Megazyme

D-MANNOSE, D-FRUCTOSE and D-GLUCOSE

ASSAY PROCEDURE

K-MANGL 08/04

(55 Assays per Kit)

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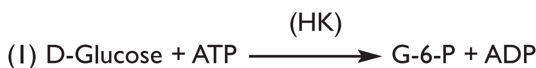
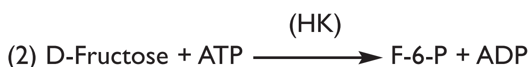
INTRODUCTION:

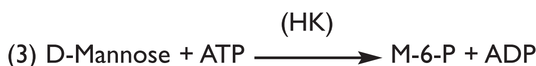
D-Mannose is the major sugar component of the so-called "mannan-type" polysaccharides, which include 1,4-β-D-mannans, D-galacto-D-mannans, D-gluco-D-mannans and D-galacto-D-gluco-D-mannans. It is a major constituent of the carbohydrate core of glycoproteins, but is rarely found free in Nature or as a constituent of simple oligosaccharides. D-Glucose and D-fructose are found in most plant and food products. In plant materials, they occur as free sugars or in a range of di-, oligo- and poly-saccharides such as fructans (inulins), starch and 1,3-1,4-β-D-glucans and cellulose. In foods, they are present in significant quantities in honey, wine and beer, and a range of solid foodstuffs such as bread and pastries, chocolate and candies.

PRINCIPLE:

D-Glucose, D-fructose and D-mannose are phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and mannose-6-phosphate (M-6-P), respectively with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1), (2) and (3).







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) (4).

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

On completion of reaction (4), F-6-P is converted to G-6-P by phosphoglucose isomerase (PGI) (5).

G-6-P reacts in turn with $NADP^+$ forming gluconate-6-phosphate and NADPH, leading to a further rise in absorbance that is stoichiometric with the amount of D-fructose.

On completion of reaction (5), M-6-P is converted to F-6-P by phosphomannose isomerase (PMI) (6) and this is then immediately converted to G-6-P by the PGI in the reaction mixture, leading to another further rise in absorbance that is stoichiometric with the amount of D-mannose.

In the analysis of the sugar composition of hydrolysates of plant and fungal polysaccharides known to contain just D-glucose and D-mannose, the separate incubation for determination of D-fructose content is not required. In such cases, the PGI and PMI incubations can be performed concurrently.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assays are specific for D-glucose, D-fructose and D-mannose.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.360/0.366 mg of D-glucose or D-mannose/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.721/0.733 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 4 to 80 μg of D-glucose or D-mannose 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 2.00 mL, this corresponds to a D-glucose concentration of approx. 0.183 to 0.366 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, D-fructose and D-mannose 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, D-fructose and D-mannose (30 μg of each in 0.1 mL) 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. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments i.e. by adding D-glucose, D-fructose or D-mannose to the sample in the initial extraction steps.

SAFETY:

The reagents used in the determination of D-glucose, D-fructose and D-mannose are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 55 determinations are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: TEA buffer (12 mL, 1 M, pH 7.6) plus magnesium chloride (100 mM) and sodium azide (0.02 % w/v) as a preservative. Stable for > 2 years at 4°C.

Bottle 2: $NADP^+$ (75 mg) plus ATP (220 mg). Stable for > 5 years at -20°C.

Bottle 3: Hexokinase (425 U/mL) plus glucose-6-phosphate dehydrogenase (212 U/mL) suspension, 1.2 mL. Stable for > 2 years at 4°C.

Bottle 4: Phosphoglucose isomerase suspension (1.2 mL, 1,000 U/mL). Stable for > 2 years at 4°C.

Bottle 5: Phosphomannose isomerase suspension (1.2 mL, 1,000 U/mL). Stable for > 2 years at 4°C.

Bottle 6: D-Glucose, D-fructose plus D-mannose standard solution (5 mL, 0.3 mg/mL of each sugar). Stable for > 2 years at 4°C.

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. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. Once dissolved, the reagent is stable for > 2 years at -20°C.

3, 4 & 5. Use the contents of bottles 3, 4 and 5 as supplied.
Stable for > 2 years at 4°C.

NOTE: If just D-glucose and D-mannose are being analysed, then the contents of bottles 4 and 5 (PGI and PMI) can be mixed. If this is done, then 40 µL of the mixture is used in the assay and this incubation is performed for 20 min.

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

NOTE: The D-glucose, D-fructose plus D-mannose standard solution is only assayed where 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 concentrations of D-glucose, D-fructose and D-mannose are determined directly from the extinction coefficient of NADPH (page 6 and 7).

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 and 100 µL).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of TEA buffer and $NADP^+$/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) and GF/A glass fibre filter papers.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.52 mL (D-glucose)
2.54 mL (D-fructose)
2.56 mL (D-mannose)
Sample solution: 4-80 µg of D-glucose, D-fructose plus D-mannose per cuvette (in 0.10-2.00 mL sample volume)

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

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C) sample solution solution 1 (TEA buffer) solution 2 ($NADP^+$/ATP)	2.10 mL - 0.20 mL 0.20 mL	2.00 mL 0.10 mL 0.20 mL 0.20 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 3 (HK/G-6-PDH)	0.02 mL	0.02 mL
Mix*, 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**. **Then add:**		
suspension 4 (PGI)	0.02 mL	0.02 mL
Mix*, read absorbances of the solutions (A_3) after 8-10 min.		
Then add:		
suspension 5 (PMI)	0.02 mL	0.02 mL
Mix*, read absorbances of the solutions (A_4) after 20 min. If the reaction has not stopped after 20 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min**.		

* for example with a plastic spatula or by gentle inversion after sealing 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.

> **NOTE:** For the measurement of just D-glucose and D-mannose, mix PGI and PMI and add 40 µL at the point where PGI is added, and allow the reaction to proceed for 20 min.

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-glucose}}$.

Determine the absorbance difference (A_3-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-fructose}}$.

Determine the absorbance difference (A_4-A_3) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-mannose}}$.

For samples containing just D-glucose and D-mannose (and where the PGI and PMI are added together), determine the absorbance difference (A_4-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-mannose}}$.

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

The concentration of D-glucose, D-fructose and D-mannose 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 D-glucose, D-fructose or D-mannose [g/mol]
ε = extinction coefficient of NADPH at 340 nm
= 6300 [$l \times mol^{-1} \times cm^{-1}$]
d = light path [cm]
v = sample volume [mL]

It follows for D-glucose:

$$c = \frac{2.52 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

$$= 0.7206 \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

for D-fructose:

$$c = \frac{2.54 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{D\text{-fructose}} \quad [g/L]$$

$$= 0.7264 \times \Delta A_{D\text{-fructose}} \quad [g/L]$$

for D-mannose:

$$c = \frac{2.56 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{D\text{-mannose}} \quad [g/L]$$

$$= 0.7321 \times \Delta A_{D\text{-mannose}} \quad [g/L]$$

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

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

Content of D-fructose

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

Content of D-mannose

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

SAMPLE PREPARATION:

1. Sample dilution.

The amount of sugar (D-glucose, D-fructose plus D-mannose) present in the cuvette (i.e. in the 0.1 mL sample being analysed) should range between 4 and 80 μg. The sample solution must therefore be diluted sufficiently to yield a sugar concentration between 0.04 and 0.8 g/L.

Dilution Table

Estimated concentration of D-glucose, D-fructose plus D-mannose (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 ΔA due to the analyte under investigation 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 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

If the determined amount of D-glucose in the sample is much larger than D-fructose or D-mannose (e.g. 10-fold higher), then the precision of the D-fructose and D-mannose determinations are impaired. In this case, reduce the content of the D-glucose using glucose oxidase/catalase reagent in the presence of atmospheric oxygen (see page 9 of Megazyme D-Fructose/D-Glucose Kit booklet at www.megazyme.com).

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. 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 (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.

SAMPLE PREPARATION EXAMPLE:

Determination of D-glucose and D-mannose in polysaccharides.

a. Removal of low degree of polymerisation (DP) sugars.

1. Mill yeast, mushroom or plant sample to pass a 0.5 mm screen using a Retsch centrifugal mill (or equivalent).
2. Add milled sample (approx. 100 mg, weighed accurately) to a 20 x 125 mm Fisher Brand culture tube (or equivalent). Tap the tube to ensure all of the sample falls to the bottom of the tube.

3. Add 8 mL of aqueous ethanol (80 % v/v) to each tube and stir the tubes vigorously on a vortex mixer. Incubate the tubes at ~ 80°C for 15 min. Ensure that the solution does not boil out of the tube. Add another 8 mL of aqueous ethanol and stir the tubes vigorously on a vortex mixer. Cool, and centrifuge at 1,500 *g* for 10 min. Carefully decant and discard the supernatant solution.

4. Resuspend the pellet in 8 mL of aqueous ethanol and stir vigorously. Add another 8 mL of aqueous ethanol and stir. Centrifuge the tubes at 1,500 *g* for 10 min. Carefully decant the supernatant. Invert the tubes on absorbent paper to ensure complete removal of all free liquid.

NOTE: For samples not containing reducing sugars (e.g. commercial yeast β-glucan preparations that are already washed), the alcohol washing steps may not be required. In this case, simply "wet" the sample with 0.2 mL of aqueous ethanol before proceeding with "b".

b. Solubilisation of polysaccharide:

1. Add a magnetic stirrer bar (5 x 15 mm) to each tube and place the tubes in a test-tube rack in an ice water bath over a magnetic stirrer. Add 2 mL of ice-cold, 60 % (v/v) sulphuric acid to each tube with vigorous stirring on a magnetic stirrer (to ensure complete dispersion/dissolution). Continue stirring for **1 hr**.

2. Add 12 mL of water to each tube and continue stirring for a few min.

3. Loosely cap the tubes and place them in a boiling water bath at ~ 100°C. Allow the remaining alcohol to boil off over 5 min, and then tighten the cap and continue the incubation for **2 hr.**

4. Cool the tubes to room temperature and carefully loosen the caps.

5. Quantitatively transfer the contents of each tube to a 100 mL beaker and adjust the volume to approx. 60 mL with distilled water. Adjust the pH to approx. 7.6 with 2 M NaOH (using a pH meter). Quantitatively transfer the solution to a 100 mL volumetric flask using a water wash bottle and adjust to volume with distilled water. Mix the contents well by inversion.

6. Filter an aliquot of the suspension through Whatman GF/A glass fibre filter paper, or centrifuge at 1,500 *g* for 10 min.

7. Analyse aliquots of this solution directly as per the procedure detailed on page 5 of this booklet. *Typically, no further dilution is required and a sample volume of 0.2 mL is satisfactory.*

REFERENCE:

Gawehn, K. (1988). D-Mannose and D-Mannose-6-Phosphate In *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 262-267, VCH Publishers (UK) Ltd., Cambridge, UK.

Figure 1. Arrangement of ice-water bath over a magnetic stirrer for dispersion/dissolution of sample material in 60 % sulphuric acid.

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