Comparison of Biochemical and Chemical Digestion and Detection Methods for Carbohydrates

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ABSTRACT

There is a multitude of chemical and biochemical detection methods for sugars. Which ones would be most practical in an undergraduate laboratory setting? How to best detect non-reducing disaccharides? How to make such lab fun for students to perform? After trying several spectrophotometric methods, it was found that chemical detection by dinitrosalicylic acid and biochemical detection by hexokinase/glucose-6-phosphate dehydrogenase reagent are most appropriate. Sucrose, a non-reducing disaccharide was digested chemically with hydrochloric acid and biochemically with invertase. It was concluded that chemical detection and biochemical detection compliment each other. Chemical digestion method was preferred over the digestion by invertase. These methods were applied for testing the validity of sugar ingredients printed on drink labels as well as the measurement of sugar levels in ripening bananas at two different conditions. The comprehensive comparison of these methods and the detection of sugar concentrations in interesting samples might serve as a basis for an undergraduate chemistry laboratory.

I. INTRODUCTION

Carbohydrates are organic compounds that are either aldehydes or ketones and have many hydroxyl groups. Carbohydrates serve a multitude of biological functions for all known organisms on Earth. They are an invaluable energy source and provide structural, storage, informational, recognition, shock absorbing and protective functions. Monosaccharides, the simplest carbohydrate units, are commonly known as 'simple sugars.' Two monosaccharides can form a glycosidic linkage by undergoing a condensation reaction, which yields a disaccharide. A disaccharide can be digested and broken down into its two original monosaccharide units. When glycosidic linkages are formed between more than two monosaccharide units, a polysaccharide is produced [1].

Sugars can be detected in natural and processed foods and drinks by chemical and biochemical methods. Such detections can be useful for not just determining amount of sugar in food or drink samples, but also in determining validity of labels. The focus of this study is to not only provide both chemical and biochemical methods of sugar digestion and detection that can be utilized in an undergraduate biochemistry laboratory, but to determine if one set of methods works more efficiently than the other.

Sucrose, commonly known as table sugar, is present in natural fruits and vegetables. Sucrose can be digested biochemically using invertase (Reaction 1, Fisher and Kohtes [2]) and chemically using hydrochloric acid (Reaction 2, Fieser and Williamson [3]) into glucose and fructose monosaccharides. The biochemical detection method using hexokinase with glucose-6-phosphate dehydrogenase is highly specific to glucose only (Reaction 3, Avigrad and England [4] and Kunst [5]). The chemical detection method, which utilizes dinitrosalicylic acid (DNS reagent), detects glucose, fructose, and other mono-

saccharides (Reaction 4, Bernfeld [6] and Miller [7]). Sucrose cannot be detected by either detection method directly. Therefore, samples containing sucrose must be digested so that the glucose and fructose products can be detected. If samples contain sucrose and some other sugars as well, monosaccharides have to be measured initially before sucrose digestion. The sucrose concentration can then be calculated when the total monosaccharides are measured after hydrolysis of the sample.

Another biochemical method alternative to hexokinase is detection using glucose oxidase and peroxidase [8-9]. Alternatives to the chemical DNS methods are the well known Nelson's method [10] and the ortho-toluidine method of Dubovski [11]. These methods were tried, but not chosen for the reasons discussed in the results section.
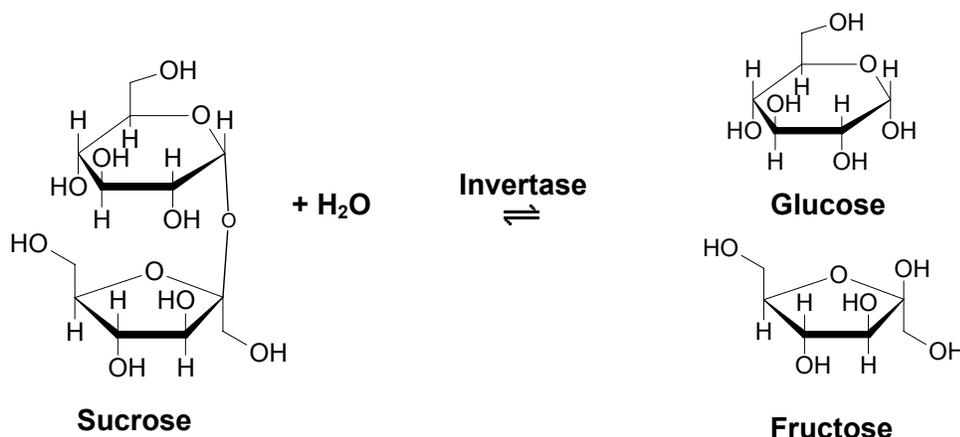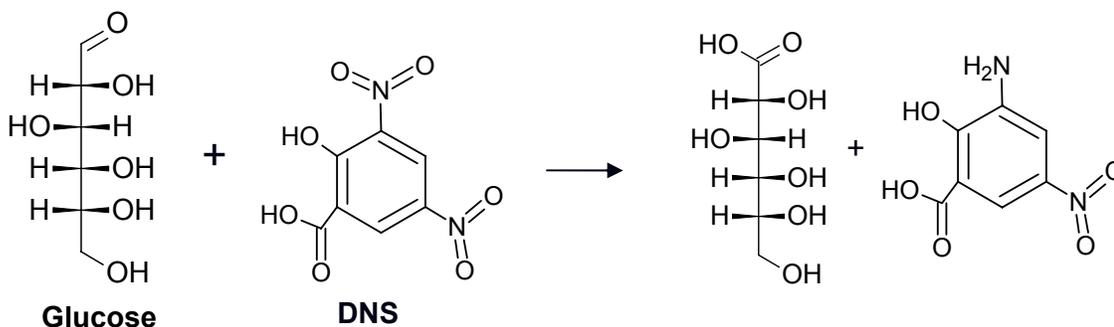

Reaction 1: Sucrose is broken down by invertase into glucose and fructose via a hydrolysis reaction.
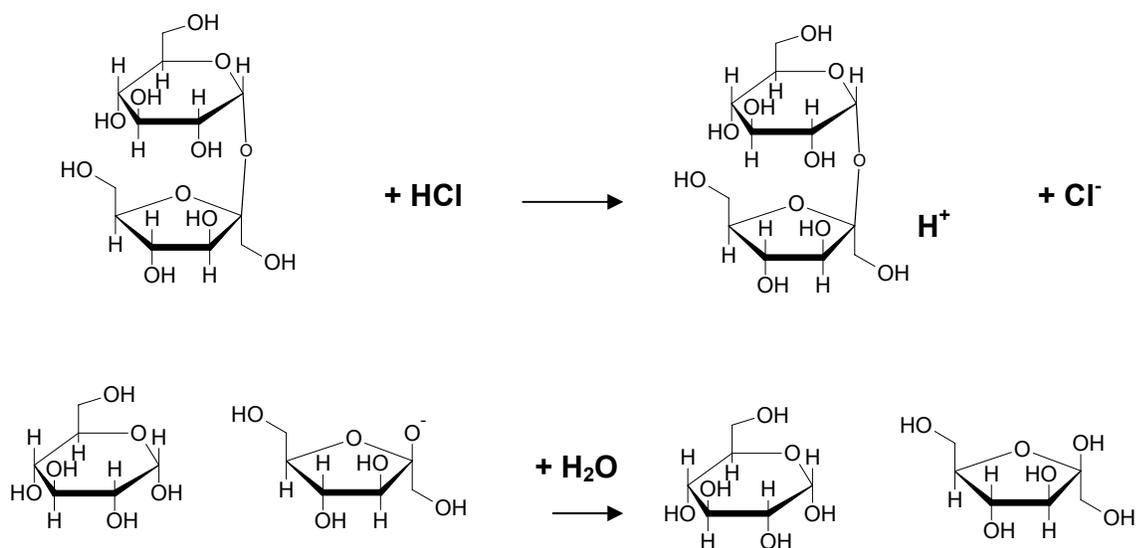
Reaction 2: Sucrose is protonated by HCl then undergoes a hydrolysis reaction to yield glucose and fructose as the final products.
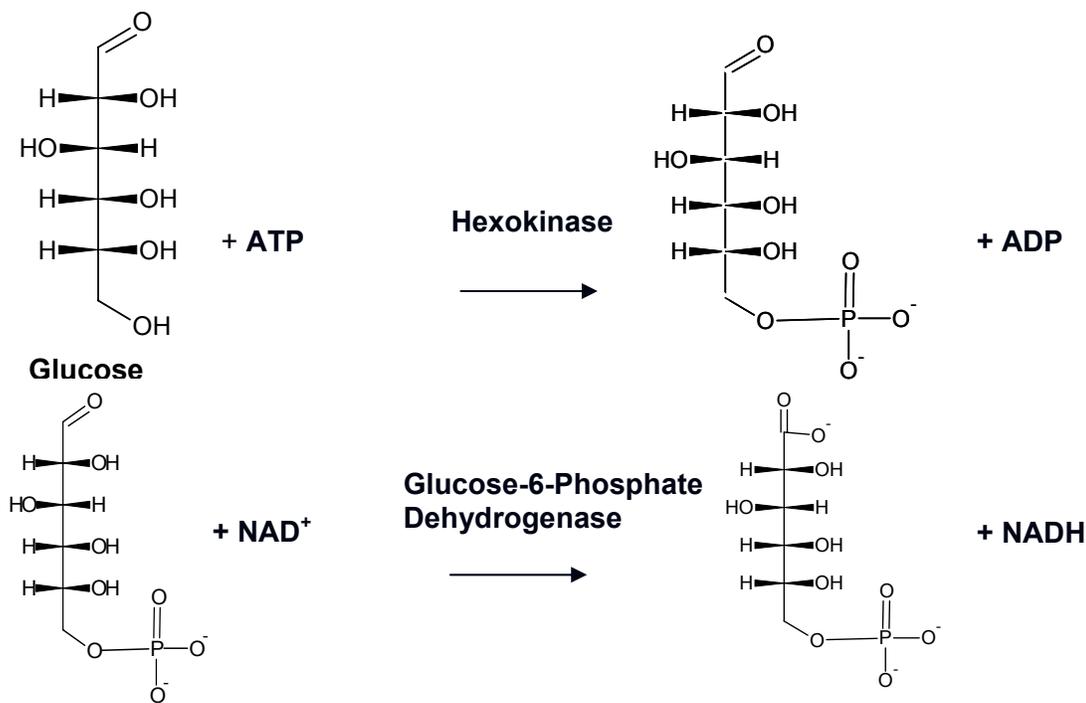
Reaction 3: Glucose is phosphorylated by ATP using hexokinase. This product is then oxidized. The absorbance of NADH is measured.

Reaction 4: Glucose (or fructose) and DNS undergo a reduction-oxidation reaction during DNS detection. The sugar is the reducing agent and DNS is the oxidizing agent.

II.   METHODS

a.   Preparation of the Standards and Standard Curves

All chemicals were purchased from Sigma-Aldrich. Stock solutions (50 mL each) of 40 mM fructose, 40 mM glucose, and 40 mM sucrose were prepared weekly and stored refrigerated until needed. Desired concentrations of samples for the standard curve for each sugar were prepared by performing dilutions of the stock solution.

b.   Preparation of the Drink Samples

Table 1 exhibits the drink samples used in this study along with information on each sample's label. It is important to choose drinks that have contrasting sugars.

Drink Sample	Sugar Ingredient(s)	Grams sugar per 240 mL serving	Sugar (mM) expected (1:20 dilution)
Sierra Mist Free	no sugar	0	0
Tea with 3 *Domino*® *sugar* packets	sucrose	12.6	7.7
Pedialyte®	glucose	6	6.9
Sprite	High-fructose corn syrup (mainly glucose and fructose)	26	
Gatorade® *Rain*	fructose, glucose, & sucrose mixture	14	

**Table 1.** Label and calculated information of each drink sample.

Carbonated drinks were degassed by opening the bottles for 30 minutes, and placing samples under the aspirator-generated vacuum for 10 minutes. Samples were diluted 1:20 with water. Expected concentrations were determined based on the grams of the sugar included, its specific molecular weight, and volume of one serving (240 mL). Actual concentration of each individual type of sugar could not be determined accurately in the samples containing sugar mixtures. The mass of sugar listed on the label is inclusive of all sugars in the entire mixture. Therefore, determining the number of grams of fructose or glucose in the sample is not possible from the label alone. For example, Sprite's ingredients contain high fructose corn syrup (HFCS). In the United States, the main source of fructose in diets is HFCS [12]. HFCS are quite similar to sucrose's composition. However, sucrose is composed of 50 % glucose and 50% fructose, whereas HFCS contains approximately 42 % fructose and the rest glucose (55 %) and other simple sugars [13]. Hence, the calculations of expected concentration per simple sugar become complicated. This is when detection methods come in handy for unknown concentrations. Using a highly glucose specific biochemical detection method, the actual grams of glucose in the sample can be found. Knowing this and the total amount of sugar in the sample, fructose can be determined.

c. Biochemical Digestion

Samples containing sucrose (specifically Gatorade Rain, tea with sugar, and the standard) were biochemically digested in triplicate via a hydrolysis reaction using invertase enzyme. The stock invertase was made by mixing 0.01 g of invertase solid with 1 mL of 50 mM sodium phosphate buffer at a pH of 7. A 180 µl aliquot of 0.2 M sodium acetate buffer (pH = 4.5) was added to 450 µL of sample, followed by an addition of 9 µL invertase stock. Water was then added to the samples to bring each to the designated volume, 900 µL. Samples were left to digest at room temperature for 30 minutes.

d. Chemical Digestion

A separate set of the samples containing sucrose was chemically digested in triplicate by adding 3 µl concentrated hydrochloric acid (HCl) to 450 µL of each sample. These samples were placed in a 90°C hot water bath for 15 minutes, removed, and left to sit at room temperature for another 15 minutes. Approximately 40 µl of sodium bicarbonate was added in 10 µL portions to each sample in order to neutralize the acid. The pH was checked with pH test strips to ensure a pH of 7. Water was then added to bring each sample to the designated volume, 900 µL.
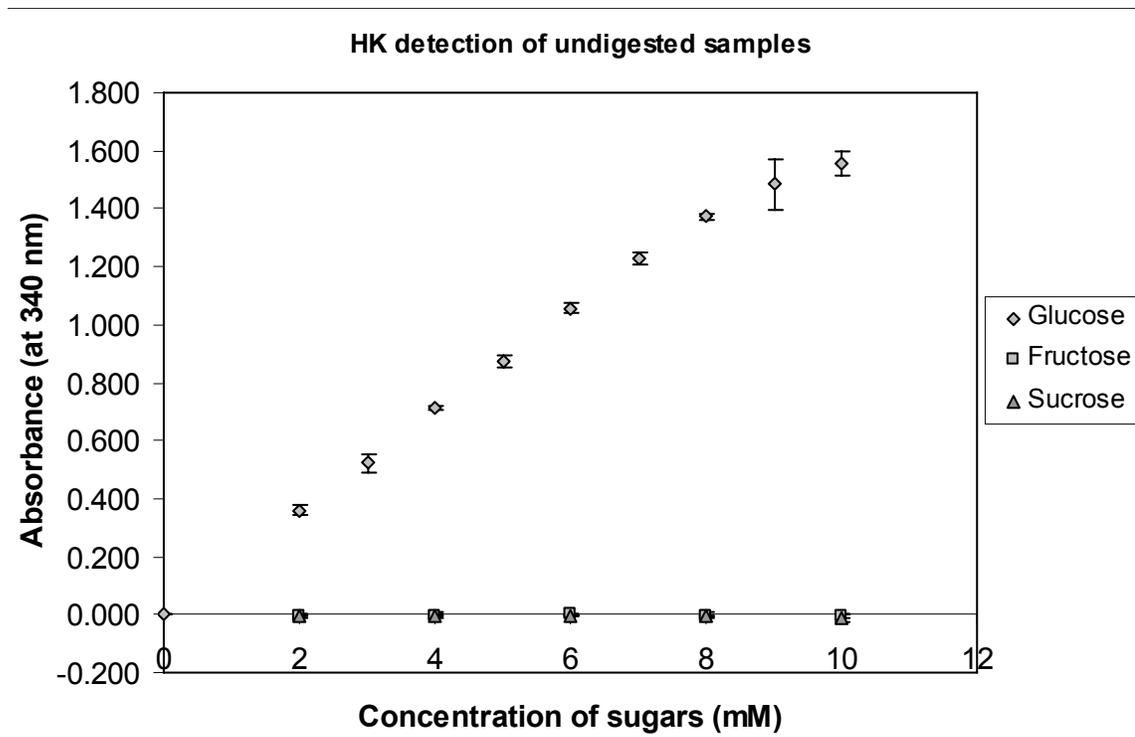
Figure 1. Hexokinase detection of undigested glucose, fructose, and sucrose.

e. Biochemical Detection

The standards, undigested drink samples, chemically and biochemically digested samples all underwent biochemical detection. A 10 µL aliquot of each sample was pipetted into the wells of a 96-well microplate. A 300 µL aliquot of Hexokinase reagent was added to each well and the plate was left at room temperature for 5 minutes. Absorbance of each sample was read at 340 nm using a 96- well microplate reader (Bio-Tek) and the average absorbance for each set of triplicate was calculated. Using the standard curve constructed from the absorbance data, the unknown concentrations of the drink samples were found.

f. Chemical Detection

Another set of chemically and biochemically digested samples, as well the other standards and drink samples underwent chemical detection in triplicate. A 450 µL aliquot of each sample was pipetted into new micro-tubes. A 450 µl aliquot of dinitrosalicylic acid (1% DNS) was added to each tube. Samples were immersed in a 90

ºC hot water bath for 10-12 minutes. It is important that samples and standards are done at the same time. After removal from the bath, 150 µl of sodium potassium tartrate (40%) was added to each tube. A 300 µL aliquot of each sample was pipetted into separate wells of the 96-well microplate. Absorbance was read at 575 nm with the plate reader and the average absorbance for each set of triplicate was calculated.

g. Banana Experiment

Two set of bananas were kept for 15 days, one at room temperature and one refrigerated at 4ºC. To determine the amount of starch, the slice was placed onto a microscope slide, dyed with 4% potassium iodide, and photographed. Amounts of sugars were determined after the mechanical lysis of 1 g of banana in 10 mL of distilled water and the centrifugation of the sample at 14,000 rpm for 5 minutes.

III. RESULTS AND DISCUSSION

Both hexokinase/ glucose-6-phos-phate dehydrogenase (HK) reagent and glucose oxidase/ peroxidase reagent are
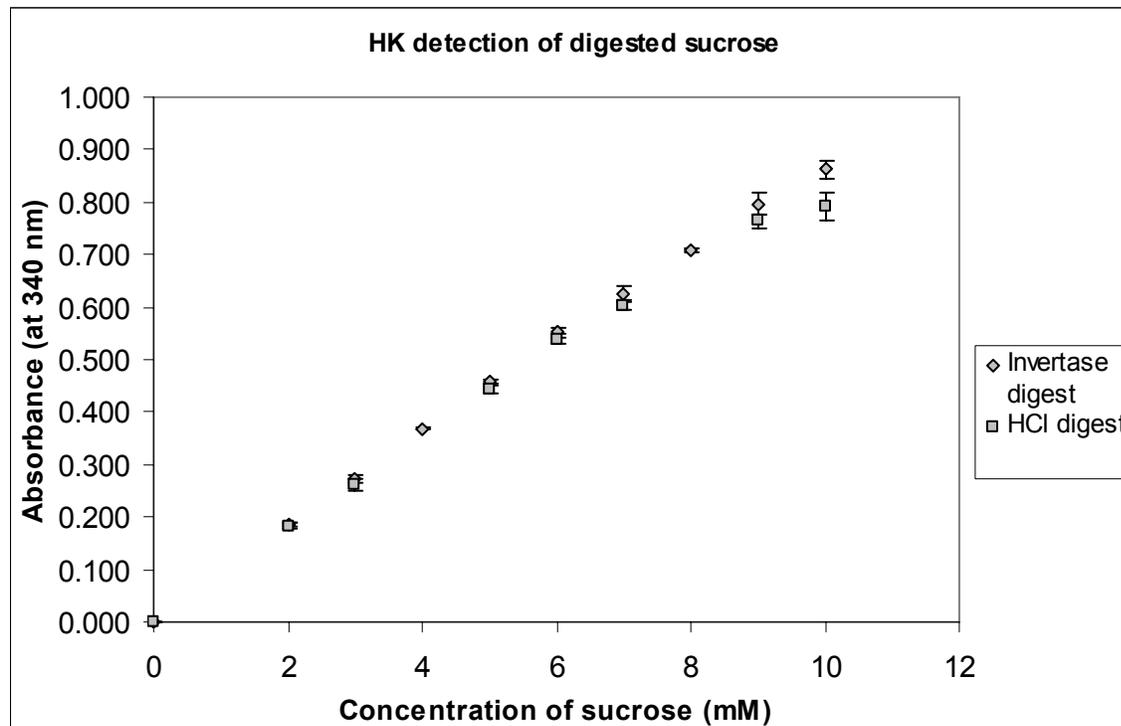
Figure 2. HK detection of sucrose digested by invertase and by hydrochloric acid.

available from Sigma at approximately same price for small quantities. The decision was made to use HK because it is available in both large and small quantities, which makes it useful in a teaching laboratory. Also, this reaction takes only 5 minutes, it is glucose specific, and the amount of sample used is 10 µL. The price, however, was 30¢ per measurement. All chemical methods were an order of the magnitude less expensive.

Among chemical methods, Nelson's assay is often used in laboratory experiments. It takes 50 minutes to perform the assay, it detects glucose and fructose equally well, and has no response for sucrose. However, it uses quite toxic cupper and arsenomolybdate reagents. Ortho-toluidine method can be used to detect glucose in about 20 minutes. However, it responds to fructose (7.6 % of glucose response) and sucrose (20.7 % of glucose response), making it completely inappropriate for complex mixtures. DNS takes 40 minutes to perform. It responds to glucose and fructose but not sucrose. Data is available but not shown for methods we chose not to use.

In Figures 1 through 4, the standard curves for both the digested and undigested standards using biochemical and chemical detection are displayed. Each sample was done in triplicate, and Y- error bars show a single standard deviation for each point.

The concentration of the glucose standard is directly proportional to the absorbance of the sample detected using the HK method, while fructose and sucrose have no absorbance as they can not serve as substrates for at least one enzyme. This shows the specificity of this reaction and its suitability for the purpose of this investigation.

Both invertase and HCl digestion methods take about 30 minutes to perform. Sucrose standard is digested and yields glucose and fructose. Glucose only is detected using hexokinase. The digest yields a two-fold dilution in this procedure. The absorbance of the samples is expected, therefore, to be half of the absorbance of the same sample of glucose, which it was. This indicates a complete digest in both cases. The only difference between the invertase and HCl digestion are procedural and fiscal,
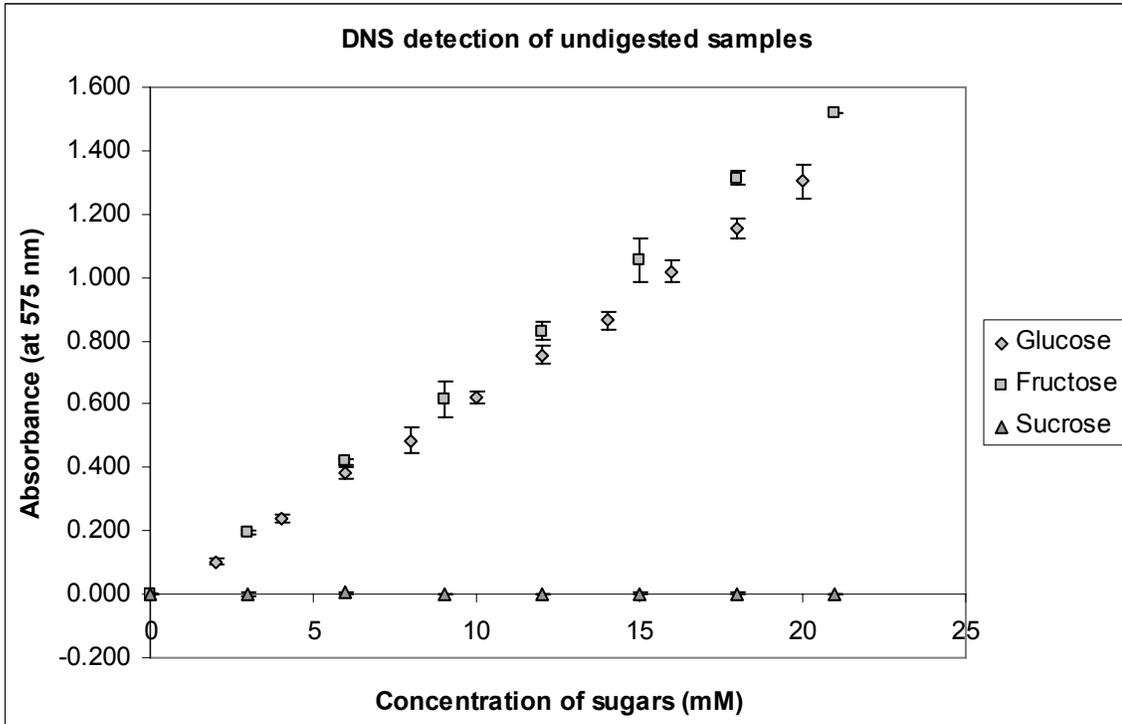
Figure 3. Hexokinase detection of undigested glucose, fructose, and sucrose.
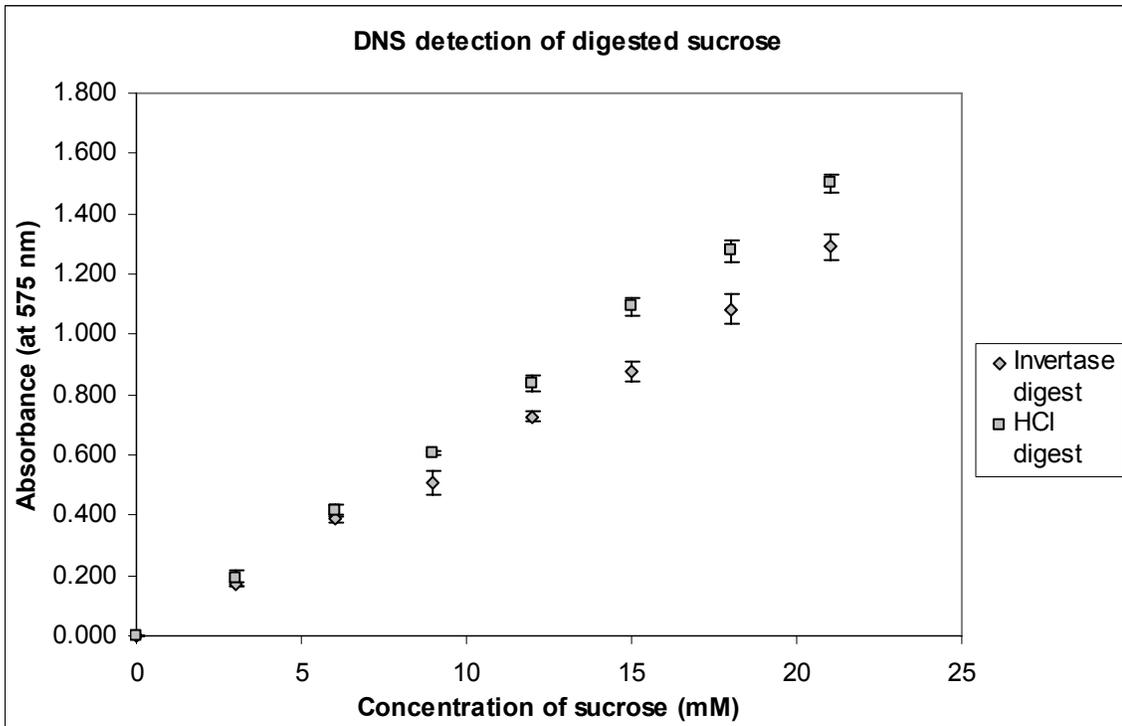
Figure 4. DNS detection of sucrose digested by invertase and by hydrochloric acid.

Drink Sample	Average Absorbance	[sugar] mM
Sprite	0.623 ± 0.019	5.31± 0.310 mM glucose, 3.278± 0.310 mM fructose
Sierra Mist Free	0.002 ± 0.002	0.050 ± 0.050
Gatorade	0.650 ± 0.007	NA
Pedialyte	0.513 ± 0.078	7.600 ± 0.900
Tea	0.009 ± 0.001	0.158 ± 0.024

Table 2. Average absorbance and concentration for each undigested drink sample using DNS detection.

Drink Sample	Average Absorbance	[glucose] mM
Sprite	0.859 ± 0.102	5.00 ± 0.400
Sierra Mist Free	0.073 ± 0.097	0.226 ± 0.371
Gatorade	1.271 ± 0.069	7.500 ± 0.200
Pedialyte	1.204 ± 0.074	7.090 ± 0.230
Tea	0.050 ± 0.093	0.089 ± 0.344

Table 3. Average absorbance and concentration of glucose in each undigested drink sample using the hexokinase detection method.

with the cost of the HCl digest being much less.

The DNS method detects both glucose and fructose to similar extent (slopes differ by 10.7 % only). This method compliments the HK detection method, which only detects glucose. Using subtraction of data from DNS and HK detection, students can determine the concentration of fructose in a mixed sample.

According to this data, invertase digest seemed to be incomplete, with slope 16.2 % below that for HCl digest. One possible explanation is that the DNS reagent stops the invertase reaction, while the HK reagent, being quite compatible with the invertase, forces the reaction to completion. Thus, DNS detection should be used in conjunction with the HCl digest. Also, since DNS detects both fructose and glucose, the 2-fold dilution upon digestion has been compensated by the production of two detectable molecules. The absorbance at the same concentration of sucrose after the digest is about the same as undigested glucose/fructose of the same concentration, not 2 times lower as in HK method.

a. Chemical Detection of Undigested Drink Samples

DNS detection was successfully run on all the drink samples (Table 2). The expected concentration of Pedialyte (6.9 mM) fell in range with the concentrations found (7.6 ± 0.9 mM). This is a less than

DNS detection		
Drink Sample	**Average Absorbance**	**[glucose and fructose] mM**
Tea	0.504 ± 0.019	7.240 ± 0.007
Gatorade	0.605 ± 0.107	NA
Hexokinase detection		
Drink Sample	**Average Absorbance**	**[glucose] mM**
Tea	0.4667 ± 0.022	5.480 ± 0.021
Gatorade	0.4594 ± 0.035	4.800± 0.170

Table 4. DNS and hexokinase detection of tea and Gatorade drink samples after HCl digestion.

DNS detection		
Drink Sample	**Average Absorbance**	**[glucose and fructose] mM**
Tea	0.313 ± 0.008	5.640 ± 0.137
Gatorade	0.449 ± 0.019	NA
Hexokinase detection		
Drink Sample	**Average Absorbance**	**[glucose] mM**
Tea	0.174 ± 0.003	2.010 ± 0.020
Gatorade	0.198 ± 0.014	2.296 ± 0.176

Table 5. DNS and hexokinase detection on tea and Gatorade drink samples after invertase digestion.

10% difference between the actual and expected concentrations of glucose in Pedialyte. Such a percent difference is acceptable in an undergraduate biochemistry lab. Sprite is a mixture of simple sugars due to HFCS and has the large overall amount of sugars in the mixture. The expected absorbance and derived sugar concentrations should be higher than the Pedialyte. This was observed. The concentrations of glucose and fructose in Sprite were estimated by a calculation using the known percentages of each sugar in HFCS (55% glucose, 42% fructose). Tea had virtually no absorbance since the sucrose was not digested and undetectable. The same result occurred for the Sierra Mist Free since there are no natural sugars in it. As for the Gatorade mix, a high absorbance was expected. However because the actual percentages of glucose, fructose, and sucrose components of the

total sugar amount are unknown, the expected concentrations of these sugars cannot be estimated. Because this method is not specific to any one specific sugar, this is a major drawback in using this detection procedure.

b. Biochemical Detection of Undigested Drink Samples

Hexokinase successfully detected glucose in the undigested samples (Table 3). The expected concentration of glucose in Pedialyte (6.9 mM) was in range of the concentration measured (7.090 ± 0.230 mM). The percent difference between the actual and expected was only 2.68%. Because of the glucose specificity, the absorbance of glucose alone in Sprite could be determined directly and not by calculation. Note that the concentration of glucose using this method (5.00 ± 0.400 mM) is similar to the concentration of glucose estimate found using the DNS method (5.31± 0.310 mM). As found in the chemical detection method, tea had no glucose detected. Sierra Mist Free had very slight glucose detection (0.226 ± 0.371 mM) but the relatively high standard deviation negates this data point. Gatorade had a high absorbance of glucose in it and thus a high concentration of glucose (7.500 ± 0.200 mM). Even though the DNS method does not differentiate between reducing sugars and their individual concentrations, the hexokinase does allow specific detection of glucose in any drink mixture.

c. Chemical Digestion of Drink Samples Containing Sucrose

HCl digestion of the drink samples containing sucrose (tea and Gatorade; see Table 4) was successful. After running DNS detection, the expected concentration of glucose and fructose combined (7.7 mM) was close to the concentration expected based on the label (7.240 ± 0.007) with a difference of 5.97%. The concentration could not be determined in Gatorade because, as stated earlier, the percentages of each sugar of the total mixture are unknown. This detection method detects other simple sugars in the Gatorade as well. The concentrations of glucose determined by the HK method after chemical digestion

of sucrose are shown in Table 4. The reason for lower readings in tea is unclear.

d. Biochemical Digestion of Drink Samples Containing Sucrose

The sucrose in tea and Gatorade drink samples was digested using the invertase method. The determined concentration of glucose and fructose in tea (5.640 ± 0.137 mM) was lower than expected (7.7 mM) with a 36% difference. This is probably due to the invertase reaction, which is a reversible reaction and it does not fully go to completion. Hence, the concentrations of sugar products are lower than expected. Glucose was also detected in the digested drink samples and the concentrations are shown in Table 5. Those readings were significantly lower than expected. The reason for lower readings is unclear.

e. Findings of the banana experiment

It was expected that bananas stored at room temperature for 15 days will ripen more quickly than those stored in a refrigerator. Bananas would be expected to have an increase in concentration of sugars with a concurrent decrease in starch. The starch and glucose results were consistent with the predicted hypothesis. Starch levels decreased over the fifteen day period. The decrease in starch in samples stored at room temperature was greater than that of samples that were refrigerated. Glucose levels, measured by HK method, increased over the fifteen day period, more quickly in room temperature samples. Data from the DNS method and digests was inconsistent. This data is available, but not provided.

IV. CONCLUSIONS

The advantage of the biochemical detection and digestion method is a relatively small amount of sample and enzyme required, compared to the larger amount of sample and DNS reagent required by chemical methods. Hexokinase is also time efficient and highly specific to glucose. Determining glucose concentrations of food or drink samples

would be highly efficient in an undergraduate biochemistry laboratory using the HK detection method. This was found to be effective both in analyzing drink samples and bananas. The drawback to biochemical methods of digestion and detection used in this investigation is that the enzymes are more expensive. Also, invertase seems to falter in achieving a complete digest under certain conditions.

Advantages regarding chemical digestion and detection are that reagents are inexpensive and readily available for students in a laboratory setting. In terms of efficiency, the chemical methods are just as efficient as the biochemical methods based the results of this research. The DNS method is nonspecific and detects all reducing sugars. This detection method is more time consuming and requires a larger sample size than the HK method. Hence, neither biochemical nor chemical detection methods are exclusively superior to one another. Rather, they compliment each other.

On a side note, if a drink sample contains HFCS or a mixture of sugars, like Sprite or Gatorade respectively, the determination of sugar concentrations becomes too complicated and discouraging for an undergraduate lab when using nonspecific detection methods, such as DNS. However, if a class was to use drink samples containing sugar mixtures, they could determine the glucose concentration in the samples quite easily using the hexokinase method. An example of possible student explorations is comparing glucose levels in multiple brands of cola. In addition, the validity of diet and regular soda labels could be investigated. Students could also be provided unknown samples and determine if the samples were of regular or diet soda.

Taking into account the time and financial constraints of a typical collegiate laboratory setting, it is recommended to use both HK and DNS methods to detect glucose and all reducing sugars prior to digestion. A chemical digestion, which uses reagents that are cheaper and more available in a typical lab, as well as yielding a more complete digestion of sucrose, should be followed by the DNS detection in order to calculate the concentration of sucrose present in the sample. The proposed lab for undergraduate curriculum can be requested from the corresponding author of this publication.
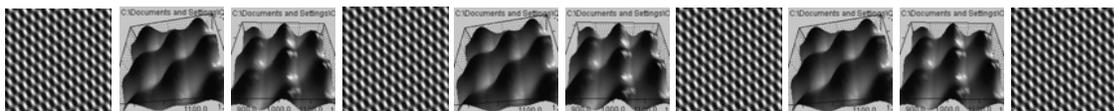
REFERENCES

1. Voet, D., Voet, J.G., Pratt C.W. *Carbohydrates, Funamentals of Biochemistry* (3rd Edn.) 2008, Wiley, 219- 244.
2. Fisher and Kohtes, 1951. Helv Chim Acta, 34, 1123.
3. Fieser, L.F. and Williamson K.L., 1979 *Hydrolysis of a Disaccharide. Organic Experiments*, D.C Heath and Company, 196-197.
4. Avigrad, G. and Englard, S., 1968. "Phosporylation of Yeast Hexokinase." *Journal of Biological Chemistry* 243:1511.
5. Kunst, A.; Draeger, B.; Zeigenhorn, J. In UV-Methods with Hexokinase and Glucose-6-Phosphate Dehydrogenase; Bergmeyer, H. U., Ed.; *Methods of. Enzymatic Analysis* (3rd. Edn) 1984; pp 163.172.
6. Bernfeld, P. In Amylases, α and β; Colowick, S. P., Kaplan, N. D., Eds.; *Methods Enzymol. 1*, 1955; pp 149.158.
7. Miller, G., 1959. "Use of dinitrosalicylic acid reagent for determination of reducing sugar." *Analytical Chemistry* 31: 426-428.
8. Eliot F. Beach and James J. Turner, "An Enzymatic Method for Glucose Determination in Body Fluids," *Clinical Chemistry*, Vol. 4, No. 6, 1958, 462-475.
9. Bergmeyer, H. U.; Bernt, E. In Determination with Glucose Oxidase and Peroxidase; Bergmeyer, H. U., Ed.; *Methods of. Enzymatic Analysis (2nd Edn)* 1974; pp 1205.1212.
10. Nelson, N., *J. Biol. Chem.* 153, 375 (1944).
11. Dubovski, K.M., "An o-toluidine method for body-fluid glucose determination 1" *Clin. Chem.* 1962 May-Jun; 8:215-35.
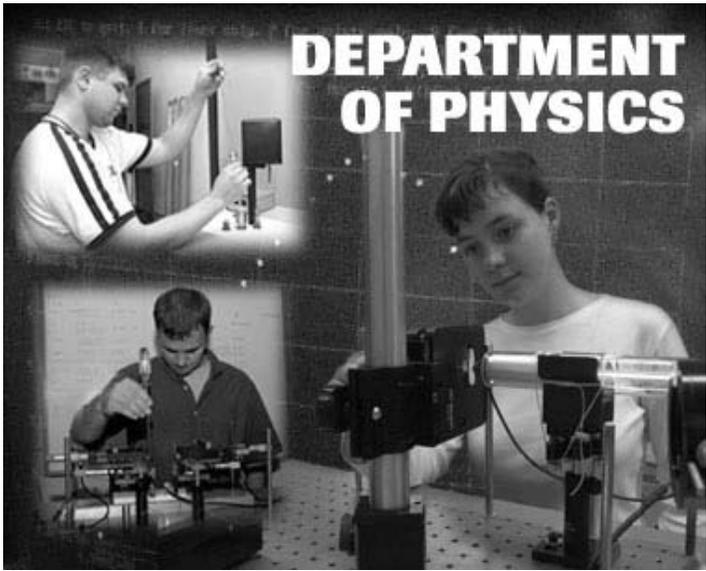
12. K. J. Melanson, L. Zukley, J. Lowndes, V. Nguyen, T. J. Angelopoulos and J. M. Rippe. "Effects of high-fructose corn syrup and sucrose consumption on circulating glucose, insulin, leptin, and ghrelin and on appetite in normal-weight women." *Nutrition*, 23, 103 (2007).

13. CRA, Corn Refiners Association. 2007. 'HFCS Facts.' Web URL www.hfcsfacts.com Accessed August 1 2008.