Enzymatic Spectrophotometric Reaction Rate Determination of Glucose in Fruit Drinks and Carbonated Beverages W

An Analytical Chemistry Laboratory Experiment for Food Science–Oriented Students

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Glucose is a major component of animal and plant carbohydrates. Quantitative determination of glucose is important in clinical chemistry, biochemistry, and food analysis. This paper describes an undergraduate analytical chemistry laboratory for the kinetic determination of glucose in fruit drinks and carbonated beverages using glucose oxidase (GOX) and horseradish peroxidase (HRP). Glucose is oxidized to δ-D-gluconolactone in the presence of GOX:

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

To measure the reaction rate, the production of H_2O_2 is coupled to an HRP-catalyzed reaction:

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

The reaction product exhibits an absorption maximum at 505 nm with a corresponding molecular extinction coefficient of 1.27×10^4 $M^{-1}cm^{-1}$ (*1*). The reaction rate is measured by monitoring the increase of absorbance with time.

Three laboratory experiments concerning the enzymatic determination of glucose have been published in this *Journal* (*2–4*). One procedure utilized *o*-dianisidine as oxygen acceptor (*2*); however this substance is of low water solubility and was found to be carcinogenic. Two other experiments stressing instrumentation issues have been published. In these, H_2O_2 produced through reaction 1 reacts with iodide to produce iodine and the reaction is followed amperometrically using a rotating platinum electrode (*3, 4*). In all three experiments, calibration was achieved using the variable-time method, and no "real" samples were analyzed.

The laboratory experiment described in this article is optimized for student use in the temperature range of 18–28 °C and avoids interference from ascorbic acid. A single mixed reagent solution containing GOX, HRP, phenol, and 4-aminoantipyrine is used. Students learn about reaction rate kinetic methods of analysis, the use of enzymes as selective analytical reagents for the determination of substrates, the kinetic masking of ascorbic acid interference, and the determination of glucose in beverages.

Reagents and Apparatus

Glucose stock solution, 0.200 M. Weigh 36.04 g of glucose in a 1000-mL volumetric flask and bring to volume with water. The solution is stable for at least 3 months when stored at 5–8 °C.

Enzyme Working Solution: Phenol, 7.50 mM; 4-Aminoantipyrine, 2.50 mM; GOX, 0.5 IU mL^{-1}; and HRP, 20 IU mL^{-1} in 0.10 M Phosphate Buffer pH 7.5. Weigh 0.706 g of phenol, 0.508 g of 4-aminoantipyrine, and 14.2 g of Na_2HPO_4 in 800 mL of water and adjust pH to 7.5. Add 500 IU of GOX and 20,000 IU of HRP to the buffered solution, transfer to a 1000-mL volumetric flask, and bring to volume. The enzyme working solution is stable for at least two months when stored at 5–8 °C.

Calibration Solutions: 2.00, 4.00, 8.00, 12.0, and 16.0 mM Glucose. Prepare by transferring 1.00, 2.00, 4.00, 6.00, and 8.00 mL of the glucose stock solution respectively into 100-mL volumetric flasks and bringing to volume with water.

Phosphate Buffer 0.10 M, pH 7.5. Dissolve 14.2 g of Na_2HPO_4 in 900 mL of water and adjust pH to 7.5 using 4 M HCl, place in a 1000-mL volumetric flask, and bring to volume with water.

Spectrophotometers. The Milton Roy Spectronic-20, Cecil CE 2343, and Jasco V550 spectrophotometers were used.

Procedure

A 20-mL sample of the carbonated beverage to be tested is transferred to a 100-mL beaker and stirred for 10 min to remove excess carbon dioxide. After stirring, 5.00 mL of the beverage is diluted to 100 mL with phosphate buffer. The wavelength is set at 505 nm and the 100% *T* of the spectrophotometer is adjusted. One milliliter of standard or diluted sample and 5.00 mL of the enzyme working solution are rapidly mixed and immediately transferred to the spectrophotometer. To eliminate ascorbic acid interference, samples are preincubated for $2\frac{1}{2}$ min with the enzyme working solution. After preincubation, the initial absorbance reading and three subsequent readings acquired at 2-min intervals are recorded. Readings are plotted versus time (Fig. 1) and reaction rates are calculated in units of m*A* min^{-1} (milli-absorbance min^{-1}). Then the calibration curve is constructed (Fig. 2) and the glucose concentration is calculated.

Optimization of the Assay for Student Use

To ensure that the measured reaction rate ($\Delta A \Delta t^{-1}$) is proportional to glucose concentration, the glucose oxidation reaction must be the rate-limiting step and the HRP-catalyzed *indicator reaction* should be fast. To achieve this, a 40-fold excess of HRP to GOX (20/0.5 IU mL^{-1}) and the highest readily soluble quantities of phenol and 4-aminoantipyrine were used. This laboratory experiment can be used at room temperatures of 18–28 °C. Increasing the temperature from 18 to 28 °C results in a 39% increase in the reaction rate (Fig. 2). The linear range was 1–16 mM; the lower concentration standard for student use was set at 2 mM. In this way, inexpensive spectrophotometers can be used.

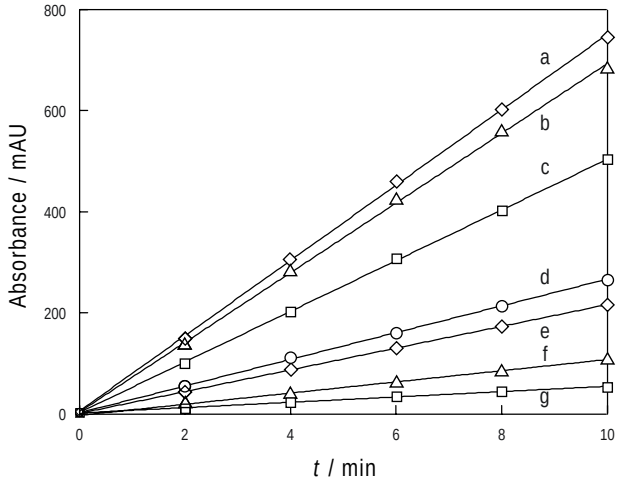

Figure 1. Reaction rate curves at various glucose concentrations. a: 16.0 mM, rate = 75.50 ± 0.04 mA min^{-1}; b: 15.0 mM, rate = 69.11 ± 0.05 mA min^{-1}; c: 10.0 mM, rate = 50.35 ± 0.04 mA min^{-1}; d: 5.00 mM, rate = 26.61 ± 0.03 mA min^{-1}; e: 4.00 mM, rate = 21.62 ± 0.01 mA min^{-1}; f: 2.00 mM, rate = 10.96 ± 0.02 mA min^{-1}; g: 1.00 mM, rate = 5.19 ± 0.02 mA min^{-1}. Correlation coefficients of fits were .996–.9999 at 23 °C.

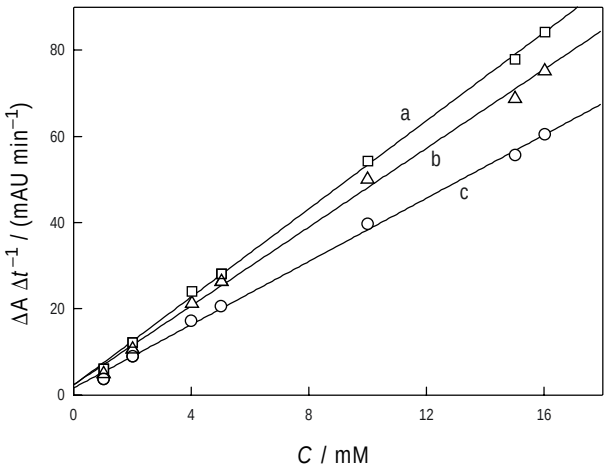

Figure 2. Calibration data obtained at various temperatures. a: 28 °C, $\Delta A\,\Delta t^{-1}$ = (2.3 ± 0.7) + (5.11 ± 0.07) × C, r = .9995; b: 23 °C, $\Delta A\,\Delta t^{-1}$ = (2 ± 1) + (4.6 ± 0.1)) × C, r = .998; c: 18 °C, $\Delta A\,\Delta t^{-1}$ = (1.7 ± 0.7) + (3.67 ± 0.07) × C, r = .999.

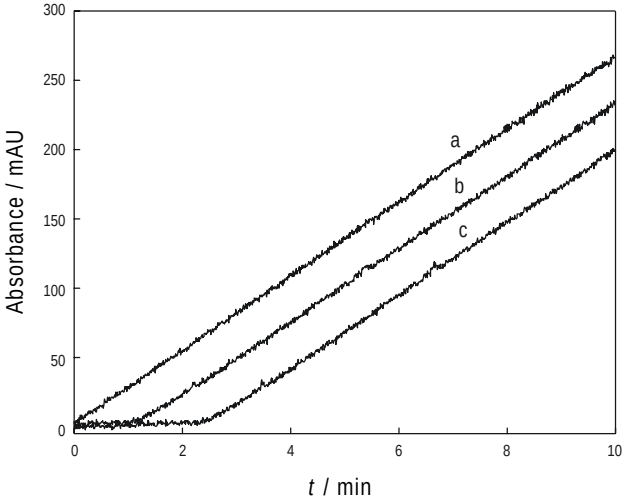

Figure 3. Effect of ascorbic acid on the signal from a 5.00 mM glucose standard at 23 °C. a: no ascorbic acid, reaction rate = 26.6 ± 0.2 mAU min^{-1}; b: 0.0500 mM ascorbic acid, reaction rate = 26.8 ± 0.4 mAU min^{-1}, lag time = 70 s; c: 0.100 mM ascorbic acid, reaction rate = 26.3 ± 0.5 mAU min^{-1}, lag time = 140 s.

Interferences

Hydrogen peroxide produced through reaction 1 oxidizes ascorbic acid (vitamin C) present in beverages, producing dehydroascorbic acid:

$$H_2O_2 + C_6H_8O_6 \rightarrow C_6H_6O_6 + 2H_2O \qquad (3)$$

Because this reaction is faster than the HRP-catalyzed reaction 2, ascorbic acid interferes in end-point assays, resulting in negative errors. This interference can be eliminated by using ascorbic acid oxidase in the enzyme working solution. In kinetic assays, the initial quantity of H_2O_2 produced through reaction 1 is consumed for ascorbic acid oxidation (reaction 3). Thus, a lag phase in the reaction rate curve is recorded (Fig. 3). When all ascorbic acid has been consumed, the reaction rate is measured. The lag time is proportional to ascorbic acid concentration and inversely proportional to glucose concentration (i.e., proportional to the ascorbic acid/glucose concentration ratio). The concentration of ascorbic acid even in vitamin C–fortified drinks is less than 6 mM (*5, 6*), while the expected glucose concentration in fruit drinks and carbonated beverages is in the range of 55–295 mM (*7*). Therefore the ascorbic acid/glucose ratio will always be less than 1/50 and the lag time less than 140 s, as shown in Figure 3c, and if samples are preincubated with the enzyme solution for $2\frac{1}{2}$ min, ascorbic acid interference is eliminated.

Hazards

Phenol and 4-aminoantipyrine are harmful if swallowed and are irritating to the eyes, respiratory tract, and skin.

Student Results

Typical results from the analysis of beverage samples are shown in Table 1. Student performance is evaluated by the analysis of aqueous samples (Table 2). The %RSDs shown in Table 2 are between-days values for a period of eight weeks. Better precision can be achieved by thermostatting the spectrophotometer cuvette.

Further Studies

A variety of special projects based on this experiment can be envisioned for interested students. Outlines of a few possible projects are mentioned below.

Determination of polymers or oligomers that contain glucose as a building unit: After hydrolysis, disaccharides and polysaccharides are quantitated by the determination of the resulting glucose. In this way amylose (*8*), sucrose, lactose, and maltose (*9, 10*) can be determined.

Table 1. Student Results for the Determination of Glucose in Beverages

Brand	C/mM
Peach nectar[a]	45.3
Apricot nectar[a]	61.2
Orange nectar[a]	69
Sprite[b]	88.2
Orange juice amita[c]	107
Gatorade[d]	135
Banana juice[e]	145
Coca-Cola[b]	166
Fanta[b]	330

Manufacturer: [a]Pepsico-IVI, [b]Coca-Cola, [c]3E, [d]Quaker Beverages, [e]Florina.

Table 2. Student Results for the Analysis of Aqueous Glucose Samples

Concentration/mM		%RSD
Given	Found ± SD[a]	
4	4.3 ± 0.5	12
8	8.4 ± 0.8	9.5
12	12.7 ± 0.7	5.5
16	15.8 ± 0.8	5.1

NOTE: Results obtained by 48 students during 8 weeks of laboratory experiments.
[a]n = 12.

Determination of other substrates: By substituting choline oxidase (*11*), uricase (*12*), cholesterol esterase and cholesterol oxidase (*13, 14*), or glutamine synthetase and pyruvate kinase (15) for GOX, methods for the determination of choline, uric acid, cholesterol and ammonia can be developed.

Determination of peroxides: Although GOX is relatively specific for β-D-glucose, the coupling reaction (reaction 2) is completely nonspecific and many oxidizing agents will give a colored product with the phenol–4-aminoantipyrine system (*16*). In this way reaction 2 can be used for the determination of hydroperoxide in seafoods (*17*) and lipid hydroperoxides in oils and fats (*18*).

Determination of phenols: Phenols react with 4-aminoantipyrine according to reaction 2 even without H_2O_2 and HRP in the presence of $Fe(CN)_6^{3-}$ or $S_2O_8^{2-}$. In this way, total phenols in water and soil (*19–21*) and pentachlorophenol in sawdust (*22*) can be determined.

WSupplemental Material

Supplemental material for this article is available in this issue of *JCE Online*.

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