Quantitative Determination of Glucose using a Coupled Enzymatic Assay Lab Report

The objective of this experiment is to determine the composition of an unknown solution of glucose by means of a coupled enzymatic assay. We will be using a set of known solutions of glucose to create a calibration curve and from there determine the unknown concentration of glucose.

In order to accomplish these goals, a working understanding of glucose oxidase, enzymatic assays, and calibration curves are required. Several key experimental design structures require sufficient understanding, such as: the wrapping of the microcentrifuge tubes, the statistical relevance of the number of samples as well as performing every sample twice, the utility of running the blank again, and the importance of adding the 2 M HCL.

We must be sure to wrap all of our microcentrifuge tubes thoroughly, such that no light interacts with our reaction mixtures. The explanation for this is two fold. First, o-Dianisidine is reactive with long term exposure to light, and more importantly glucose oxidase is inactivated by light.

It is very important that we have a sufficient number of samples. This of course is important for the precision of our data analysis. By having 8 samples we get 6 degrees of freedom for our statistical analysis. With fewer degrees of freedom, say 3 or 4, drawing conclusions from our data would prove dubious at best because random error could give us good data despite clear experimental flaws or vice versa.

There are several good reasons to run every sample twice. The first and most obvious is we measure more data, that is we get more information and the ability to choose one of two datasets to analyze. This could counteract some systematic errors on one of the data sets, but also certainly reduces random error. More importantly, running two sets of measurements allows us to

determine if the reactions continued to proceed during data collection. If this is the case our second data set will presumably show a higher absorbance that needs to be accounted for in analysis.

Running the blank a second time serves a similar purpose as measuring a second set of data. It ensures that instrument readings did not shift significantly throughout the duration of the data collection. A baseline shift typically results from malfunctions in the instruments and this will also certainly affect our data analysis.

Lastly, it is important to understand the importance of adding the 2 M HCl. The HCl is acidic enough to denature the glucose oxidase and peroxidase, stopping the reaction. This is critical to the design of the experiment because without adding HCl, measurements taken later would skew more towards higher absorbances. The difference between the first measurement taken of sample 1 and the last measurement taken of unknown 3 would be very significant, simply due to the extra reaction time throughout data collection. This is not what we want if we are trying to determine the concentration of the unknowns using the change in absorption. We want the absorption to change as a function of glucose concentration. It is not desirable for time to become a variable in this analysis.

Methods:

1. Prepare 12 microcentrifuge tubes for your glucose stock solutions.
    a. Wrap all twelve microcentrifuge tubes in aluminum foil.
2. Prepare blank and the eight varying glucose solutions described in the table below.
3. Prepare the three unknown solutions by adding 100 μL of the unknown solution to three of the wrapped microcentrifuge tubes.

4. Add 1 mL of the combined enzyme-color solution to all 12 microcentrifuge tubes. Stir with care.
5. Incubate the tubes at room temperature for 45 min.
6. Add 50 µL of 2 M HCl solution to all the microcentrifuge tubes. Stir well.
7. Perform a baseline correction in the spectrophotometer using the blank at 400-500 nm and 0.0 to 1.2 absorbance.
8. Wash the cuvette with 160 µL of the first microcentrifuge tube twice, fill the cuvette with 160 µL and then measure the first absorbance.
9. Repeat with samples 2-8.
10. Repeat with the three unknowns.
11. Repeat with samples 1-8 and then the unknowns one more time. Lastly run the blank at the end, such that each sample has been measured twice. The order should be: Blank ⇒ samples 1-8 ⇒ Unknowns 1-3 ⇒ samples 1-8 ⇒ Unknowns 1-3 ⇒ Blank

Results:

1. The Standard Concentrations of glucose vs the Measured Absorbance:

	[Glucose] (µM)	Absorbance of First Calibration	Absorbance of Second Calibration
Blank	0	Baseline	Baseline
Standard 1	10	0.03230	0.04497
Standard 2	20	0.07858	0.09040
Standard 3	30	0.11630	0.11400
Standard 4	40	0.15517	0.15347
Standard 5	50	0.20409	0.19685
Standard 6	65	0.25405	0.24742
Standard 7	80	0.30339	0.31225
Standard 8	100	0.39282	0.39751

2. There is some discrepancy between some of the absorbance values in the two calibrations, but they are extremely similar overall. I will still perform two linear regressions and perform most of the calculations with both in order to compare the resulting data.

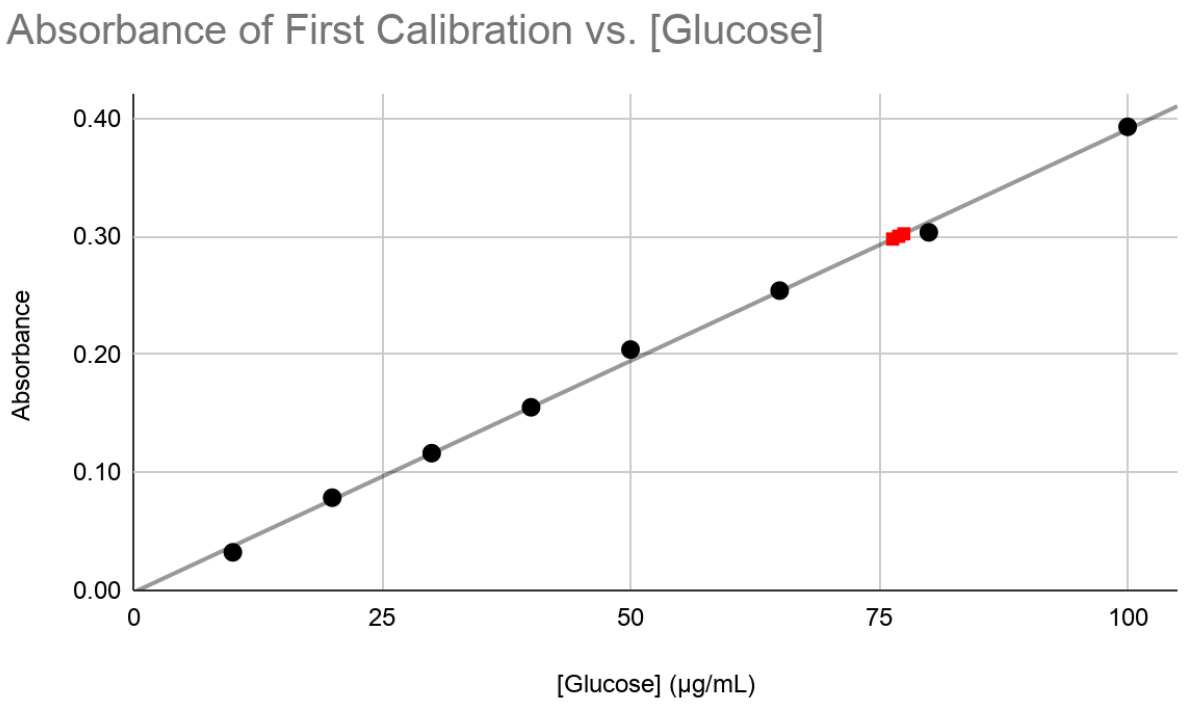
3. These are the two linear regressions for the two calibrations:

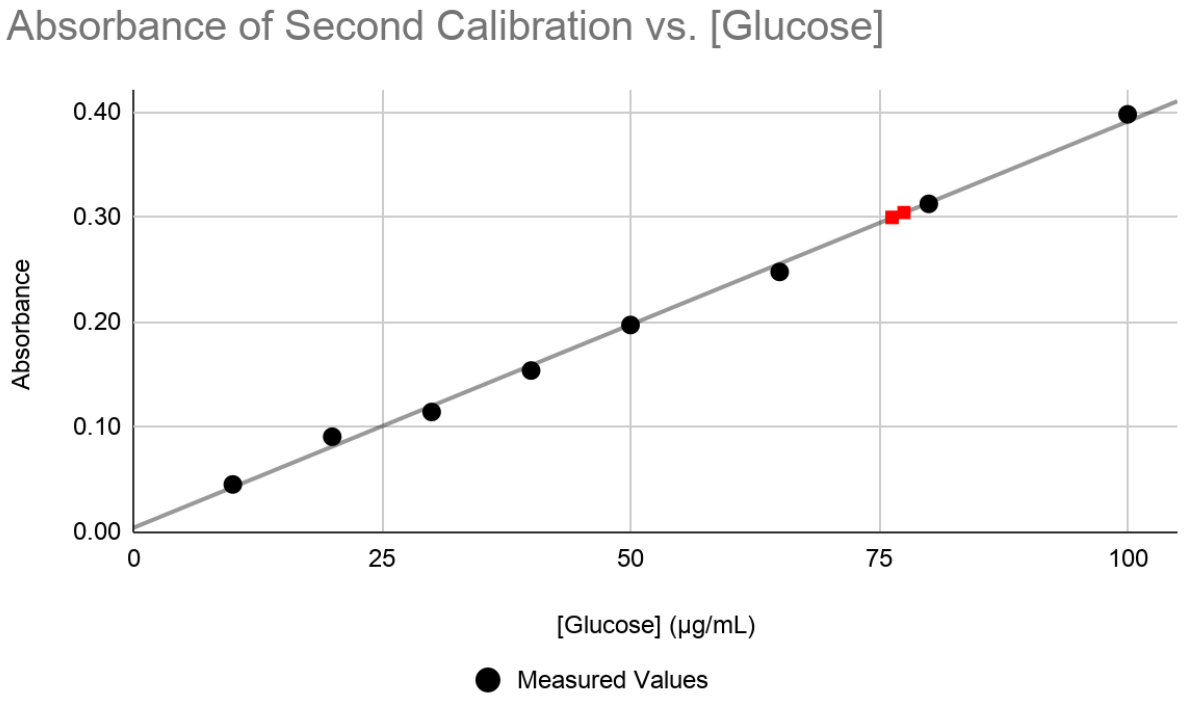
Calibration 1	Calibration 2
Absorbance = 0.003899 x [Glucose]	Absorbance = 0.003922 x [Glucose]

4. The standard error of regressions, the standard error of slopes, and the coefficients of determination are:

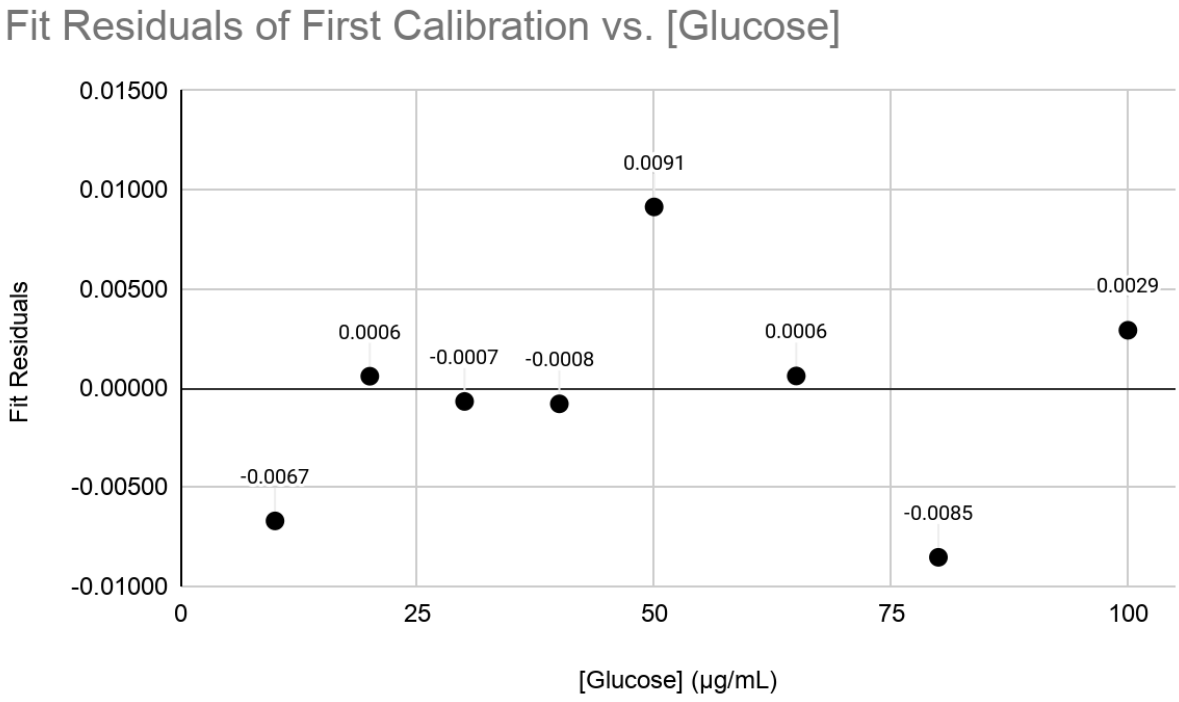
	Calibration 1	Calibration 2
Standard Error of Regression	0.12083	0.11907
Standard Error of Slope	0.00427	0.00424
Coefficient of Determination	0.99787	0.99782

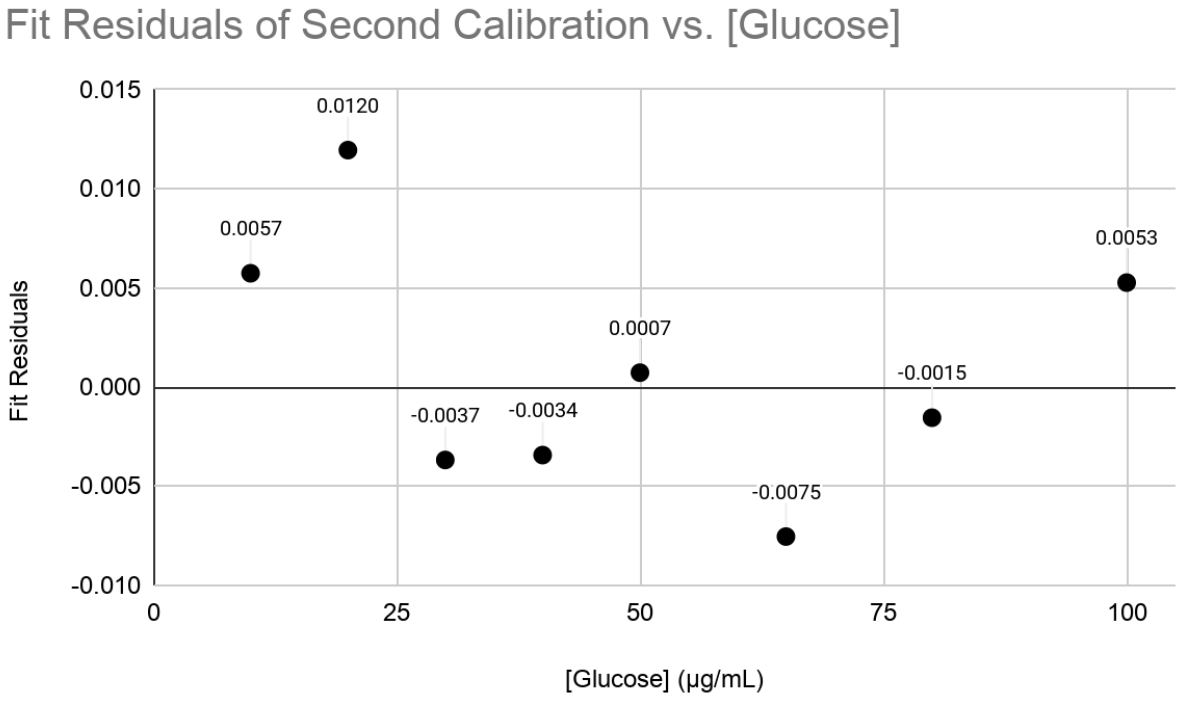
5. These are two calibration curves obtained from the linear regression from each set of data:





6. These are the fit residuals vs [Glucose] plots corresponding to the two calibration curves above. No statistically significant outliers were found:





7. A chart showing the calculated glucose concentrations based on the measurement number and calibration equation used. Various averages and standard deviations are shown as well. The overall calibration equation averages are also given. The data points corresponding to the appropriate measurement and calibration curve are marked as red squares in the calibration curve of results section (5).

Data Summary Chart		Measured Absorbance	[Glucose] (µg/mL) from Calibration Equation 1 [Glucose] = Absorbance/0.003899	[Glucose] (µg/mL)from Calibration Equation 2 [Glucose] = Absorbance/0.003922
First Measurement	Unknown 1	0.30212	77.4865	77.0321
	Unknown 2	0.30010	76.9685	76.5171
	Unknown 3	0.29774	76.3632	75.9153
Average [Glucose] from First Measurement			76.9394	76.4882
Average Deviation from First Measurement			0.5622	0.5589
Second Measurement	Unknown 1	0.29933	76.7710	76.3208
	Unknown 2	0.29924	76.7479	76.2978
	Unknown 3	0.30399	77.9661	77.5089
Average [Glucose] from Second Measurement			77.1617	76.7092
Average Deviation from Second Measurement			0.6968	0.6927

Average [Glucose] (µg/mL) Using Calibration Equation 1	Average [Glucose] (µg/mL)Using Calibration Equation 2
77.05052578	76.59867415

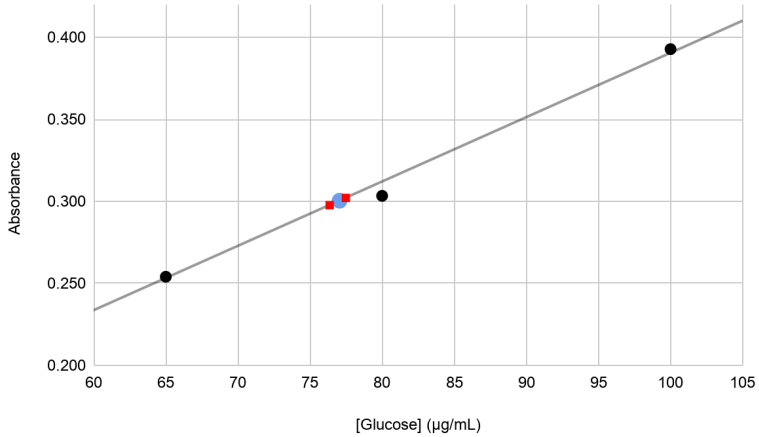
8. A chart of average values and standard deviations of the unknown samples:

Average Absorbance from First Measurement	0.29999
Average Absorbance from Second Measurement	0.30085
Standard Deviation from the First Measurement	0.00219
Standard Deviation from the Second Measurement	0.00272
Average [Glucose] Value of Unknown Sample 1 (µg/mL)	76.9026
Average [Glucose] Value of Unknown Sample 2 (µg/mL)	76.6328
Average [Glucose] Value of Unknown Sample 3 (µg/mL)	76.9384
Overall Standard Deviation	0.00226

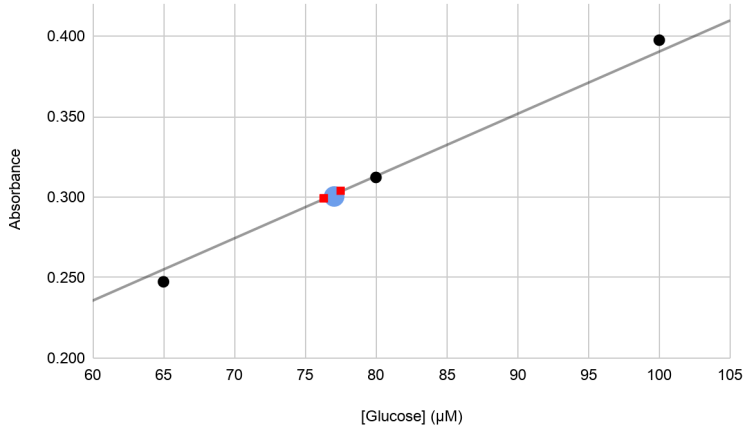
9. Here is a chart of glucose concentrations from various average absorbance values along with a zoomed in view of the calibration curves so as to see the average values of the first and second measurements (Blue circle):

	Absorbance	[Glucose] (µg/mL) from Calibration Equation 1	[Glucose] (µg/mL) from Calibration Equation 2
Average of First Measurement	0.3000	76.93939	76.48819
Average of Second Measurement	0.3009	77.16167	76.70916
Average of Unknown 1	0.3007	77.12875	76.67644
Average of Unknown 2	0.2997	76.85817	76.40745
Average of Unknown 3	0.3009	77.16466	76.71214
Overall Average of All Unknown Samples	0.3004	77.05053	76.59867

Calibration Curve One Showing the Average Value of the [Glucose] from All Measurements for the Unknown:



Calibration Curve Two Showing the Average Value of the [Glucose] from All Measurements for the Unknown:



10. The standard error of concentration is 1.09578 (µg/mL). This was calculated from the slope, the standard error for slope, the standard deviation of absorption, and the average value of the absorbance. Thus, the concentration of glucose in the unknown is 77.05053 ± 1.09578 (µg/mL).

Discussion of Results:

In the end, we determined the concentration of glucose to be 77.05053 (µg/mL) with a standard error of concentration of 1.09578 (µg/mL) using a calibration curve of measured absorbance values as a function of the change of known glucose concentrations. We also calculated a 95% confidence interval of ± 2.816 (µg/mL). With the data provided by the professor, we can be very confident that our calculated glucose concentration is within about 5 (µg/mL) of the actual glucose concentration. The duplication of each measurement resulted in redundant data. One alternative would be to average the two values at each known concentration, such that analysis is only performed on a single set of data. That being said, some benefits of the data analysis performed are outlined below.

Performing analysis on two data sets gave me more calculations to practice and it also showed me how despite having very similar r^2 values, there are still appreciable differences in the calculated data at the end. The first calibration curve had a slightly higher r^2 value and thus

was slightly more linear than calibration curve two. So any final values should come from the calibration equation one. Both, however, yielded good data.

The statistical analysis of this data went very smoothly, and this can primarily be attributed to the quality of data collected. The linear regression gave a very good impression of how the absorbance varies with the concentration of glucose. At zero concentration of glucose there should be zero absorbance, and there was. This is because our blank nullified other impurities that were in all of the samples. Our only known variable was the concentration of glucose and this yielded calibration equations with a 0 y-intercept and a constant slope. We also had no significant outliers.

I did not see the utility in the final calculations of the data analysis so I did not do them the exact way described. Calculating concentration based on each of the unknown samples makes sense in two cases: the first is if the samples were prepared separately, and the second would be if we collected more data for each unknown sample. As it stands, it does not make sense to calculate our objective three times with such small sample sizes. Not to mention that each measurement of the unknown was taken a minute or two apart. Instead, I calculated the glucose concentration for each unknown to best determine my final calculation. In the end, in order to best determine the final concentration value the average of all of the unknown absorbances were divided by the best fit value for the molar absorptivity from the calibration equation. I did this because there was a strange discrepancy between the first and second measurement taken of each unknown. It was not terribly large, but it was large enough to result in a difference of about one (μg/mL) for each unknown sample.

I would alter two aspects of this experiment in order to improve results and reduce ambiguity in our analysis. The first change would be to increase the number of samples of

unknown and to not bother with having three unknowns because they were all the same. I would run ten unique samples of the unknown, such that I would have nine degrees of freedom and my t-distribution would decrease from 2.57 to 2.26. This further simplifies analysis as well. A simple average of the unknowns could be compared to the calibration curve of the known concentrations of glucose. We could analyze each unknown data point individually as well, so that we could remove outliers. The second error of our experiment was only preparing one batch of the combined-enzyme reagent. The activity of the glucose oxidase and peroxidase is a potential source of error that could have significantly affected our data. We do not know with certainty whether the active enzyme quantity was sufficient to consume our glucose appropriately. There are two solutions to this. The first is to run the second set of standard concentrations and another ten unknowns on a separate enzyme preparation. The second and presumably better solution is to let the reaction proceed for longer. If some of the enzyme is not active then it will just take more time to reach completion. Waiting overnight to ensure the reaction goes to completion would give better results for the original concentration of glucose added to the unknowns.

At the end of the day, the experiment was a great success. We determined the concentration of an unknown sample of glucose to be 77.05053 ± 2.816 (µg/mL) with a standard error of only 1.09578 (µg/mL). We accomplished this by fitting a calibration curve to a set of known glucose concentrations and by designing a coupled enzymatic assay that produced a compound that we could measure using Beer's Law and a spectrophotometer.

Calculations:

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(* Standard Error of Regression: sy *)
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$$s_{y1} = \sqrt{\frac{0.10219}{7}}$$

$$s_{y2} = \sqrt{\frac{0.09924}{7}}$$

$$s_y = \sqrt{\frac{\sum_i \left(y_i - \hat{y}_i\right)^2}{n-1}} = \sqrt{\frac{\sum_i y_i^2 - \frac{\left(\sum_i x_i y_i\right)^2}{\sum_i x_i^2}}{n-1}}$$

0.120825

0.119068

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(* Standard Error of Slope: sa *)
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$$s_{a1} = \sqrt{\frac{0.12082454812070033}{6621.875}}$$

$$s_{a2} = \sqrt{\frac{0.11906780781194745}{6621.875}}$$

$$s_a = \frac{s_y}{\sqrt{\sum_i x_i^2}}$$

0.00427157

0.0042404

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(* Coefficient of Determination: r1 *)
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$$r_1 = \left(\frac{25.9868}{\sqrt{6621.875 * 0.1022}}\right)^2$$

$$r_2 = \left(\frac{25.6019}{\sqrt{6621.875 * 0.0992}}\right)^2$$

$$r^2 = \frac{\sum_i \left(\hat{y}_i - \bar{y}\right)^2}{\sum_i \left(y_i - \bar{y}\right)^2}$$

0.997869

0.997819

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(* Standard Error of Concentration: sx *)
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$$s_{x1} = \sqrt{\left(\frac{0.00226}{0.102189}\right)^2 + \left(\frac{0.004271565798867639}{0.003899}\right)^2}$$

$$s_{x1} = \sqrt{\left(\frac{0.00226}{0.099244}\right)^2 + \left(\frac{0.004240398670777018}{0.00392238}\right)^2}$$

$$\frac{s_x}{\bar{x}} = \sqrt{\left(\frac{s_{sample}}{\bar{y}_{sample}}\right)^2 + \left(\frac{s_a}{a}\right)^2}$$

1.09578

1.08132