BI002

The kinetics and mechanisms of *Brassica olereacea*–derived cyanidin-3-glucoside in inhibiting deoxyribonucleic acid oxidation by reactive oxygen species

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Abstract

Extraction of both *Allium cepa* deoxyribonucleic acid and purple *Brassica olereacea*-derived cyanidin-3-glucoside anthocyanin were carried out through solvent extraction and filtration. Assay 1 investigated the kinetics and energetics of the reaction between cyanidin-3-glucoside and hydrogen peroxide (H_2O_2) oxidant, a reactive oxygen species precursor, through an illuminance analysis with changes in concentration and temperature variables. Reaction rates were calculated through a change in 535 nm wavelength absorbance, measured by a do-it-yourself and Vernier spectrophotometer. Do-it-yourself spectrophotometer was constructed with iPhone lux sensor, flashlight and 535 nm filter. Using linearized Arrhenius and Eyring equations, thermodynamic and kinetic properties were calculated. Values such as activation energy, pre-exponential factor, rate orders, and rate laws were derived through Arrhenius plots. Rate determining step of Gibbs free energy change, change in enthalpy and change in entropy were determined through Eyring analysis. Assay 2 investigated the efficacy of cyanidin-3-glucoside as an oxidative inhibitor between the oxidant-antioxidant interactions of reactive oxygen species and deoxyribonucleic acid. Methods to determine such behaviour were carried out through reaction quenching of reactive oxygen species, cyanidin-3-glucoside and deoxyribonucleic acid, with the use of cold ethanol. Changes in concentration and temperature variables in assay 2 allowed for possible correlation investigations between the 2 assays. The aim of this study was to determine the efficacy of cyanidin-3-glucoside as an antioxidant or oxidation inhibiting species by investigating correlations between kinetics of cyanidin-3-glucoside interactions and role of cyanidin-3-glucoside in extent of inhibiting oxidative deoxyribonucleic acid damage.

Report

1. Research Objectives:

To determine the activation energy, reaction order, pre-exponential factor, rate constant, rate determining step properties including change of Gibbs free energy, enthalpy and entropy through reaction of hydrogen peroxide and cyanidin-3-glucoside in Purple *Brassica oleracea var. capitata f. rubra* by using illuminance analysis of 535 nm wavelength absorbance in measuring reaction rate and Arrhenius plot and Eyring analysis and to investigate the efficacy of cyanidin-3-glucoside as a deoxyribonucleic acid oxidation inhibitor.

2. Research Background

Anthocyanins and associated flavonoids have been presented with the ability to protect humans from a myriad of diseases.[1] However, they have been extremely difficult to study with regards to human health. Anthocyanins frequently interact with other phytochemicals to potentiate biological effects, and undergo complex reactions within the human biological system such as the neutralization of reactive oxygen species within the body.[2] Hence, its individual contributions are difficult to analyse.[3] Most notably, however, are its interactions with reactive oxygen species. Reactive oxygen species (ROS) are a group of molecules produced in human cells through the metabolism of oxygen. Though serving as cell signalling molecules for normal biological processes, elevated levels of different ROS can lead to molecular damage, commonly known as oxidative stress.[4] Anthocyanins in their original forms can transfer electrons to ROS and hence, provide protection to important oxidizable molecules such as deoxyribonucleic acid (DNA).[5] Hence, a shift in the oxidant – antioxidant balance in the body could have gradual health complications with effects on signalling pathways, impairment of immune systems and an increased likelihood of developing malignancies by means of direct cell and DNA damage.[6] As such, this research aims to investigate anthocyanin's efficacy in mitigating oxidative stress.

3. Hypothesis

Assay 1: An increase in both temperature and reactant concentration will result in an increase in the rate of reaction. Endothermic reactions towards transition states are expected with anticipated mechanisms of bimolecular rate determining steps.

Assay 2: An increase in hydrogen peroxide concentration or a decrease in anthocyanin concentration will result in more extensive DNA oxidation. An increase in temperature will result in more extensive DNA oxidation due to degradation of both anthocyanin and DNA.

4. Methods:

4.1. Extraction Procedure:

1. 150 g of red cabbage was chopped
2. Red cabbage was added to 5 cm^3 of 0.1 mol dm^{-3} hydrochloric acid and 495 cm^3 of water in a 1000 ml beaker for 10 minutes.
3. Solution was heated at 50°C for 10 minutes, until the solution turned purple in colour.
4. Mixture was left for 20-30 minutes to cool down to room temperature.
5. Mixture was filtered into a 500 ml conical flask.

4.2. Assay 1: Kinetic study of antioxidant behaviour

4.2.1. Change in concentration (with DIY spectrophotometer): Methods

1. 3 ml of anthocyanin stock solution was added into cuvette, and placed into the spectrophotometer to record absorbance at λ_{max} to determine concentration
2. 100ml of the following solutions were prepared, via dilution

   X, 2X concentration of hydrogen peroxide (X≈0.44 mol dm^{-3}); Y, 2Y, 3Y concentration of anthocyanin (Y≈5.39033 x 10^{-6} mol dm^{-3})
3. Volumetric flasks containing the solutions were placed in a water bath at 25°C
4. Each solution mixture were mixed (20ml for hydrogen peroxide, 40ml for anthocyanin) in the 100ml beaker.

   Solution X and Y; Solution 2X and Y; Solution X and 2Y; Solution X and 3Y
5. Beaker was placed in the set-up immediately after mixing and recording started with the Arduino Science Journal. (Figure 1) This was repeated three times.

4.2.2 Change in concentration: Results

The Arduino Science Journal was used to graph the variations of the intensity of light at a wavelength of 535nm, providing 2 sets of data, *Relative_time* and *Brightness/EV*:

Relative _time	Brightness /EV	Time/s	Illuminance /lux	Control illuminance /lux	Transmittance	Absorbance	Calibrated Absorbance	Concentration/mol dm^{-3}
A	B	$t = \frac{A}{1000}$	$I = 2.5 \times 2\text{^}B$	$c = 17.480205$	$T = I/c$	$A = -\lg T$	$A_c = kA$	$C = \frac{A_c}{\varepsilon l}$

$\varepsilon = 26900\, mol^{-1}\, dm^{-3}\, cm^{-1},\ l = 4cm$. *$k$ is an arbitrary value that ensures that A_c will be equal for all data sets of the same concentration.*[7]

The initial rate of reaction is determined by taking the gradient of the secant between the first 6 and the subsequent 6 readings:

$$\frac{dC}{dt}|_{t=0} = \frac{\frac{\sum Concentration\ (7\ \text{to}\ 12)}{6} - \frac{\sum Concentration\ (1\ \text{to}\ 6)}{6}}{t_{midpoint\ from\ (7\ to\ 12)} - t_{midpoint\ from\ (1\ to\ 6)}}$$

The mean initial rates of reaction for varying concentrations of reactants are tabulated below:

C3G Concentration/ mol dm^{-3}	H_2O_2 Concentration/ % (w/v)	Mean Instantaneous Initial Rate of Change of Absorption	Mean Instantaneous Initial Rate of Reaction/10^{-7} mol dm^{-3} s^{-1}
$3.5936*10^{-6}$	0.5	0.0030827	1.146
$3.5936*10^{-6}$	1.0	0.0062435	2.321
$7.4349*10^{-6}$	1.0	0.010467	3.891
$1.4870*10^{-5}$	1.0	0.024482	9.101

*2:1 mixture ratio changes the concentration of the reactants in a solution mixture.

4.2.3. Change in temperature (with Vernier spectrophotometer): Methods

1. 20 ml each of 2X and 4Y solution were placed in differing water baths of 8°C, 25°C, 45°C and 58°C.
2. 1 ml of 2X solution and 2 ml of 4Y solution were mixed in a cuvette.
3. The cuvette was immediately put into the spectrophotometer with an absorption analysis at 535 nm.
4. The experiment was repeated 5 times and an absorption over time graph was plotted.

4.2.4. Change in temperature: Results

The Vernier Spectrophotometer was used to plot variations in the reaction solution's absorption of light at 535nm. The instantaneous initial rate for each run was calculated through the process of power regression, followed by differentiation. Power graphs have an asymptote at t=0, as such, the initial rate was estimated by taking the derivative at $t \approx 0$.

Temperature /K	Mean Instantaneous Initial Rate of Change of Absorption	Mean Initial Instantaneous Rate of Reaction/10^{-7} mol dm^{-3} s^{-1}
281.15	0.026669	9.9140
298.15	0.10479	38.955
318.15	0.40710	151.67
331.15	0.949044	352.80

4.3. Assay 2: Efficacy of DNA oxidation inhibition by antioxidant

4.3.1. Extraction of DNA: Methods

1. Mix 10 ml of detergent, 1.5 g of sodium chloride and water to a final volume of 100 ml.
2. Roughly chop one large *Allium cepa* in a 1000 ml beaker and pour the solution into it.
3. Place the 1000 ml beaker in a 60°C water bath, squeezing the mixture lightly.
4. Cool the mixture in an ice bath for 5 minutes, continually squeezing the mixture lightly.
5. Filter the mixture and dispense the filtrate into test tubes, not exceeding 1/3 of the tubes.
6. Cold ethanol was added to the bottom of the test tube to a level of 1 cm.
7. After 2 minutes, white precipitated DNA can be spooled out with a hook.

4.3.2. Change in concentration: Methods

1. 50 ml each of solution X, 2X, Y, 2Y and 4Y were prepared in a conical flask.
2. Solution mixtures were mixed in the following manner (2 ml of hydrogen peroxide, 4 ml of anthocyanin and 1 ml of DNA) in a measuring cylinder:
   Solution X, Y and DNA; X, 2Y and DNA; X, 4Y, DNA; 2X, Y and DNA
3. After 1 minute of mixing, pour cold ethanol to form a 1 cm layer.
4. After 2-3 minutes, spool out the white precipitated DNA strands, dry it, and place it into a measuring cylinder with a fixed volume of water. Calculate the displaced volume of water and record the final volume of DNA precipitated from the reaction mixture.

4.3.3. Change in concentration: Results

Cyanidin-3-glucoside concentration / mol dm^{-3}	H_2O_2 Concentration / % (w/v)	Initial volume of DNA / ml	Final mean volume of DNA / ml	Change in mean volume of DNA / ml	Percentage change in mean volume of DNA / %
3.5936×10^{-6}	0.5	1.0	0.4	-0.6	-60%
3.5936×10^{-6}	1.0	1.0	0.2	-0.8	-80%
7.4349×10^{-6}	1.0	1.0	0.6	-0.4	-40%
1.4870×10^{-5}	1.0	1.0	0.9	-0.1	-10%

4.3.2. Change in temperature: Methods

1. 20 ml each of 2X, 4Y and 5 ml of DNA solution were placed in differing water baths of 8°C, 25°C, 45°C and 58°C.
2. Mix 2 ml of hydrogen peroxide, 4 ml of anthocyanin and 1 ml of DNA of different temperatures in a measuring cylinder.

3. After 1 minute of mixing, pour cold ethanol to form a 1 cm layer.
4. After 2-3 minutes, spool out the white precipitated DNA strands, dry it, and place it into a measuring cylinder with a fixed volume of water. Calculate the displaced volume of water and record the final volume of DNA precipitated from the reaction mixture.

4.3.3. Change in temperature: Results

Temperature / K	Initial volume of DNA / ml	Final mean volume of DNA / ml	Change in mean volume of DNA / ml	Percentage change in volume of DNA / %
281.15	1.0	0.2	-0.8	-80%
298.15	1.0	0.3	-0.7	-70%
318.15	1.0	0.8	-0.2	-20%
331.15	1.0	0.6	-0.4	-40%

5. Analysis:

5.1. Deriving Rate Equation:

Rate= $k[C3G]^m[H_2O_2]^n$

5.1.1. Order of reaction with respect to C3G:

$$\left(\frac{7.4349\times10^{-6}}{1.4870*10^{-5}}\right)^m = \left(\frac{3.891\times10^{-7}}{9.101\times10^{-7}}\right),\ 0.49999^m = 0.42754\ \therefore m \approx 1$$

5.1.2. Order of reaction with respect to H_2O_2:

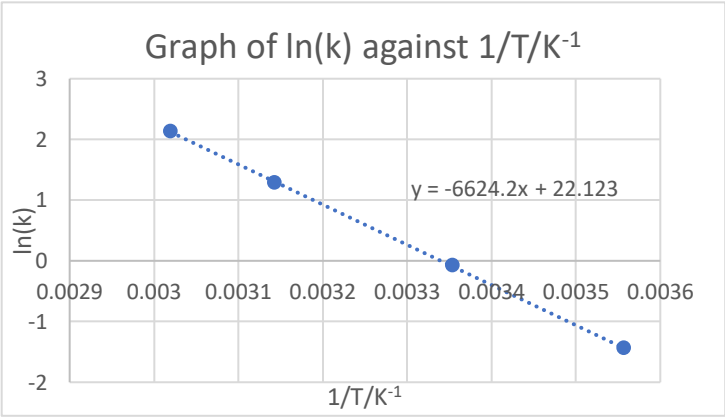
$$\left(\frac{0.5\%}{1.0\%}\right)^n = \left(\frac{1.146\times10^{-7}}{2.321\times10^{-7}}\right),\ 0.5^n = 0.49375\ \therefore n \approx 1$$

∴ Rate = $k[C3G][H_2O_2]$

5.2. Calculating Activation Energy and Pre-exponential Factor using an Arrhenius Plot:

Linearized Arrhenius equation:

$$\ln k = \frac{-E_a}{R}\left(\frac{1}{T}\right) + \ln A$$



Equation: -6642.2x + 22.123

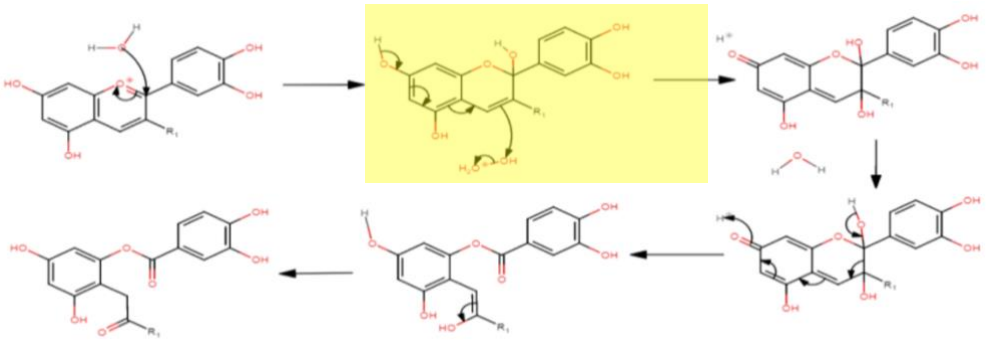
Slope: $\frac{-E_a}{R} = -6624.2$

$\boldsymbol{E_a = -55.074\ kJ\ mol^{-1}}$

Y-intercept: $\ln A = 22.123$

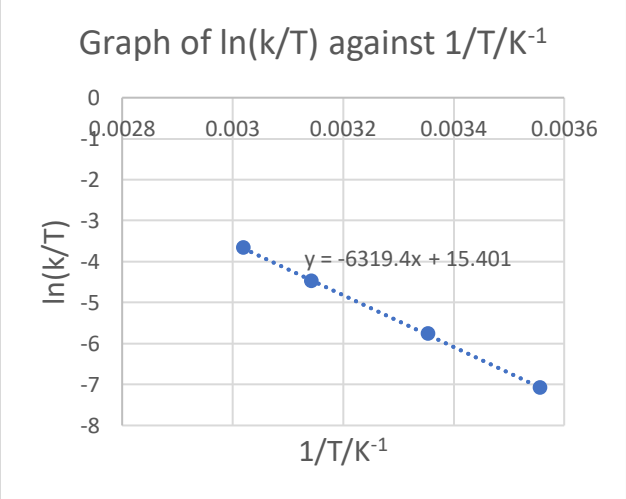
$\boldsymbol{A = 4.0541 \times 10^9}$

5.3. Determining $\Delta G^{\ddagger}, \Delta S^{\ddagger}, \Delta H^{\ddagger}$ using Eyring Analysis



The bimolecular rate determining step which was determined from calculations, is highlighted. An Eyring Analysis can be employed to find the ΔH, ΔS, ΔG of the rate determining step (RDS). Linearized Eyring equation:

$$\ln\frac{k}{T} = \frac{-\Delta H^{\ddagger}}{R}\cdot\left(\frac{1}{T}\right) + ln\frac{k_B}{h} + \frac{\Delta S^{\ddagger}}{R}$$



Equation: y = -6319.4x + 15.401

Slope: $\frac{-\Delta H^{\ddagger}}{R} = -6319.4$

$\Delta \boldsymbol{H^{\ddagger}} = \boldsymbol{+52.539\ kJ\ mol^{-1}}$

Y-intercept: $ln\frac{k_B}{h} + \frac{\Delta S^{\ddagger}}{R} = 15.401$

$\Delta \boldsymbol{S^{\ddagger}} = \boldsymbol{-69.500 J\ K^{-1}\ mol^{-1}}$

$\Delta G = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$

$\Delta \boldsymbol{G^{\ddagger}} = \boldsymbol{52.539 + 69.500T}$

6. Discussion:

Hydroxyl and Peroxyl radicals are formed from H_2O_2, because of the homolytic cleavage of O-O bonds or O-H bonds present in the H_2O_2 molecule[10]. These highly reactive free radicals readily bind to and oxidise C3G molecules, hence preventing the formation of the coloured flavylium cation. This results in the decolourization of the anthocyanin solution, and a decrease in the solution's absorption of light at 535nm. In assay 1, as temperature increases linearly, the initial rate of reaction increases exponentially in agreeance with the Maxwell-Boltzmann distribution

curve, where the number of particles with kinetic energy greater or equal to the E_a doubles for every 10K increase in temperature. At elevated temperatures, reacting particles have more kinetic energy and move at greater speeds, resulting in an increase in the frequency of successful collisions and hence, increased reaction rate. With reference to the derived rate law, the initial rate of reaction is directly proportional to the concentration of either reactant. At higher concentration, there will be more reacting particles per unit volume, increasing the frequency of successful collisions between reacting particles. In assay 2, an increase in anthocyanin concentration resulted in a lesser change in mean DNA volume. As such, a correlation between the kinetics of anthocyanins quenching reactive oxygen species and a less extensive oxidative stress on DNA is present. Furthermore, although the presence of Iron(II) ions in the human body catalyzes oxidative stress on DNA by ROS, it is notable that quenching of ROS by anthocyanins are catalysed by the same molecule too. As such, in vitro research suggests a notable efficacy of oxidative stress protection by cyanidin-3-glucoside derived from purple *Brassica oleareacea*.

7. Conclusion

In conclusion, when temperature increases linearly from 281.15K to 331.15K, the rate of reaction increases exponentially from $9.9140*10^{-7}$ mol dm^{-3} s^{-1} to $352.80*10^{-7}$ mol dm^{-3} s^{-1}. As the concentration of H_2O_2 increases from 0.5 to 1.0 % (w/v), the mean instantaneous initial rate of reaction increases from $1.146*10^{-7}$ to $2.321*10^{-7}$ mol dm^{-3} s^{-1}. As the concentration of C3G increases from $3.5936*10^{-6}$ to $1.4870*10^{-5}$ mol dm^{-3}, the instantaneous initial rate of reaction increases from $2.321*10^{-7}$ to $9.101*10^{-7}$ mol dm^{-3} s^{-1}. As inferred from the results of assay 1, anthocyanins can react with and neutralise hydroxy radicals with increasing efficacy as the concentration of the anthocyanin increases. The presence of anthocyanins can minimize the damage done to DNA by hydroxy radicals, as seen from assay 2, possibly by reacting preferentially over the DNA. The sensitivity of anthocyanins to temperature is highlighted in both assays, whereby an increase in temperature up to 331.15 K generally results in an increase in the protective mechanism of the anthocyanin. Hence, it can also be concluded that anthocyanins are effective to a significant extent in vitro in neutralising hydroxy radicals and minimizing the free radical oxidation of DNA molecules.

From further calculations, the rate equation was derived to be Rate = k[C3G][H_2O_2]. The following values were also determined: $E_a = -55.074\ \text{kJ mol}^{-1}$, $A = 4.0541 \times 10^9$, $\Delta H^{\ddagger} = +52.539\ \text{kJ mol}^{-1}$, $\Delta S^{\ddagger} = -69.500\ \text{J K}^{-1}\ \text{mol}^{-1}$, $\Delta G^{\ddagger} = 52.539 + 69.500T$.

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2. Ibid

3. Ibid

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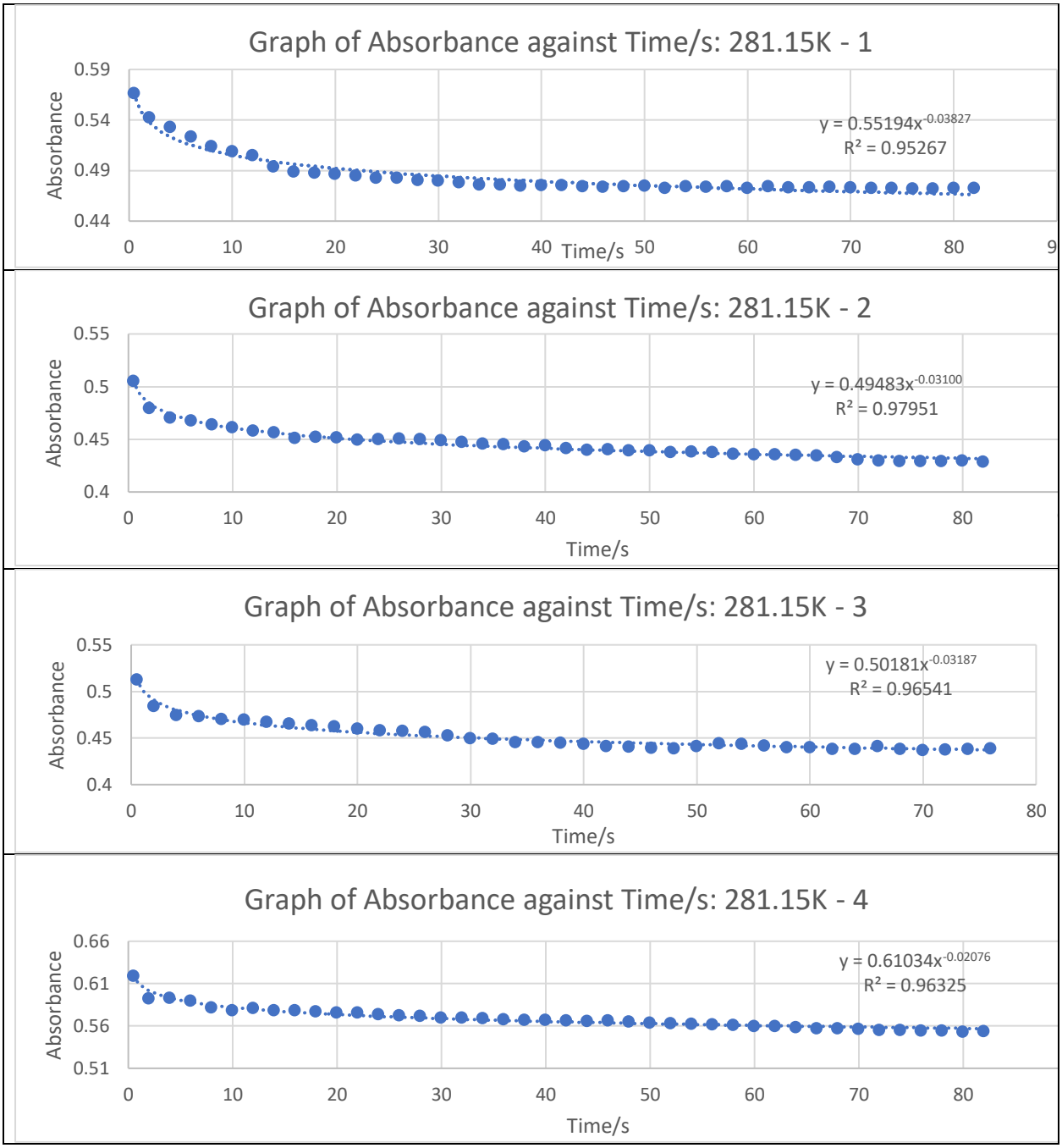
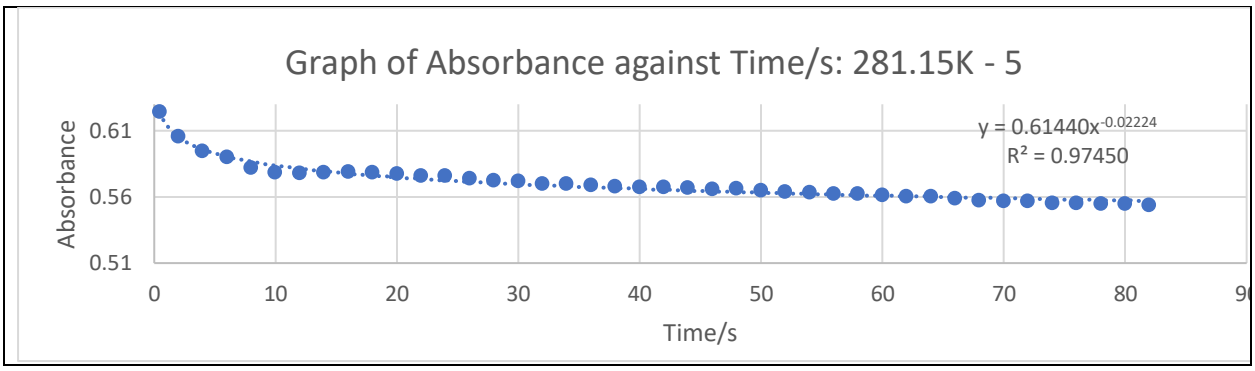
Appendix

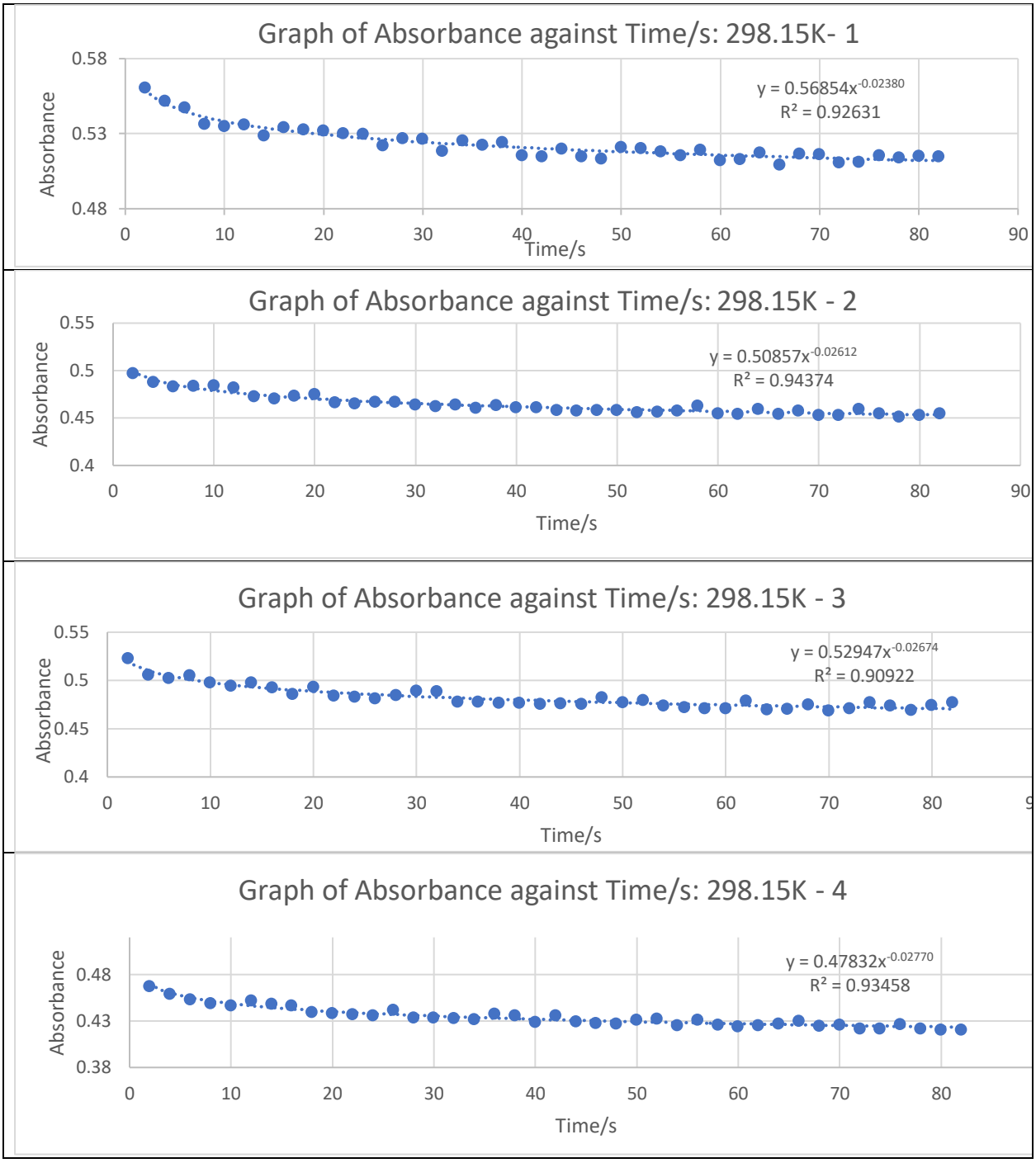
Assay 1: Change in temperature

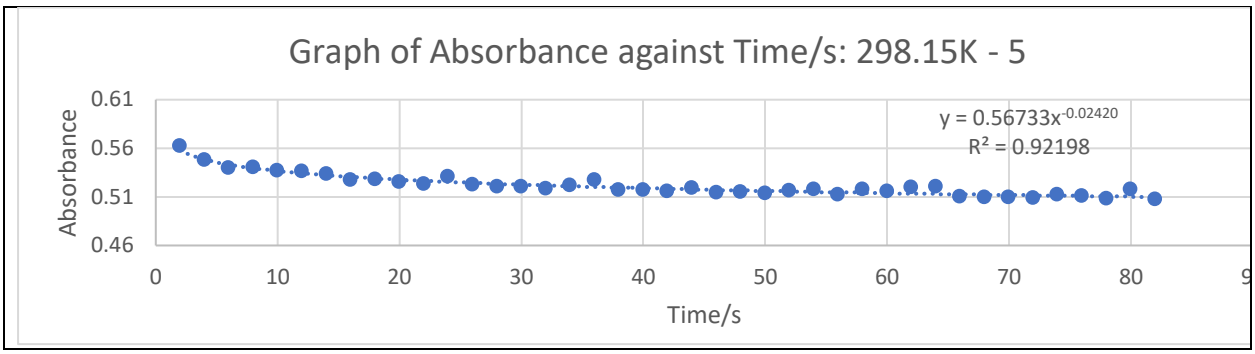
1.a. Graphs of Absorbance Against Time/s - 281.15K



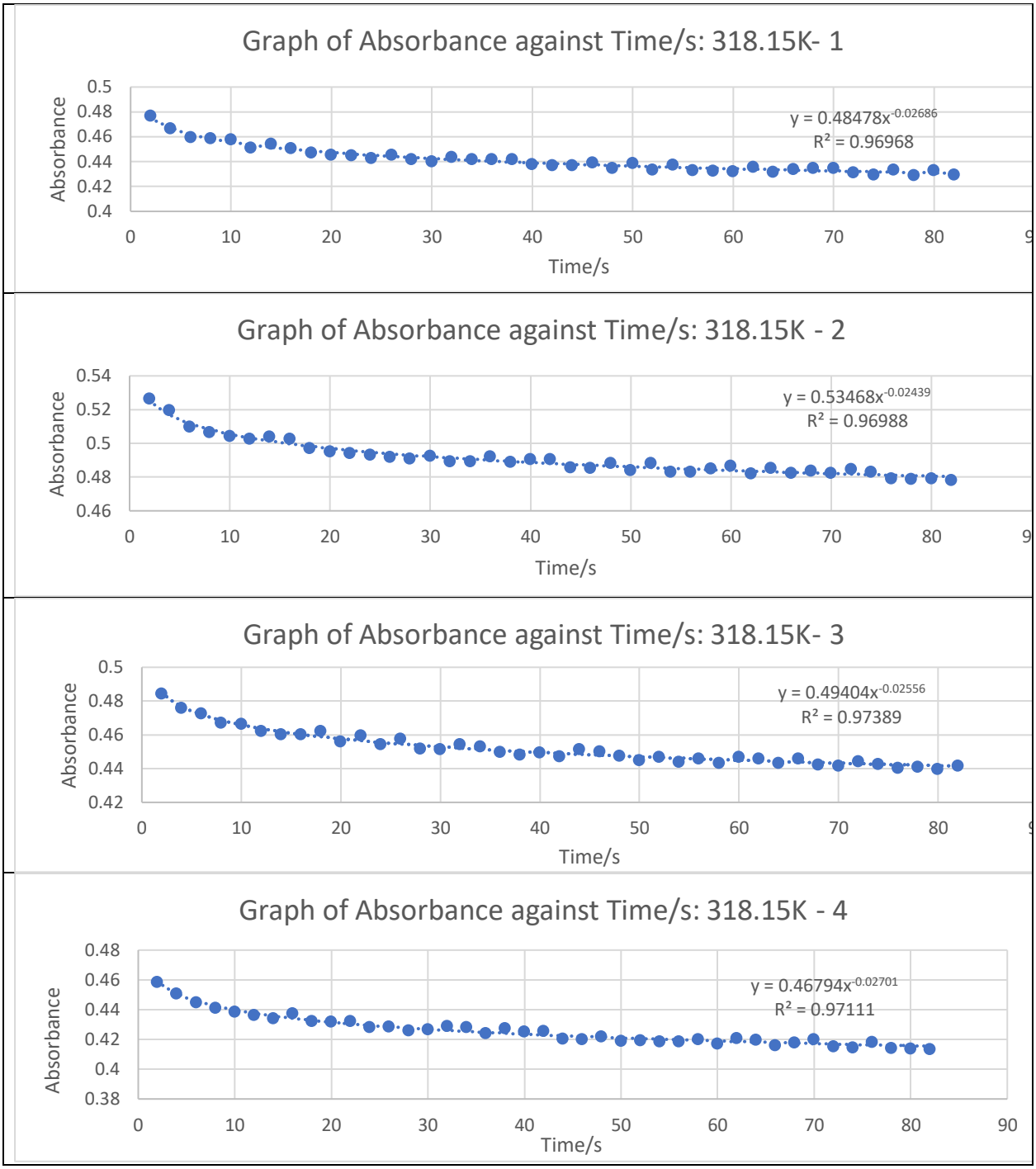

Graph of Absorbance against Time/s: 281.15K - 5
Absorbance
0.61
0.56
0.51
0
10
20
30
40
50
60
70
80
Time/s
y = 0.61440x^-0.02224
R² = 0.97450

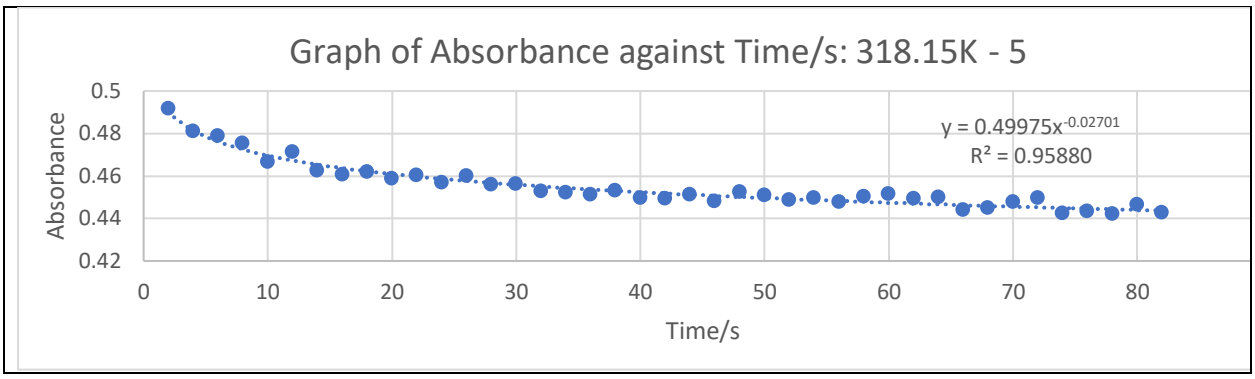
1.b. Graphs of Absorbance Against Time/s - 298.15K



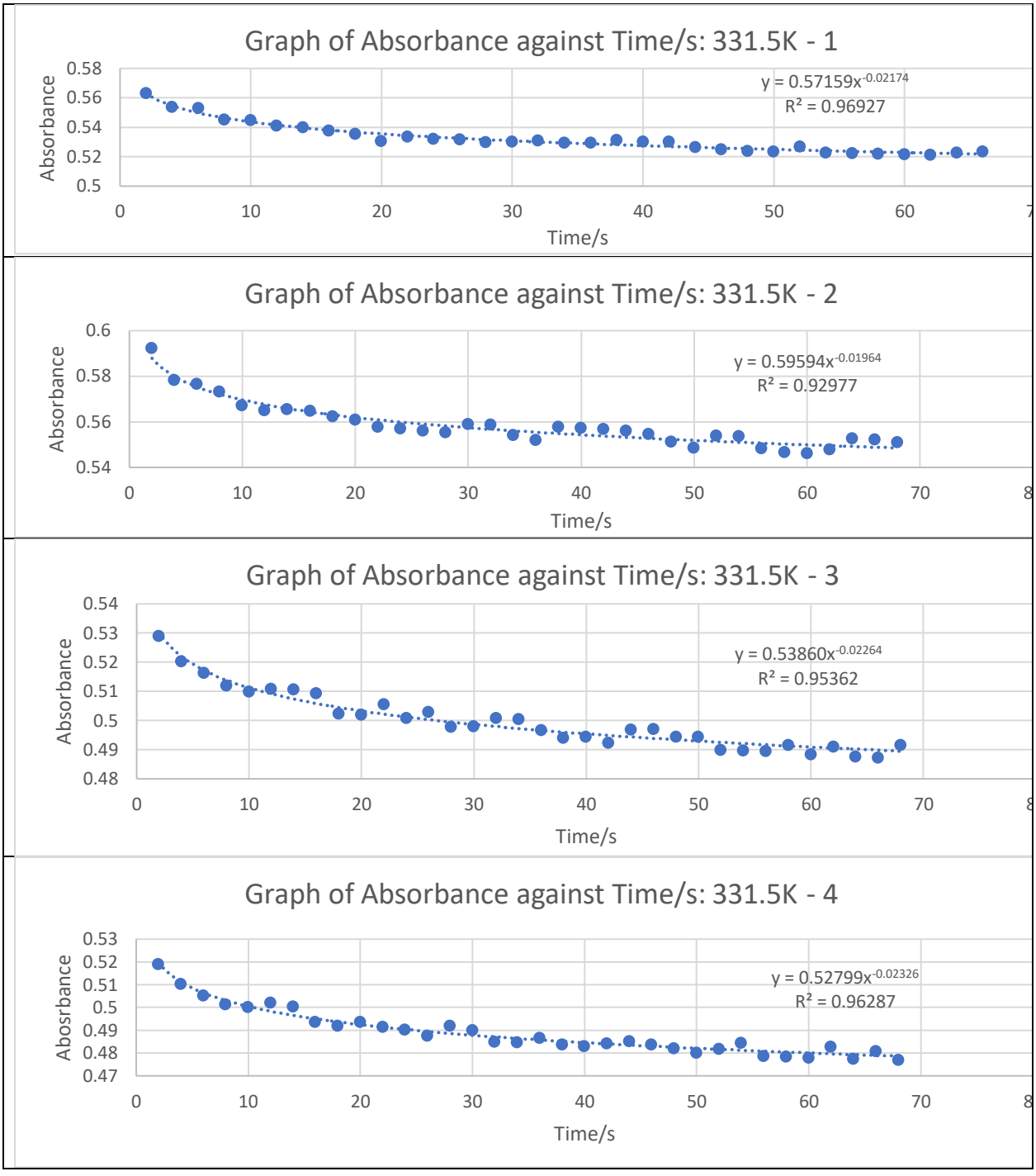

Graph of Absorbance against Time/s: 298.15K - 5
Absorbance
0.61
0.56
0.51
0.46
0
10
20
30
40
50
60
70
80
Time/s
y = 0.56733x-0.02420
R² = 0.92198

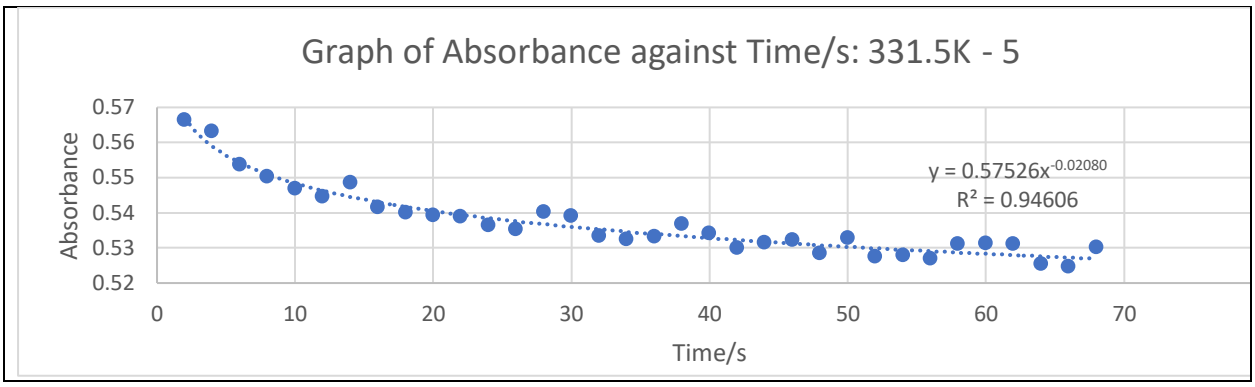
1.c. Graph of Absorbance Against Time/s - 318.15K




Graph of Absorbance against Time/s: 318.15K - 5
Absorbance
0.5
0.48
0.46
0.44
0.42
0
10
20
30
40
50
60
70
80
Time/s
y = 0.49975x-0.02701
R² = 0.95880

1.d. Graph of Absorbance Against Time/s - 331.15K




Graph of Absorbance against Time/s: 331.5K - 5
Absorbance
0.57
0.56
0.55
0.54
0.53
0.52
0
10
20
30
40
50
60
70
Time/s
y = 0.57526x-0.02080
R² = 0.94606

1.e. Table of Instantaneous Initial Rates Against Temperature

Temperature /K	Graph no.	Instantaneous Initial Rate of Change of Absorption	Instantaneous Initial Rate of Reaction/10^{-7} mol dm^{-3} s^{-1}	Mean Instantaneous Initial Rate of Change of Absorption/10^{-7}	Mean Instantaneous Initial Rate of Reaction/10^{-7} mol dm^{-3} s^{-1}	Standard Deviation of Initial Rate of Reaction/10^{-7}
281.15	1	-0.0359	-13.3455	-9.91398	-0.026668617	2.0983
	2	-0.02597	-9.65585			
	3	-0.02709	-10.0714			
	4	-0.02134	-7.93432			
	5	-0.02303	-8.56284			
298.15	1	-0.10394	-38.6394	-38.9554	-0.10479	1.06081
	2	-0.10251	-38.1078			
	3	-0.10939	-40.6654			
	4	-0.10257	-38.1301			
	5	-0.10554	-39.2342			
318.15	1	-0.4107	-152.677	-151.672	-0.407998	4.33956
	2	-0.40792	-151.643			
	3	-0.39655	-147.416			
	4	-0.39885	-148.271			
	5	-0.42597	-158.353			
331.15	1	-0.97391	-362.048	-3521804	-0.949044	10.0661
	2	-0.90913	-337.967			
	3	-0.95937	-356.643			
	4	-0.96878	-360.141			
	5	-0.93403	-347.223			

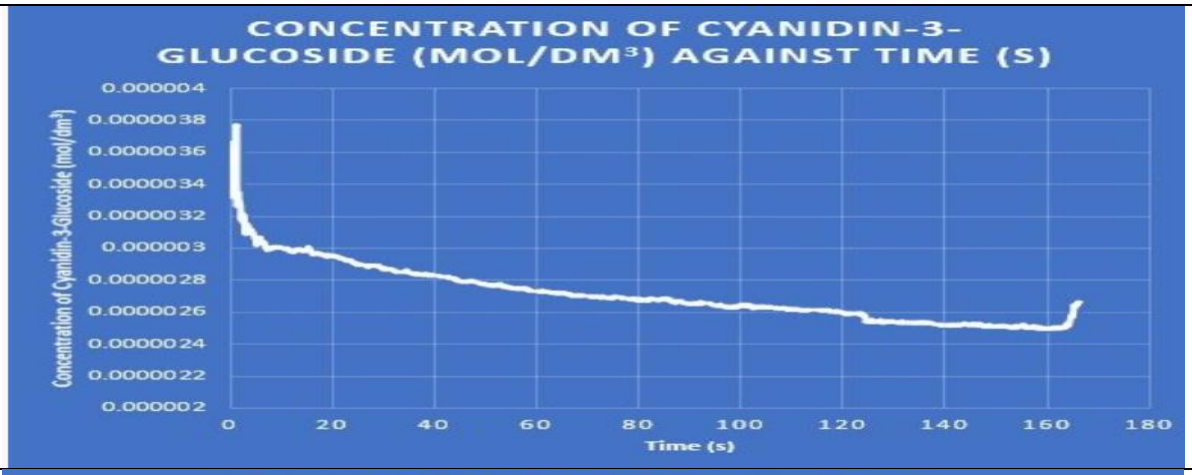
Assay 1: Change in concentration

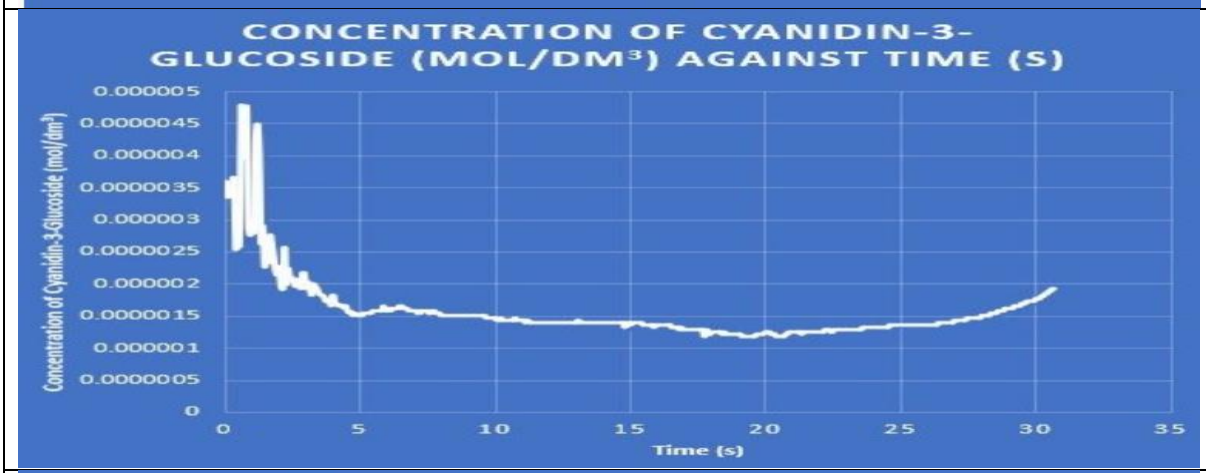
2.a. Arduino Science Journal Processed Data

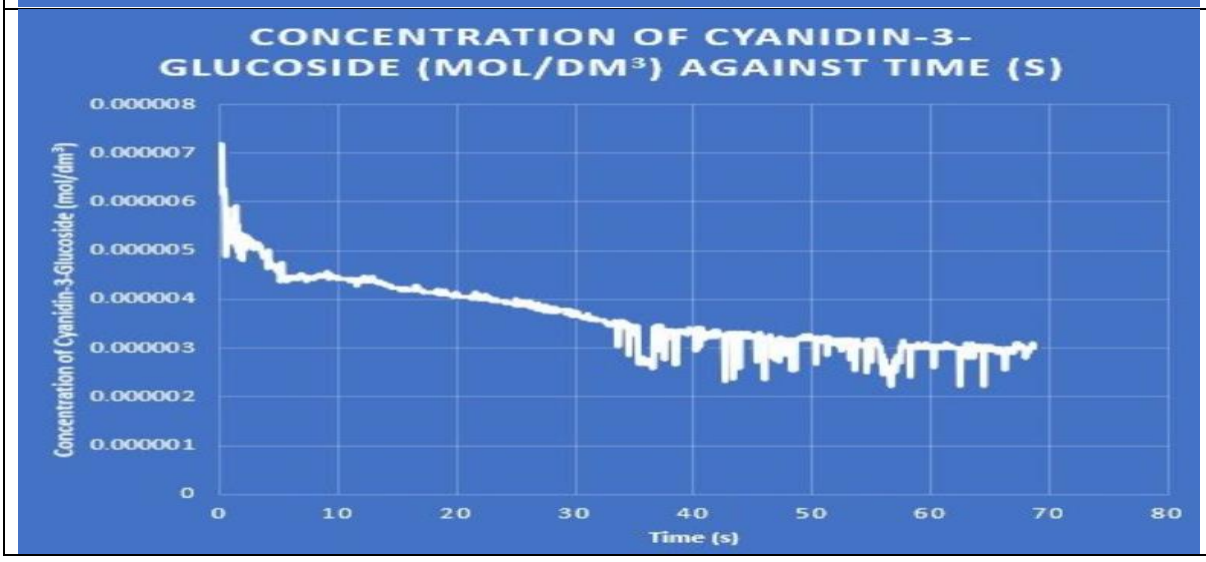
Section Number	C3G Concentration/ mol dm^{-3}	H_2O_2 Concentration/% (w/v)
2.b	$3.5936*10^{-6}$	0.5
2.c	$3.5936*10^{-6}$	1.0
2.d	$7.4349*10^{-6}$	1.0
2.e	$1.4870*10^{-5}$	1.0

2.b.

C3G Concentration/ mol dm^{-3}	H_2O_2 Concentration/% (w/v)
$3.5936*10^{-6}$	0.5

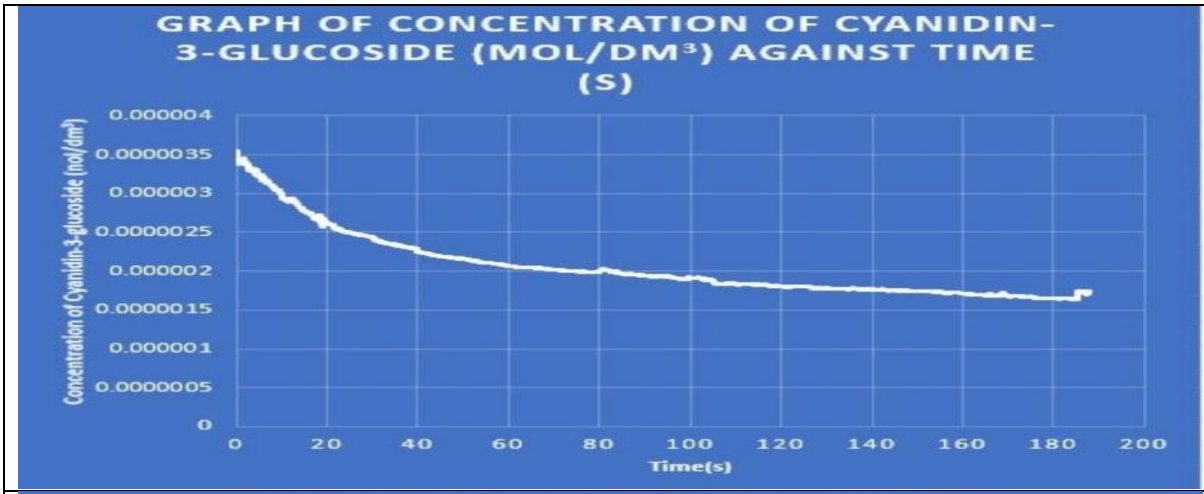


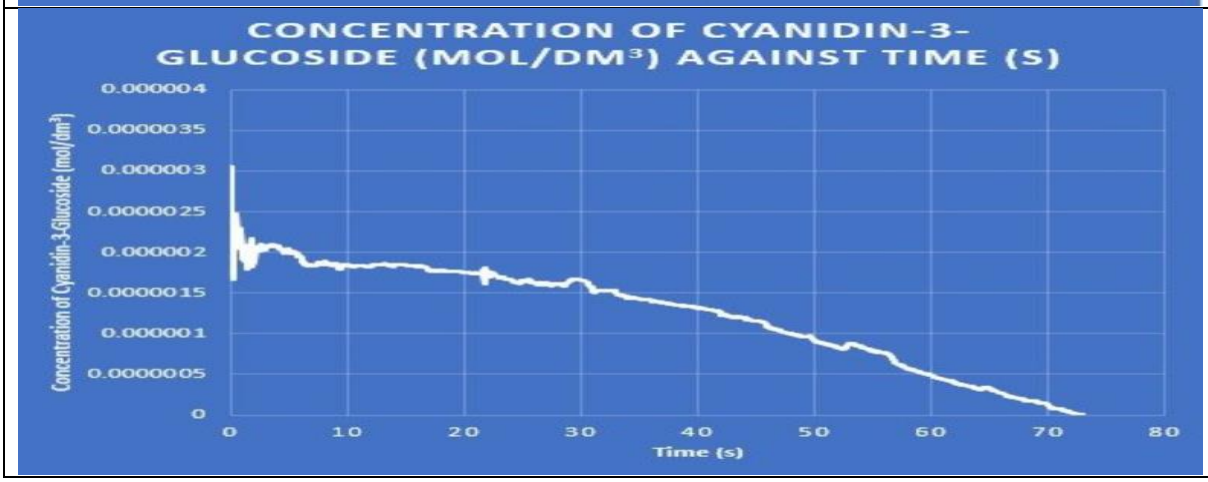


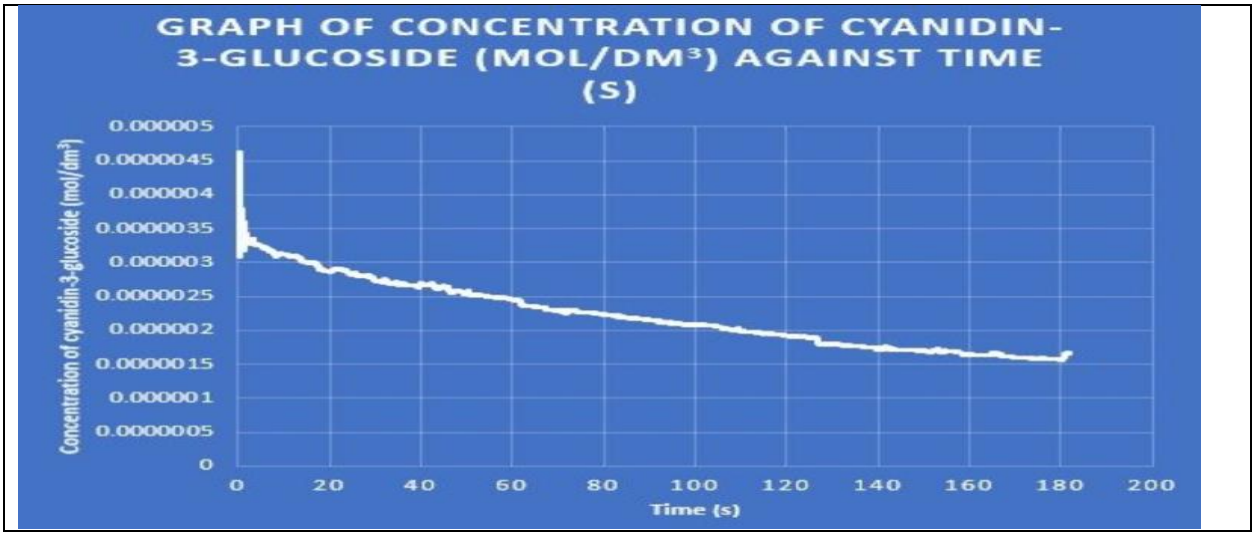


2.c.

C3G Concentration/ mol dm^{-3}	H_2O_2 Concentration/% (w/v)
$3.5936*10^{-6}$	1.0

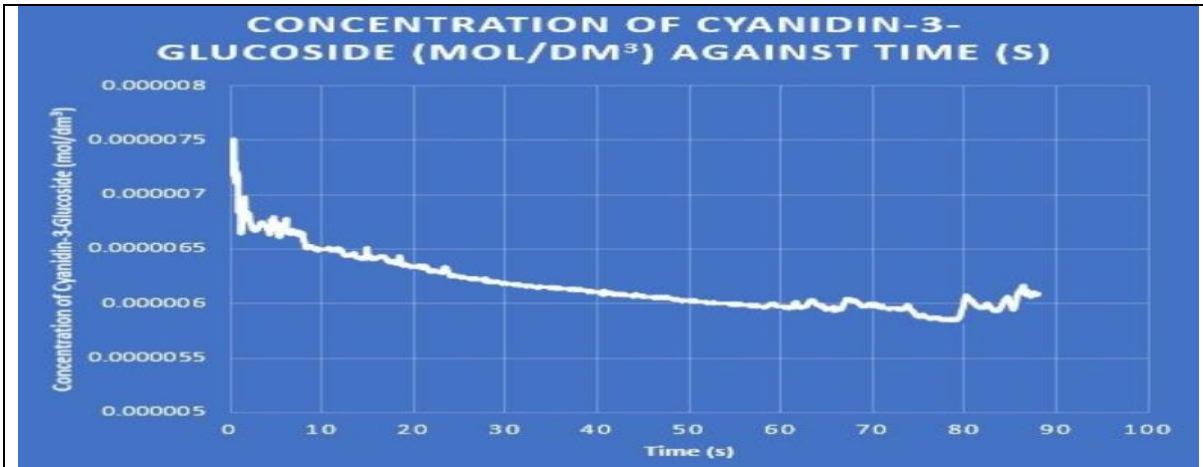


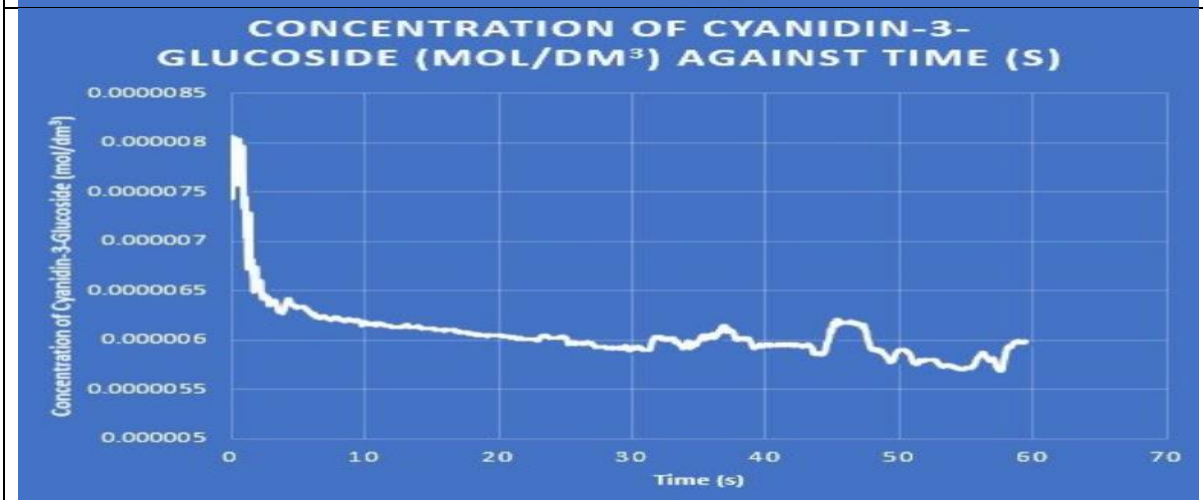


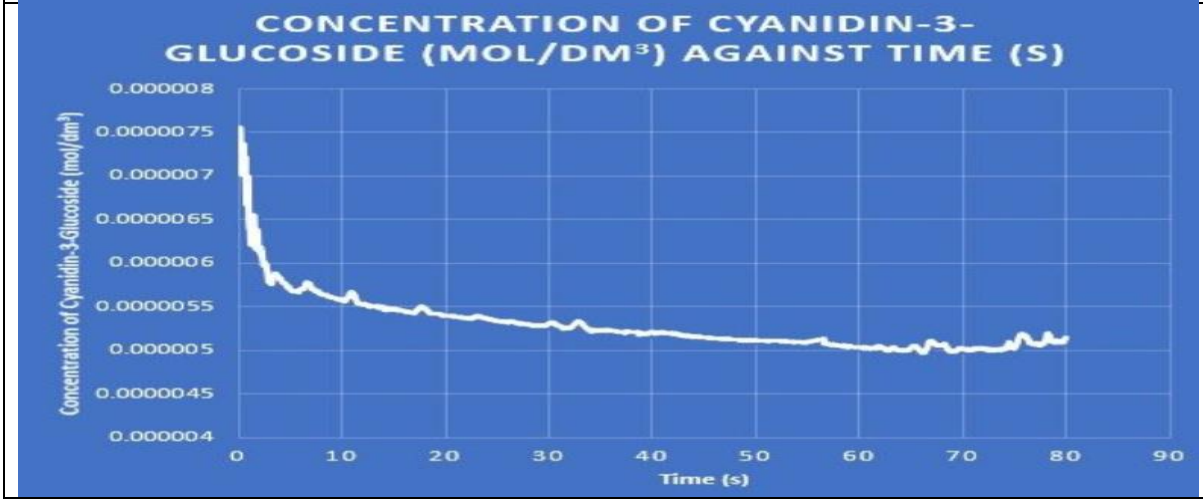

GRAPH OF CONCENTRATION OF CYANIDIN-3-GLUCOSIDE (MOL/DM³) AGAINST TIME (S)
Concentration of cyanidin-3-glucoside (mol/dm³)
0.000005
0.0000045
0.000004
0.0000035
0.000003
0.0000025
0.000002
0.0000015
0.000001
0.0000005
0
0
20
40
60
80
100
120
140
160
180
200
Time (s)

2.d.

C3G Concentration/ mol dm^{-3}	H_2O_2 Concentration/% (w/v)
$7.4349*10^{-6}$	1.0

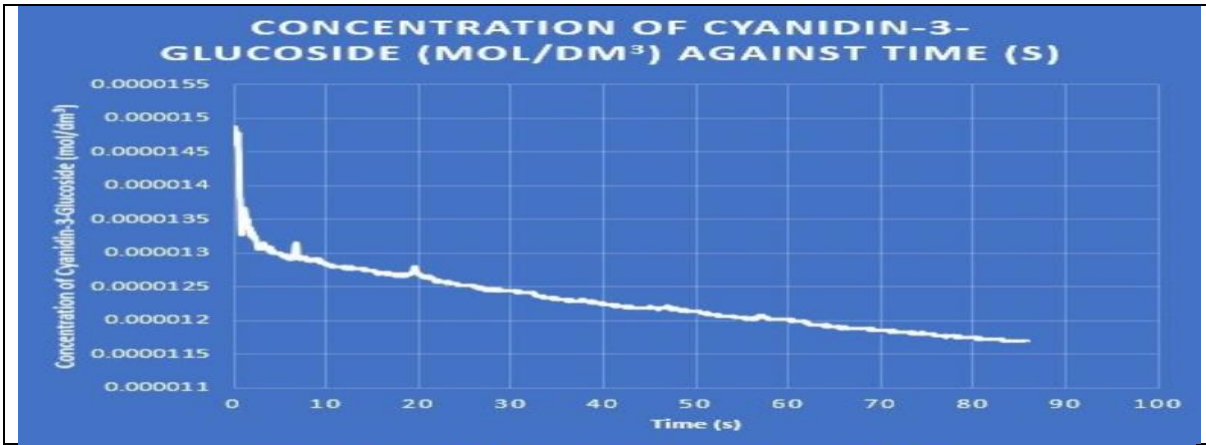


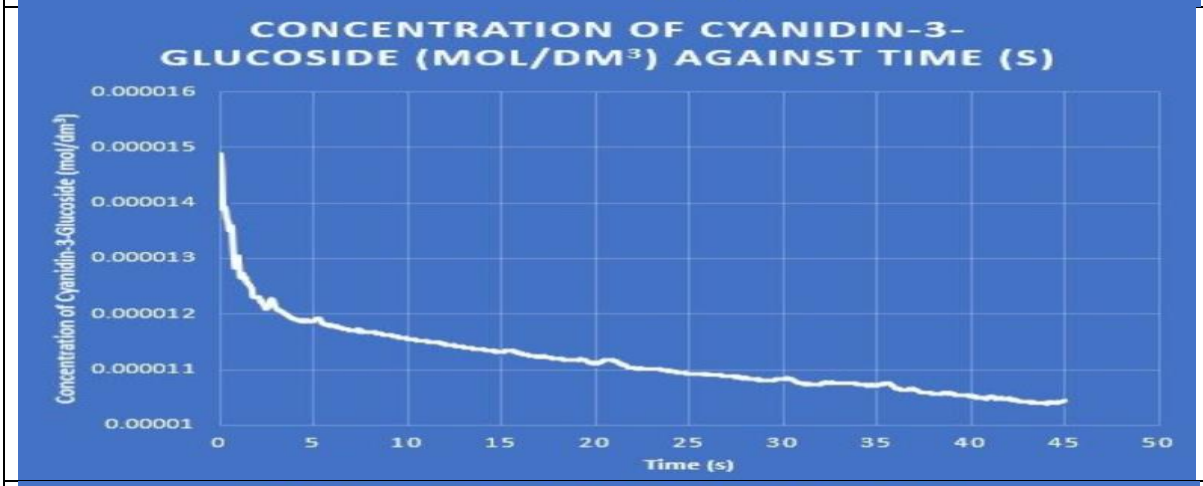


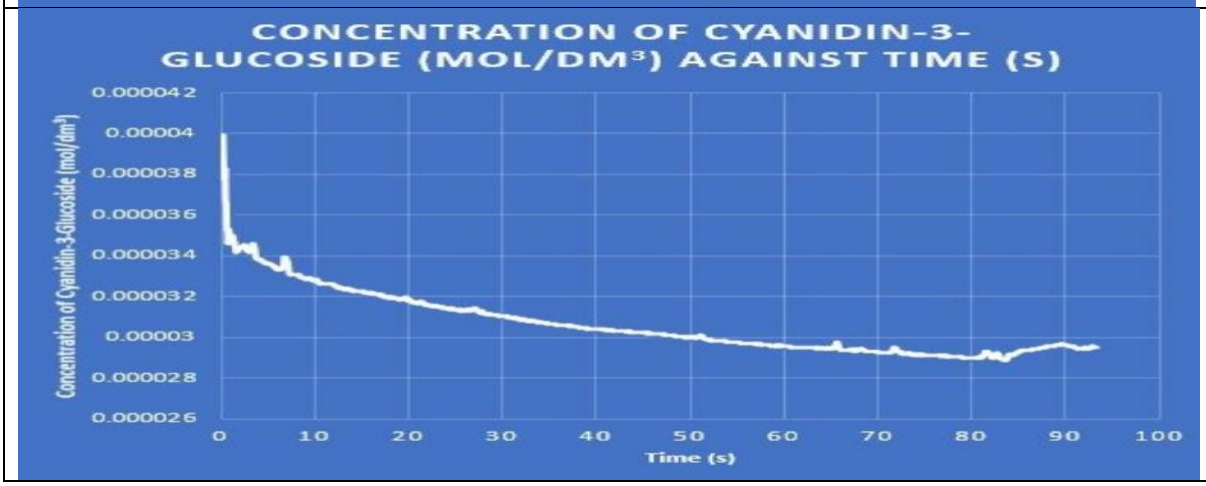


2.e.

C3G Concentration/ mol dm^{-3}	H_2O_2 Concentration/% (w/v)
$1.4870*10^{-5}$	1.0







3. Assay 2 Results

3.a. Change in concentration: Results

Cyanidin-3-glucoside concentration / mol dm^{-3}	H_2O_2 Concentration / % (w/v)	Initial volume of DNA / ml	Try 1	Try 2	Try 3	Try 4	Try 5	Final mean volume of DNA / ml	Change in mean volume of DNA / ml	Percentage change in mean volume of DNA / %
3.5936×10^{-6}	0.5	1.0	0.3	0.4	0.2	0.4	0.7	0.4	-0.6	-60%
3.5936×10^{-6}	1.0	1.0	0.1	0.1	0.4	0.2	0.3	0.2	-0.8	-80%
7.4349×10^{-6}	1.0	1.0	0.5	0.4	0.6	0.7	0.6	0.6	-0.4	-40%
1.4870×10^{-5}	1.0	1.0	0.9	0.8	0.9	0.9	0.8	0.9	-0.1	-10%

3.b. Change in temperature: Results

Temperature / K	Initial mean volume of DNA / ml	Try 1	Try 2	Try 3	Try 4	Try 5	Final volume of DNA / ml	Change in mean volume of DNA / ml	Percentage change in volume of DNA / %
281.15	1.0	0.2	0.2	0.4	0.3	0.1	0.2	-0.8	-80%
298.15	1.0	0.3	0.4	0.3	0.1	0.4	0.3	-0.7	-70%
318.15	1.0	0.6	0.8	0.8	0.7	0.9	0.8	-0.2	-20%
331.15	1.0	0.7	0.5	0.6	0.7	0.6	0.6	-0.4	-40%

4. Table of Constants used

Name	Symbol	Value	Units
Molar Absorption Coefficient (C3G)	ε	26900	$mol^{-1}\ dm^{-3}\ cm^{-1}$
Molar Gas Constant	R	8.31454598	$m^2\ kg\ s^{-2}\ K^{-1}\ mol^{-1}$
Plank Constant	h	$6.6261*10^{-34}$	$m^2\ kg\ s^{-1}$
Boltzmann Constant	k_B	$1.3806*10^{-23}$	$J\ K^{-1}$