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Laboratory Experiment

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Bioelectroanalysis in a Drop: Construction of a Glucose Biosensor

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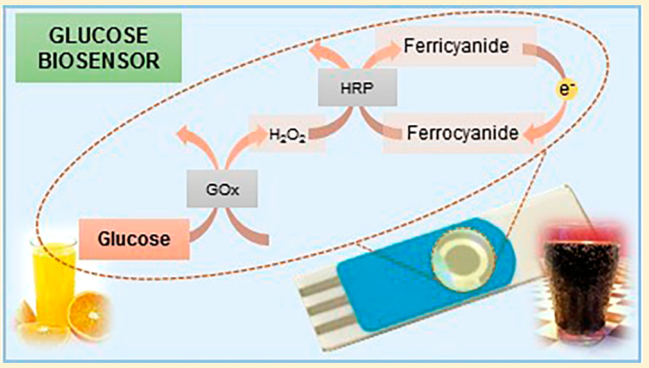
Supporting Information

ABSTRACT: This lab experiment describes a complete method to fabricate an enzymatic glucose electroanalytical biosensor by students. Using miniaturized and disposable screen-printed electrodes (SPEs), students learn how to use them as transducers and understand the importance SPEs have acquired in sensor development during the last years. Students can also revise concepts related to enzymatic assays, with glucose oxidase and horseradish peroxidase involved in subsequent reactions. Moreover, they learn the trends that current analytical chemistry follows presently such as miniaturization, portability, and low cost. At the same time, this experiment serves to teach basic analytical concepts (accuracy, precision, sensitivity, and selectivity) in a practical way. The high clinical interest of glucose, due to a large number of diabetes patients around the world, and the application of the sensor to analysis of real food samples make this experiment very attractive to students. The questions set out along this experiment help students to acquire skills for solving analytical problems from the very beginning.



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Miniaturization is presently one of the most important trends in analytical chemistry. The reduction of the size of analytical systems[1] involves their simplification as well as a decrease in costs, reagents, and sample volume. Furthermore, it is related to many principles of Green Analytical Chemistry.[2] Electrochemical detection closely connects with these aims because of its inherent ease of miniaturization. Moreover, an improvement in productivity-related properties such as analysis time or cost as well as in others related to environmental benefits like waste production or energy consumption is very advantageous. Other basic analytical properties (e.g., accuracy, precision, sensitivity, and selectivity) are generally not compromised since electrochemical analysis is among the most sensitive detection techniques (as demonstrated by its leading use in biosensing)[3] and mass production increases the precision of disposable devices.

During the past few years, screen-printing technology has been increasingly used in the fabrication of low-cost thick-film electrodes with small size and good analytical characteristics. During the last years, they have been the basis of many biosensors[4−6] because of their low cost and simplicity. Another advantage of screen-printed electrodes (SPEs) is the possibility of doing in situ analysis.[7] Apart from the electrodes, electrochemical equipment (potentiostats) is also being miniaturized.

The aim of this experiment is to build an enzymatic electrochemical biosensor to measure glucose in real food samples. Biosensing is a field that is growing continuously, as demonstrated by the leading place of the journal *Biosensors & Bioelectronics*.[8] Glucose is probably one of the most important biological compounds because of its engagement in a multitude of reactions.[9] Glucose analysis in blood is very important and common because of diabetes mellitus, a disease that is suffered by approximately 150 million people around the world.[10,11] This disease is produced when the pancreas does not generate enough insulin or when the body cannot use the insulin it produces in an effective way. This leads to an increased level of glucose in the blood. Thus, determination of glucose is one of the most important analytical problems in food science and clinical analysis, so much so that glucose biosensors account for approximately 85% of the entire biosensor market.[12,13]

In this experiment, the students develop an amperometric glucose sensor using the bienzymatic system glucose oxidase (GOx)/horseradish peroxidase (HRP) and ferrocyanide as an electron-transfer mediator.[14,15] Moreover, since the sensor is fabricated using screen-printed carbon electrodes (SPCEs), students are introduced to the miniaturization of analytical devices.

The fabrication of this glucose sensor is based on a very simple procedure reported by our research group,[14,16,17] and it is addressed to undergraduate students of advanced analytical chemistry. The high educational content related to biosensor principles and new contemporary trends in analytical chemistry

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also makes this experiment very attractive for the training of chemistry, biotechnology, or biochemistry students.

There are many laboratory experiments about detection of glucose using the enzyme GOx, but to the best of our knowledge this is the first undergraduate lab experiment that uses screen-printed electrodes to develop a glucose sensor. Moreover, the combination of a simple procedure with the use of SPCEs eliminates time-consuming maintenance of conventional electrodes commonly required with other reported sensors.[18−20]

This laboratory experiment is very useful to introduce students to both electrochemical and biosensor methodologies and provides students with several objectives:

- Learn the principles of important electrochemical techniques such as cyclic voltammetry and chronoamperometry.
- Use low-cost, disposable, and miniaturized electrodes.
- Fabricate a glucose biosensor, optimize the parameters influencing the analytical signal, and study the analytical characteristics of the methodology.
- Analyze real food samples and learn how to validate the methodology.

EXPERIMENTAL SECTION

This lab experiment is designed for a maximum of 15 undergraduate or Master's students working in groups of three during four sessions of 4 h (the lab experiment planning is more detailed in the Supporting Information). The laboratory experiment consists of the following steps:

(i) Evaluation of the ferro/ferricyanide system using cyclic voltammetry to set the detection potential.
(ii) Optimization of the concentrations of enzymes and mediator.
(iii) Calibration of the biosensor and evaluation of the sensitivity.
(iv) Study of the precision.
(v) Evaluation of the selectivity.
(vi) Determination of glucose in real food samples.

Instrumentation

Electrochemical measurements were carried out with a μAUTOLAB potentiostat (Metrohm, Switzerland) interfaced with a computer system and controlled by Autolab GPES 4.9 software. Commercial screen-printed carbon electrodes (ref. DRP-110; Figure 1) and the connector to the potentiostat (ref. DRP-DSC) were purchased from DropSens (Spain). More information on SPEs can be found in the student handout in the Supporting Information.

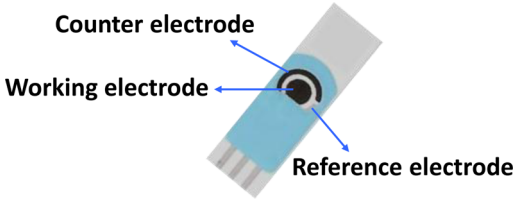


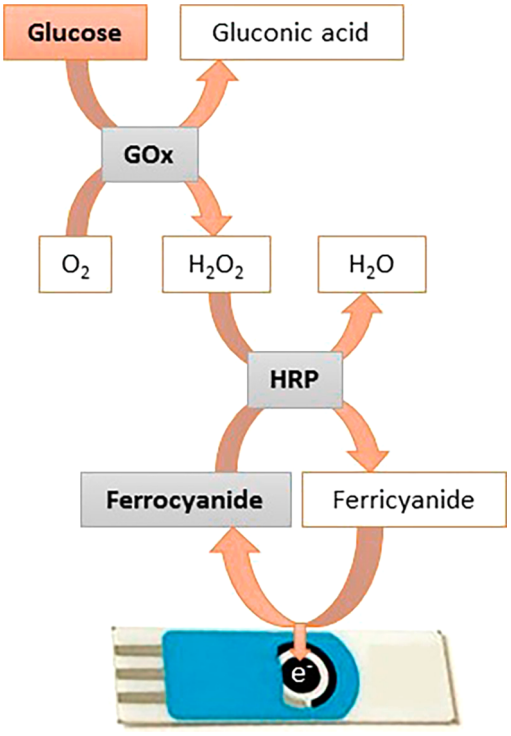
Figure 1. Picture of a screen-printed carbon electrode.

Sensor Phase

The biosensor constructed in this experiment has a bienzymatic sensor phase consisting of glucose oxidase and horseradish peroxidase, with ferrocyanide as an electron-transfer mediator. The combination of these enzymes produces an enzymatic cascade of reactions in which GOx and HRP are catalytically linked.[21] These types of cascade schemes may produce signal amplification and therefore enhance the sensitivity of the biosensor. Another advantage is that by removal of the hydrogen peroxide (H_2O_2) generated, peroxide-induced degradation of the GOx enzyme could be reduced.[22] On the other hand, redox mediators are frequently employed in bienzymatic sensors because of their lower detection potentials.[10,23,24] This is very interesting since it improves the selectivity: at lower potentials (in absolute value), fewer compounds present in the sample are exposed to being oxidized or reduced.

In the proposed enzymatic cycle, GOx catalyzes the oxidation of glucose by oxygen, generating gluconic acid and H_2O_2. Then HRP catalyzes the oxidation of ferrocyanide to ferricyanide, consuming the H_2O_2 previously generated. The analytical signal is the current intensity due to the electrochemical reduction of the enzymatically generated ferricyanide. Scheme 1 shows the reactions involved. Since glucose produces hydrogen peroxide stoichiometrically, and this in turn produces ferricyanide, the concentration of glucose in the measuring solution can be calculated by measuring the concentration of reduced ferricyanide.

Scheme 1. Diagram of the Catalytic Reactions and the Reduction of Ferricyanide Produced on the Electrode Surface, Where GOx, HRP, and Ferrocyanide Are Immobilized[a]



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Procedure

Students prepare the biosensors by depositing onto the surface of the working electrode 10 μL of a mixture containing the enzymes and the mediator at the adequate concentrations,

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prepared in a 0.1 M Tris-HNO_3 buffer (pH 7.0). Then, after a drying step at room temperature (approximately 40−60 min), the sensors are ready to use. If they are going to be employed in the following days or weeks, they must be kept protected from light and at 4 °C.

All of the measurements are carried out at room temperature with all three electrodes of the SPCE (working, counter, and pseudoreference) covered with 40 μL of the measuring solution.

The analytical signal is the current intensity measured after recording a chronoamperogram (current vs time) at a potential of −0.1 V vs Ag pseudoreference electrode for 100 s (Figure 2).

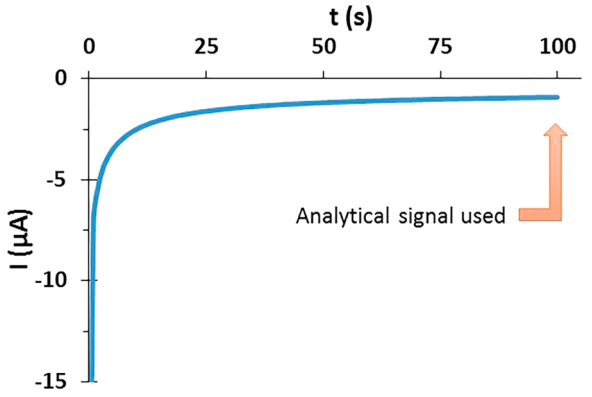

Figure 2. Chronoamperogram obtained at −0.1 V vs Ag pseudoreference electrode, recorded in a 0.5 mM glucose solution with 1.6 units/μL GOx, 2.5 units/μL HRP, and 0.05 M ferrocyanide (in 0.1 M Tris-HNO_3 buffer (pH 7.0)) immobilized on the working electrode (10 μL).

A negative current due to the reduction of ferricyanide is obtained, as shown in Figure 2. SPCEs are considered as disposable, and a different sensor is used for each measurement.

HAZARDS

Nitric acid, used for the preparation of the Tris-HNO_3 buffer, is corrosive and causes serious burns, so it must be handled with appropriate gloves, safety glasses, and protective clothing. The main hazard of potassium ferrocyanide is that it releases a very toxic gas when it is in contact with acids.

RESULTS

Electrochemical Behavior of Ferrocyanide

After learning about the cascade of enzymatic reactions, students knew that they had to measure the current intensity due to the reduction of ferricyanide. Thus, a potential for ferricyanide reduction had to be applied. Then students investigated the process of the ferro/ferricyanide system, recording a cyclic voltammogram (CV) (one CV can be recorded by each group) in a drop of 0.01 M ferrocyanide solution from −0.2 to 0.8 V at a scan rate of 50 mV/s (Figure 3). Previously, a CV was recorded in the background electrolyte to confirm that there was no interference in the potential window scanned. Ferrocyanide shows an electrochemical process according to the following reaction:

$$[Fe(CN)_6]^{4-} \rightleftarrows [Fe(CN)_6]^{3-} + e^-$$

Looking at the voltammogram, students discussed the reversibility of the process. In this case, the system was

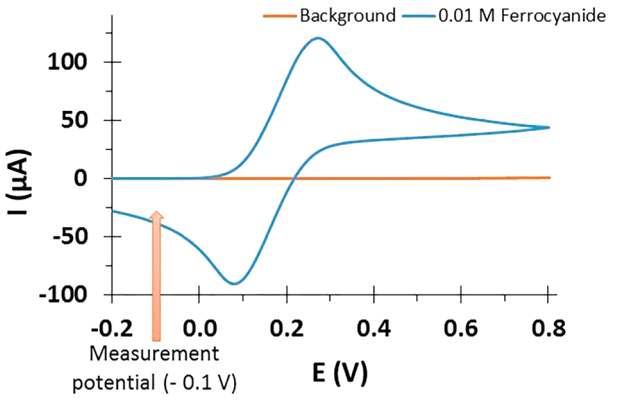

Figure 3. Cyclic voltammogram for 0.1 M Tris-HNO_3 buffer solution (pH 7.0) (background, orange) and for 0.01 M ferrocyanide (blue).

considered to be quasireversible since the difference in potentials is 183 mV and the ratio of peak currents is 1.1.[25]

To set the adequate potential for recording the chronoamperograms used for glucose determination, students looked at the process recorded in the CV for the ferrocyanide solution (Figure 3) and chose the potential they considered better (−0.1 V in this case). They argued that at this potential ferricyanide can be reduced to obtain the initial product, ferrocyanide, which is maintained as a complex of Fe(II) on the electrode, and therefore, all of the current intensity comes from the reduction of the ferricyanide (Fe(III)) enzymatically produced. Thus, higher glucose concentrations produce higher current intensities (in absolute value).

Optimization of the Enzyme Concentrations

Students had to know that introducing an analytical methodology requires that once the analytical signal has been identified, the different variables involved must be optimized before the analytical properties (sensitivity, precision, etc.) can be studied. Then students were requested to identify which were the different variables that can influence the signal. After discussion, variables such as pH, electrolyte, and enzyme and mediator concentrations were mentioned. The instructor explained that a 0.1 M Tris-HNO_3 buffer (pH 7.0) was chosen since it was used for similar reported glucose sensors.[14,26] Thus, the enzyme and ferrocyanide concentrations were identified as relevant variables that should be optimized. With this aim, students prepared sensors with various concentrations of the enzymes, using 10 μL of mixtures with different concentrations of the enzymes and a constant concentration of ferrocyanide. For each mixture, chronoamperograms were recorded in the background electrolyte (buffer solution) and in a 0.4 mM glucose solution.

Figure 4 shows results the students obtained for the different concentrations of enzymes studied. It can be noted that the intensities for glucose solutions were very similar for all of the mixtures, whereas the intensities for the background increased with the concentration of HRP, with the lowest obtained for 2.5 units/μL HRP. For this concentration of HRP, the lowest background was obtained for 1.6 units/μL GOx. Therefore, students chose those enzyme concentrations for the construction of the biosensor.

Optimization of the Ferrocyanide Concentration

The following step was the optimization of the concentration of ferrocyanide. Different concentrations of the electron-transfer mediator (0.05, 0.1, and 0.2 M) were studied using the enzyme

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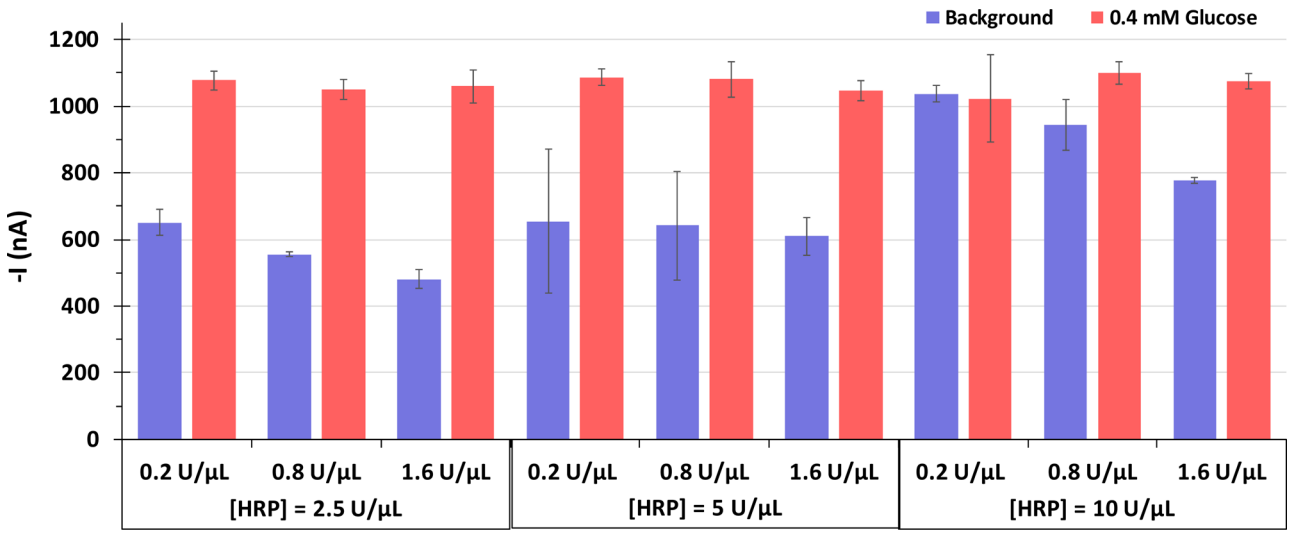

Figure 4. Current intensities recorded at −0.1 V vs Ag pseudoreference electrode after 100 s in buffer solution (background, blue) and a 0.4 mM glucose solution (red). [HRP] = 2.5, 5, or 10 units/μL; [GOx] = 0.2, 0.8, or 1.6 units/μL; [ferrocyanide] = 0.1 M. Data are given as mean ± standard deviation (SD) (n = 3).

concentrations optimized in the previous section (1.6 units/μL GOx and 2.5 units/μL HRP).

Figure 5 presents the current intensities obtained by the students using different concentrations of ferrocyanide. As can

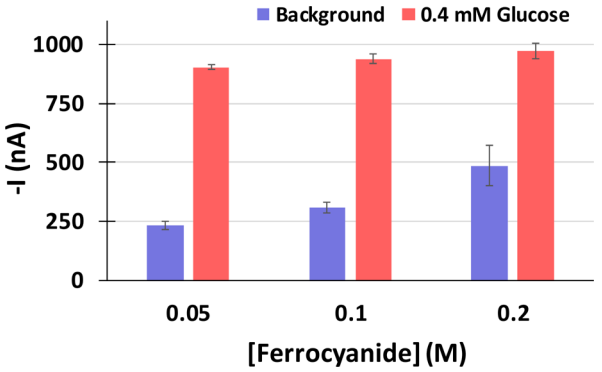

Figure 5. Current intensities recorded at −0.1 V vs Ag pseudoreference electrode after 100 s in buffer solution (background, blue) and 0.4 mM glucose solution (red) with ferrocyanide concentrations of 0.05, 0.1, and 0.2 M using 1.6 units/μL GOx and 2.5 units/μL HRP (deposition of a 10 μL drop). Data are given as mean ± SD (n = 3).

be seen, the analytical signal for a 0.4 mM glucose solution increased very slightly with the ferrocyanide concentration. However, the intensities in the background decreased when the concentration of the mediator was reduced. Therefore, students concluded that 0.05 M ferrocyanide was the best concentration because it gave a higher signal-to-noise ratio.

Calibration of the Biosensor

Once the sensor phase had been optimized, students carried out a calibration plot in order to know how the biosensor responded to increasing glucose concentration and to revise some analytical characteristics of the methodology, namely, capital (e.g., accuracy, representativeness), basic (e.g., sensitivity, precision), and productivity-related properties (e.g., analysis time and cost). As shown in Figure 6, they found a linear relationship between the current intensity and glucose

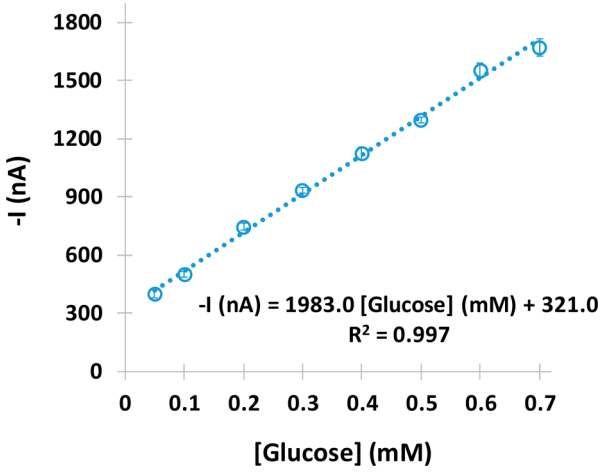

Figure 6. Calibration plot obtained with the glucose biosensor fabricated by immobilizing 10 μL of 1.6 units/μL GOx, 2.5 units/μL HRP, and 0.05 M ferrocyanide solution. Data are given as mean ± SD (n = 3).

concentration in the range between 0.05 and 0.7 mM with a sensitivity of 1983.0 nA/mM. Students also calculated important parameters such as the limit of detection (LOD) and the limit of quantification (LOQ) according to the following equations: LOD = $3s_b/m$ and LOQ = $10s_b/m$, where m is the slope of the calibration curve and s_b is the standard deviation of the intercept.[27,28] For this biosensor, the LOD and LOQ values thus calculated were 0.03 and 0.1 mM, respectively. They were also motivated to discuss other glucose sensors found in the literature[14,22,26,29,30] and to compare their analytical characteristics (sensitivity, precision, linear range, LOD, and LOQ) as well as the procedure of construction (simplicity and fabrication time).

Precision

The precision of the calibration curve is very important, especially when dealing with an analyte as important as glucose. In order to evaluate it, three calibration curves measured on different days by different groups of students using different solutions were compared (data are shown in Table 1). They

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Table 1. Equations of the Calibration Plots for Three Glucose Biosensor Series

Calibration Curve	Slope, nA/mM[a]	Intercept, nA[b]	R^2	Linear Range, mM
1	1983.0	321.0	0.996	0.05−0.70
2	2136.0	168.0	0.997	0.05−0.70
3	2105.0	230.0	0.991	0.05−0.70

[a]Mean ± SD = 2080 ± 81. [b]Mean ± SD = 240 ± 77.

showed very good reproducibility, with a relative standard deviation of the slopes of 3.9% (n = 3). In this way, students understood that a biosensor must present good reproducibility notwithstanding the fabrication day, the day of use, and the operator. Since the temperature is not controlled (experiments were done at room temperature), this was also indicative of the robustness of the methodology.

Selectivity

Selectivity is another important property of a biosensor that has to be taken into account. Students evaluated how the presence of some species affected the analytical signal. In this case, fructose and ascorbic acid were chosen as possible interferences that could be found in real samples. Thus, different biosensors were constructed for determination in mixtures of glucose/fructose and glucose/ascorbic acid. The results obtained are reported in Table 2.

Table 2. Study of Fructose and Ascorbic Acid Interferences in the Glucose Sensor

Sample	−I, nA	±SD, nA[b]
Background	386	11
Glucose 0.3 mM	791	21
Glucose 0.3 mM/Fructose 0.3 mM	852	30
Glucose 0.3 mM/Ascorbic Acid 0.3 mM	538	25
Glucose 0.3 mM/Ascorbic Acid 6 μM[a]	829	29

[a]Ratio in real samples. [b]n = 3.

As can be seen in Table 2, fructose and ascorbic acid produced the opposite effect on the analytical signal. In the case of fructose, a slight increase in the signal was seen; meanwhile, ascorbic acid (usually employed as an antioxidant) produced a decrease in the signal. The effect is not so important when the glucose:ascorbic acid ratio is similar to that found in real samples (e.g., orange juice[31]). Students were encouraged to look for possible solutions to avoid the interference produced by ascorbic acid and incorporated their ideas in the lab report. Students indicated as a good idea coating the sensor with a Nafion film since it is a negatively charged polymer that repels anions.[32,33]

Application to Real Samples

The final aim of the biosensor developed here was to determine glucose concentrations in real samples. Thus, students determined glucose concentrations in a cola beverage and orange juice purchased in the market. The only pretreatment needed was dilution of the sample to obtain a concentration within the linear range of the biosensor (dilutions were made in 0.1 M Tris-HNO_3 buffer solution, pH 7.0). Previous degassing by stirring was required for cola samples.

The samples were validated previously by the instructor using an alternative method (a commercial glucose enzymatic assay kit with spectrophotometric detection), and the results were given to the students after they analyzed the samples. In this way, students compared the results obtained using the biosensor with the results given by a "reference method". The values for glucose concentration obtained with the sensor and the commercial kit are summarized in Table 3. Students

Table 3. Application of the Biosensor to Analysis of Real Samples

	Glucose Concentration Determination, g/100 mL[a]	
Sample	Electrochemical Sensor	Spectrophotometric Assay
Cola beverage	3.3 ± 0.3	3.42 ± 0.03
Orange juice	3.4 ± 0.2	3.47 ± 0.04

[a]Data are given as mean ± SD calculated with two degrees of freedom and p = 0.05; n = 3.

statistically compared the mean values obtained using the two methodologies through a Student's t test.[34] The t values calculated for the cola beverage and orange juice were less than the t value tabulated for two degrees of freedom and a 0.05 significance level. Thus, there were no significant differences between the glucose concentrations given by the biosensor and the enzymatic assay.

DISCUSSION

After constructing the glucose biosensor, the students learned about different electrochemical techniques and their application to the development of an enzymatic biosensor. They also realized that the use of low-cost, disposable, miniaturized, and portable equipment is of paramount importance today, especially when real samples are analyzed.

The experiment was completed with discussions on the following topics:

(i) The analytical problem: types of samples and levels of glucose.
(ii) The state of the art: previous works obtained from a bibliographic search on glucose electrochemical enzymatic sensors, discussing also the different generations of sensors, the role of nanomaterials, and nonenzymatic approaches.
(iii) Evolution of electroanalysis from conventional cells to miniaturized designs.
(iv) Analytical properties (accuracy, precision, sensitivity, selectivity, and especially those related to productivity: analysis of time and cost—see the Supporting Information) and approaches for improving them.

CONCLUSIONS

This experiment served as a practical introduction to biosensor technology and to the challenge of glucose determination. The high number of diabetes patients worldwide increased its relevance, and the application of the developed sensor in analysis of real samples stimulated the students' interest. This lab experiment also introduced students to the miniaturization and simplification of analytical devices and methodologies, some of the most important trends in modern analytical chemistry. Biosensors are an excellent example of simple and promising analytical tools, and students could become familiarized with their two components (sensing zone and transducer), understanding concepts of enzymatic analysis and electroanalysis at the same time. Moreover, they learned how to use screen-printed electrodes, which are widely used today in

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the development of sensors. They also discussed analytical properties (e.g., accuracy, precision, sensitivity, and selectivity) and productivity-related features (e.g., analysis time and cost). In summary, this lab experiment allowed students to acquire problem-solving skills, to reach a high level of critical thought, and to be more confident in facing real-world analytical problems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.6b00948.

Notes for instructors and a suggested student handout (PDF, DOCX)

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The authors declare no competing financial interest.

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