Megazyme

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SUCROSE/ D-GLUCOSE

ASSAY PROCEDURE

K-SUCGL 04/18

(250 Assays per Kit)

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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. D-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, D-glucose in plant extracts usually occurs together with maltose, maltosaccharides, starch, sucrose and/or β-linked D-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 D-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/D-Glucose Test Kit employs high purity glucose oxidase, peroxidase and β-fructosidase (invertase) and can be used with confidence for the specific measurement of D-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:

(1)

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconate} + H_2O_2$$

(2)

$$2H_2O_2 + \textit{p}\text{-hydroxybenzoic acid} + 4\ \text{aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + 4H_2O$$

The reactions involved are:

(3)

$$\text{Sucrose} + H_2O \xrightarrow{\beta\text{-fructosidase}} \text{D-glucose} + \text{D-fructose}$$

Free D-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 D-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.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 250 determinations are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: **Buffer** (20 mL, pH 4.6).
Stable for > 2 years at 4°C.

Bottle 2: **β-Fructosidase (invertase) solution** (yeast; 5 mL) plus sodium azide as a preservative (0.02% w/v).
Stable for > 2 years at 4°C.

Bottle 3: **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.095% w/v).
Stable for > 4 years at 4°C.

Bottle 4: **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine.
Freeze-dried powder.
Stable for > 5 years below -10°C.

Bottle 5: **D-Glucose standard solution** (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years at room temperature.

Bottle 6: **Control flour sample.** Sucrose and D-glucose

contents shown on vial label.
Stable for > 5 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dilute the contents of bottle 1 to 400 mL with distilled water before use (this is Solution 1).
Stable for > 1 year at 4°C.
(To increase stability, store in a 400 mL Duran® bottle, and overlay the solution with 2 drops of toluene).

2. Dilute 1.0 mL of the contents of bottle 2 to 10 mL with Solution 1 (this is Solution 2).
In dispensing this viscous liquid, a positive displacement dispenser is recommended (however, this is not essential as the enzyme is in excess).
Stable for > 2 years below -10°C.

3. Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water (this is Solution 3). Use immediately.

NOTE:

1. On storage, salt crystals may form in the concentrated buffer. These must be completely dissolved when this buffer is diluted to 1 L with distilled water.
2. This buffer contains 0.095% (w/v) sodium azide.
This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of bottle 4 in 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is Glucose Determination Reagent **(GOPOD Reagent)**.
Stable for ~ 3 months at 2-5°C or > 12 months below -10°C.

If this reagent is to be stored in the frozen state, it should be divided into aliquots (e.g. 200 mL in polypropylene containers). Do not freeze/thaw more than once.

When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink colour over 2-3 months at 4°C. The absorbance of this solution should be less than 0.05 when read against distilled water.

5 & 6. Use the contents of bottles 5 and 6 as supplied.
Stable for > 5 years at room temperature.

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. P9387) 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. Z4750) in 100 mL of distilled water. Store at room temperature.

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

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm and 18 x 150 mm).
2. Micro-pipettors, e.g. Gilson Pipetman® 100 µL, 200 µL and 500 µL.
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of diluted β-fructosidase and buffer).
   - with 12.5 Combitip® (to dispense 1.0 mL aliquots of β-fructosidase solution).
4. Analytical balance.
5. Spectrophotometer set at 510 nm.
6. Vortex mixer (e.g. IKA® YellowLab Test Tube Shaker TTS).
7. Thermostated water bath (set at 50°C).
8. Boiling water bath (set at 85-90°C).
9. Stop clock.
10. Whatman No. 1 (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. Include reagent blanks and D-glucose controls (100 µg quadruplicate) with each set of determinations.
3. Analyse an extract from the control powder with each set of determinations.

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

b. The D-**glucose control** consists of 0.1 mL of D-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 D-glucose standard should be checked. This is usually approx. 15 min.

ASSAY PROCEDURE:

Assay for Glucose and Sucrose:

1. Add 0.2 mL of sample extract (containing D-glucose + sucrose at a concentration of 0.02-0.5 mg/mL) to the **bottom** of four 16 x 100 mm glass test tubes. Add either Solution 1 or β-fructosidase (Solution 2) to duplicate tubes as follows:

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

2. Incubate all tubes, including the Reagent Blanks and D-glucose controls at 50°C for 20 min.

3. Add 3.0 mL of GOPOD Reagent to all tubes and incubate these at 50°C for 20 min.

4. Measure all absorbances at 510 nm against the reagent blank.

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

CALCULATIONS:

D-Glucose; g/L of sample solution:

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

$$= \Delta A \times F \times 0.0050 \times \text{Dilution}$$

Sucrose; g/L 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:

$\Delta A/0.2$ and $\Delta B/0.2$

= absorbances (510 nm) (GOPOD Reagent) for 0.2 mL of sample treated with Solution 1 (ΔA) (free D-glucose); or β-fructosidase (ΔB) (free D-glucose plus D-glucose from sucrose).

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

1/1000 = conversion from µg to mg.

1000/1000 = conversion from mg/mL to g/L.

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

Dilution = dilution of the original sample solution.

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

Content of sucrose

$$= \frac{c_{sucrose}\ (g/L\ sample\ solution)}{weight_{sample}\ (g/L\ sample\ solution)} \times 100 \quad [g/100\ g]$$

SAMPLE DILUTION:

The amount of sucrose and D-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, colourless or slightly coloured 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 coloured solutions which are used undiluted for the assay because of their low sucrose and D-glucose concentrations must be decolourised 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. 0.02-0.50 g/L. The diluted sample solution can be used for assay even if slightly coloured. With highly coloured solutions, decolourise as follows:

Mix 10 mL of juice and approx. 0.2 g of polyvinylpolypyrrolidone, stir for 1 min and filter. Use the clear, slightly coloured 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 I solution, 5 mL of Carrez II 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 to 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.

Accurately weigh approx. 1 g of finely grated chocolate 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/A). 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 coloured filtrate for the assay.

3. Pastry products.
Homogenise semi-solid samples, extract with water or dissolve, filter if necessary, clarify with Carrez reagents or decolourise.

Examples:

Determination of sucrose and D-glucose in jam.
Homogenize approx. 10 g of jam in an electric mixer. Weigh approx. 0.5 g of the homogenised 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% v/v 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:

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

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 below -10°C.

Procedure for D-glucose oxidation

Pipette into a 25 mL volumetric flask	Volume
300 mM phosphate buffer solution (1) Sample solution (up to approx. 5 mg/mL D-glucose) Enzyme solution (2)	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.

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2. Kunst, A., Draeger, B. & Ziegenhorn, J. (1988). "*Methods of Enzymatic Analysis"* (Bergmeyer, H. U. ed), 3rd ed., vol. **6,** pp. 163-172. VCH Verlagsgesellschaft mbH, Weinheim, Germany.

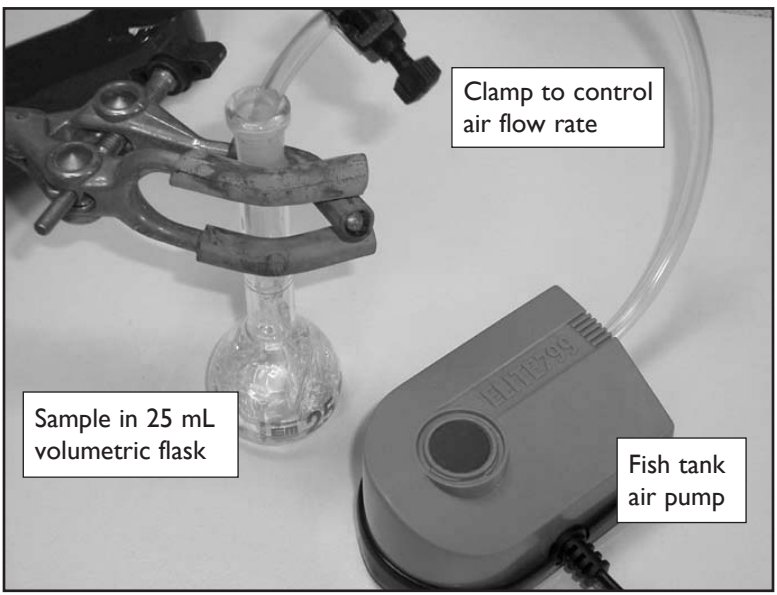

Figure 1. Arrangement for the oxidation of glucose by glucose oxidase plus catalase in the presence of a stream of air.

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