EXPERIMENT 4
Determination of Glucose in Sprite and Coke[1]

Key Experimental Concepts: To study a reaction rate kinetic methods of analysis and the use of enzymes as ***selective*** analytical reagents for the determination of substrates

Analysis Goals: Application of standard curve, % recovery, and error propagation of linear least squares line fitting

BACKGROUND

Literally billions of clinical chemistry tests are performed annually in the United States. The bulk of these analyses are performed by use of spectrophotometric methods. Of these, ordinary absorption spectroscopy in the visible region is the one most commonly used. The instrumentation for visible absorption spectroscopy is simple, stable, relatively inexpensive, and easy to automate, thus it can handle the large numbers and variety of tests to be done. The method has a large number of advantages and very wide applicability. Many substances absorb light, and if the analyte itself does not absorb, it can often be treated with reagents to generate a product with suitable absorbance characteristics.

The vast majority of chemical analyses are performed on systems that are at equilibrium, clinical or otherwise. The chemistry is "over." This is true whether the analysis method is spectrometric, electrochemical, chromatographic, calorimetric, or even radiochemical in nature. If not technically at complete equilibrium, as is the case in redox electrochemistry and radiochemistry, the analyses performed are under quasi steady-state conditions. When chemistry is performed to prepare samples for analysis, even with use of enzymes, the samples are normally allowed to react or to "incubate" for a sufficient period of time that the system is at or very near equilibrium.

However, kinetic methods of analysis, wherein one measures the rate of an ongoing chemical reaction to determine the quantity or concentration of the analyte of interest, are becoming increasingly popular. The first, and probably most important, advantage of kinetic methods in general is speed. You don't have to wait for the reaction to go to completion to make your measurement. Kinetic methods based on reaction-rate measurements are usually done only during the first 3 to 10% of the reaction, before possible back-reactions start to become significant. In this situation, one measures the so-called initial reaction-rate with little error.

Kinetic methods are also particularly useful in samples in which some interferent is present, even if in large and variable concentrations from sample to sample. For example, take absorption spectroscopy as the method and a colored sample such as whole blood or bottled/canned soft drinks and juices. If you can develop a kinetic method that reacts only with the analyte, or at least does not react with the interferent, modern instruments with microprocessors can often succeed in providing you with a decent or even very good analysis by measuring the rate of change in absorbance of a sample undergoing a reaction without having to do complicated and time-consuming chemistry to eliminate the interfering colored background or to resort to some separation method.

The primary objective of this experiment is two-fold: To provide you experience with a kinetic method of analysis and to acquaint you with the use of enzymes as an analytical tool or "reagent".

THE ENZYMATIC REACTION

Glucose is a major component of animal and plant carbohydrates. Quantitative determination of glucose is important in clinical chemistry, biochemistry, and food science. In this experiment you will measure glucose levels using enzymatic reactions and visible absorption spectrophotometry. While there are several variants in enzymatic determinations of glucose, this one will use glucose oxidase(GOX) and horseradish peroxidase (HRP) enzymes. However, we will flood the reaction with HRP so that the GOX reaction will be the rate-determining process -- and therefore the basis for quantitation.

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[1] Vasilarou, A-M.G., Georgious, C. A., *J. Chem. Ed.* **77**, 1327 (2000).

In the first step, glucose is oxidized to δ-D-gluconolactone, catalyzed by GOX:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{GOX}} \delta\text{-D-gluconolactone} + H_2O_2 \quad (1)$$

In the second step, H_2O_2 produced in step 1 is used to generate a light-absorbing product, catalyzed by HRP:

$$2H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{HRP}} \text{4-}N\text{-}(p\text{-benzoquinoneimine)-antipyrine} + 4H_2O \quad (2)$$

The reaction product exhibits an absorption maximum at 505 nm with a corresponding molecular extinction coefficient $\varepsilon = 1.27 \times 10^4\ M^{-1}cm^{-1}$

A Note on Enzyme Concentrations
Enzyme "concentrations" are usually expressed in units of IU. IU is an acronym for International Unit, which is essentially an enzymatic "activity" –how well the enzyme does its catalysis. An IU is typically defined as the amount of an enzyme that will produce 1 μmol of product per minute or that will consume 1 μmol of reactant per minute. This, of course, is highly dependent on reaction conditions such as temperature, pH, and other solution components. While the total molarity of the enzyme itself would stay constant under different experimental conditions (except for the effect of volume changes with change in temperature), its activity can change markedly. For example, the activities of almost all enzymes are highly dependent on the pH. The rates of chemical reactions typically double for a 5°C increase in temperature, but eventually raising the temperature too high will denature (unfold) an enzyme, rendering it totally inactive. For example, think about a hardboiled egg. Proteins in their natural form are called "native proteins." Any significant change in the secondary, tertiary, and quaternary structures of a native protein is called denaturation. For kinetic enzyme assays, giving enzyme "concentrations" in IUs is a useful and function definition.

Prelab Assignment:
Read the background material and answer the questions in the ELN.

Procedure: (work in pairs unless otherwise noted)

Reagents to prepare
Glucose stock solution. Prepare 25.00 mL of a 0.200 M glucose solution.

Calibration solutions. Prepare 25.00 mL of each of the following calibration solutions from your stock solution: 2.00, 4.00, 8.00, 12.0, and 16.0 mM glucose.

Phosphate pH 7.5 buffer solution used for diluting the Sprite unknown. Place 7 g of Na_2HPO_4 in 400 mL of water in a large beaker and adjust the pH to 7.5. Transfer the solution to a 500 mL volumetric flask and bring to volume with DI water. After mixing, place this solution in two labeled bottles, and give one to the instructor (for making the following solution).

Enzyme working solution (this will be prepared once by the class, for the entire class): phenol, 7.50 mM; 4-aminoantipyrine, 2.50 mM; GOX, 0.5 IU mL^{-1}; and HRP, 20 IU mL^{-1} in 0.10 M phosphate buffer pH 7.5.
Place 0.706 g of phenol and 0.508 g of 4-aminoantipyrine in 800 mL of pH 7.5 phosphate buffer in a 1 L beaker. Add 500 IU of GOX and 20,000 IU of HRP to the buffered solution, transfer to a 1000-mL volumetric flask, and bring to volume with pH 7.5 phosphate buffer. Be sure to record the actual masses used by the class in your notebook.

Preparing the Unknowns: It is important that the beverage be stirred to decrease carbonation for at least 30 minutes prior to analysis. For the analysis, 1.00 mL of the unknown is diluted to 25 mL with phosphate buffer. Working with your lab partner prepare two unknown solutions as assigned by your lab professor.

Enzyme Reactions

Set the wavelength at 505 nm and adjust the spectrophotometer to zero absorbance with enzyme solution as the blank. One milliliter of sample and 5.00 mL of the enzyme working solution are rapidly mixed and immediately transferred to the spectrophotometer. To eliminate ascorbic acid interference, samples are preincubated for 1 min with the enzyme working solution (in the spectrophotometer), after which the initial absorbance reading ("time 0") and four minutes of subsequent readings are recorded (at intervals of 1 min or less). This procedure is performed for each calibration solution and each diluted unknown sample.

Spike Recovery Test: Add one mL diluted unknown sample *and* 60 uL of the 0.200 M glucose stock solution to 5.00 mL of the enzyme working solution, mix rapidly, and analyze as usual. By adding in the extra glucose stock solution, you have "spiked" your unknown with an additional 2.0 mM glucose. You can verify this by solving the following dilution equation for x, the additional glucose concentration added:

$$(0.06\ \text{mL})*(0.200\ \text{M}) = (6.05\ \text{mL})*(x)$$

Results and Analysis

1. Plot absorbance readings versus time to determine reactions rates (in units of m*A* min^{-1} or milli-absorbance min^{-1}). Convert these slopes to M min^{-1} of glucose lost, like we did in class before break.
2. Construct a summary table of reaction rates for each standard and soda / juice sample, including R^2 values.
3. Using the Jupyter notebook, construct a calibration curve of reaction rates vs. glucose concentration (and glucose squared) in the standards. Is the reaction 1st or 2nd order in glucose?
4. Input the reaction rates (measured y) for the each unknown solution – and the spiked unknown solution -- to the linear regression equation to determine the glucose concentration (x) in the diluted beverages. Next week in lab, we'll use Python to determine the error that comes from using your calibration curve ($x \pm s_x$).
5. Calculate the % recovery of the spiked glucose.
6. Take into account your dilution factors, and calculate the (grams of sugar)/serving in the original, undiluted samples. Compare to the Nutritional Facts label and the pre-lab info, and calculate relative errors. Next week, we will propagate error through this calculation, so show your work today to make next week's task easier. Once you have estimated the random error in your final concentrations next week, you'll be able to do the following things and answer the following questions:
7. Calculate a 95% CI for grams of glucose per serving for each unknown. Does the CI include the Nutritional Facts value?
8. Include a brief discussion/conclusion about the quality of the results and the goal of the experiment. Some questions to consider:
    a. Since this technique is selective, how do think this might affect your results?
    b. How do your results compare to the manufacturer's Nutritional Facts label? Why might they be different? Consider the difference between glucose, fructose, sucrose and high fructose corn syrup.
    c. What other errors or interferences might have impacted your results? Which of these errors have been included in the confidence interval, and which have not?