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The Enzymatic Determination of Glucose in Carbonated Beverages: a Useful Tool for the Undergraduate Students to Learn the Basis of Enzymatic Analysis and the Comparison of Two Analytical Methods

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Abstract

The importance of enzymatic analysis in biochemistry, clinical chemistry and food chemistry is undoubted. The course "Applied Biochemistry" in our Faculty is aimed to undergraduate students of Chemistry and Biochemistry. In this subject, the principles and applications of enzymatic analysis are presented to the students, who receive a theoretical introductory lecture in the classroom before they carry out an experiment that should be feasible to be solved in a short laboratory period. The enzymatic determination of glucose in carbonated beverages, has been implemented at the University of Málaga and it has been optimized according to the students' results and commentaries along the last years. It aims to illustrate basic issues relating enzymatic analysis, including its potential application to food chemistry.

Although there are several enzymatic methods that can be used for the determination of glucose, the one based on the coupled reactions of glucose oxidase and peroxidase allow the mentioned enzymatic reactions to be used in both, the end point and the kinetic enzymatic analysis methods. In this way, data for two different protocols for the determination of glucose concentration are obtained by the students from a single reaction mixture. This allows the comparison of both methods in terms of sensitivity, accuracy, and time consumed.

In addition, the evaluation of glucose concentration in four carbonated beverages: coloured coke and uncoloured tonic sodas (regular or sugarless in both cases) makes student to recognise the appearance of interferences that should be either avoided or eliminated.

Undergraduate students having performed this experiment in our laboratories have found it formative, interesting and challenging.

1. The Teaching of *Enzymatic Analysis* within the Grades in Biochemistry and Chemistry at the University of Málaga (Spain)

The Grades in Biochemistry and Chemistry at the University of Málaga (Spain) are organized around 8 semesters along with students must complete a total of 240 ECTS, among compulsory and optative subjects. For Biochemistry students, ranging from 60 to 80 per year, the principles and practices of enzymatic analysis are covered in third course, after they have become familiarized with the structure and function of enzymes in several subjects, including *Fundamentals of Biochemistry*, *Metabolism*, and *Enzymology*, among others. They have also learned some basic principles of *Analytical Chemistry*, although have not had the opportunity to bring them into practice.

In the case of Chemistry students, ranging from 40 to 60 per year, the principles and practices of enzymatic analysis are treated in the fourth, and last, course. Along the previous years, those students have become aware of principles of *Structural Biochemistry*, *Enzymology* or *Metabolism*, and they have strong foundations of *Analytical Chemistry*, being familiar with a number of traditional chemical methods of quantitative analysis.

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In both cases, *Applied Biochemistry* has been designed as a biochemistry laboratory course, where students are forced to face experimental situations that resembles those problems that they could find in a laboratory of chemistry or biochemistry, to awake their critical thinking, and to give an introduction to the world of research. The students are divided into laboratory groups (about 20 students each) for experimental work, which consists of a protocol to investigate a problem that must be feasible to be solved in a short laboratory period. At the same time, they have to evaluate the convenience of one protocol against others considering sensitivity, selectivity, cost and time consumed. At the beginning of the course, they receive some introductory lectures in the classroom for the major theoretical and experimental issues they will need to understand in order to solve the problems proposed. At the same time, they are encouraged to find alternative protocols using the necessary bibliography.

2. The enzymatic determination of glucose as a tool to illustrate the principles enzymatic analysis

Hans Ulrich Bergmeyer has defined enzymatic analysis as "*a branch of analytical chemistry, measuring the catalytic activity of enzymes or using them for analysis*" [1]. The main advantage of the use of enzymes in analysis relies on their specificity, what makes them to react specifically with individual components of a complex mixture, avoiding the need for separations and shortening the time required for the assay to complete.

The importance of enzymatic analysis is undoubted, with a vast and continuously growing field of application. Besides biochemistry itself, probably the most widespread use of enzymatic analysis is found in food chemistry and clinical chemistry.

There are several enzymatic methods that can be used for the determination of glucose. The one based on the coupled reactions of glucose oxidase and peroxidase is especially interesting because the kinetic constants of glucose oxidase allow the mentioned enzymatic reactions to be used in both, the end-point and the kinetic enzymatic analysis methods [2]. In this method β-D-Glucose is oxidized by glucose oxidase (GOD) to D-glucono-δ-lactone, and the hydrogen peroxide produced in the GOD reaction is determined by means of peroxidase (POD) reaction, yielding a coloured product (oxidized o-dianisidine) that can be measured at 440 nm (Fig. 1).

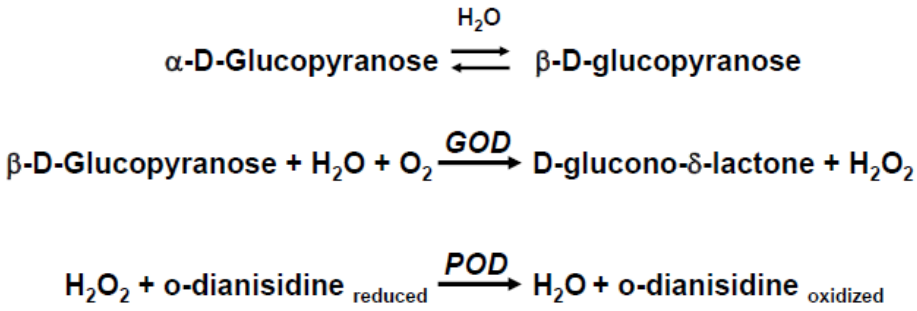

Fig. 1. Scheme of enzymatic reactions used for the determination of glucose
GOD= Glucose oxidase; POD = Peroxidase

The use of cheap and commercially available enzymes for the detection of glucose in foodstuffs helps to the widespread application of this protocol and avoids the inconveniences derived from the use of samples like blood, that are more difficult to handle.

2.1 The Coupled Reactions of Glucose Oxidase and Peroxidase Can Be Used for both, the End-Point and the Kinetic Methods

Any substance which takes part in an enzyme-catalyzed reaction can be determined quantitatively by means of that reaction. If the conversion of the substrate is practically complete (end point methods), the enzymatic analysis is simple, and the result can easily be calculated by the determination of the extent of the primary enzyme-catalyzed reaction by means of physical or chemical procedures, or by

further enzymatic analysis (coupled reactions). In this assay, GOD and POD reactions are carried out in a single assay mixture (coupled reactions), their equilibria lie completely to the right, and under the assay conditions, quantitative oxidization of glucose is obtained within 30 min at room temperature, making these coupled reactions suitable for the end-point method of enzymatic analysis. In this case, a calibration curve, constructed by plotting final absorbance versus glucose concentrations, will be used to interpolate the values of the problem solutions.

Nevertheless, in kinetic methods for the determination of substrate concentrations, the parameter measured is the reaction rate. Kinetic analysis are of great importance for the automated laboratory, allowing a drastic reduction of the time required for analysis, and they are generally less sensitive to interferences (e.g. turbidity, intrinsic colour of the sample…) than end point methods [3]. For the kinetic determination of substrate concentrations, enzymes with high Michaelis constant are required. That is the case for the Michaelis constant of GOD for glucose (0.11 M) [4], which allows the use of kinetic enzymatic method for the determination of a wide range of glucose concentrations, since the oxidation of glucose for GOD obeys pseudo-first order kinetics with respect to β-glucose. For this method a calibration curve, constructed by plotting absorbance changes per time unit versus glucose concentrations, will be used to interpolate the values of the problem solutions.

2.2 The Comparison of End Point and Kinetic Methods Allows a Critical Discussion of their Advantages and Drawbacks

All the students carry out both enzymatic analysis procedures and plot the experimental values. They are asked to determine the linear dynamic range and the sensitivity for each assay. Next, students calculate and discuss the results obtained with a simple glucose problem sample (glucose in water). They give the value of glucose concentration of the solution as a mean of 4 replicates, and discuss which method is more precise (expressed as the agreement between replicate measurements) and which has a higher accuracy (by comparison with the real value of concentration, as it was prepared by the instructor). As for students, when they were required to choose a method for the glucose determination in this sample, most of them preferred the end-point method, because of its easier handling that avoids the cumbersome measurement of reaction rates.

Afterwards, students are asked to determine the glucose concentration in more complex samples. For this, two different coke sodas, regular and sugarless, are chosen, because their colour interferes with that of the coloured product of the enzymatic reactions (oxidized o-dianisidine). In this case, results obtained by the kinetic method differ from those obtained with the end-point method, especially in the sugarless coke analysis, which due to its low glucose content had not been diluted before being assayed, and therefore presented a maximum interference by the intrinsic colour of the sample. This interference was greatly reduced in the regular soda, which needed to be diluted about a thousand times to get a value that could be interpolated in the calibration curve.

Nevertheless, when the glucose concentrations in uncoloured sodas (regular and sugarless tonic water) are determined, no discrepancies between results of glucose concentrations obtained from end-point and kinetic method are found.

Students are asked to explain this discrepancy in the results, and to make comments about the suitability of both assays for the determination of glucose concentration in those samples. In this case, comparison of the results obtained by using each protocol makes them to conclude for glucose determination in a coloured sample, the kinetic method should be chosen in order to avoid colour interferences.

3. Conclusions

The enzymatic determination of glucose in carbonated beverages by the GOD/POD method is a useful tool for the undergraduate students to learn the principles of enzymatic analysis and its potential application to food chemistry.

From the instructor point of view, the experiment requires minimal preliminary preparation and it is inexpensive.

The simultaneous measurement of the parameters used for both methods (absorbance increase per minute for kinetic method; final absorbance for end-point method) in the same reaction mixtures shortens and cheapens the protocols, allowing both of them to be performed by students in a 3-4 hours session.

The comparison of end-point and kinetic methods allows a critical discussion of their advantages and drawbacks.

This practical experiment illustrates how the appearance of interferences may do a given analytical method unsuitable for a specific practical problem, gets students to do calculation in the laboratory, and provides them with some training in choosing the best method according to specific purposes.
Chemistry and Biochemistry undergraduate students having performed this experiment in our laboratories for the last years have found it formative, interesting and challenging.

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