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Megazyme

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LACTOSE/ SUCROSE/D-GLUCOSE

ASSAY PROCEDURE

FOR THE MEASUREMENT OF LACTOSE, SUCROSE AND D-GLUCOSE IN FLOURS

K-LACSU 08/18

(100 Assays of each per Kit)

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INTRODUCTION:

Sucrose and milk solids (containing lactose) are major components of many flour mixtures used in the production of cakes, biscuits, breads and confectionery goods. Traditionally these components are measured by titrametric methods,[1] however, high performance liquid chromatographic methods have also been developed.

Enzymic methods for the measurement of sucrose and lactose are well known, but are not widely used in the cereals industries. With these procedures, sucrose is hydrolysed by invertase to D-glucose and fructose, and lactose is hydrolysed by β-galactosidase to D-glucose plus D-galactose. The D-glucose released from sucrose and lactose, and the free D-glucose in the sample, are determined enzymically [usually with glucose oxidase/peroxidase reagent (GOPOD Reagent)].

PRINCIPLE:

The Megazyme Sucrose/Lactose/D-Glucose Kit has been designed specifically for the measurement of D-glucose, sucrose and lactose in flour mixtures, however it is applicable to any materials containing these sugars. Before extraction of the sugars, endogenous enzymes are inactivated by heating the flour in boiling ethanol for 5 min.

Sucrose is hydrolysed to D-glucose and D-fructose with invertase (1).

$$\text{(1) Sucrose} + H_2O \xrightarrow{\text{(invertase)}} \text{D-glucose} + \text{D-fructose}$$

Lactose is hydrolysed to D-glucose and D-galactose by *Aspergillus niger* β-galactosidase (lactase) (2).

$$\text{(2) Lactose} + H_2O \xrightarrow{\text{(β-galactosidase)}} \text{D-glucose} + \text{D-galactose}$$

D-glucose is converted to D-gluconate by the enzyme glucose oxidase in the presence of oxygen and water (3).

$$\text{(3) D-Glucose} + H_2O + O_2 \xrightarrow{\text{(glucose oxidase)}} \text{D-gluconate} + H_2O_2$$

The enzyme, peroxidase (POD) catalyses the reaction involving hydrogen peroxide (H_2O_2), *p*-hydroxybenzoic acid and 4-aminoantipyrine with quantitative formation of quinoneimine dye (4).

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

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The amount of quinoneimine dye formed in this reaction is stoichiometric with the amount of D-glucose. It is the quinoneimine dye that is measured by the increase in absorbance at 510 nm.

ACCURACY:

Standard errors of less than 5% are achieved routinely.

KITS:

Kits contain sufficient reagents for performing 100 measurements of D-glucose, sucrose and lactose, i.e. they contain the full assay method plus:

Bottle 1: β-Fructosidase solution (invertase) (yeast; 5 mL) plus sodium benzoate (0.2% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: β-Galactosidase (lactase; *A. niger*, 1 mL) suspension.
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 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, lactose and D-glucose contents shown on vial label.
Stable for > 5 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

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

2. Dissolve the contents of bottle 2 in 19 mL of Buffer 1 (this is solution 2). Divide into 5 mL aliquots and store frozen in polypropylene tubes between use.
Stable for > 2 years below -10°C.

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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 back into 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.

BUFFER (NOT SUPPLIED):

Sodium acetate buffer (50 mM, pH 4.5)

Add 2.9 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.5 by careful addition of 1 M (4 g/100 mL) sodium hydroxide solution. Adjust the volume to 1 L and store the buffer at 4°C. Sodium azide (0.2 g) can be added as a preservative. Stable for > 6 months at 4°C.

NOTE:
Sodium azide is a toxic chemical and should be treated accordingly. It is added to buffers solely as a preservative. It can be deleted from buffer recipes but buffers should then be stored at 4°C.

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EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm and 18 x 150 mm).
2. Micro-pipettors, e.g. Gilson Pipetman (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 invertase and β–galactosidase).
   - with 12.5 Combitip® (to dispense 1.0 mL aliquots of invertase solution).
4. Analytical balance.
5. Spectrophotometer set at 510 nm.
6. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
7. Thermostated water bath (set at 50°C).
8. Boiling water bath (set at 85-90°C).
9. Stop clock.
10. Whatman GF/C (9cm) 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.

   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 D-glucose standard solution (100 µg/0.1 mL) + 0.3 mL of distilled water + 3.0 mL GOPOD Reagent.

3. Analyse an extract from the control powder with each set of determinations.
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 about 15 min.

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ASSAY PROCEDURE:

Enzyme Inactivation and Sugar Extraction:

1. Accurately weigh 0.5 g of milled sample into a glass test-tube (18 x 150 mm) and add 5 mL of ethanol (95% v/v).
2. Incubate the tube in a water bath at 85-90°C and allow to reflux for 5 min (This inactivates endogenous enzymes). Take care to avoid the tube contents boiling from the top of the tube.
3. Quantitatively transfer the tube contents to a 50 mL volumetric flask using 50 mM sodium acetate buffer from a wash bottle to ensure complete transfer. Adjust to volume (50 mL) with 50 mM sodium acetate buffer (pH 4.5) and thoroughly mix the solution.
4. Filter an aliquot of this solution through Whatman GF/C glass fibre filter paper or Whatman No. 1 (9 cm) filter paper.
5. Add 1.0 mL of the filtrate to 3 mL of distilled water and mix thoroughly (**Solution X**).

Assay for D-Glucose, Sucrose and Lactose:

1. Add 0.2 mL of sample extract (Solution X; containing D-glucose + sucrose + lactose at a concentration of 0.02-0.5 mg/mL) to the **bottom** of six 16 x 100 mm glass test tubes. Add one of the following, to each of the tubes (in duplicate):
   A. 50 mM sodium acetate buffer (pH 4.5)
   B. β-fructosidase, or
   C. β-galactosidase to duplicate tubes, as follows:

   - 0.2 mL of Solution X + 0.2 mL buffer (pH 4.5) [D-Glucose] . . .**A**
   - 0.2 mL of Solution X + 0.2 mL β-fructosidase [Sucrose + D-Glucose] . . . .**B**
   - 0.2 mL of Solution X + 0.2 mL β-galactosidase [Lactose + D-Glucose] . . . . .**C**

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 (zero the spectrophotmeter using the reagent blank).**

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

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CALCULATIONS:

D-Glucose, % (w/w):

$$= \frac{\Delta A}{0.2} \times F \times \frac{1}{1000} \times \frac{50}{500} \times 4 \times 100$$

$$= \Delta A \times F \times 0.2$$

Sucrose, % (w/w):

$$= \frac{\Delta B - \Delta A}{0.2} \times F \times \frac{1}{1000} \times \frac{50}{500} \times 4 \times \frac{342}{180} \times 100$$

$$= (\Delta B - \Delta A) \times F \times 0.38$$

Lactose, % (w/w):

$$= \frac{\Delta C - \Delta A}{0.2} \times F \times \frac{1}{1000} \times \frac{50}{500} \times 4 \times \frac{342}{180} \times 100$$

$$= (\Delta C - \Delta A) \times F \times 0.38$$

where:

$\Delta A/0.2$, $\Delta B/0.2$ and $\Delta C/0.2$
= absorbances (510 nm) (GOPOD Reagent) for 0.2 mL of sample incubated with acetate buffer (ΔA), β-fructosidase (ΔB) or β-galactosidase (ΔC), respectively.

$F = \frac{100\ (\mu g\ of\ glucose)}{absorbance\ for\ 100\ \mu g\ of\ glucose}$ (conversion from absorbance to µg)

$\frac{1}{1000}$ = conversion from micrograms to milligrams.

$\frac{50}{500}$ = 500 mg of sample was extracted with 50 mL of buffer.

4 = dilution factor for the filtered extract.

$\frac{342}{180}$ = conversion from D-glucose as measured to sucrose or lactose.

100 = factor to express D-glucose, sucrose and lactose as a percentage of flour weight.

REFERENCES:

1. American Association of Cereal Chemists. 1983. *Approved Methods of the AACC*, 8th edition. Methods 80-68, 80-31 and 80-50. The Association; St. Paul, MN.

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