Megazyme

SUCROSE / GLUCOSE

ASSAY PROCEDURE

FOR THE MEASUREMENT OF
SUCROSE and GLUCOSE

KSUCGL 8/04

(250 Assays per Kit)

INTRODUCTION:

Sucrose and D-glucose are two of the most commonly occurring sugars in plant and food products and have serious impacts on human nutrition. Glucose can be conveniently measured in body fluids using commercially available kits based on the glucose oxidase/peroxidase or on the hexokinase/G6PDH enzymic procedures. However, glucose in plant extracts usually occurs together with maltose, maltosaccharides, starch, sucrose and/or β-linked gluco-oligosaccharides. Consequently, more stringent requirements are placed on the purity of the assay reagents. The reagents must be essentially devoid of starch degrading enzymes, sucrose degrading enzymes and β-glucosidase, as these can lead to either an overestimation or an underestimation of free glucose present in the extract or derived by specific enzymic degradation of glucose containing oligosaccharides or polysaccharides (e.g. barley β-glucan). The Megazyme Sucrose/Glucose Test Kit employs high purity glucose oxidase, peroxidase and β-fructosidase (invertase) and can be used with confidence for the specific measurement of glucose and sucrose in plant and food extracts. The colour which forms is stable at room temperature for at least two hours after development.

PRINCIPLE:

The reactions involved are:

(1)

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

(2)

$$2H_2O_2 + p\text{-Hydroxybenzoic acid} + 4\ \text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$

(3)

$$\text{Sucrose} + H_2O \xrightarrow{\beta\text{-fructosidase}} \text{D-Glucose} + \text{D-Fructose}$$

Free glucose in the sample extract is determined by conversion to a red coloured quinoneimine dye compound through the action of glucose oxidase (1) and peroxidase (2) at pH 7.4, and employing *p*-hydroxybenzoic acid and 4-aminoantipyrine.

At pH 4.6, sucrose is hydrolysed by the enzyme β-fructosidase to D-glucose and D-fructose (3). The determination of D-glucose after inversion (total glucose) is carried out simultaneously according to the principle outlined above. The sucrose content is calculated from the difference of the D-glucose concentrations before and after enzymatic inversion.

ACCURACY:

Standard errors of less than 5% are achieved routinely.

REFERENCES:

1. Outlaw, W.H. Jr. and Tarczynski, M.C., (1988) in **Methods of Enzymatic Analysis** (Bergmeyer, H.U. ed) 3rd ed., **vol. 6**, pp 96-103. VCH Verlagsgesellschaft mbH, Weinheim, Germany.
2. Kunst, A., Draeger, B. & Ziegenhorn, J. (1988) in **Methods of Enzymatic Analysis** (Bergmeyer, H.U. ed) 3rd ed., **vol. 6,** pp. 163-172. VCH Verlagsgesellschaft mbH, Weinheim, Germany.

KIT CONTENTS:

Kits contain sufficient reagents for performing 250 measurements of glucose and sucrose i.e. they contain the full assay method, plus:

1. β-Fructosidase (invertase) (yeast; 5 ml; 100 U/ml on sucrose) in 50% glycerol plus bovine serum albumin (BSA; 1 mg/ml) and sodium benzoate (0.2%). Store at 4°C.
2. Sodium acetate buffer (20 ml, 2 M, pH 4.6). Store at 4°C.
3. Glucose Determination Reagent (GOPOD; high purity). Store dry at 4°C. Long term storage at -20°C.
4. Glucose Reagent Buffer (concentrate). Store at 4°C.
5. Glucose Standard Solution. Store at room temperature.
6. Control Flour Sample (containing glucose and sucrose). Store dry at room temperature.

ENCLOSED ENZYME:

β-Fructosidase (5 ml, 100 U/ml on sucrose).

Dilute an aliquot (1.0 mL) to 10 mL with sodium acetate buffer (100 mM, pH 4.6). For dispensing this viscous liquid, it is recommended that a positive displacement dispenser is used (however, this is not essential, as the enzyme is in excess). Store frozen between use.

ENCLOSED REAGENTS:

A. Glucose Determination Reagent (GOPOD) (for 1 litre).
Reagent concentrations after dissolution in buffer:

Glucose oxidase	> 12,000 U/litre.
Peroxidase	> 650 U/litre.
4-Aminoantipyrine	0.4 m**M**.

B. Glucose Reagent Buffer (concentrate containing 0.2% sodium azide) (50 ml).
Dilute the entire contents to 1 litre with distilled water and use to dissolve the Glucose Determination Reagent (GOPOD).

Divide this reagent (**GOPOD Reagent**) into aliquots of desired volume for storage.
Stability: 2-3 months at 4°C; 12 months at -20°C

NOTE:
Sodium azide is a toxic chemical and should be treated accordingly. It is added to the Glucose Reagent Buffer to stabilise the ready to use GOPOD reagent against microbial infection. Protective gloves should be worn while diluting the concentrate.

ENCLOSED BUFFER:

2 M sodium acetate buffer (20 ml, pH 4.6). Dilute to 200 ml with distilled water before use to give a final concentration of 100 mM. Store at 4°C. Stable for ~ 1 year. To increase stability period, store in a 200 ml Duran bottle, and overlay the solution with 2 drops of toluene.

ENCLOSED STANDARD:

Glucose Standard Solution (1 mg/ml in 0.2% benzoic acid).
Stable at room temperature for > 5 years.

ENCLOSED CONTROL:

Powder mixture containing glucose and sucrose. The exact levels are shown on the vial label. Stable at room temperature for > 5 years.

SOLUTIONS FOR SAMPLE CLARIFICATION:

***Carrez I solution*.-** Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P-9387) in 100 mL of distilled water. Store at room temperature.

***Carrez II solution*.-** Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z-4750) in 100 mL of distilled water. Store at room temperature.

***Sodium Hydroxide (100 mM)*.-** Dissolve 4 g of sodium hydroxide in 1 litre of distilled water. Store at room temperature.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100mm and 18 x 150mm).
2. Micro-pipettors, e.g. Gilson Pipetman 200 µl and 500 µl.
3. Positive displacement pipettor e.g. Eppendorf Multipette®
   - with 12.5 Combitip® [to dispense 1.0 mL aliquots of β-fructosidase (in 50% glycerol)].
   - with 5.0 ml Combitip® (to dispense 0.2 ml aliquots of diluted β-fructosidase and buffer).
4. Analytical balance.
5. Spectrophotometer set at 510 nm.
6. Vortex mixer (e.g. IKA YellowLab Test Tube Shaker TTS).
7. Thermostatted water bath (set at 50.0°C).
8. Boiling water bath (set at 85-90°C).
9. Stop clock.
10. Whatman GF/C (9 cm) glass fibre filter papers.

CONTROLS AND PRECAUTIONS:

1. The time of incubation with GOPOD reagent is not critical, but should be at least 20 min.
2. With each set of determinations, reagent blanks and glucose controls (100 µg quadruplicate) should be included.
3. With each set of determinations an extract from the control powder is included.

a. The **reagent blank** consists of 0.4 mL of distilled water + 3.0 ml GOPOD Reagent.

b. The **glucose control** consists of 0.1 ml of glucose standard solution (1 mg/ml) + 0.3 mL of distilled water + 3.0 ml GOPOD Reagent.

4. With each new batch of GOPOD Reagent, the time for maximum colour formation with 100 μg of glucose standard should be checked. This is usually about 15 min.

ASSAY PROCEDURE:

Assay for Glucose and Sucrose:

1. Add 0.2 mL of sample extract (containing glucose + sucrose at a concentration of 0.02 - 0.5 mg/ml) to the **bottoms** of four 16 x 100 mm glass test tubes. Add either 100 mM sodium acetate buffer or β-fructosidase to duplicate tubes as follows:

   - 0.2 ml of sample + 0.2 ml buffer [Glucose] . . . . . **A**
   - 0.2 ml of sample + 0.2 ml β-fructosidase [Sucrose + Glucose]. . . . . **B**

2. Incubate all tubes at 50°C for 20 min.

3. Add 3.0 ml of GOPOD Reagent to tubes A and B and incubate these at 50°C for 20 min.
4. Measure the absorbance for each sample, the enclosed control and the glucose standard at 510 nm against the reagent blank:

Absorbances: ΔA = GOPOD absorbance for A
ΔB = GOPOD absorbance for B

CALCULATIONS:

Glucose; grams/litre of sample solution:

$$= \frac{\Delta A}{0.2} \times F \times \frac{1}{1000} \times \frac{1000}{1000} \times \text{Dilution}$$

$$= \Delta A \times F \times 0.0050$$

Sucrose; grams/litre of sample solution:

$$= \frac{\Delta B - \Delta A}{0.2} \times F \times \frac{1}{1000} \times \frac{1000}{1000} \times \frac{342}{180} \times \text{Dilution}$$

$$= (\Delta B - \Delta A) \times F \times \text{Dilution} \times 0.0095.$$

where:

ΔA/0.2 and ΔB/0.2
= absorbances (510 nm) (GOPOD Reagent) for 0.2 ml of sample treated with acetate buffer (ΔA) (free glucose); or β-fructosidase (ΔB)(free glucose plus glucose from sucrose).

F = factor to convert from absorbance to μg for 100 μg of glucose (= 100/absorbance for 100 μg glucose).

1/1000 = conversion from micrograms to milligrams.

1000/1000 = conversion from mg/ml to g/litre.

342/180 = conversion from μg of glucose (as measured) to μg of sucrose.

Dilution = dilution of the original sample solution.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is calculated from the amount weighed:

$$\text{Content}_{\text{sucrose}} = \frac{c_{\text{sucrose}}\ (\text{g/l sample solution})}{\text{weight}_{\text{sample}}\ (\text{g/l sample solution})} \times 100\ [\text{g}/100\ \text{g}]$$

PERFORMANCE OF ASSAY:

The amount of sucrose and glucose present in the cuvette should range between 10 μg and 100 μg. The sample solution must therefore be diluted sufficiently to yield a sugar concentration between 0.02 and 0.5 g/l.

Dilution table

estimated amount of sucrose + glucose per litre	dilution with water	dilution factor
< 0.5 g	-	1
0.5 - 5.0 g	1 + 9	10
5.0 - 50 g	1 + 99	100
> 50 g	1 + 999	1000

SAMPLE PREPARATION:

1. Liquid foodstuffs

Use clear, colorless or slightly colored solutions directly or after dilution according to the dilution table for the assay. Filter turbid solutions (Whatman GF/C glass fibre filter papers) or clarify with Carrez reagents. Strongly colored solutions which are use undiluted for the assay because of their low sucrose and D-glucose concentrations must be decolorized with polyvinylpolypyrrolidone (PVPP). Beverages containing gas should be degassed under vacuum.

Examples:

Determination of D-glucose and sucrose in fruit juices and similar beverages

Filter turbid juices (alternatively clarify with Carrez reagents) and dilute sufficiently to yield a sucrose and D-glucose concentration of approx. 0.02 - 0.5 g/l. The diluted sample solution can be used for assay even if slightly colored. With highly colored solutions, decolorize as follows:
Mix 10 ml of juice and approx. 0.1 g of polyvinylpolypyrrolidone, stir for 1 min and filter. Use the clear, slightly colored solution for the assay.

Determination of sucrose in sweetened condensed milk and ice-cream

Weigh approx. 1 g sample accurately into a 100 ml volumetric flask, add approx. 60 ml water and incubate for 15 min at approx. 70°C; shake from time to time. For protein precipitation, add 5 ml Carrez 1 solution, 5 ml of Carrez 2 solution and 10 ml of NaOH (100 mM), shake vigorously after each addition, adjust to room temperature and fill up to the mark with water, filter. Use the clear, possibly slightly opalescent solution diluted according the dilution table for the assay.

2. Solid Foodstuffs

Mince solid and semi-solid foodstuffs (e.g. bread and pastries, fruit, vegetables, meat and milk products) in an electric mixer, meat grinder or mortar. Weigh out the well mixed sample and extract with water, heated to 60°C if necessary. Transfer quantitatively into a volumetric flask and fill up to the mark with water. Filter, clarify with Carrez reagents, if necessary, and use the clear solution diluted, if necessary, for the assay.

Examples:

Determination of sucrose in chocolate

Weigh approx. 1 g of chocolate, finely grated, accurately into a 100 ml volumetric flask, add approx 70 ml water, and heat in a water bath at 60-65°C for 20 min. Shake from time to time. After the chocolate has been completely suspended, allow to cool and fill up to the mark with water. To separate the fat, place in a refrigerator for at least 20 min. Filter the cold solution through a glass fibre filter paper (Whatman GF/C). Use the clear filtrate diluted according to the dilution table, if necessary, for the assay. Alternatively, clarify with the Carrez reagents.

Determination of sucrose and D-glucose in (roast) coffee

Weigh approx. 1 g ground coffee into a 100 ml volumetric flask and add 60 ml hot water (90°C). Stir for 5 min on a magnetic stirrer. Allow to cool to room temperature and remove the magnetic stirrer bar. Clarify with Carrez reagents as for "sweetened condensed milk and ice-cream (as above). Use the clear, possibly slightly colored filtrate for the assay.

3. Pasty products

Homogenize semi-solid samples, extract with water or dissolve, filter if necessary, clarify with Carrez reagents or decolorize.

Examples:

Determination of sucrose and D-glucose in jam

Homogenize approx. 10 g of jam in an electric mixer. Weigh approximately 0.5 g of the homogenized jam accurately into a 100 ml volumetric flask, mix with water, and fill up to the mark. Filter through glass fibre filter paper. Use the clear filtrate diluted according to the dilution table, if necessary, for the assay.

Determination of sucrose and D-glucose in honey

Thoroughly stir the honey with a spatula. Take approx. 10 g of the viscous or crystalline honey, heat in a beaker for 15 min at approx. 60°C, and stir occasionally with a spatula (there is no need to heat liquid honey). Allow to cool. Weigh approx. 1 g of the liquid sample accurately into a 100 ml volumetric flask. Dissolve at first with only a small portion of water, and then fill to the mark.

a) Determination of D-glucose

Dilute the 1% honey solution in a ratio of 1:20 (1+19) and use for the assay.

b) Determination of sucrose

If the estimated sucrose content in the honey lies between 5 and 10%, dilute the 1% solution in a ratio of 1:5 (1+4) and use for the assay. If the estimated sucrose content in the honey lies between 0.5 and 5%, the excess D-glucose should be removed as much as possible before sucrose is determined. D-Glucose is oxidised to D-gluconate in the presence of the enzymes glucose oxidase (GOD) and catalase.

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{(GOD)}} \text{D-gluconate} + H_2O_2$$

The hydrogen peroxide is destroyed by catalase:

$$2\,H_2O_2 \xrightarrow{\text{(catalase)}} 2\,H_2O + O_2$$

Reagents.

1. Sodium phosphate buffer (300 mM, pH 7.6) plus 5 mM $MgCl_2.7H_2O$.

Add 53.4 g of di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) to 900 mL of distilled water and dissolve by stirring. Add 1.11 g of $MgCl_2.7H_2O$ and dissolve. Adjust the pH to 7.6 with 1 M NaOH (40 g/L) and adjust the volume to 1 L with distilled water. Store at 4°C in a well sealed Duran® bottle. To prevent microbial contamination, overlay the solution with 2 drops of toluene.

2. Glucose oxidase (12,000 U) plus Catalase (300,000 U). (Megazyme cat. no. E-GOXCA).

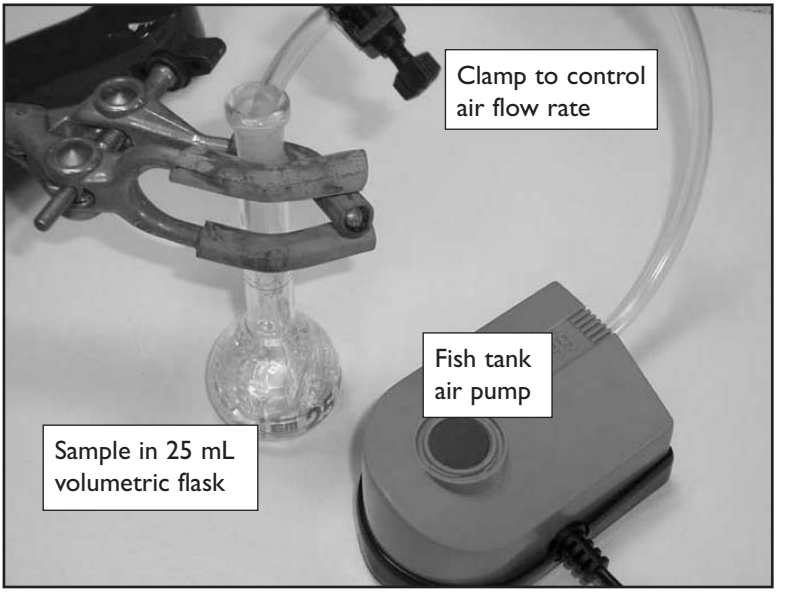
Dissolve the contents of 1 vial in 20 mL of 300 mM sodium phosphate buffer (pH 7.6) plus 5 mM $MgCl_2.2H_2O$. Divide this solution into 2.0 mL aliquots. Stable for > 3 years at -20°C.

Procedure for D-glucose oxidation

Pipette into a 25 mL volumetric flask	Volume
300 mM phosphate buffer solution Sample solution (up to approx. 5 mg/mL D-glucose) Enzyme solution	5.0 mL 5.0 mL 0.2 mL
Incubate the flask at ~ 25°C and pass a current of air (O_2) through the mixture for 1 h (see Figure 1). While this oxidation could theoretically lead to a decrease in pH, no significant changes are observed in solutions containing D-glucose at concentrations of up to 5 mg/mL (due to the buffering capacity of the phosphate buffer used).	

To inactivate the glucose oxidase plus catalase, incubate the volumetric flask in a boiling water bath for 15 min, allow it to cool to room temperature and dilute the contents to the mark with distilled water. Mix and filter. Use 0.5 mL of the clear solution for the determination of D-fructose. Determine the residual D-glucose as usual.

Figure 1. Arrangement for the oxidation of glucose by glucose oxidase plus catalase in the presence of a stream of air.



NOTES:

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