,3-dicarboxylate **15** (3.80 g, 81 %) as a bright orange powder without any further purification required.[2]

δ_H (500 MHz, $CDCl_3$) 9.16 (2H, d, *J* 10.2 Hz, H^4, H^8), 7.79 (2H, br. s, NH_2), 7.55 (2H, t, *J* 10.3 Hz, H^5, H^7), 7.47 – 7.41 (1H, m, H^6), 4.47 (4H, q, *J* 7.1 Hz, CH_2), 1.49 (6H, t, *J* 7.1 Hz, CH_3). δ_C (125 MHz, $CDCl_3$) 166.7 (C=O), 162.6 (C^2), 146.3 (C^{3a}, C^{8a}), 133.0 (C^6), 132.7 (C^5, C^7), 131.6 (C^4, C^8), 99.9 (C^1, C^3), 60.0 (CH_2), 14.8 (CH_3). HRMS (ESI+) *m/z* calcd for $(C_{16}H_{17}NO_4+Na)^+$, 310.1050; found 310.1063. Analytical data in agreement with those previously reported.[3]

Diethyl 2-amino-6-bromoazulene-1,3-dicarboxylate (9)

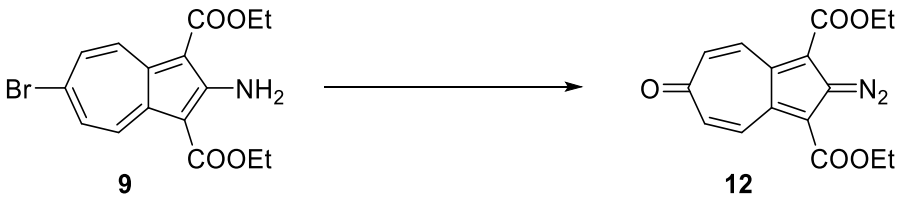


15 **9**

Diethyl 2-aminoazulene-1,3-dicarboxylate **15** (1.00 g, 3.48 mmol, 1.0 eqv) was dissolved in anhydrous CH_2Cl_2 (20 mL) and cooled to 0 °C, into which bromine (0.20 mL, 3.83 mmol, 1.1 eqv) was added over a 20 min period. The solution was warmed to room temperature and stir for 1.5 hours. The reaction was quenched with water (150 mL), separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 50 mL). The collected organic extracts were dried with $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was recrystallized from toluene/ hexane to give diethyl 2-amino-6-bromoazulene-1,3-dicarboxylate **9** as a dark brown crystalline solid (1.14 g, 89%).

δ_H (500 MHz, $CDCl_3$) 8.86 (2H, d, *J* 11.1 Hz, H^4, H^8), 7.82 (4H, m, H^5, H^7, NH_2), 4.46 (4H, q, *J* 7.1 Hz, CH_2), 1.47 (6H, t, *J* 7.1 Hz, CH_3). δ_C (125 MHz, $CDCl_3$) 166.4 (C=O), 162.5 (C^2), 144.5 (C^{3a}, C^{8a}), 135.5 (C^5, C^7), 129.7 (C^4, C^8), 128.6 (CBr), 101.2 (C^1, C^3), 60.3 (CH_2), 14.8 (CH_3). HRMS (ESI+) *m/z* calcd for $(C_{16}H_{16}BrNO_4+Na)^+$, 388.0155; found 388.0149. Analytical data in agreement with those previously reported.[4]

Diethyl 2-diazo-6-oxo-2,6-dihydroazulene-1,3-dicarboxylate (12)



Diethyl 2-amino-6-bromoazulene-1,3-dicarboxylate **9** (0.300 g, 0.822 mmol, 1.00 eqv) was dissolved in dioxane (7 mL) and conc. H_2SO_4 (1 mL) and cooled to 0 °C to form a yellow mixture. A solution of $NaNO_2$ (0.150 g, 2.200 mmol, 2.70 eqv) in water (1 mL) was added to the solution of **9** over a period of 3 hours. The mixture was warmed to room temperature and stirred for a further 2 hours, after which it had turned orange and water (20 mL) was added. The mixture was extracted CH_2Cl_2 (3 × 20 mL), and the collected organics were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was recrystalised from toluene/ petroleum ether to give **12** as an orange powder (46 mg, 18%).[5] δ_H (500 MHz, $CDCl_3$) 8.36 (2H, dt, *J* 12.9, 1.5 Hz, H^4, H^8), 6.68 (2H, dt, 12.9, 1.4 Hz, H^5, H^7), 4.46 (4H, q, *J* 7.1 Hz, CH_2), 1.44 (6H, t, *J* 7.1 Hz, CH_3). δ_C (125 MHz, $CDCl_3$) 189.8 (CH<u>C</u>(O)CH), 161.9 (<u>C</u>(O)OEt), 133.9 (C^5, C^7), 133.4 (C^{3a}, C^{8a}), 132.0 (C^4, C^8), 121.1 (C^1, C^3), 61.9 (CH_2), 14.3 (CH_3). HRMS (ESI+) *m/z* calcd for $(C_{16}H_{14}N_2O_5+Na)^+$, 337.0795; found 337.0799. Analytical data in agreement with those previously reported.[6]

Job Plot and Calibration Curve

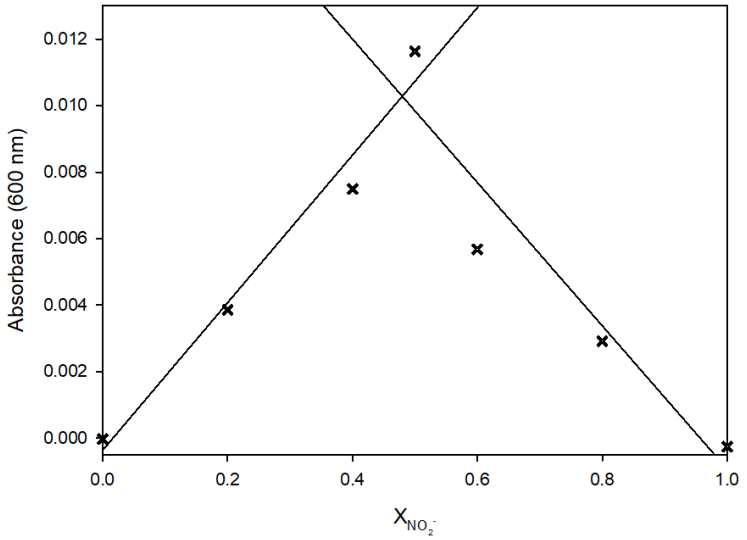


Figure S1. Absorbance of sensor **9** against $NaNO_2$ at 600 nm, reaching a maximum at 0.5 X_{NO^-}, suggesting a 1:1 ratio of sensor and analyte. The peak at λ=600 nm was used due to the requirement of a Job plot to start with an intensity of absorbance equal to 0.

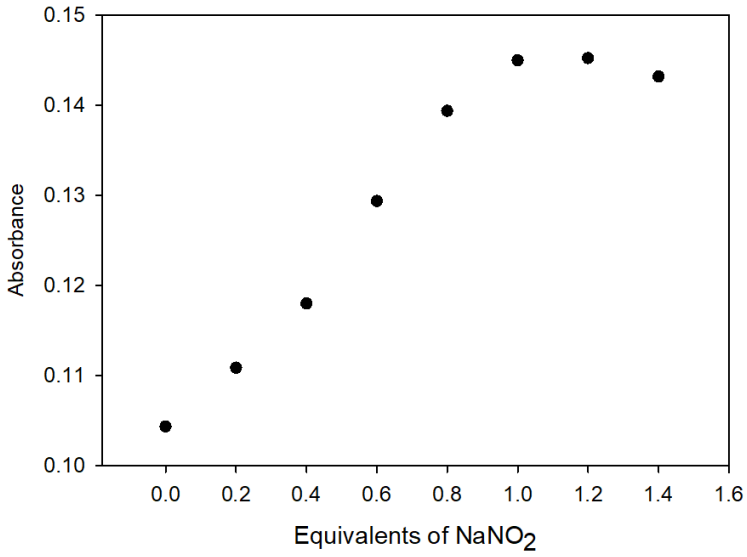


Figure S2. Calibration curve of sensor **9** (λ=395 nm) against increasing equivalents of $NaNO_2$.

pH Study

The phosphate buffer (pH 5.8), PBS buffer (pH 7.4) and acetate buffer (pH 3.6) were prepared at their given pHs. Each sample contained a solution as tabulated below and was left to react for 30 min before each UV/vis absorbance was measured.

	MeCN	DI Water	Buffer	Analyte	Sensor **9**
Volume/ µL	4950	1950	3000	50	50

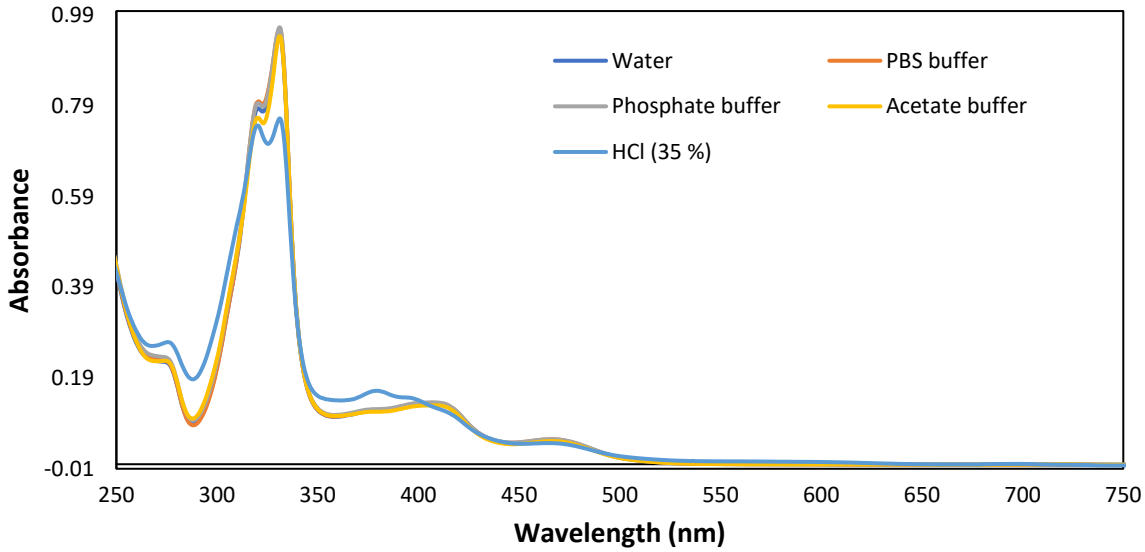


Figure S3. Comparison of absorbance of sensor **9** for nitrite at varying acidic pHs. Sensor **9** has a λ_{max} at 332 nm, whilst diazoquinone species **12** has a λ_{max} at 395 nm.

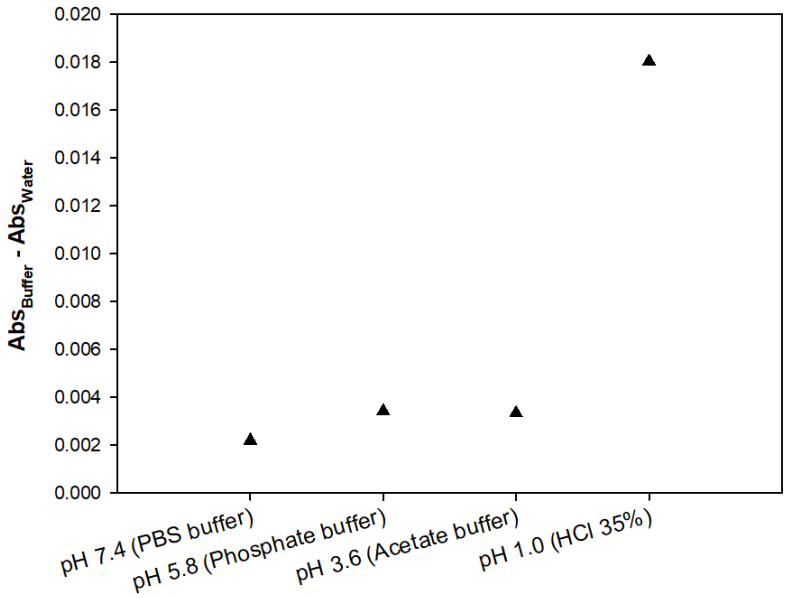


Figure S4. Relative change in absorbance of sensor **9** for nitrite at varying acidic pHs, measured at λ = 395 nm.

Kinetic Studies

High analyte concentration (1.0 equivalents $NaNO_2$)

	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	$NaNO_2$	Sensor **9**
Volume/ µL	4950	1950	3000	50	50

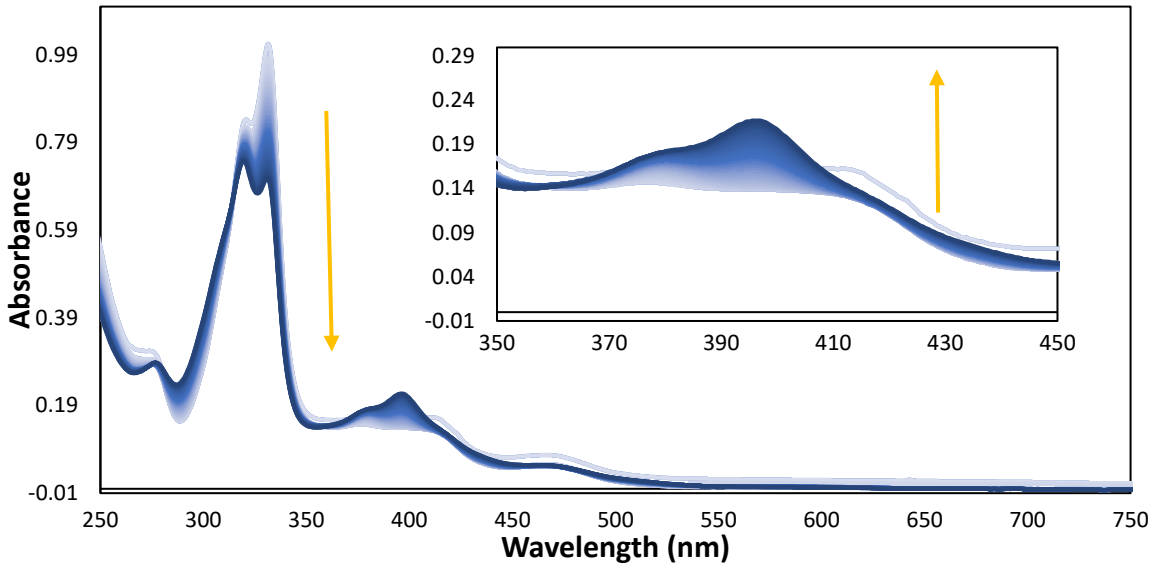


Figure S5. Change in absorbance of sensor **9** with a stoichiometric amount of $NaNO_2$ over the period of an hour.

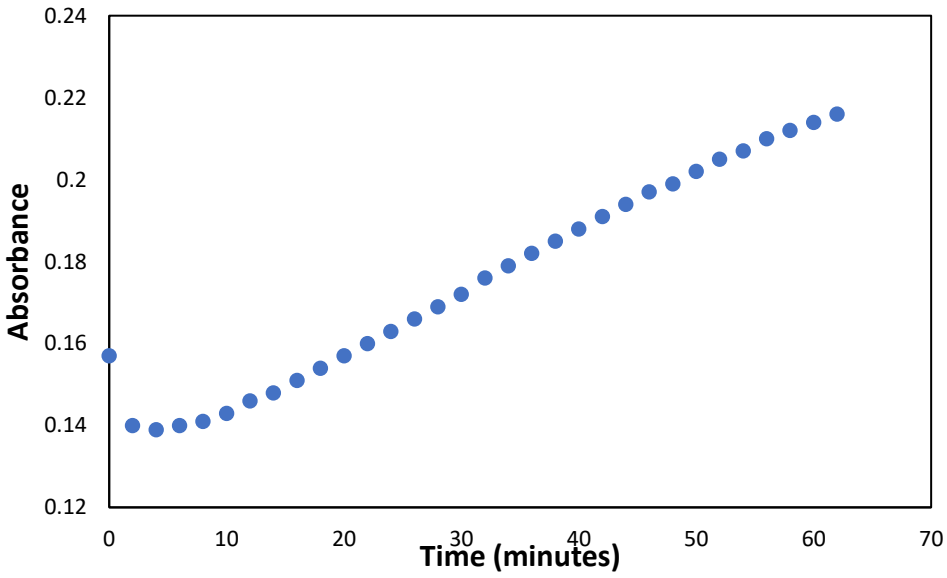


Figure S6. Change in absorbance of sensor **9** with a stoichiometric amount of $NaNO_2$, measured at λ = 395 nm, over the period of an hour.

$$Slope = \frac{Abs_2 - Abs_1}{t_2 - t_1} = \frac{0.197 - 0.151}{46 - 16} = 1.53\text{x}10^{-3}$$

Low analyte concentration (0.2 equivalents $NaNO_2$)

	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	$NaNO_2$	Sensor **9**
Volume/ µL	4950	1990	3000	10	50

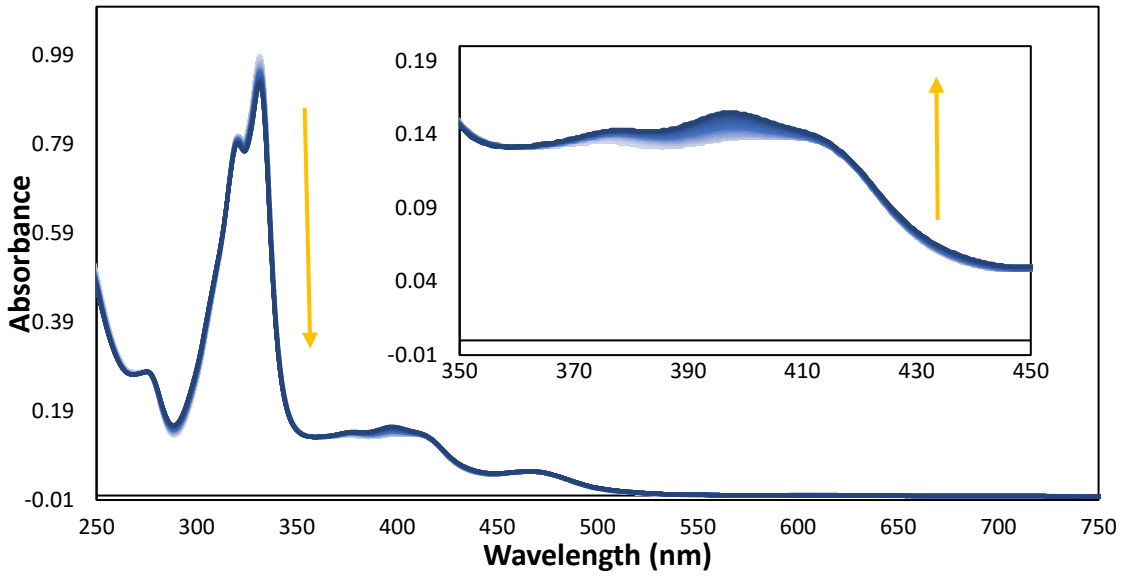


Figure S7. Change in absorbance of sensor **9** with 0.2 equivalents of $NaNO_2$ over the period of an hour.

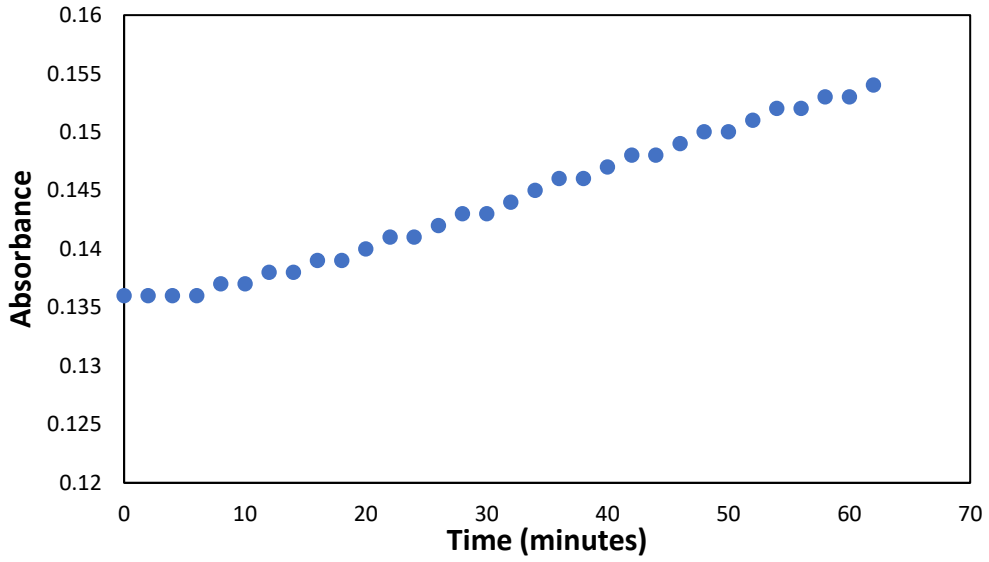


Figure S8. Change in absorbance of sensor **9** with 0.2 equivalents of $NaNO_2$, measured at λ = 395 nm, over the period of an hour.

$$Slope = \frac{Abs_2 - Abs_1}{t_2 - t_1} = \frac{0.149 - 0.139}{46 - 16} = 3.33\text{x}10^{-4}$$

Naked Eye Detection Limit Determination of Sensor 9

$NaNO_2$ solutions were generated in DI water, at concentrations of 800, 400, 200, 100, 50, 25, and 12.5 mg/L. Each sample contained a solution as tabulated below.

	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	$NaNO_2$	Sensor **9**
Volume/ μL	450	100	300	100	50

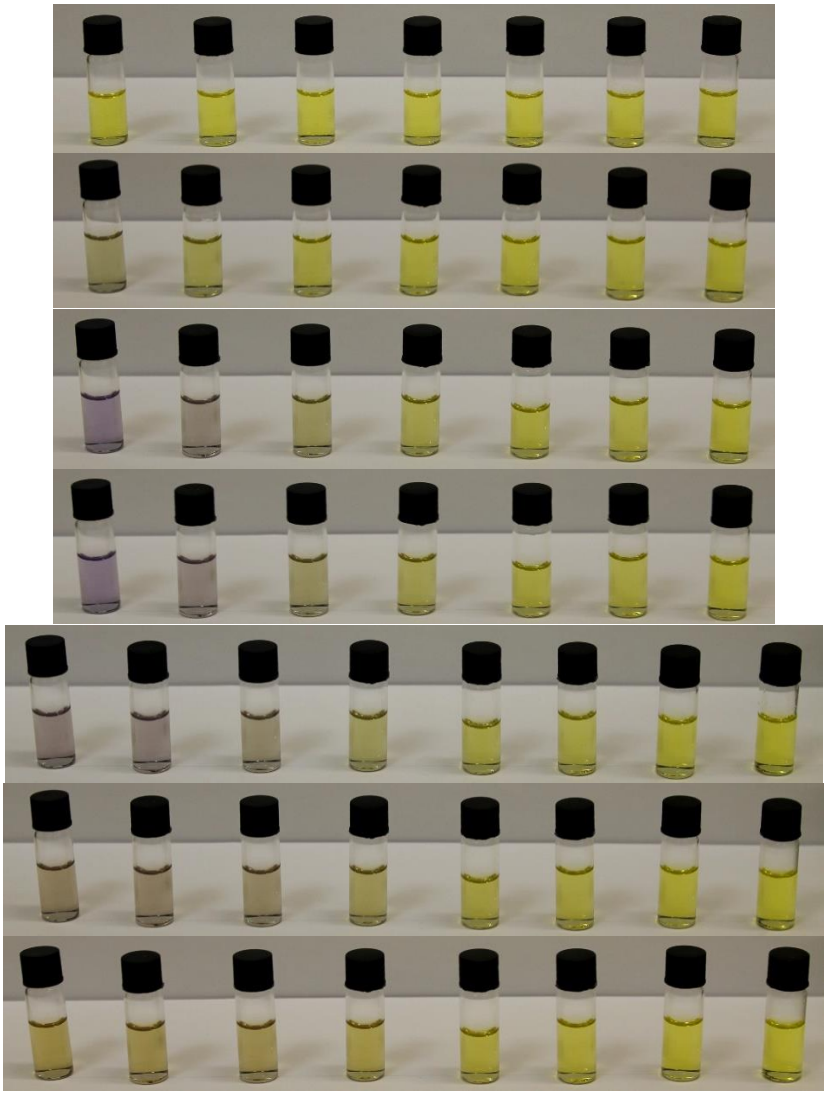


Figure S9. Naked eye detection limit determination of sensor **9**. From left to right, each vial contains a decreasing concentration of $NaNO_2$ in DI water as tabulated above. The top image was taken before nitrite addition. From top to bottom, the other images were taken at 0, 60, 120, 300, 600, and 1200 s after nitrite addition. The bottom three images contain a nitrite-free sample (on the right) for comparison.

Procedure for determining nitrite content in cured meat

The following procedures were based upon the British Standard Method of test: Meat and meat products – Part 8: Determination of nitrite content (BS 4401-8:1976).

Preparation of required solutions

Solution I: Disodium tetraborate decahydrate (5.0 g) was added to a 100 mL volumetric flask, diluted to the mark with DI water and mixed thoroughly to create a saturated solution.

Solution II: Potassium ferrocyanide trihydrate (10.6 g) was added to a 100 mL volumetric flask, diluted to the mark with DI water and mixed thoroughly.

Solution III: Zinc acetate dihydrate (22.0 g) and glacial acetic acid (30 mL) were added to a 100 mL volumetric flask, diluted to the mark with DI water and mixed thoroughly.

Solution IV: Sulphanilamide (0.20 g) was dissolved in DI water (80 mL) in a 100 mL volumetric flask with moderate heating. After cooling to room temperature, the solution was diluted to the mark with DI water and mixed thoroughly.

Solution V: Concentrated $HCl_{(aq)}$ (44.5 mL, 35% in water) was added to a 100 mL volumetric flask, diluted to the line with water and mixed thoroughly.

Solution VI *N*-(1-napthyl)ethylenediamine dihydrochloride was added to a 25 mL volumetric flask and diluted to the mark with DI water and mixed thoroughly.

Nitrite Extraction Procedure

Store-bought pepperoni (70 g) was diced and added to an Argos-brand XJ-10402 liquidiser, into which DI water (30 g) was added. The contents were blended over 10 minutes in 30 second intervals (avoiding heating the mixture) to afford a homogenised thick paste. The meat sample (15 g) was transferred to a round bottomed flask, into which solution I (5 mL) and DI water (100 mL at 75 °C) was added. The mixture was refluxed for 15 minutes and allowed to cool to room temperature, after which solution II (2 mL) and solution III (2 mL) were added and stirred for 5 minutes. The mixture was transferred to a 250 mL volumetric flask, diluted to the line, mixed thoroughly and left for 30 minutes to allow the proteins to precipitate. The solution was

decanted and filtered into a 250 mL volumetric flask and diluted to the mark to afford a clear solution.

UV/vis calculations for nitrite content

Calibration Curve of Azulene Chemodosimeter 9: Four standard solutions of sodium nitrite in DI water (0, 2.5, 5.0 and 10.0 mg/L) were generated, into which **9** was added (Table S1). After 20 minutes, the absorbance of each sample at 600 nm was measured and plotted against concentration of $NaNO_2$ (Figure S10). Using the same procedure, the nitrite extract from the meat was analysed, for which the measured absorbance was used to extrapolate the nitrite concentration from Figure S3. The test was repeated three times and the mean result was calculated, giving a nitrite concentration of 0.434 mg/L.

Nitrite Solution/ µL	MeCN/ µL	$HCl_{(aq)}$ (35% in water)/ µL	Sensor **9** (0.1 mM MeCN)/ µL
2000	4000	3000	1000

Table S1

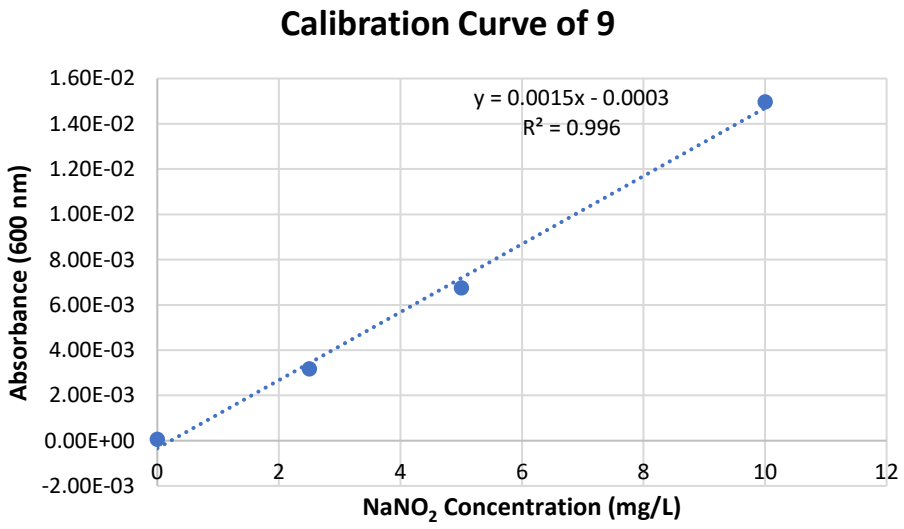


Figure S10. Calibration curve of sensor **9** for concentration of nitrate (mg/ L).

Calibration Curve for British Standard Test Procedure: Four standard solutions of sodium nitrite in DI water (0, 2.5, 5.0 and 10.0 mg/L) were generated, into which Solution IV and

Solution V were added (Table S2) and left for 5 minutes. Solution VI was then added, left for 10 minutes, and diluted to 100 mL. The absorbance of each sample was measure at 538 nm and plotted against concentration of $NaNO_2$ (Figure S11). Using the same procedure, the nitrite extract from the meat was analysed, for which the measured absorbance was used to extrapolate the nitrite concentration from Figure S4. The test was repeated three times and the mean result was calculated, giving a nitrite concentration of 0.452 mg/L.

Nitrite Solution/ mL	Solution IV/ mL	Solution V/ mL	Solution VI/ mL
10	10	6	2

Table S2

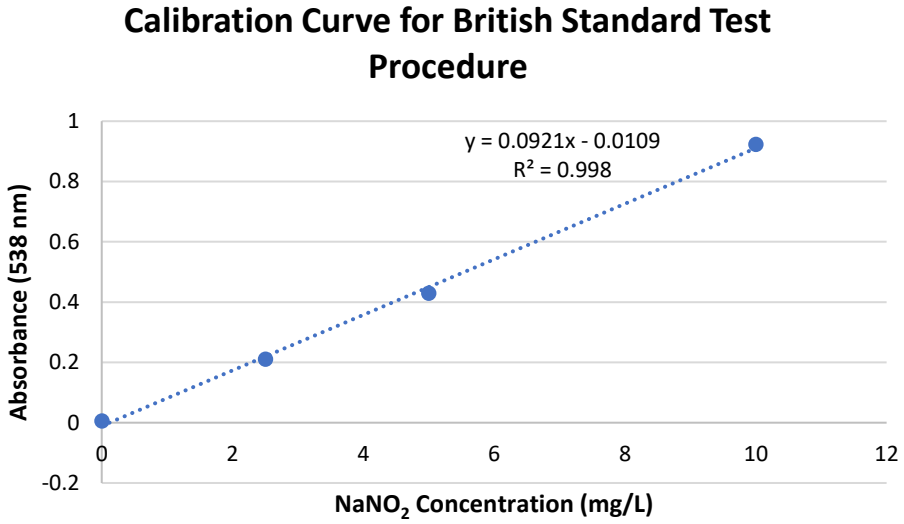


Figure S11. Calibration curve of the sensor used in British Standard 4401-8:1976 for concentration of nitrate (mg/ L).

Using Equation 1 and the concentrations obtained from Figure S10 and Figure S11, the total nitrite concentration of the assessed pepperoni was calculated, giving a total nitrite concentration of 10.3 mg/kg for sensor **9** and 10.8 mg/kg for the British Standard procedure.

$$N = \frac{Vx}{m} = \frac{250x}{10.5} \qquad \textbf{(1)}$$

Where N= amount of nitrite in meat sample (in mg/kg), V= total volume of water nitrite extract was dissolved in (in litres), x= nitrite concentration determined from calibration curved (in mg/L), and m=total mass of meat sample measured.*

*15 g of homogenised sample of which 30% was added water, 70% original sample.

General Sample Preparation

Probe **9** and DQ **12** were diluted in acetonitrile to obtain 5 mM solutions. The tested analyte(s) were diluted in DI water to obtain 5 mM solutions (except for the detection limit study). Spectroscopic data were acquired 20 minutes after addition of analyte.

Table for Figure 2: Comparison of Sensor 9, Sensor 9 + $NaNO_2$, and Diazoquinone 12 (Volume/ µL)

	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	$NaNO_2$	Sensor **9**	DQ
DQ (**12**) (Black Line)	4950	2000	3000	0	0	50
S (**9**) (Red Line)	4950	2000	3000	0	50	0
S+$NaNO_2$ (Green Line)	4950	1950	3000	50	50	0

Table for Figure 3: Titration of $NaNO_2$ and Sensor 9 (Volume/ µL)

	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	$NaNO_2$	Sensor **9**
Solution	4950	2000	3000	10	50

Table for Figures 4 and 5: Naked Eye Selectivity Test of Sensor 9 (Volume/ µL)

	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	Analyte	Sensor **9**
Solution	400	100	300	100	100

Table for Figure 6: UV/Vis Selectivity Test of Sensor 9 (Volume/ µL)

	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	Analyte	Sensor **9**
Solution	4950	1950	3000	50	50

Table for Figure S1: Job plot (Volume/ µL)

$NaNO_2$ (%)	MeCN	DI Water	$HCl_{(aq)}$ (35% in water)	NaNO2	Sensor **9**
0	4900	2000	3000	0	100
20	4920	1980	3000	20	80
40	4940	1960	3000	40	60
50	4950	1950	3000	50	50
60	4960	1940	3000	60	40
80	4980	1920	3000	80	20
100	5000	1900	3000	100	0

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