THE USE OF INVERTASE FOR DETERMINATION OF SUCROSE. APPLICATION TO CEREALS, CEREAL PRODUCTS, AND OTHER PLANT MATERIALS

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ABSTRACT

A specific enzymatic method for the determination of sucrose in cereals, cereal products, and other plant materials is described. Sucrose, after repeated extraction with hot 80% ethanol, is hydrolyzed by invertase. The liberated glucose is estimated by glucose oxidase. Free glucose, if present, can be determined before inversion. The method is flexible in that sucrose can be determined in ethanol extracts as well as in water extracts. The procedure is simple and rapid and does not require sophisticated laboratory equipment. Sucrose can be determined specifically in the presence of large amounts of other sugars. In many cases the usual clarification procedure required for the removal of nonsugar reducing substances is not necessary.

The need for simpler and more specific methods for the quantitative determination of sugars in cereals and other plant materials is well recognized. Common methods for sugar analysis involve chemical (1–4), chromatographic (4–8), and enzymatic (9–13) procedures. For complete analysis of individual sugars, a combination of these techniques (14–16) is often required. A major limitation of chemical methods is their lack of specificity. Chromatographic procedures (paper and thin-layer) work well for sugar analysis, but are time consuming when sugars are determined by a colorimetric method after elution. Other methods require sophisticated equipment (column chromatography, Auto-Analyzer) not always available in control laboratories (17).

The purpose of the present work was to examine the possible application of invertase for the determination of sucrose and to provide a method for estimation of sucrose in cereals, cereal products, and other plant materials. The method may be useful also for quality assurance and regulatory purposes of sucrose-sweetened food products such as composite flours, or cake, or pastry ready-mixes.

MATERIALS AND METHODS

The method is based on the enzymatic hydrolysis of sucrose by invertase and the specific estimation of liberated glucose by glucose oxidase. If free glucose is present, it can be determined by the same procedure before hydrolysis of sucrose.

Reagents

1) *Standard D-Glucose Solution.* Dissolve 400 mg of pure anhydrous D-glucose in distilled water and dilute to a volume of 1,000 ml with water. This solution must be prepared 4 hr before use to allow time for complete mutarotation. Store at 4°C.

2) *Standard Sucrose Solution.* Dissolve 500 mg of pure anhydrous sucrose in distilled water and dilute to a volume of 500 ml with water. Store at 4°C.

3) *"Tris" Buffer.* Dissolve 61 g of tris(hydroxymethyl)aminomethane in 85 ml

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$5N$ hydrochloric acid and dilute to a volume of 1,000 ml with distilled water. Store at 4° C.

4) *Enzyme-Buffer-Chromogen Mixture.* a) Glucose oxidase solution—dissolve 25 mg of glucose oxidase (Type II from *Aspergillus niger*, Sigma Chemical Co., St. Louis, Mo.) in 25 ml of "tris" buffer; b) Peroxidase solution—dissolve 15 mg of peroxidase (Type I from horseradish, Sigma) in 25 ml of "tris" buffer; c) Chromogen solution—dissolve 50 mg of *o*-anisidine dihydrochloride in 10 ml of 95% ethanol.

Solutions a and b can be kept at −5° C and are stable, without loss of activity, for at least 10 days. The chromogen can be stored at 4° C.

The reagent mixture is prepared just prior to use by mixing 20 ml of solution a, 5 ml of solution b, and 0.5 ml of solution c, and diluting to 125 ml with "tris" buffer.

5) *Invertase Solution.* Prepare a solution containing 1 mg of invertase (Type VI from yeast, Sigma) per 1 ml.

6) *Acetate Buffer* ($2M$, pH 4.7): dissolve 164 g of anhydrous sodium acetate in 120 ml of acetic acid. Dilute to a volume of 1,000 ml with distilled water.

7) *Hydrochloric Acid, 5N.*

8) *95% and 80% Ethanol.*

9) *Carrez Solution I and II.*

Carrez solution I: dissolve 23.8 g of zinc acetate trihydrate and 3 g of glacial acetic acid in water and dilute to a volume of 100 ml with water. Carrez solution II: dissolve 10.6 g of potassium ferricyanide in water and dilute to a volume of 100 ml with distilled water.

Method

Sugar Extraction—Weigh 2.5 g of product in a 60-ml centrifuge tube and add 40 ml of 80% ethanol. Connect to a condenser and gently boil in a water bath for 30 min with occasional stirring. Cool, centrifuge for 15 min at 3500 rpm and collect the supernatant solution in a 200-ml volumetric flask. Repeat the extraction once. Then make two additional washings with 40 ml 80% ethanol at 25° C. Centrifuge, collect the supernatant solutions in the 200-ml volumetric flask, and dilute to volume with 80% ethanol.

For cereals and cereal products, sucrose can be determined directly in the ethanol extract. However, it may be necessary to purify the extract if total sugars or sucrose in protein-rich seeds are to be determined. If free glucose, usually present in much smaller amounts than sucrose, has to be estimated it may be necessary to reduce the volume of the extract. In that case, evaporate the ethanol extract almost to dryness under reduced pressure, dissolve the residue with 30 ml of distilled water, and transfer quantitatively to a 50-ml volumetric flask. Add 1.5 ml of Carrez solution I and then 1.5 ml of Carrez solution II. Shake, and dilute with water to a volume of 50 ml. Shake and filter, when necessary.

Enzymatic Hydrolysis of Sucrose—Pipet aliquots of the extracts (or extracts diluted in water or ethanol) containing between 0.5 and 1.5 mg of total glucose into a 25-ml volumetric flask. Add 2.5 ml of acetate buffer previously diluted to a concentration of $0.1M$ and 1 ml of the invertase solution. Dilute to volume with water or with 30% ethanol (for ethanol extracts). The final ethanol concentration depends on the volume of the aliquots used for hydrolysis. The control (2.5 ml of

sucrose solution, 2.5 ml of acetate buffer, 1 ml of invertase solution in 25 ml volume) should have the same ethanol concentration as the extract. Allow the digests to stand overnight at room temperature.

Determination of Liberated Glucose—Pipet 1 ml of the digests or dilutions of the digest (20 to 60 μg glucose/ml) into test tubes. At zero time initiate the reaction by adding 5 ml of the enzyme-buffer-chromogen mixture. Shake the tubes and place in the dark at 20° C ± 2° C for exactly 45 min to develop the color. Stop the reaction with 0.25 ml 5*N* hydrochloric acid and measure the absorbances at 400 nm. Include a blank (water or ethanol in the concentration used for the digest).

The glucose in the sample is determined with a standard curve and the results are expressed in the following manner

$$\text{Sucrose} = (\text{total glucose} - \text{free glucose}) \times 1.90$$

Standard Curve—Pipet 5 ml, 10 ml, and 15 ml of the standard glucose solution into 100-ml volumetric flasks. Add ethanol in such amount as to obtain the same concentration as that of the digest. Of each of these dilutions, 1 ml constitutes the standard for the calibration curve (20, 40, and 60 μg).

For the determination of free glucose, if present, remove aliquots of the extracts or diluted extracts (20 to 60 μg glucose) and perform the colorimetric determination as described above.

Precision—The mean reproducibility with respect to the relative value is 3% including the error inherent in extraction of the sample as well as in the analysis.

RESULTS AND DISCUSSION

Assays with Pure Sugar Solutions

Sugars are usually extracted with aqueous ethanol. It is possible to determine total sugars by the anthrone method, and glucose by glucose oxidase directly in the ethanol extracts (15,16). Therefore, it seemed of interest, at least for routine work, to ascertain whether sucrose could also be determined directly in ethanol extracts. This is the reason why all enzymatic reactions are accomplished at room temperature. Under the experimental conditions described, pure sucrose was

TABLE I
Reaction of Invertase on Pure Sucrose Dissolved in Water and Ethanol of Varying Concentrations at Room Temperature (20° C ± 2° C), Incubation Time 20 hr, pH 4.7

Sucrose (theoretical) mg	Sucrose (recovered)[a] mg					
		Ethanol				
	Water	30%	50%	60%	70%	80%
10	10.1	9.9	9.9	9.9	10.0	10.2
10	9.9	10.1	10.2	10.2	10.0	10.1
10	10.0	10.1	10.1	10.1	9.9	9.8

[a]Mean value of four determinations.

incubated with invertase in the presence of varying concentrations of ethanol. The results of assays performed at intervals of several weeks are given in Table I. It is obvious that invertase hydrolyzes sucrose even when the ethanol concentration is as high as 80%. However, such ethanol concentrations (80%) do not occur normally in sucrose determinations since a buffer and an invertase solution is added to an aliquot of the sample extract for incubation.

TABLE II

Reaction of Invertase on Sucrose in the Presence of Varying Amounts of Glucose and Fructose at Room Temperature (20°C ± 2°C), Incubation Time 20 hr, pH 4.7

		Glucose[a] mg		
			Assay Medium	
Sugars	Amount mg	Theoretical	Water	30% Ethanol
Sucrose	10	5.26	5.39	5.32
Sucrose Glucose Fructose	5 2.5 2.5	5.13	5.04	5.17
Sucrose Glucose Fructose	10 10 10	15.26	15.1	15.2
Sucrose Glucose Fructose	1 4 4	4.53	4.44	4.45

[a]Mean value of three determinations.

TABLE III

Reaction of Invertase on Sucrose in the Presence of Varying Amounts of Raffinose and Stachyose at Room Temperature (20°C ± 2°C), Incubation Time 20 hr, pH 4.7

Sugars	Amount mg	Glucose Determined[a] mg
Sucrose	10	5.2
Sucrose + Raffinose + Stachyose	10 0.6 0.6	5.16
Sucrose + Raffinose + Stachyose	10 3 3	5.26
Sucrose + Raffinose + Stachyose	10 6 6	4.74

[a]Mean value of two determinations.

Ethanol extracts from cereal products and other plant materials frequently contain varying amounts of sugars other than sucrose, such as glucose, fructose, raffinose, and stachyose. In order to check for interference or for inhibition by these sugars, differing amounts of glucose, fructose, raffinose and stachyose were added to the sucrose assays. Results indicated no significant differences in sucrose values due to the presence of these sugars (Tables II and III).

It was verified by thin-layer chromatography that the invertase used had no α-galactosidase or α-glucosidase activity; which would have resulted in the liberation of galactose from raffinose and stachyose, and of glucose from maltose. The presence of maltose and maltosaccharides does, however, interfere in the estimation of glucose. In fact, glucose oxidase preparations frequently contain carbohydrases (α-glucosidases) which liberate glucose from glucose-containing polymers making determination of glucose impossible in the presence of such saccharides (10–12). However, the "tris" buffer used is known to inhibit completely the action of maltase (18,19), and we verified that amounts of maltose up to 100% of the glucose present did not interfere in the glucose assay under the experimental conditions described in this paper.

TABLE IV
Comparative Data of Determination of Sucrose and Glucose in an Ethanol (80%) Extract of Potatoes by the Enzymatic Procedure and by Paper Chromatography[a]

	Enzymatic Procedure	Paper Chromatography[b]	
	Unpurified extract	Unpurified extract	Purified extract[c]
Glucose	0.225[d]	0.223	0.225
Sucrose	0.205	0.194	0.195

[a]Mean value of two determinations.
[b]Recovery of pure glucose and sucrose from paper chromatograms was 97 and 97.5%, respectively.
[c]Dowex®-50 resin.
[d]Values are per cent of dry matter.

TABLE V
Comparative Data of Determination of Sucrose in Wheat Flours and Horse Bean Samples by the Enzymatic Procedure and by Paper Chromatography[a]

Sample	Enzymatic Procedure	Paper Chromatography	Difference[b] %
Patent flour	0.49[c]	0.50	2.0
Coarse fraction	0.67	0.65	3.1
Medium fraction	0.66	0.64	3.1
High-protein fine	0.48	0.49	2.4
Horse beans	2.10	2.15	4.1
Horse beans	2.20	2.30	2.2
Horse beans	1.50	1.45	3.4

[a]Mean value of two determinations.
[b]Difference between the two methods with respect to the relative value.
[c]Values are per cent of dry matter.

It is imperative to establish a calibration curve with glucose standards that have been prepared so that they are comparable in all their constituents to the sample to be analyzed. Conditions should be arranged so that the buffer concentration in the solution to be analyzed does not exceed 0.01*M*.

Application of the Proposed Method to Various Plant Materials

In order to investigate possible applications of the method and to check precision and accuracy, sucrose was determined in various materials by both the invertase assay and by quantitative paper chromatography (4). Table IV shows results of the assays of a plant material which contains a relatively-simple sugar composition (glucose, fructose, sucrose). The data show that the enzymatic procedure is in good agreement with the chromatographic procedure. Furthermore, the values obtained demonstrate that purification of the ethanol extracts, *i.e.*, elimination of nonsugar substances such as proteins, is not necessary. It seems relevant to mention that the results obtained by paper chromatography are based on the recovery of the applied amounts of pure-sugar solutions, which was 97.0 to 97.5% as measured by anthrone after elution (4,16).

The values in Table V illustrate that the method also gives satisfactory results when applied to wheat flour and legume seeds. Ethanol extracts of wheat and barley contain a certain amount of glucofructans (7,20). Williams and Bevenue (21) reported that the glucose unit from this carbohydrate fraction is likely to be hydrolyzed by invertase. However, since the sucrose values obtained by paper chromatography and by the enzymatic procedure are in close agreement, it is assumed that the hydrolysis, if such exists, is negligibly small.

In order to ascertain that invertase was not inhibited by ethanol-extracted material from the sample itself, pure sucrose was added to wheat and corn extracts. For this purpose, samples with widely—varying chemical compositions

TABLE VI
Recovery of Sucrose Added to Ethanol Extracts of Corn and Wheat[a]

Ethanol Extracts	Sucrose in the initial extract μg	Sucrose in the extract with added (50 μg) sucrose μg	Difference[b] μg	Error[c] μg
Corn (immature)	62.7	113.9	51.2	+1.2
Corn (mature)	62.7	112.7	50.0	0
Wheat (immature)	25.0	72.8	47.8	−2.2
Wheat (mature)	32.7	81.9	50.2	+0.2

[a]Mean value of two determinations.
[b]Difference between the sucrose determination in the initial extract and extract with added sucrose.
[c]Error calculated based on 50 μg of added sucrose.

TABLE VII
Comparative Data of the Determination of Sucrose by the Enzymatic Procedure and by Estimating Nonreducing Sugars (% Dry Matter)[a]

	Nonreducing sugars expressed as sucrose	Sucrose by the enzymatic procedure
Wheat flour	1.4[a,b]	0.59[a]
Composite flour[c]	57.1	51.0
Composite flour[d]	55.0	50.6

[a]Mean value of two determinations.
[b]Values are per cent of dry matter.
[c]Ready mix containing wheat flour, wheat starch, defatted milk powder, and 50% sucrose.
[d]Ready mix containing soluble gums, pregelatinized starch, fat, defatted milk powder, and 51% sucrose.

were chosen (immature and mature). Table VI illustrates the results of these experiments. As seen in all four cases, the sucrose added to the incubation of the cereal extracts is recovered with a mean reproducibility of about 1.8% with respect to the relative value. Thus, it may be presumed that no interference from the ethanol-extracted material occurs in the sucrose assay.

Finally, the proposed method was applied to commercial composite flour samples designed for the production of cakes and desserts (Table VII). The composition of these mixtures is rather complex in that they contain, other than wheat flour, native or pregelatinized starch, fat and defatted milk powder, and known amounts of sucrose. Usually, sucrose is the desired value for quality control. Great difficulties are encountered when sucrose is determined by the conventional procedure which involves both the determination of reducing and nonreducing sugars. In this procedure, errors are introduced by the noncarbohydrate reducing components as well as by the presence of ethanol-extractable higher-molecular-weight carbohydrates ($\overline{DP} \rightarrow 12$) such as maltosaccharides and glucofructans. The latter are hydrolyzed, to a certain extent, during acid-catalyzed inversion procedures, thus resulting in excessive sucrose values based on reducing and nonreducing sugar values (Table VII). The amounts of sucrose in the ingredients are only about 0.1%, but 50% added sucrose has to be determined. The enzyme procedure comes very close to these values, demonstrating the practical application of the proposed sucrose assay.

DISCUSSION

The use of invertase provides a very accurate method for determining sucrose specifically in the presence of other sugars in various materials. Of particular interest is the fact that hydrolysis of sucrose by invertase and determination of the liberated glucose by glucose oxidase can be accomplished directly in ethanol extracts. The equipment requirements are modest; the method is simple, specific, and reasonably rapid, and may be useful to determine added sucrose in sweetened products, such as ready cake mixes.

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