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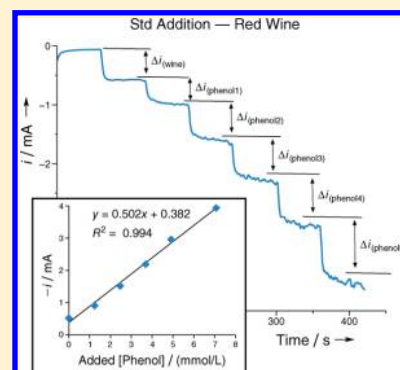
# Using the Tyrosinase-Based Biosensor To Determine the Concentration of Phenolics in Wine

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**S** Supporting Information

**ABSTRACT:** A tyrosinase-linked biosensor project described previously in this *Journal* has been expanded for upper-division undergraduate instrumentation courses; students use the biosensor that they fabricate to measure the concentration of phenolics in wine. The original project involved the use of a calibration curve and exposed students to the instruments and principles of electrochemical measurements. This extension, performed in three, 3-h laboratory periods, addresses three further topics of discussion: matrix effects in chemical analysis, the biochemical significance of antioxidants, and the use of statistical analysis techniques, such as the Student's *t* test and ANOVA.



**KEYWORDS:** Upper-Division Undergraduate, Analytical Chemistry, Biochemistry, Laboratory Instruction, Oxidation/Reduction, Instrumental Methods, Potentiometry, Electrochemistry, Phenols, Enzymes

Recently, Njagi et al.<sup>1</sup> described an excellent enzyme-linked biosensor experiment that was the basis for a project in advanced chemistry and advanced biochemistry laboratories. Here, a further<sup>2</sup> extension of the experiment is described in which students use the biosensor that they fabricate to measure the concentration of phenolics in wine, an application first described by Carralero Sanz et al.<sup>3</sup> Having students analyze wine opens up two key topics of discussion, namely, the importance of matrix effects in chemical analysis and the biochemical significance of antioxidants.

Oxidants, especially reactive oxygen species such as peroxide, superoxide, and hydroxyl radicals, cause oxidative damage in living cells by oxidizing all four major types of biomolecules: proteins, nucleotides, lipids, and carbohydrates.<sup>4,5</sup> This damage is believed to figure prominently in many diseases, including cancer, diabetes, cardiovascular disease, Alzheimer's, and scurvy, in addition to the "normal" aging process. Antioxidants, such as ascorbic acid (vitamin C), quinols (e.g., vitamins E and K, coenzyme Q), and phenolics, are essentially avid reducing agents, donating electrons to reduce damaging reactive oxygen species to water.<sup>5,6</sup> For this reason, the high phenolic content of red wines is believed to be protective against the diseases of old age.

## PROJECT GOALS AND LEARNING OUTCOMES

The goals of this adaptation are to incorporate an electrochemical calibration curve, matrix effects, statistical analysis including Student's *t* test and ANOVA, and the biochemical significance of antioxidants. The learning objective of the first laboratory period is for students to reductively plate gold nanoparticles onto a glassy carbon electrode (GCE) and use

this modified Au-GCE for cyclic-voltammetry on ferricyanide; students then cross-link tyrosinase (TYR) onto the Au-GCE. In a second period, students employ direct current potential amperometry to follow three titrations with a standard phenol, an unknown phenol, and wine. Using their calibration curve with standard phenol, students determine the sensitivity, detection limit, and linear range of the TYR-Au-GCE biosensor, as well as the concentration of an unknown phenol solution. In a third period, students carry out statistical analyses using Student's *t* test and ANOVA on concentration results for all class electrodes. Finally, students examine matrix effects by using the standard addition method to analyze a wine sample. Students compare their results to literature values for the TYR-Au-GCE biosensor, as well as the standard Folin-Ciocalteu method, and discuss the biochemical significance of oxidative degradation and antioxidants. Electrochemical techniques are important in analytical chemistry and biochemistry; thus, this project is an excellent way to expose students to advanced aspects of data analysis, statistical analysis, and redox chemistry applied to a biochemical system.

## EXPERIMENTAL OVERVIEW

This laboratory experiment takes three, 3-h laboratory periods; students work in pairs to fabricate and characterize the biosensor electrode in one-half of a 3-h lab period. The following week they take an entire 3-h period to perform titrations with a standard phenol, an unknown phenol, and

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wine. A final 3-h lab period is used to perform their data analysis.

## ■ ANALYSIS OF PHENOL AND POLYPHENOL IN WINE

Wine is a mixture of many solutes, and this complex matrix can dramatically alter the response of analytical methods applied to the solution. When a calibration curve cannot be made that mimics the complex matrix of a sample, the method of standard additions is appropriate to use. Experience with the tyrosinase-based biosensor confirms that the standard addition method is required, as the slope of the pure phenol calibration curve can differ by 2- to 3-fold from the slope of a wine–standard addition line. To assay a sample of wine, an aliquot of the beverage is added to an electrochemical cell in which the amperometric current is measured at a constant reducing potential (e.g.,  $-0.15$  V); after the current stabilizes, a number of aliquots of standard phenol solution of known concentration are added to the cell and the current measured. Replicate titrations of wine ( $\geq 4$ ) are done.

## ■ DATA ANALYSIS

The standard addition plot (Supporting Information, Project Information and Background, Figure S1) is generated from the amperometric data, the known concentration of the phenol standard, and the volumes of all solutions used. The concentration of antioxidants in the wine, given as the phenol–“equivalent” concentration [PE], is calculated from the slope and intercept of the best-fit line and the known volume of the wine aliquot. Details of the procedure are given in the Supporting Information.

## ■ 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; it is toxic by inhalation and ingestion and is a skin sensitizer; hence, 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 premade aqueous solutions of these two compounds. In case of skin contact, wash thoroughly with large volumes of water.

## ■ RESULTS

The tyrosinase-based biosensor responds to phenolic compounds that bind productively to the enzyme. Caffeic acid and gallic acid are a significant fraction of the phenolics found in wine<sup>7</sup> and tea,<sup>8</sup> and are often used as standards in measuring phenolics.<sup>3,9</sup> However, caffeic acid and gallic acid are no more representative of all phenolics than phenol itself, and therefore, phenol was used as the standard<sup>1</sup> for measuring phenolics in this experiment. Furthermore, the Tyr–Au–GCE biosensor is orders of magnitude more sensitive to phenol ( $0.1$ – $1$  A/M) than it is to gallic acid ( $\approx 0.0002$  A/M) or caffeic acid ( $0.01$  A/M).<sup>3</sup> Because of this, the phenolics concentration is expressed as [PE] and may well be an underestimation of the total phenolics concentration; this is discussed further in the Supporting Information. On the other hand, the standard Folin–Ciocalteu method measures the total concentration of reductants (i.e., antioxidants) that react with heteropolyphosphotungstate–molybdate ( $\text{Mo}^{\text{VI}}\text{O}_3 \cdot (\text{W}^{\text{VI}}\text{O}_3)_{11} \cdot \text{PO}_4^{3-}$ ) as oxi-

dizing agent; upon reduction, this complex forms a “blue oxide” (e.g.,  $\text{Mo}^{\text{VO}}\text{O}_3 \cdot \text{H} \cdot (\text{W}^{\text{VI}}\text{O}_3)_{11} \cdot \text{PO}_4^{3-}$ ) with  $\lambda_{\text{max}} \approx 750$  nm.<sup>10</sup> It has been known for decades that this significantly overestimates phenolics because the reagent oxidizes many functional groups besides phenolics.<sup>9</sup> Carralero Sanz et al.<sup>3</sup> confirmed a linear relationship ( $R^2 = 0.98$ ) between phenol concentrations determined by the two methods, with Folin–Ciocalteu more responsive by a factor of 55.5; on the other hand, the Folin–Ciocalteu method has a detection limit of  $70$   $\mu\text{M}$ ,<sup>3</sup> whereas the biosensor’s detection limit is over 2 orders of magnitude lower at  $0.06$ – $0.21$   $\mu\text{M}$  (see Supporting Information, Project Information and Background, Table S1).

The experiment has been run in upper-division Experimental Biochemistry and Experimental Chemistry instrumental courses with approximately 60 students in 10 sections (2–4 pairs of students per section) over five years; of approximately 30 biosensors prepared, only 2–3 failed, all due to using tyrosinase stock solutions frozen for a year or more. Biosensor results from the five most recent laboratory sections (2–4 pairs of students per section, see Supporting Information), using two different vintages of Oregon Pinot Noir, yielded [PE] =  $0.19 \pm 0.13$  and  $0.28 \pm 0.05$  mM (range:  $0.07$ – $0.42$  mM). Caffeic acid was used by Carralero Sanz et al.<sup>3</sup> as a standard; multiplying [PE] (mol phenol-equivalents/L) by the molecular weight of caffeic acid (180.16) converted [PE] to units of mass of caffeic acid equivalents (CAE) per L. The results for the two vintages of wine converted to  $34 \pm 23$  and  $50 \pm 9$  mg CAE/L and matched the range of red wine results from Carralero Sanz et al.,<sup>3</sup> 35–50 mg CAE/L.

Using the linear relationship confirmed by Carralero Sanz et al.<sup>3</sup> between the biosensor and Folin–Ciocalteu methods, our laboratory results corresponded to Folin–Ciocalteu antioxidant capacity [AC] of  $8 \pm 5$  and  $11.7 \pm 2.0$  mM (range:  $3$ – $17$  mM). This compared quite well with literature values<sup>3,11,12</sup> for red wines of [AC] =  $12 \pm 4$  mM (range:  $6$ – $17$  mM). The 40-fold difference between [PE] and [AC] here was due to the fact that, as mentioned above, the biosensor probably underestimated, whereas Folin–Ciocalteu overestimated, phenolics content. It was therefore important to make students aware that results from the two methods were not directly comparable. This highlighted the importance of the published work of Carralero Sanz et al.,<sup>3</sup> who demonstrated a linear relationship between results from the two methods. Literature comparisons showed that, compared to red wine, white wine had about 10 times less antioxidant capacity: [AC] =  $1.3 \pm 0.5$  mM (range,  $0.6$ – $1.8$  mM); this difference may explain why health benefits seem to accrue only to red wine.

Although this project was aimed at upper-division laboratories, there may be some situations in which wine is difficult to obtain. Grape juice has not been tried as an analyte, but it should work. Tea is also known to be high in phenolics.<sup>8</sup>

Finally, students used the results from this experiment to gain further experience with three different statistical comparisons and arguments: (a) the use of propagation of error equations to calculate the error when two experimental values, each with its own associated error, were subtracted (or added), as opposed to divided (or multiplied); (b) the use of *P* values obtained from the *t* test to determine whether differences between wines were statistically significant; and (c) the use of ANOVA on replicate phenol-equivalent concentrations obtained from multiple electrodes to determine if any of the electrodes were outliers (see the Supporting Information).

## ■ CONCLUSION

The tyrosinase-based biosensor experiment allowed students to explore concepts in instrumental analysis, analytical chemistry, electrochemistry, biochemistry, and statistical analysis, and the goals and learning objectives enumerated above were achieved by this project. Furthermore, it fit in perfectly with an upper-division unified laboratory that combines methods from instrumental analysis and analytical chemistry with projects derived from upper-division courses, such as biochemistry.<sup>13–18</sup>

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

(1) Instructor Notes includes hazards, and a reference to Njagi et al.'s original notes to instructors; (2) Student Handout is the entire laboratory manual for this biosensor project; and (3) Project Information/Background includes a plot of typical student results for the wine titration, as well as background on the project including goals, student learning objectives, history of the project, and a summary of student results from five laboratory sections over two years (2012–2013). This material is available via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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