The Spectral Photometric Determination of Sucrose in Sugar Beets and Sugar Beet Products Via Specific Enzyme Systems

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Summary and Recommendations

Two enzyme systems (a) invertase hexokinase-G-6-PH and (b) invertase hexokinase-PGI-G-6-PDH were investigated and adapted for the determination of sucrose in sugar beets and sugar beet liquors.

The former is specific for native glucose as well as glucose derived from inverting sucrose via invertase. The difference between the two readings relates directly to sucrose concentration.

Trace impurities of PGI in the hexokinase introduce a small error through conversion of some fructose, which causes high values. This interference can be stabilized by strictly adhering to the methodology. The precision of ±.7% relative standard deviation compares very well with polarimetry. Since there are no interfering reactions aside from the above-mentioned fructose conversion, accuracy is about the same as precision making it superior to polarimetry and nearly equal to gas chromatography.

The inclusion of PGI in the methodology eliminates PGI interference and improves precision by giving a relative standard deviation of ±.54%. This is as good as gas chromatography. Interferences to this method originate with the presence of raffinose which is cleaved into melibiose and fructose during the inversion process. The response to raffinose is directly proportional to the amount of fructose cleaved; hence, each weight unit of raffinose will inflate the sucrose values by .339 weight units. Accuracy for this method is synonymous with precision when raffinose is not present. Reagent cost for either method is under seventeen cents per sample with the required operator time of about twenty minutes if carried out routinely in large batches. Instrument cost is lower than either polarimetry or gas chromatography.

Since extensive automation of this method is feasible, requirements for the skill of the technicians are about the same as for polarimetry and not nearly as stringent as for gas chromatography.

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Time requirements per sample would be further reduced by measuring rate of change for the final reaction. Such an approach could reduce the time requirements from twenty minutes to mere seconds. It is recommended that the enzymatic method of analysis be used for determining sucrose in the presence of optically active non-sucrose components when initial investment costs or lack of trained personnel prohibit the use of gas chromatography.

Introduction

In recent years the need for a new method of sucrose analysis has been recognized. The present method of polarimetry is not accurate when determining the concentration of sugar in the presence of optically active non-sucrose components. The sugar industry has suspected that a great deal of the unknown sugar loss in factory operations can be explained by this intrinsic error of polarimetry. The goal of Amalgamated Sugar's research department was to develop a method that was precise and specific only for sucrose. Beginning in 1972, an enzymatic procedure for the determination of sucrose was investigated.

The use of enzymes in analytical chemistry has become increasingly more popular in recent years because of the specificity of enzymes in difficult analytical problems. Clinical laboratories and many food processors routinely use enzymatic methods for the determination of glucose in samples of biological origin.

This report will be concerned with the modification of a popular clinical method in order to determine sucrose in beets and beet products. It will cover the enzyme reaction mechanism, conditions which influence the reaction rate, comparison of two enzyme systems, modification of the clinical procedure, and the precision and accuracy of the method.

Results and Discussion

Enzyme reaction mechanism

The basic reaction mechanism consists of the hydrolysis of sucrose by invertase to glucose and fructose.

$$\text{SUCROSE} \xrightarrow[H_2O]{\text{INVERTASE}} \text{GLUCOSE} + \text{FRUCTOSE} \qquad (1)$$

The two hexoses are then phosphorelated by the coenzyme, ADENOSINE-5-TRIPHOSPHATE (ATP), to their corresponding hexose-6-phosphates.

$$\text{FRUCTOSE} + \text{ATP} \xrightarrow{\text{HEXOKINASE}} \text{FRUCTOSE-6-PHOSPHATE} + \text{ADP} \qquad (2)$$

$$\text{GLUCOSE} + \text{ATP} \xrightarrow{\text{HEXOKINASE}} \text{GLUCOSE-6-PHOSPHATE} + \text{ADP} \qquad (3)$$

Although both the glucose and fructose are phosphorelated, only the glucose-6-phosphate is oxidized by the enzyme glucose-6-phosphate dehydrogenase (G-6-PDH) to produce the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH).

$$\text{GLUCOSE-6-PHOSPHATE} + \text{NADP} \xrightarrow{\text{G-6-PDH}} \text{GLUCONATE-6-PHOSPHATE} + \text{NADPH} \quad (4)$$

The NADPH is determined by virtue of its optical density at 340mu.

In the absence of contaminating enzymes, this mechanism is specific for glucose. In addition, if the glucose present before inversion is accounted for, the method will be specific for sucrose.

There is, however, an isomerase impurity found in the hexokinase preparation which enters into the reaction mechanism. Phosphoglucose Isomerase (PGI) isomerizes fructose-6-phosphate to glucose-6-phosphate.

$$\text{FRUCTOSE-6-PHOSPHATE} \xrightarrow{\text{PGI}} \text{GLUCOSE-6-PHOSPHATE} \quad (5)$$

The glucose-6-phosphate reacts according to equation (4) to produce additional NADPH. The error from PGI contamination is minimal (.003% of the hexokinase activity), but it can be eliminated completely by adding sufficient PGI to the system to isomerize all the fructose-6-phosphate. Sucrose then can be determined by two enzyme systems. First, using the enzymes hexokinase and G-6-PDH and determining the sucrose concentration from the amount of glucose formed during the inversion step; or secondly, by using the enzymes hexokinase, PGI and G-6-PDH and determining the sucrose concentration from the amount of glucose and fructose formed during the inversion step.

A second interfering reaction is the hydrolysis of raffinose by yeast invertase according to the equation:

$$\text{RAFFINOSE} \xrightarrow[\text{H}_2\text{O}]{\text{INVERTASE}} \text{FRUCTOSE} + \text{MELIBIOSE} \quad (6)$$

Fructose formed from the hydrolysis of raffinose enters into the reaction mechanism according to equation (2) and will finally produce NADPH when the enzyme PGI is present.

Rate determining conditions

All tests were conducted using the enzyme system which does not require PGI. The conditions which influence the rate of reaction include pH, temperature, and enzyme concentration. The coenzymes ATP and NADP were always used in sufficiently large excess to drive the reaction to completion. These conditions then, except for pH, were optimized for our specific procedure. Literature references and

enzyme manufacturers were in agreement on a pH of 7.6 for the optimum reaction rate.

The optimum temperature was determined by observing the reaction rate at different temperatures holding all other variables constant. Figure 1 shows the reaction rate to be a maximum between 30°C and 35°C.

The optimum enzyme concentration was defined as the lowest possible which would complete the reaction in reasonable length of time. In most enzyme systems, the rate of reaction falls off as the reaction proceeds. This is because of the inhibitions of the products of reaction; as they gradually accumulate they cause a slowing down of the reaction. In the hexokinase-PGI-G-6-PDH system, this is true only for the last enzyme step catylized by G-6-PDH. In the enzyme reactions using invertase, hexokinase, and PGI, the products do not interfere with the reaction rate because they are continually being removed by the succeeding reaction. It is the G-6-PDH determinative step, then, which governs the overall rate of reaction. Figure 2 indicates that a concentration of four to five units of G-6-PDH per mg sucrose is necessary for the reaction to be essentially complete in twenty minutes.

Changes in the hexokinase concentration do not markedly affect the initial reaction rate, as indicated by Figure 3.

There is, however, a noticeable change in the amount of drift after the reaction is essentially complete. This drift is a direct result of PGI which is present as an impurity in the hexokinase preparation. When using the hexokinase-G-6-PDH system, it is advantageous to limit the PGI contamination by using a minimal amount of hexokinase. A hexokinase concentration of .25 units per mg sucrose was determined as the minimum concentration which did not hinder the overall reaction rate.

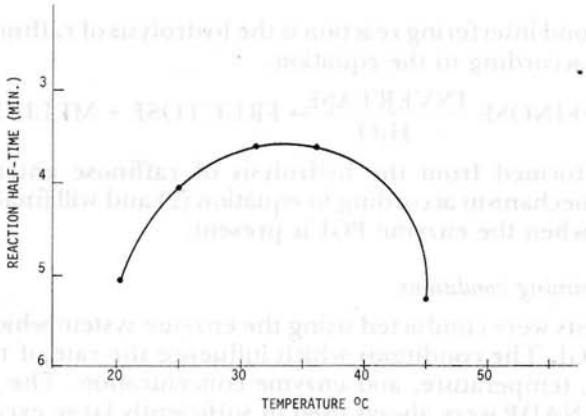

Figure 1.—Effect of temperature on reaction rate.

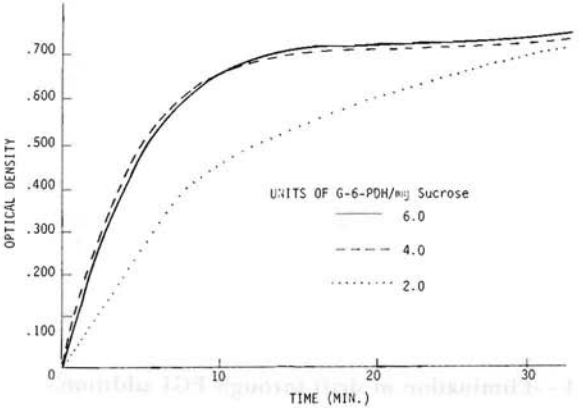

Figure 2.—Effect of G-6-PDH concentration on reaction rate.

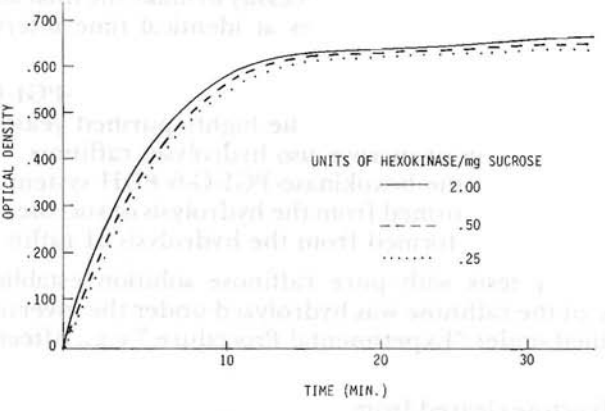

Figure 3.—Effect of hexokinase concentration on reaction rate.

When determining sucrose by the hexokinase-PGI-G-6-PDH system, it was found that a PGI concentration equal to the hexokinase concentration was adequate.

Comparison of the two enzyme systems

Determining sucrose using PGI and measuring the amount of both fructose and glucose formed by inversion is more precise than determining sucrose by measuring glucose formation alone for two reasons. First, the stoichiometric ratio between sucrose and the NADPH produced is doubled, resulting in better precision because of the increased molar absorbtivity of sucrose. Second, when PGI is used the drift in final absorbance which occurs when PGI is present only as an impurity is eliminated. Figure 4 shows two reaction curves, one with PGI present only as an impurity and the other using half the sucrose concentration and sufficient PGI to isomerize all the fructose-6-phos-

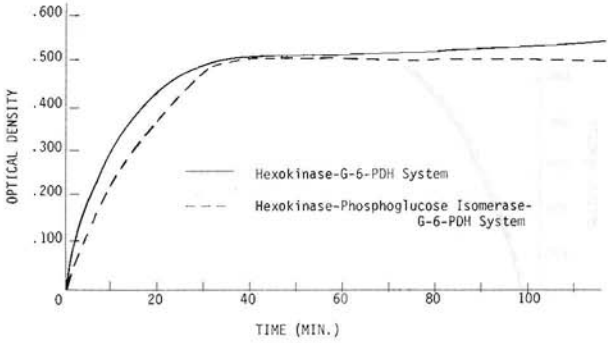

Figure 4.—Elimination of drift through PGI addition.

phate. The small but steady increase in absorbance is eliminated when PGI is added. This eliminates the necessity to make the final absorbance readings of standards and samples at identical time intervals after enzyme addition.

The major drawback to using the hexokinase-PGI-G-6-PDH system is its raffinose sensitivity. The highly purified yeast invertase used in the hydrolysis of sucrose also hydrolyses raffinose to fructose and melibiose. Since the hexokinase-PGI-G-6-PDH system measures glucose and fructose formed from the hydrolysis of sucrose, it will also measure the fructose formed from the hydrolysis of raffinose.

Laboratory tests with pure raffinose solution established that about 75% of the raffinose was hydrolyzed under the inversion conditions outlined under "Experimental Procedure;" e.g., fifteen minutes at 55°C.

The fructose cleaved from the raffinose was accurately accounted for under the conditions of the procedure as described under "Experimental Procedure" and using the hexokinase-PGI-G-6-PDH system.

It must be expected that the raffinose will be completely hydrolyzed at the low concentration germane to our sugar beet liquors and the inversion conditions required for the hex-PGI-G-6-PDH system. From this study, we know that any raffinose will increase the sucrose reading by 34% of the raffinose quantity when using the hex-PGI-G-6-PDH system. The parallel application of both methods would allow the determination of the raffinose by difference.

At the very low raffinose levels below 1% on sugar, however, the increase due to raffinose is within the error limits of the method and would allow differentiation only with several replications per test.

It should be emphasized that raffinose interferes only when sucrose is determined via the hexokinase-PGI-G-6-PDH system. When raffinose levels in the sample to be analyzed are high and impractical to account for, the hexokinase-G-6-PDH system should be used.

Procedure modifications

A number of modifications of the standard clinical method have been made to increase the precision, accuracy, and economy. One important change that reduces the cost per analysis considerably is the substitution of the coenzyme NADP with NAD. A new form of G-6-PDH, recently introduced on the market by Worthington Biochemicals Corporation, allows the use of NAD rather than NADP as a coenzyme. This substitute coenzyme is approximately one-tenth of the cost of NADP and will reduce the cost per analysis from 23.6c to 16.5c.

Replacement of the conventional spectrophotometer cuvetts with flow-through cells proved to be beneficial in simplifying the method, increasing the number of samples which can be analyzed in a day, and reducing error from cell corrections and repositioning of cells. Two flow-through cells were used for sample and reference solutions. Because the final reaction mixture is only three mls, it was necessary that the cells hold a minimum volume. Spectrosile cells purchased from Precision Glass Products which have a 0.3 ml capacity and a 1.00 cm optical path length worked well.

The basic method called for separate dispensing of the various enzymes, coenzymes, and buffers directly into a cuvett. In the course of refining the analytical technique, it was found that the various reagents could be combined into two working solutions without decreasing the activity of the enzymes or coenzymes. Combining of the reagents made it possible to reduce the number of reagent additions and therefore, the error associated with dispensing a number of small volumes.

Special consideration was given in the development of this method to the accurate dispensing of small volumes, since reagent cost considerations dictated a minimum use of reagents. A Brinkmann Brand Dilutor was found to give the precision and convenience necessary to dispense the sample to the nearest 0.1ul and the coenzyme working solution to the nearest 1.0ul. An Eppendorf pipet was determined sufficient for dispensing the enzyme working solution.

Experimental

Apparatus. The instrumentation includes a Varian 635 Double Beam Spectrophotometer equipped with two flow-through cuvetts, a Brinkmann Brand dilutor, and a 20ul Eppendorf pipet.

Reagent Preparation.

Inversion Buffer
13.3 gram NaOH
40.0 ml acidic acid
0.75 gram analytical grade invertase
Dilute to 10 liters with distilled H_2O

The inversion solution will have a pH of 4.6 and is stable for approximately one week.

The enzyme and coenzyme working solutions are dependent upon which enzyme system is used. The following table gives the information required to prepare working solutions for both methods.

Table 1.—Preparation of working solutions.

Enzyme system	Enzyme working solution	Coenzyme working solution
Hexokinase, G-6-PDH	125 units Hexokinase 500 units G-6-PDH Dilute to 2.5ml with double distilled H_2O	0.8 grams ATP 0.08 grams NAD .45 grams $MgCl_2$* .$6H_2O$ 225ml 0.25 Triethanol amine HCl adjusted to pH 7.6 with NaOH
Hexokinase, PGI G-6-PDH	125 units Hexokinase 125 units PGI 500 units G-6-PDH Dilute to 2.5ml with double distilled H_2O	0.4 grams ATP 0.08 grams NAD .45 grams $MgCl_2$* .$6H_2O$ 225ml 0.25m Triethanol amine HCl adjusted to pH 7.6 with NaOH

*The $MgCl_2$ is added to enhance the enzymatic reaction rate.

The coenzyme solution is stable for approximately two weeks. The enzyme solution should be prepared fresh daily to protect against loss in activity from bacterial contamination. Reagents and enzymes are listed in the appendix along with their suppliers and prices.

Standards. Standards containing 0.10, 0.15, 0.20, 0.25, and 0.30 mg sucrose/ml are prepared fresh daily in inversion solution. The standards should be doubled if the hexokinase-G-6-PDH system is used. Standards should be run daily or everytime a new enzyme working solution is prepared to insure proper enzyme activity.

Sample preparation. The sample preparations described below are to be used with the hexokinase-PGI-G-6-PDH system. If the hexokinase-G-6-PDH system is used the concentration of the stock sample solution should be doubled.

Beet brei or ground cossettes are prepared by homogenizing 60gm of the sample in a high-speed blender with 40gm of solvent. Dimethylformamide (DMF) is preferred as a solvent since it will produce relatively stable homogenates, minimize the inversion of sucrose during the homogenizing procedure, and inhibit sucrose breakdown.[2]

The stock sample solution is prepared by weighing approximately two grams of homogenate, to the nearest 0.1mg, rinsing into a 200ml flask, and diluting to the mark with distilled water. Stock sample solutions of molasses, raw juice, and other various beet juices may be

[2]DMF homogenates from fresh beets have been stored for over six months at normal room temperature without a noticeable change in sucrose content.

analyzed by preparing a stock sample solution containing approximately 80mg sucrose/100ml solution.

Procedure. Two 25ml aliquots of the stock sample solution are pipetted into two 100ml volumetric flasks. The reference solution is prepared by diluting one flask to the mark with distilled water. The inverted sample solution is prepared by diluting the second volumetric flask to the mark with inversion buffer. Both solutions are then thermostated at 55°C for fifteen minutes and cooled to room temperature. At this point, it may be necessary to centrifuge a portion of the solutions to settle any suspended solids that would interfere with the absorbance readings. The Brinkmann Brand dilutor is then used to dispense 0.2ml of inverted sample and 2.8ml of the coenzyme working solution into a 13 × 100mm test tube. The procedure is repeated using the uninverted reference solution in place of the inverted sample solution. Next, 20ul of the enzyme working solution is added to both sample and reference test tubes. Both test tubes are thermostated for twenty minutes from time of enzyme addition or until the reaction is complete in a 32°C water bath. The sample and reference solutions are then drawn into their respective flow-through cells, which have previously been zeroed with distilled water, and the absorbance read at 340mu.

Using this procedure the reference cell corrects for any glucose and fructose present before inversion and for any color in the sample which absorbs at 340mu.

Evaluation

The precision of both enzyme systems was tested by replicate readings of sucrose standards over a period of days using a different enzyme preparation each day. The results are shown in the following table.

Table 2.—Precision of the enzyme methods.

Enzyme system	Hexokinase-G-6-PDH	Hexokinase-PGI-G-6-PDH
Concentration of standard	.40mg/ml	.30mg/ml
Number of replicates	26	24
Mean absorbance	.5050	.7234
Standard deviation	±.00355	±.00389
Relative standard deviation	±0.703%	±0.538%

The data from Table 2 indicate that of the two enzyme systems, the hexokinase-PGI-G-6-PDH system is slightly more precise and that a relative standard deviation of ±0.54% can be expected.

Sucrose was determined by polarimetry, gas chromatography, and the hexokinase-G-6-PDH enzyme method on 1200 beet samples to compare methods. The correlation coefficients are listed below:

Table 3.—Correlation between enzyme GC and polarimetry.

	Enzyme	GC
Polarimetry	.976	.995
Enzyme	--	.977

It can be seen that the enzyme method correlates equally well with gas chromatography and polarimetry but not as well as gas chromatography does with polarimetry. It should be noted that all gas chromatography analyses were performed by a single experienced chemist while the enzyme method of analysis was performed by several different technicians with limited experience.

The two enzyme systems were compared to the popular method of gas chromatography to learn if, on the average, there is a difference between methods. Different groups of 60 beets were analyzed by each enzyme system and by gas chromatography. The results are given in Table 4. The sixty samples analyzed via hexokinase-G-6-PDH were taken at random from the program of 1200 samples.

Table 4.—Comparison of enzyme methods with gas chromatography.

Enzyme system	Hex-PGI-G-6-PDH	Hex-G-6-PDH
Number of beet samples	60	60
Average % sugar by enzyme	16.0423	14.9312
Average % sugar by GC	16.0003	14.9385
Difference between means	+0.042	−0.0073
Standard deviation of difference	±0.3780	±0.61556
T value (calculated)	.8610	.0919
Correlation coefficient	.623	.917

The means of both enzyme systems compare very closely with gas chromatography as indicated by the small t values. (The critical value is $t_{0.05} = 2.00$ for 60 df.) The standard deviation of the difference between samples is much larger for the hexokinase-G-6-PDH system. This is probably a result of several different technicians with limited experience performing the analysis.

The higher mean for the hexokinase-PGI-G-6-PDH may be traced to the presence of raffinose in the beet. The difference between the mean accounts for about .15% raffinose on beets. The inferior correlation coefficient for the PGI method has its explanation in the narrow range of its population (14.88 – 16.83 vs 11.82 – 17.85 for the hexokinase-G-6-PDH method). Varying raffinose concentration may also contribute to the inaccuracy of the PGI method even though the PGI method has a better precision.

Conclusions and Recommendations

An accurate and reliable enzyme method for the determination of sucrose in beets and beet products has been developed. The analytical procedure has been refined and optimized in terms of kinetics, cost, speed of analysis, precision, and accuracy.

Sucrose may be determined by one of two enzyme systems. At the present time, the hexokinase-G-6-PDH system has the advantage of being specific for sucrose in the presence of raffinose, but requires more attention to analytical procedure and is not as precise as the

hexokinase-PGI-G-6-PDH enzyme system. The hexokinase-G-6-PDH system would be the most advantageous overall if enzyme preparations could be purchased absolutely free of hexose isomerase impurities. As it now stands, the choice of the proper enzyme system will depend on the raffinose content in the sample being analyzed.

Compared to polarimetry, the enzyme method is more expensive, but much more specific for sucrose. The enzyme system has an extra advantage over polarimetry of practically eliminating the error due to insoluble matter (mark) in the analysis of beets which plagues the hot digestion method used with polarimetry.

When comparing the enzyme method to the popular gas chromatography method, it was found that gas chromatography had the higher correlation with polarimetry. The main advantages of the enzyme methods are the lower initial investment cost and the simplicity of sample preparation and analysis which results in a less skilled technician being required.

The insignificant difference found between the method of standard addition and the method of standard comparison indicates that the various components of beet homogenates do not significantly enhance or suppress the enzyme reaction mechanism.

Appendix I

Reagent	Supplier	Cat. No.	Cost as of May 1975
ATP	NBC		$3.83/10 gm
NAD	Sigma	N-7004	$10.00/gm
Hexokinase F-300	Sigma	N-4502	$8.00/1000 units
G-6-PDH (use with NAD)	Worthington	ZFLD	$55.00/1800 units
Triethanolamine HCl	Sigma	T1502	$4.75/500 gm
Invertase	NBC		$4.75/5 gm
PGI	Sigma	P-9010	$5.00/1000 units

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