Megazyme

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MALTOSE, SUCROSE and D-GLUCOSE

ASSAY PROCEDURE

K-MASUG 08/18

(*34 Assays of each per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

Maltose, sucrose and D-glucose are found in most plant and food products. In plant materials D-glucose occurs as a free sugar in sucrose, and in a range of oligosaccharides (galactosyl-sucrose oligosaccharides and fructo-oligosaccharides) and polysaccharides, such as starch and 1,3-1,4-β-D-glucans. Maltose (1,4-α-D-glucobiose) is produced on hydrolysis of starch by α-amylase and β-amylase. In some food products, these three sugars occur in admixture.

PRINCIPLE:

Maltose is hydrolysed by α-glucosidase (maltase) at pH 6.6 to two molecules of D-glucose (1). This enzyme concurrently hydrolyses sucrose to D-glucose and D-fructose (2).

$$\text{(1)}\quad \text{Maltose} + H_2O \xrightarrow{(\alpha\text{-glucosidase})} 2\text{D-glucose}$$

$$\text{(2)}\quad \text{Sucrose} + H_2O \xrightarrow{(\alpha\text{-glucosidase})} \text{D-glucose} + \text{D-fructose}$$

Sucrose (but not maltose) is hydrolysed by the enzyme β-fructosidase (invertase) at pH 4.6 to D-glucose and D-fructose (3).

$$\text{(3)}\quad \text{Sucrose} + H_2O \xrightarrow{(\beta\text{-fructosidase})} \text{D-glucose} + \text{D-fructose}$$

At pH 7.6, D-glucose is phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (4).

$$\text{(4)}\quad \text{D-Glucose} + \text{ATP} \xrightarrow{(\text{HK})} \text{G-6-P} + \text{ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (5).

$$\text{(5)}\quad \text{G-6-P} + NADP^+ \xrightarrow{(\text{G6P-DH})} \text{gluconate-6-phosphate} + \text{NADPH} + H^+$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of sucrose, D-glucose and half the amount of maltose. It is the NADPH which is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

α-Glucosidase hydrolyses α-glucosidic bonds in a number of α-glucosides including maltose, maltotriose, sucrose, turanose and maltitol. Under the assay conditions employed, α-glucosidase has insignificant action on α-trehalose, maltopentaose and higher dextrins and starch. Sugars with β-glycosidic bonds such as lactose and cellobiose are not hydrolysed.

Since β-fructosidase also hydrolyses low molecular weight fructans (e.g. kestose) the method is not absolutely specific for sucrose. Some indication of the presence of fructo-oligosaccharides will be given by the ratio of D-glucose to D-fructose in the determination after hydrolysis by β-fructosidase. Deviation from 1:1 (increasing proportion of D-fructose) would indicate the presence of fructan.

The determination of D-glucose is specific.

In the analysis of commercial sucrose, values of 100% w/w can be expected. However, in the analysis of D-glucose and maltose, values of less than 100% w/w will be obtained due to the rapid absorption of water by these sugars.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.75 mg/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 1.50 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 1.00 mL.

The assay is linear over the range of 4 to 80 µg of D-glucose, or maltose per assay (8 to 160 µg of sucrose per assay). In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 1.00 mL, this corresponds to a D-glucose concentration of approx. 0.37 to 0.75 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of D-glucose has been completed within the time specified in the assay, it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-glucose (approx. 40 µg in 0.1 mL), but not sucrose or maltose, to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed. Interfering substances in the sample being analysed can be identified by including

an internal standard (sucrose or maltose). Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding maltose, sucrose or D-glucose to the sample in the initial extraction steps.

To confirm that sucrose and maltose are completely hydrolysed by α-glucosidase, perform the incubation for the recommended time and for twice the recommended incubation time. The final determined values for sucrose or maltose should be the same. Perform similar studies with β-fructosidase on sucrose.

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 34 assays of maltose, sucrose and D-glucose are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (25 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: $NADP^+$ plus ATP.
Stable for > 5 years below -10°C.

Bottle 3: Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.25 mL.
Stable for > 2 years at 4°C.

Bottle 4: β-Fructosidase in sodium citrate buffer (pH 4.6), lyophilised powder.
Stable for > 2 years below -10°C.

Bottle 5: α-Glucosidase in sodium citrate buffer (pH 6.6), lyophilised powder.
Stable for > 2 years below -10°C.

Bottle 6: D-Glucose standard solution (5 mL, 0.4 mg/mL).
Stable for > 2 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 12 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottles to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position.
Stable for > 2 years at 4°C.

4. Dissolve the contents of bottle 4 with 14 mL of distilled water. Divide into aliquots of approx. 5 mL. Store below -10°C in polypropylene tubes between use and on ice during use.
Stable for > 2 years below -10°C.

5. Dissolve the contents of bottle 5 with 14 mL of distilled water. Divide into aliquots of approx. 5 mL. Store below -10°C in polypropylene tubes between use and on ice during use.
Stable for > 2 years below -10°C.

6. Use the contents of bottle 6 as supplied.
Stable for > 2 years at room temperature.

NOTE: The D-glucose standard solution is only assayed when there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of D-glucose is determined directly from the extinction coefficient of NADPH (pages 7 and 8).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL, 100 mL and 500 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 100 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of buffer and 0.1 mL aliquots of $NADP^+$/ATP solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

PROCEDURE (for D-glucose and sucrose):

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.62 mL
Sample solution: 4-80 µg of D-glucose (D-Glc) (or 8-160 µg of sucrose) per cuvette (in 0.10-1.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank sucrose + free D-Glc	Sucrose + free D-Glc sample	Blank free D-glucose	Free D-glucose sample
solution 4* (β-fructosidase)	0.20 mL	0.20 mL	-	-
sample solution	-	0.10 mL	-	0.10 mL
Mix** and incubate for 20 min (NOTE: Before pipetting solution 4 first warm it to 25-30°C). **Then add:**				
distilled water (at ~ 25°C)	2.10 mL	2.00 mL	2.30 mL	2.20 mL
solution 1 (buffer)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
solution 2 ($NADP^+$/ATP)	0.10 mL	0.10 mL	0.10 mL	0.10 mL
Mix**, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
suspension 3 (HK/G6P-DH)	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min***.				

* pipette both solution 4 and sample solution into the bottom of the cuvette and mix by gentle swirling.

** for example with a plastic spatula or by gentle inversion after closing the cuvette with a cuvette cap or Parafilm®.

*** if the absorbance continues to increase, this may be due to the effects of colour compounds or enzymes in the sample. These interfering substances may be removed during sample preparation.

NOTE: If the sample is also to be analysed for maltose, include incubations with α-glucosidase (sample and blank) as shown in the following table.

PROCEDURE (for D-glucose and maltose plus sucrose):

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.62 mL
Sample solution: 4-80 µg of D-glucose (D-Glc) + maltose per cuvette (in 0.10-1.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank maltose + sucrose + free D-Glc	Maltose + sucrose + free D-Glc sample	Blank free D-glucose	Free D-glucose sample
solution 5* (α-glucososidase)	0.20 mL	0.20 mL	-	-
sample solution	-	0.10 mL	-	0.10 mL
Mix** and incubate for 20 min (NOTE: Before pipetting solution 5, first warm it to 25-30°C). **Then add:**				
solution 1 (buffer)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
solution 2 ($NADP^+$/ATP)	0.10 mL	0.10 mL	0.10 mL	0.10 mL
distilled water (at ~ 25°C)	2.10 mL	2.00 mL	2.30 mL	2.20 mL
Mix**, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
suspension 3 (HK/G6P-DH)	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min***.				

* pipette both solution 5 and sample solution into the bottom of the cuvette and mix by gentle swirling.

** for example with a plastic spatula or by gentle inversion after closing the cuvette with a cuvette cap or Parafilm®.

*** if the absorbance continues to increase, this may be due to effects of colour compounds or enzymes in the sample. These interfering substances may be removed during sample preparation.

CALCULATIONS:

Determine the absorbance differences (A_2-A_1) for blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample as follows:

$$\Delta A = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$$

$\Delta A_{sucrose + free\ D\text{-}glucose}$ is obtained from the "sucrose + free D-Glc" sample (page 5).

$\Delta A_{maltose + sucrose + free\ D\text{-}glucose}$ is obtained from the "maltose + sucrose + free D-Glc" sample (page 6).

$\Delta A_{free\ D\text{-}glucose}$ is obtained from the "free D-glucose sample" (page 5 or 6).

Determination of sucrose:

$$\Delta A_{sucrose} = \Delta A_{sucrose + free\ D\text{-}glucose} - \Delta A_{free\ D\text{-}glucose}$$

Determination of maltose:

$$\Delta A_{maltose + sucrose} = \Delta A_{maltose + sucrose + free\ D\text{-}glucose} - \Delta A_{free\ D\text{-}glucose}$$

$$\Delta A_{maltose} = \Delta A_{maltose + sucrose} - \Delta A_{sucrose}$$

The values of $\Delta A_{free\ D\text{-}glucose}$, $\Delta A_{sucrose + free\ D\text{-}glucose}$ and $\Delta A_{sucrose + maltose + free\ D\text{-}glucose}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-glucose, sucrose and maltose can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of the substance assayed [g/mol]
ε = extinction coefficient of NADPH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]
2 = 2 molecules of D-glucose released from each molecule of maltose hydrolysed

It follows for D-glucose:

$$c = \frac{2.62 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{\text{D-glucose}} \quad [g/L]$$

$$= 0.7492 \times \Delta A_{\text{D-glucose}} \quad [g/L]$$

for sucrose:

$$c = \frac{2.62 \times 342.3}{6300 \times 1.0 \times 0.1} \times \Delta A_{\text{sucrose}} \quad [g/L]$$

$$= 1.4235 \times \Delta A_{\text{sucrose}} \quad [g/L]$$

for maltose:

$$c = \frac{2.62 \times 342.3}{6300 \times 1.0 \times 0.1 \times 2} \times \Delta A_{\text{maltose}} \quad [g/L]$$

$$= 0.7118 \times \Delta A_{\text{maltose}} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of D-glucose

$$= \frac{c_{\text{D-glucose}}\ [g/L \text{ sample solution}]}{\text{weight}_{\text{sample}}\ [g/L \text{ sample solution}]} \times 100 \quad [g/100\ g]$$

Content of sucrose

$$= \frac{c_{\text{sucrose}}\ [g/L \text{ sample solution}]}{\text{weight}_{\text{sample}}\ [g/L \text{ sample solution}]} \times 100 \quad [g/100\ g]$$

Content of D-maltose

$$= \frac{c_{\text{maltose}}\ [g/L \text{ sample solution}]}{\text{weight}_{\text{sample}}\ [g/L \text{ sample solution}]} \times 100 \quad [g/100\ g]$$

SAMPLE PREPARATION:

1. Sample dilution.

The amount of D-glucose or maltose present in the cuvette (i.e. in the 0.2 mL of sample being analysed) should range between 4 and 80 µg (sucrose in the sample taken should range between approx. 8 and 160 µg). The sample solution must therefore be diluted sufficiently to yield a D-glucose concentration between 0.04 and 0.8 g/L.

Dilution Table

Estimated concentration of D-glucose (g/L)	Dilution with water	Dilution factor (F)
< 0.8	No dilution required	1
0.8-8.0	1 + 9	10
8.0-80	1 + 99	100
> 80	1 + 999	1000

If the value of $\Delta A_{\text{free D-glucose}}$, $\Delta A_{\text{sucrose + free D-glucose}}$ or $\Delta A_{\text{maltose + sucrose + free D-glucose}}$, is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 1.00 mL making sure that the sum of the sample, distilled water and solution 4 (or solution 5) components in the reaction is 2.30 mL, and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

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 (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if an acidic sample is to be used undiluted (such as red wine or coloured fruit juice), the pH of the solution should be increased to approx. 7.6 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing carbon dioxide should be degassed by increasing the pH to approx. 7.6 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no HK/G6P-DH should be performed in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 1 g/100 mL of polyvinylpolypyrrolidone (PVPP). Stir for 2 min and then filter.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to 20°C and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

SPECIAL SAMPLE PREPARATION FOR THE DETERMINATION OF MALTOSE AND SUCROSE IN THE PRESENCE OF EXCESS D-GLUCOSE:

Sample preparation involves the removal of excess D-glucose using a glucose oxidase/catalase mixture supplied by Megazyme (Megazyme cat. no. **E-GOXCA**). This procedure is performed as follows:

D-Glucose is oxidised to D-gluconate in the presence of glucose oxidase (GOD) and oxygen from the air (1).

(1) D-Glucose + O_2 + H_2O $\xrightarrow{\text{(GOD)}}$ D-Gluconate + H_2O_2

The hydrogen peroxide produced is decomposed by catalase (2).

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

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).

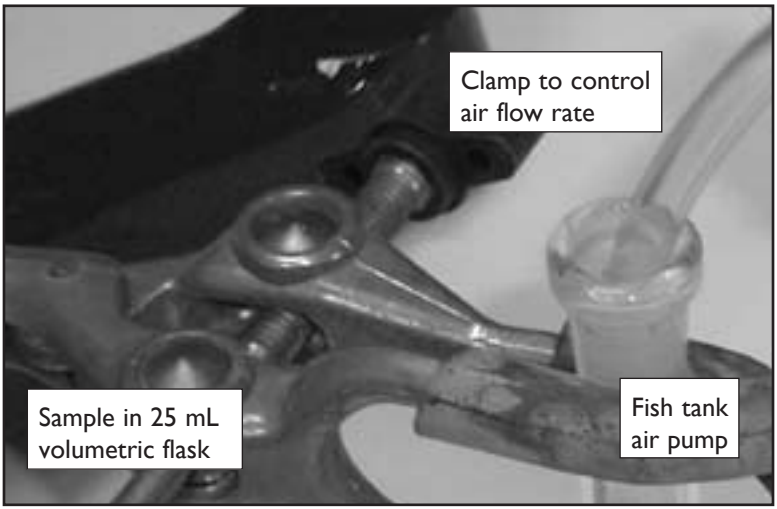
Dissolve the contents of 1 vial in 20 mL of 300 mM sodium phosphate buffer (pH 7.6) plus 5 mM $MgCl_2.2H_2O$. Divide this solution into 2.0 mL aliquots. Stable for > 3 years below -10°C.

Procedure for D-glucose oxidation

Pipette into a 25 mL volumetric flask	Volume
300 mM phosphate buffer solution 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 25-30°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 maltose and sucrose. 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.



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