Project 7B: Analysis of Phenols by Electrochemical Biosensor

LABORATORY REPORT: Informal Report

PRE-LAB ASSIGNMENT

- Read the entire laboratory project described in the following pages. Also read the source paper by Njagi *et al*.[1]

- Prepare, on a typed sheet of paper, the Project Objectives of this lab; on the same sheet, complete the assignment below:

1) Using ChemDraw for the aromatic compounds, give balanced chemical equations for the complete (4e-) oxidation of phenol to quinone, and the electrochemical reduction of quinone to the diol catechol.

2) Phenolics in wine can be classified as mono-, di-, tri-, or poly-phenols.
   a. tannic acid is a _____-phenol;
   b. catechin and resveratrol are _____-phenols;
   c. quercetin, cyanidin, and malvidin are _____-phenols
   d. vanillin, 4-OH-cinnamic acid, caffeic acid, and guaiacol are _____-phenols.

3) Anti-oxidants are good _____ing agents. Phenolics are good anti-oxidants because they are easily _____ed to _____.

4) Is phenolic content higher in red or white wines? Cite your source.

5) Review, as necessary, the sections on Molecular Selective Electrodes (Section 23F) and cyclic voltammetry (Section 25D) in Skoog, *et al*.[2] Review especially the key features of cyclic voltammograms in Skoog, *et al.* (Figure 25-24) and in Njagi *et al*. (Fig. 3A). Sketch the CV shown in Figure 25-24 but use **IUPAC** conventions (see below) and label the important features such as parameters that: (a) are sensitive to the concentration of redox-active analyte; (b) allow determination of the reduction potential; and (c) allow determination of the reversibility of the redox reaction at the electrode.

---

[1] Njagi, J. et al "A bioanalytical chemistry experiment for undergraduate students: Biosensors based on metal nanoparticles" *J. Chem. Educ.* 2007, *84,* 1180-1182.

[2] Skoog, Holler, and Crouch, *Principles of Instrumental Analysis*, 6th ed., Thomson Brooks/Cole, Belmont, CA, 2007.

INTRODUCTION

In the parlance of analytical chemistry, a biosensor is a device that is capable of monitoring specific chemical species using a biochemical probe, usually an enzyme, coupled with a suitable transducer. In this experiment the redox enzyme tyrosinase (TYR) will be immobilized on a gold-modified glassy carbon electrode (Au-GCE). This biosensor combines the selectivity of enzyme-catalyzed reactions and the sensitivity of electrochemical analysis to allow analysis of phenolic compounds of biochemical interest in the complex biological matrix, wine.

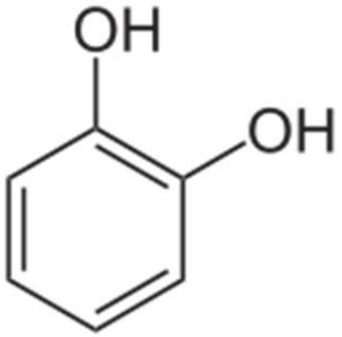
The biosensor for this experiment responds only to phenolic substrates that can be bound and readily oxidized by tyrosinase. The biosensor is fabricated according to the procedure of Njagi *et. al*[1]: A glassy carbon electrode is modified by coating the surface with gold nanoparticles; tyrosinase is then adsorbed to the gold surface and cross-linked using glutaraldehyde. The immobilized tyrosinase will oxidize phenols to quinones at the electrode surface. If a sufficiently negative potential is applied to the electrode, the quinones will then be reduced to diols such as catechol. [See Fig. 1 in Njagi et al, but note the errors in the unbalanced equation.[3]] Electrons flowing through the electrode to reduce bound quinones register as a current that is proportional to the concentration of phenolic compounds.

The tyrosinase-based electrode has been applied to a number of analyses, including the measurement of phenolic content in environmental samples and in wine.[4] Phenol is the parent compound of a large family of phenolics; members of this family are categorized as mono-, di-, tri, tetra-, or poly-phenols, based on the number of phenol rings (not the number of hydroxyl groups) in the compound. For example, catechol, hydroquinone, and pyrogallol are all monophenols, whereas tellimagrandin II is a pentaphenol (Scheme 1).

Scheme 1: Some common phenolic compounds.

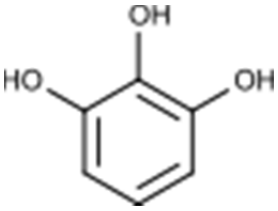
[3] In the second oxidation catalyzed by tyrosinase, catechol is oxidized by ½ O_2, yielding both *o*-quinone and H_2O. Also, the reduction of quinone to catechol requires 2 H^+ along with 2 e-.

[4] Svitel, J.; Miertus, S. *Environ. Sci. Technol.*, 1998, *32*, 828-832; Carralero Sanz, V.; Luz Mena, M.; Gonzalez-Cortes, A.; Yanez-Sedeno, P.; Pingarron, J. M. *Anal. Chim. Acta*, 2005, *528*, 1-8.

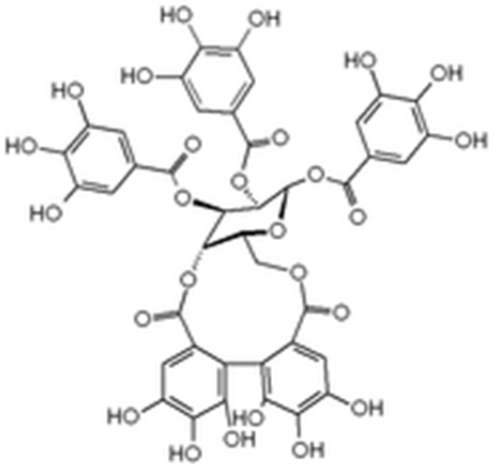


catechol

1,4-hydroquinone



pyrogallol



tellimagrandin II

Phenolics are naturally occurring antioxidants that are promoted by wine makers as providing beneficial effects. The fact that oxidants, especially reactive oxygen species like peroxide, superoxide, and hydroxyl radicals, cause oxidative damage in living cells has been known for decades, and is discussed in most biochemistry and biology textbooks.[5,6] Oxidative damage accrues to all four major types of biomolecules (proteins, nucleotides, lipids, and carbohydrates), and figures importantly in a number of diseases, including cancer, diabetes, cardiovascular disease, and scurvy, in addition to the "normal" aging process. Cells have devised many pathways to counter oxidative damage, and antioxidants are key substrates in these pathways.

[5] Mathews, C.K.; van Holde, K.E.; Appling, D.R.; Anthony-Cahill, S.J. *Biochemistry,* 4th edn. Pearson, Toronto, 2012, pp. 663-668.
[6] Berg, J.M.; Tymoczko, J.L.; Stryer, L. *Biochemistry*, 6th edn., W.H. Freeman & Co., NY, 2007, pp. 517-519, 779, 805.

Antioxidants like ascorbic acid (vitamin C), quinols (e.g., vitamins E and K, coenzyme Q), and phenols are essentially avid reducing agents, donating electrons to reduce damaging reactive oxygen species to water.[6,7] For this reason, the high phenolic content of red wines is believed protect against the diseases of old age.

In this experiment you will fabricate a biosensor and measure phenolic content in an unknown phenol solution, and in wine. The instrument you will use to measure electrical current is the BAS Epsilon potentiostat-galvanostat attached to an electrochemical cell. The electrochemical cell has three electrodes: (a) a Ag/AgCl reference electrode; (b) a platinum wire counter electrode; and (c) a working electrode. You will use the Au-GCE or the biosensor as working electrodes in a direct-current potential amperometry (DCPA) system. When electroactive solutes are present in the solution, a current passes between the working electrode surface and the counter electrode. The potential of the working electrode is controlled and measured vs. the reference electrode.

Another electrochemical method used to characterize redox reactions and electrodes is cyclic voltammetry (CV). The reduction potential and reversibility of a redox couple at an electrode surface is characterized by CV; for example, the ferro-/ferricyanide redox couple [$Fe^{II}(CN)_6^{4-}$/ $Fe^{III}(CN)_6^{3-}$] is well-behaved and is often used to characterize electrodes, as you will do in this experiment. A typical CV can be found in Skoog et al, and also in Njagi et al's Fig. 3A.

Important note: Skoog *et al*. use electrochemical sign conventions common to the United States. For this laboratory you will use the IUPAC conventions in which oxidizing currents are taken as positive, and voltammograms are drawn with potentials increasing to the right. In other words, in a typical CV, the potential (in V) is scanned from negative (left side) to positive (right side); at a certain potential related to the $E°$ of the redox couple, oxidation takes place and positive current is recorded on the *y*-axis. Once a suitably high positive potential is reached, the potential is scanned back in reverse, from positive (right) to negative (left), whereupon reduction takes place and negative current is recorded. The resulting CV voltammogram is sometimes said to

[7] Metzler, D.E. *Biochemistry: The Chemical Reactions of Living Cells*, 2nd edn., Harcourt Academic Press, San Diego, 2001, pp. 818-826.

resemble a duck, whose head (high potential/upper right) represents oxidation (positive current) and whose belly (low potential/lower left) represents reduction.

EXPERIMENTAL: DAILY PLAN

Prior to beginning laboratory work, students should have in their notebooks a daily plan based on pre-lab discussions. Have the instructor check your plan every day before you begin work. On the first laboratory day, you will work in pairs and rely on the paper by Njagi *et. al*[1] for specifics. Each pair of students will prepare a biosensor, i.e., a gold-modified/tyrosinase cross-linked electrode (TYR-Au-GCE). Team A will also prepare a second gold modified glassy carbon electrode that lacks TYR (Au-GCE), to be used as a control electrode by all class teams. Prepare your notebook accordingly.

SAFETY AND HAZARDS

Safety glasses must be worn during this experiment. Phenol is toxic by inhalation, ingestion, and skin absorption; it is also a mutagen. Therefore, protective gloves and safety glasses should be worn when handling it. Chlorauric acid is a strong oxidizing agent, and is toxic by inhalation and ingestion, and is a skin sensitizer; hence hand gloves should be worn when handling it. Solid phenol and chlorauric acid should be handled in a fume hood, preferably by the instructor; students should use only pre-made aqueous solutions of these two compounds. In case of skin contact, wash thoroughly with large volumes of water.

EXPERIMENTAL: ELECTRODE PREPARATION

Polish your glassy carbon electrode (GCE):

Obtain a roughly 2" length of aluminum oxide (3 micron) polishing strip, and place this on the lab bench, shiny side down/dull side up. Place a small drop of deionized water on the left side of the strip, and hold your GCE firmly in the middle of the water drop, flat against the polishing strip. Polish for 5 min. by exerting moderate pressure and grinding in a circular motion in the left half of the strip. After polishing, rinse well the GCE surface (at the bottom of the electrode) with deionized water.

Any electroactive compounds remaining on the GCE surface can be removed by electrochemical oxidative stripping. Add approximately 4 mL of 1 M KNO_3 supporting electrolyte to an electrochemical cell without a stir bar, and place the cell under the Teflon electrode holder disk. At this point, familiarize yourself with the basic electrode array in the BAS electrochemical instrument: (a) the Pt wire counter electrode is connected to the red lead; (b) the Ag/AgCl reference electrode is connected to the white lead; (c) your modified GCE working electrode is connected to the black lead; and (d) a plastic tube is present for bubbling $N_{2(g)}$ into the solution. Current passes between the working electrode and electroactive solutes in solution, and potential is measured vs. the reference electrode. Place your GCE in the central hole of the Teflon disk (leaving the hole closest to you open for aliquot additions) and carry out the oxidative stripping process using DCPA and the following settings. (Please see Appendix 1 at the end of this project for details on the use of the BAS electrochemical instrument.)

Applied potential:	+100 mV (oxidizing)	Sample interval:	0.5 s
Time limit:	60 s	Full scale:	1 μA

After stripping, rinse your GCE well with deionized water and perform a final polish. Place another small drop of deionized water on the right side of the strip, and polish for about 5 min. Be sure to make good contact between the electrode's bottom surface and the alumina strip. Rinse the electrode's bottom GCE surface well with deionized water, and place in the bath sonicator for 10 min. From this point on, take care ***not to touch*** the bottom surface of the GCE with anything except a plastic pipet tip. Rinse the GCE bottom surface well with deionized water, then methanol, and air dry for a few minutes. Note the color of the glassy carbon circle at the bottom of the electrode – it should be black and shiny.

Cyclic Voltammetry with ferri-/ferrocyanide:

Characterize the bare GCE by running a cyclic voltammogram (CV) on 2.0 mM potassium ferricyanide(III) (prepared in 1 M KNO_3) using the polished GCE as the working electrode. Deaerate 4 mL of the ferricyanide solution by purging with $N_{2(g)}$ for about 4-5 minutes; do NOT stir during the run. Set-up the CV to scan from -200 to +700 mV and back, as follows:

Initial potential:	-200 mV;	Switching potential:	+700 mV
Final potential:	-200 mV;	# of segments:	2
Scan rate:	50 mV/s;	full scale:	100 μA

Examine the voltammogram: For both the oxidative peak (upper right, duck's "head") and the reductive peak (lower left, duck's "belly"), record the potential (in mV) and the current (in μA). Save the CV in your data folder. Remove the ferricyanide cell, cover it with Parafilm, and save it for later. Rinse the electrode thoroughly and electrodeposit gold as described in the next step.

Gold-plating the GCE (Au-GCE):

You will reductively plate gold onto your GCE using the DC potential amperometry operation mode (DCPA) with the following electrochemical parameters:

Applied potential:	-200 mV (reducing)	Sample interval:	0.2 s
Time limit:	60 s	Full scale:	100 μA

Place 4 mL of 0.49 mM (0.167 mg/mL) chloroauric acid stock solution, $HAuCl_{4(aq)}$, in a cell and deaerate the solution by purging with nitrogen for ~5 min.; do NOT stir during the run. When the run is completed, remove the $HAuCl_4$ cell and pour the solution back into the stock bottle. Rinse the electrode with distilled water and allow it to dry. At the end of the deposition, notice the presence of gold on the surface of the working electrode (yellowish color).

Run a CV of 2.0 mM potassium ferricyanide(III) (in 1 M KNO_3) using the gold-modified GCE (Au-GCE) as the working electrode, and the potential and current settings as above. Record the potential (in mV) and the current (in μA) of the oxidative and reductive peaks; save the CV in your data folder. (Later you will compare the GCE CV to that with the Au-GCE.) Discard the ferricyanide in the waste beaker, rinse the electrode thoroughly with water and allow it to dry.

Tyrosinase immobilization (TYR-Au-GCE):

The final electrode preparation step involves immobilizing the enzyme tyrosinase (TYR) onto the Au-GCE by cross-linking with glutaraldehyde. Use a micropipettor to deposit 5 μL of a

TYR stock solution (100 IU/μL) over the entire gold surface of the Au-GCE, and allow it to dry for 30 minutes. Place about 1 mL of glutaraldehyde (25% aqueous v/v) in a small test tube, and suspend the electrode so its bottom surface is about 0.5 cm above the liquid. Seal with Parafilm and incubate for 30 minutes. Glutaraldehyde is quite toxic, so dispose of it in a separate, labeled, sealed plastic container. Rinse the electrode thoroughly with distilled water; store it in 0.10 M phosphate buffer, pH 6.5. We will refer to this electrode, your biosensor, as the TYR-Au-GCE; the control electrode prepared by Team A will be referred to as Au-GCE.

EXPERIMENTAL: CHARACTERIZATION AND USE OF ELECTRODES

You will now characterize the Tyr-Au-GCE with respect to analytical performance, measuring its sensitivity, detection limit, linear range, and operational stability. Amperometric measurements are carried out in *stirred* solutions to ensure the free transport of substrate and products to and from the electrode surface. Because oxygen is a second substrate in the phenol oxidation catalyzed by tyrosinase, you will NOT deaerate these solutions.

Titrations with standard phenol:

Use a volumetric pipet to add 4.00 mL of 0.10 M phosphate buffer, pH 6.5, to an electrochemical cell; add a small stir bar. For this standard phenol titration, you will need the standard phenol stock solution (record its concentration) and a 20 μL pipettor (yellow plunger, small white tips); check that the pipettor can be inserted into the open hole in the Teflon electrode holder disk with the pipet tip below the surface of the solution in the cell. During the titration you will add successive phenol aliquots to the cell at 30-60 s intervals, and measure the electrode response.

With the electrochemical analyzer in the DC potential amperometry mode, set the applied potential at -150 mV, 10 μA full scale, and the full monitoring time at 20 minutes. Start the experiment; allow 60 sec for stabilization, then record the initial current from the computer screen. Add 4 μL of standard phenol solution into the stirred electrochemical cell. Observe the digital readout of the current; it should stabilize after 20-30 sec. Record[8] the aliquot volume added and the stabilized current value (final current). Repeat the procedure at 30-60 sec intervals

[8] These current values read from the computer screen are only approximate. For your final data analysis you will use averages of the accurate, digital values recorded over time.

by adding several 4 μL aliquots of standard phenol solution and recording the final current. At a certain point you may observe that the electrode response saturates, that is, addition of a 4 μL aliquot gives a smaller and smaller current response. At this point, increase aliquot volumes to 8 μL, then finally, 16 μL. Continue until the response to a 16 μL aliquot is significantly saturated, i.e., current response is severely curtailed. Save the data set for later data analysis. It should resemble Fig. 4A in Njagi et al. After this first titration is complete, lower the cell away from the electrodes, discard the solution in the waste beaker, and rinse the electrodes with water.

Because the TYR-Au-GCE surface may be conditioned by its first exposure to phenol, you must do at least two more full titrations with standard phenol solution in order to get reproducible results. For each titration, add 4.00 mL of fresh phosphate buffer, titrate with at least six 4 μL aliquot additions; stop at or before the electrode response begins to saturate. Rinse the electrode between titrations, and save all data sets.

Titration with unknown phenol:

Add 4.00 mL of fresh phosphate buffer to a stirred cell, and titrate with at least six 5 μL aliquots of the unknown (stockroom-prepared) phenol solution; stop at or before the electrode response begins to saturate. Discard the cell solution, rinse the electrodes, and repeat until you have at least four full titrations of the unknown phenol solution. Save all data sets.

Response of the control Au-GCE:

Perform one standard phenol titration as above using the control Au-GCE instead of the biosensor TYR-Au-GCE. Add successive 4.00 μL aliquots of standard 1.00 mM phenol solution, and measure current changes (if any). Stop after 4-5 additions; save the data set.

EXPERIMENTAL: ANALYSIS OF PHENOL AND POLYPHENOLS IN WINE

Analyze samples of red or white wine using a *standard-addition* method, as follows. Carefully rinse the electrodes and the electrochemical cell and add 4.00 mL of fresh phosphate buffer. Use the same electrochemical parameters as in the phenol titrations above. Thus, each titration will start with 4.00 mL of buffer for the initial current, then the wine sample will be added and the current measured, followed by five or six aliquots (5.00 μL) of the 1.00 mM standard phenol.

You will have to determine the appropriate volume for the wine sample – make your first measurement using a 50 µL of wine, and adjust subsequent aliquot volumes up if the current response is too small, or down if the response is too large. After each titration, discard the phenol-titrated wine sample, rinse the cell, and load with fresh buffer. Run enough wine replicates (≥ 4) for good statistics. Save all data sets.

DATA ANALYSIS: ELECTRODE PREPARATION

Compare the ferricyanide CV for the bare GCE to that of the Au-GCE. For a perfectly reversible one-electron redox reaction at 25°C, the difference between the potentials of the oxidation and reduction peak should be 59 mV (=2.3*RT/nF*).

1. *Describe the electrochemical process that takes place during the deposition of gold onto the electrode surface. Give a balanced equation for this process.*

2. *Based on the CVs, how reversible is the ferricyanide redox reaction at the surface of the GCE vs. the Au-GCE?*

3. *The peak current is a measure of how much sample is oxidized or reduced. How does gold-plating alter the electrochemical properties of the electrode?*

4. *Specify the types of counter, reference, and working electrodes that you used in this project. Summarize briefly the general function of each of the three electrodes.*

5. *Comment on the importance of enzyme immobilization and the differences between the TYR-Au-GCE vs. control Au-GCE electrodes. How important is the enzyme? What does the enzyme actually do?*

DATA ANALYSIS: CALIBRATION CURVES

Find all of your DCPA titration data sets on the BAS computer at MyComputer/Local Disk(C:)/Epsilon EC2000/Data/ExpBio 20xx, and copy them (Ctrl-c) to a flash drive (Ctrl-v). Remove any periods from filenames. Open the Epsilon EC program and select "File/Convert to Text File". In the middle left box, select DCPA format. In the middle right box, select your flash

drive (:f) as the source directory. Select all of your DCPA files and click "Convert". All files should now be on your flash drive in both .DCPAO and .DAT format. Start Excel and use the "Text Import Wizard" to convert the .dat files to Excel files; if you need explicit instructions, see Appendix 2. Convert negative current values in amps (A) in Column B, to positive current values in μA in column C.

Make a current (μA) vs. time (s) scatter plot of all of the data in the first titration of standard phenol. You may wish to label each current plateau on the plot with aliquot number, starting with zero for the initial plateau (no phenol). It also may help you in column D to label all measurements with the appropriate aliquot number: 0 for the initial values, 1 for the first aliquot, 2 for the second, etc. Examine the results for several aliquot additions.

6. *Regarding response time, how long does it take for the current to stabilize?*

Calculate the measured current for each aliquot number (including zero) by averaging the values from the initial point of current stabilization until just before the next addition; for each average value, obtain the standard deviation as well. Calculate also, for each aliquot addition: moles of phenol added (= $V_{\text{aliquot}} \times M_{\text{phenol}}$); total moles of phenol in solution (additive for each aliquot); total volume of solution (also additive); and finally, [phenol]. Set up a table in your spreadsheet with columns for: aliquot number (starting with zero); total concentration of phenol (μM) in solution after aliquot addition; average current (μA); standard deviation (μA); and finally, baseline-corrected average current ($i_{\text{aliquot}} - i_0$). Note that the concentration and corrected current readings will be zero for aliquot zero, but they will be additive for each subsequent aliquot.

Generate a calibration curve by plotting corrected average current (μA) vs. total phenol concentration (μM). Use the appropriate propagation of error equation to calculate the standard deviation of the corrected average current[9]; insert these values as *y*-error bars. You should see a linear region of the calibration curve, which may be flanked by nonlinear points showing electrode conditioning (at low concentration) and/or saturation (at high concentration). If necessary, plot a second series that includes only the linear points, and add a trendline forced through the origin (i.e., zero") to these "linear-only" points. Also, perform a statistical linear

[9] $= \sqrt{s.d.(aliquot\ x)^2 + s.d.(aliquot\ 0)^2}$

regression of the "linear-only" points (under Tools/Data Analysis (at the bottom)/Regression; again forcing the fit line through the origin (check the box "constant is zero"); insert the results in your spreadsheet. This will give you the R^2 value, the standard error of the regression S_r, and the slope (x-variable 1) with its uncertainty.

7. *Select a particular point on your calibration curve, and provide a reference to the exact pages in your laboratory notebook where sample calculations can be found. Calculations of interest for your chosen point will include: moles of phenol in aliquot, total moles in solution, total [phenol] in solution, corrected average current, and standard deviation of corrected average current.*

8. *Include in your informal report the calibration curve for the first titration, with current error bars, regression equation of the linear region, and* R^2.

Analyze subsequent standard phenol titrations in the same fashion. These should all be linear over at least part of the concentration range, with the first point at the origin.

In characterizing your biosensor, you now can report:

(a) the **sensitivity** in units of amps/molar (or μA/μM);
(b) the **linear range**, in units of total phenol concentration in μM; and
(c) the **detection limit** (in μM) equals three times the standard deviation of the blank (aliquot zero) divided by the slope.

9. *Prepare a data table in your informal report that includes sensitivity, linear range, and detection limit for all of your standard phenol titration runs. Provide a reference to the exact pages in your laboratory notebook where sample calculations can be found.*

10. *Does run 1 seem to be an outlier? For runs that are similar, calculate the average and standard deviation of the slopes, and report these in the table above under sensitivity.*

11. *Add your electrode characterization table to the class Google doc spreadsheet; be sure to complete your data entry before the deadline listed on your class schedule sheet.*

Your first standard phenol titration curve most likely shows noticeable saturation at high concentration. This could be due to the enzyme saturation modeled by Michaelis-Menten kinetics, but it could also be due to substrate/product diffusion limitations.[10] To test this question using data from your first standard phenol titration, fit the corrected current vs. [phenol] to hyperbolic saturation[11]. It will be easiest to do this by converting your current values (*i,* in μA) to positive values.

12. *Include in your informal report the hyperbolic saturation plot. Calculate and plot the set of residuals, $i_{exptl} - i_{fit}$, for each point: Is the fit good?*

13. *Also make a Lineweaver-Burke double reciprocal plot: 1/current vs. 1/concentration. Is this linear?*

14. *Does it seem as if the enzyme is following Michaelis-Menten kinetics? If not, what is a better description of biosensor behavior?*

DATA ANALYSIS: UNKNOWN PHENOL SOLUTION

For your first titration of the unknown phenol solution, you have at least six aliquot additions. Calculate the average initial current (i_0), as well as the average current after each addition (i_1, i_2, i_3, etc.; see Appendix 4A for an explanatory figure and further details). Next calculate the current response, Δi, to each aliquot ($\Delta i_x = i_x - i_{x-1}$), and use the average sensitivity of your electrode to convert each of these to a concentration change. From these concentration changes and the volume of solution in the cell after each addition, calculate moles of phenolics added to the cell each time. Finally, use the aliquot volume (generally, 5.0 μL) to obtain replicate values for the phenol concentration in the unknown solution. Calculate the average and standard deviation for the replicate concentration values from your first titration. Perform the same set of calculations for each unknown phenol titration run that you did.

[10] Silverstein, T.P. and Goodney, D.E. "Enzyme-Linked Biosensors: Michaelis-Menten Kinetics Need Not Apply" *J. Chem. Educ.*, 2010, *87,* 905-906.

[11] If your curve goes through the origin, the fit equation is: i_{max}*[phenol]/([phenol] + K_d).

14. *Select a particular unknown phenol solution aliquot addition, and provide a reference to the exact pages in your laboratory notebook where sample calculations can be found. Calculations of interest for your chosen aliquot will include: current change, change in [phenol], moles of phenol in aliquot, [phenol] in unknown phenol stock solution.*

15. *Include the following, both in your informal report and in the class Google doc spreadsheet: average [phenol] ± standard deviation (in mM), for each unknown phenol titration run. Finish your data entry in the class Google doc by the deadline stipulated on your class schedule sheet.*

It is possible that one or more of the electrodes functions significantly differently than the others, due to a problem in its preparation. If this is the case, then the systematic error from this electrode should be excluded from the data set. ANOVA, combined with pair-wise t-tests, can be used to determine if any of the electrodes is an outlier. Please see Appendix 3 for details on the use of ANOVA.

15. *Report the results of the ANOVA comparison of all electrodes. If one or more electrode results seem to be outliers, are there indications in the electrode characterization table that might explain this?*

DATA ANALYSIS: ANALYSIS OF PHENOL AND POLYPHENOL IN WINE

There are several methods for analyzing data from *standard addition* experiments; those described in Skoog et al[2] assume that all samples have identical volumes. In our biosensor titrations the total volume increases. In this case, the best way to determine the concentration in the unknown (wine) is the standard addition plot described in detail in Appendix 4B.

Briefly, starting with 4000 µL of phosphate buffer, determine the average initial current (i_0) upon stabilization. Next, determine the average stabilized current after addition of the wine aliquot (i_{wine}), as well as the average stabilized current after each standard phenol aliquot addition (i_1, i_2, i_3, etc.). Calculate corrected current ($i - i_0$) and create a *standard addition* plot: current vs. final diluted concentration of standard phenol in the cell (total moles/total volume), as you did for the phenol standard curve. Note that due to i_{wine}, you will have a distinct non-zero intercept. Fit the

points to a linear trendline, and from the Excel regression statistical analysis, obtain the standard error of the regression (S_r), and the slope and intercept with their standard errors.

For the standard addition plot above, the y-intercept represents the electrode response due to the wine, and the slope is the electrode sensitivity to the standard phenol aliquots. Thus the concentration of oxidizable phenolics in each wine titration (M_f) is calculated from the intercept (in μA) divided by the slope (in μA/μM). The uncertainty in this concentration is calculated from the following equation:

$$uncerty = \frac{S_r}{m}\sqrt{\frac{1}{1}+\frac{1}{n}+\frac{(b-i_{avg})^2}{m^2\sum_{i=0}^{n}(c_i-c_{avg})^2}}$$

where S_r = standard error of the regression;

m = slope; b = y-intercept; n = number of points in the line, including the i_{wine};

c_i = [standard phenol] at each point i; c_{avg} = average of [standard phenol] for all n points;

i_{avg} = average of current for all n points

To calculate the concentration of oxidizable phenols in each *original* wine sample, you must correct for the dilution effect. The dilution factor for a titration with a 50 μL wine sample addition would be = V_f/V_i = 4050 μL/50.0 μL = 81.0. The concentration of phenolics in the original wine sample would then be the concentration calculated above (M_f) times the dilution factor (81.0). Likewise, the uncertainty in this concentration is calculated from the above uncertainty multiplied by the same dilution factor.

16. *Select one of your wine titrations, and provide a reference to the exact pages in your laboratory notebook where sample calculations can be found. Calculations of interest for your chosen wine titration will include: [phenol] in cell solution (M_f), uncertainty in M_f, dilution factor, [phenol] in undiluted wine.*

17. *Report the conc. of oxidizable phenols ± uncertainty for each <u>undiluted</u> wine sample.*

18. *From your replicate wine samples, calculate the average and standard deviation of the*

concentration of oxidizable phenols.

19. *How does your average [phenols] in wine compare to literature values*[12] *obtained by enzyme-linked biosensor? Note that most literature reports, including Vinson and Hontz (1995), use the Folin-Ciocalteau method for assaying phenolics; because this method responds to many redox-active analytes besides phenolics, it is less selective than the biosensor. Is Carralero-Sanz et al (2005) a better literature source for your comparison?*

20. *Provide a Summary paragraph as described in the introductory section of the lab manual, "Writing in the Chemistry Laboratory: The Informal Report."*

FURTHER READING:

"A bioanalytical chemistry experiment for undergraduate students: Biosensors based on metal nanoparticles"
J. Njagi, J. Warner, and S. Andreescu (2007) *J. Chem. Educ.* **84***,* 1180-1182.

"Development of a tyrosinase biosensor based on gold nanoparticles-modified glassy carbon electrodes Application to the measurement of a bioelectrochemical polyphenols index in wines"
V. Carralero Sanz, M. Luz Mena, A. Gonzalez-Corte, P. Yanez-Sedeno, J.M. Pingarron (2006) *Anal. Chim. Acta* **528,** 1-8

"Phenol Antioxidant Index: Comparative Antioxidant Effectiveness of Red and White Wines"
J. A. Vinson and B. A. Hontz (1995) *J. Agric. Food Chem.* **43,** 401-403

"Development of Tyrosinase-Based Biosensor and Its Application for Monitoring of Bioremediation of Phenol and Phenolic Compounds"
J. SVITEL, S. MIERTUS (1998) *Env. Sci. Technol.* **32,** 828-832.

"Automatic Method for the Determination of Folin−Ciocalteu Reducing Capacity in Food Products"
L. M. MAGALHAES, M. A. SEGUNDO, S. REIS, J. L. F. C. LIMA, A.O. S. S. RANGEL (2006) *J. Agric. Food Chem.* **54,** 5241-5246.

"Volatile Phenol Determination in Wine"

[12] In addition to a Google search, you may start with these two articles:
Carralero-Sanz, V. et al "Development of a tyrosinase biosensor based on gold nanoparticles-modified glassy carbon electrodes: Application to the measurement of a bioelectrochemical polyphenols index in wines" *Anal. Chim. Acta*, 2005, *528,* 1-8.
Vinson, J.A. and Hontz, B.A. "Phenol antioxidant index: Comparative antioxidant effectiveness of red and white wines" *J. Agric. Food Chem,* 1995, *43,* 401-403.

P. X. Etievant (1981) *J. Agric. Food Chem.* **29,** 65-67.

APPENDIX 1: USE OF THE BAS ELECTROCHEMICAL INSTRUMENT

Electrode Stripping: Make sure that the BAS electrochemical "CGME" control tower is on (switch: rear middle right), as well as the Epsilon power tower (switch: rear bottom right). On the Dell WU# 9861 desktop screen, double click on the Epsilon EC-20 icon. The subsequent "CS Link Dialog" box should eventually register a successful connection between the computer and the BAS system.

Under "Experiment/Select New Experiment", select "DC Potential Amperometry (DCPA)", the 5th selection, under "Potentiostat". Input the following electrochemical parameters: applied potential: +100 mV; sample interval: 1 sec; time limit: 60 sec; full scale: 1 μA. Click "Apply" and "Run". After the 60 s oxidative stripping run, you can expand the amperogram to view the oxidative current more clearly, if necessary: Select "Graph Display/Manual Zoom" and input appropriate smaller values for "Y upper" and "Y lower", then "Apply" and "Exit".

Ferricyanide CV scan: Under "Experiment/Select New Experiment", select "Cyclic Voltammetry (CV)", the first selection under "Potentiostat". Set the scanning potential range between -200 mV and 700 mV: initial potential = -200 mV; switching potential = +700 mV; final potential = -200 mV. Also set # segments =2, scan rate = 50 mV/s, and full scale = 100 μA; click "Apply".

APPENDIX 2: DATA ANALYSIS – CONVERTING DCPA.DAT FILES TO EXCEL.XLS FILES

If the DCPA file is greyed out under Excel's "Open" menu item, then select "Enable: All Documents." Open the DCPA.dat file, and in the first "Text Import Wizard" box, select "Delimited" and "Next". In the next "Text Import Wizard" box, select "Tab" and "Commas" as Delimiters, and click "Next". In the last "Text Import Wizard" box, the "Column Data Format" should be "General"; click "Finished". This will give you an Excel spreadsheet with the time (in sec) of each measurement in column A (it might be easier to change this numerical format to number, no decimals: Format/Number/Category: Number; Decimal Places: 0/OK); in column B you have the current measurement at each time, in amps. In column C, convert negative current values in amps to positive values in μA.

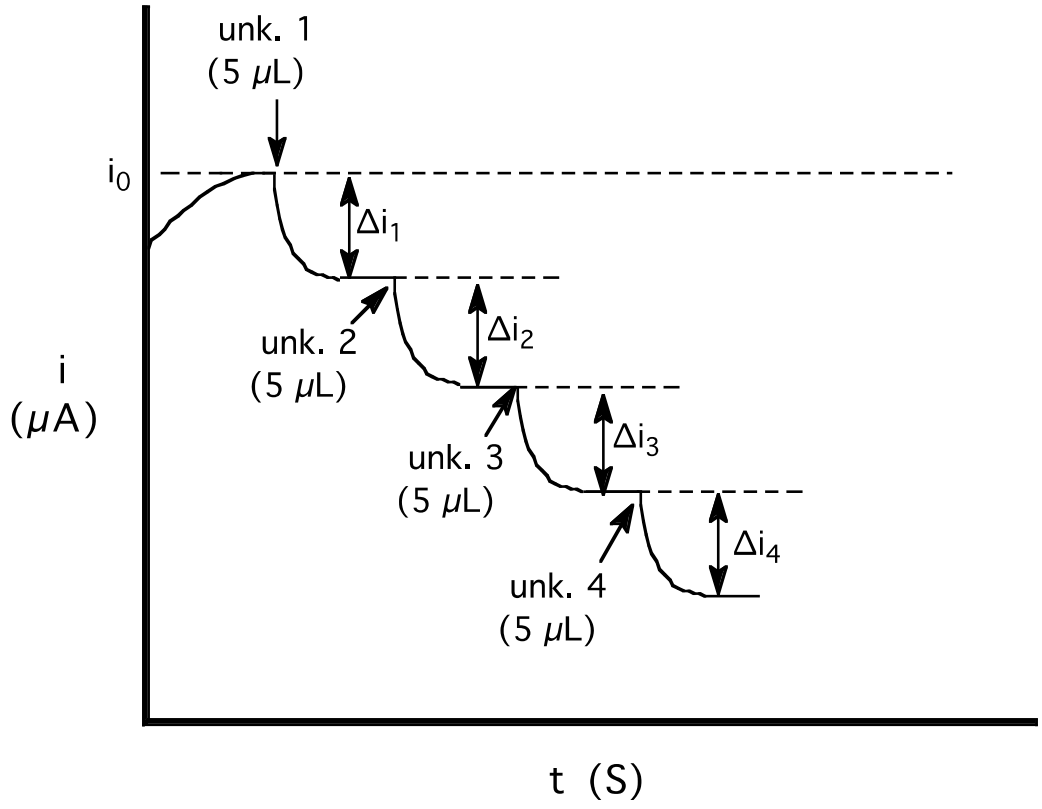
APPENDIX 3: DATA ANALYSIS – ANOVA AND PAIR-WISE *t*-TESTS

When doing quantitative experiments, there is uncertainty due to random error in the measurement, as well as differences caused by changes in experimental conditions. If one has only two data sets, e.g., control vs. experimental samples, then a t-test is used to determine whether any difference between the two data sets is statistically significant. Consider unknown phenol concentration results for electrode #1: 0.265, 0.247, 0.252, and 0.271 μM, as opposed to electrode #2: 0.266, 0.244, 0.275, 0.281 μM. The average for electrode #2, 0.267 ± 0.016 μM, is higher than that for electrode #1, 0.259 ± 0.011 μM; however, the data sets clearly overlap, and the difference is less than the uncertainty. Using a t-test to compare these two data sets (Excel/Tools/Data Analysis/"t-test assuming equal variances"), gives P(two-tailed)[13] = 0.46. This signifies that the probability is 46% that the 0.008 μM difference between these two average slopes is due to random error; this probability is considered to be quite high. Generally, two data sets are not deemed to be statistically different unless $P < 0.05$.

If one wishes to compare more than two data sets simultaneously, then ANalysis Of VAriance (ANOVA) is used. For example, consider concentration results for electrode #3: 0.255, 0.241, 0.259, and 0.270 μM, in addition to electrode #4: 0.296, 0.284, 0.295, 0.281 μM. The average for electrode #4, 0.289 ± 0.008 μM, is higher than that for electrode #3, 0.256 ± 0.012 μM; the difference is about three standard deviations, hence it seems significant. One can test this directly by using Excel's "ANOVA: single factor" analysis. For this data set comprising all four electrodes, each with four measurements, the F-factor of 6.035 is greater than the F-critical of 3.49, hence at least one of the electrodes is significantly different from the others; this conclusion is also corroborated by the low P-value of 0.0095. To identify electrode #4 unambiguously as the outlier, you may do an ANOVA analysis on the other three electrodes, and/or you may do a *t*-test on electrode #4 as compared to the entire data set for all three other electrodes.

[13] Use the two-tailed P value if there is no theoretical reason to expect data set #1 to be systematically higher (or lower) than data set #2. Use the one-tailed P value if there is a theoretical reason to expect data set #1 to be systematically higher (or lower) than data set #2.

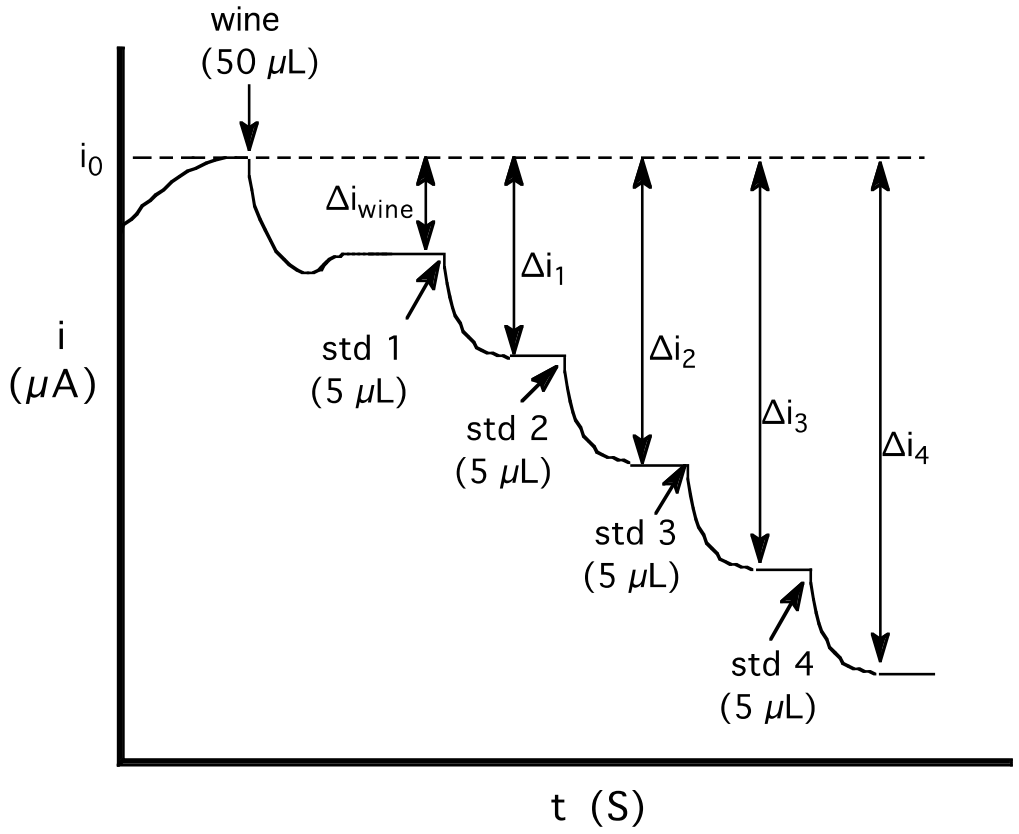
APPENDIX 4A: DATA ANALYSIS—UNKNOWN PHENOL SOLUTION



Perform the following series of calculations to determine the [phenol] in the unknown solution:

1. *Calculate the average initial current (i_0), as well as the average current after each addition (i_1, i_2, i_3, etc.). These are depicted as dotted lines in the figure above.*
2. *Next calculate the current response, Δi, to each aliquot ($\Delta i_x = i_x - i_{x-1}$).*
3. *Divide each replicate electrode response, Δi (μA), by the average sensitivity of your electrode (μA/μM) to convert each of these responses to a concentration change (μM).*
4. *Calculate moles of phenol added to the cell: Multiply each replicate concentration change by the total volume of solution in the cell after each addition (e.g., 4005, 4010, 4015 μL).*
5. *Calculate replicate values for the phenol concentration in the unknown solution: Divide the moles added by the aliquot volume (generally, 5.0 μL).*
6. *Use the replicate phenol concentration values to calculate the average and standard deviation for the concentration of phenol in the unknown solution.*
7. *Do the same for each titration of the unknown phenol solution.*

APPENDIX 4B: DATA ANALYSIS—PHENOLICS IN WINE



1. *Starting with 4000 μL of phosphate buffer, determine the average initial current (i_0) after stabilization; this corresponds to the dotted line in the figure above. Determine the standard deviation of this average value.*
2. *Similarly, determine the average stabilized current after addition of the wine aliquot (i_{wine}), and the average stabilized current after each standard phenol aliquot addition (i_1, i_2, i_3,...). For each average value, determine the standard deviation.*
3. *Calculate the corrected current ($i - i_0$) for each aliquot. These values correspond to the Δi_{wine}, Δi_1, Δi_2, etc. values in the figure above.*
4. *Calculate the moles of phenol added with each aliquot of standard phenol, e.g., 5.00 μL × 0.97 mM = 4.85 nmol. Note that this value is zero for the wine aliquot.*
5. *Calculate the total moles of phenol in the cell after each aliquot of standard phenol.*
6. *Calculate the total solution volume in the cell after each aliquot addition, e.g., 4050 μL after the wine aliquot, 4055 μL after the first standard phenol addition, etc.*

7. *Calculate the total concentration of standard phenol in the cell after each aliquot addition, e.g., 4.85 nmol/4055 µL = 1.2 µM.*
8. *Create a standard addition plot: corrected current vs. final diluted concentration of standard phenol in the cell. Note that due to i_{wine}, you will have a distinct non-zero intercept.*
9. *Fit the points to a linear trendline, and from the Excel regression statistical analysis, obtain: the standard error of the regression (S_r); the slope and intercept with their standard errors.*
10. *Calculate the concentration of oxidizable phenols in each wine titration (M_f) = the intercept (in µA) divided by the slope (in µA/µM).*
11. *Calculate the uncertainty in this concentration from the following equation:*

$$uncerty = \frac{S_r}{m}\sqrt{\frac{1}{1}+\frac{1}{n}+\frac{(b-i_{avg})^2}{m^2\sum_{i=0}^{n}(c_i-c_{avg})^2}}$$

where S_r = standard error of the regression; m = slope; b = y-intercept;
n = number of points in the line, including the i_{wine} point;
c_i = [standard phenol] at each point i; c_{avg} = average of [standard phenol] for all n points;
i_{avg} = average of current for all n points

12. *Calculate the wine sample dilution factor, V_f/V_i: for a titration with a 50 µL wine sample addition, V_f/V_i = 4050 µL/50.0 µL = 81.0.*
13. *Calculate the concentration of oxidizable phenols in each original wine sample: phenols concentration calculated above (M_f) times the dilution factor (81.0).*
14. *Calculate the uncertainty in this concentration: the above uncertainty (step 10) multiplied by the same dilution factor.*