Megazyme

D-FRUCTOSE
and
D-GLUCOSE

ASSAY PROCEDURE

K-FRUGL 04/04

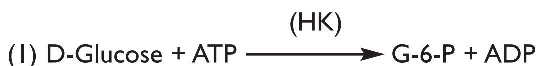
(110 Determinations per Kit)

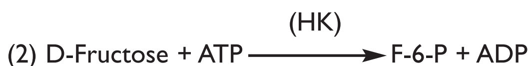
INTRODUCTION:

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





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) (3).

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 (3), F-6-P is converted to G-6-P by phosphoglucose isomerase (PGI) (4).

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.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

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

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.486 mg/L of sample solution

at the maximum sample volume of 1.60 mL. The detection limit is 0.972 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 1.60 mL.

The assay is linear over the range of 4 to 80 μg of D-glucose or D-fructose 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 1.60 mL, this corresponds to a D-glucose concentration of approx. 0.243-0.486 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 0.02-0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of D-glucose or D-fructose 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 and/or D-fructose (approx. 50 μg 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 or D-fructose to the sample in the initial extraction steps.

SAFETY:

The reagents used in the determination of D-glucose and D-fructose are not hazardous materials in the sense of the Hazardous Substances Regulations. However the general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 110 determinations are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Imidazole buffer (25 mL, 2 M, pH 7.6) plus magnesium chloride (100 mM). Stable for > 2 years at 4°C.

Bottle 2: $NADP^+$ (150 mg) plus ATP (440 mg). Stable for > 5 years at -20°C.

Bottle 3: Hexokinase (425 U/mL) plus glucose-6-phosphate dehydrogenase (212 U/mL) suspension, 2.25 mL. Stable for > 2 years at 4°C.

Bottle 4: Phosphoglucose isomerase suspension (2.25 mL, 1,000 U/mL). Stable for > 2 years at 4°C.

Bottle 5: D-Glucose plus D-fructose standard solution (5 mL, 0.5 mg/mL of each).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dilute the contents of bottle 1 to 200 mL with distilled water. Check pH and, if necessary, adjust to pH 7.6 using either 1 M HCl or 1 M NaOH. Adjust the volume to 250 mL and store in a well sealed Duran® bottle. Store at 4°C between use. Stable for 12 months at 4°C if 2 drops of toluene are added to prevent microbial contamination, or for 2 months at 4°C if toluene is not added. Preferably, store the diluted buffer in aliquots in polypropylene containers at -20°C, under which conditions it is stable for > 2 years.

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

5. Use the contents of bottle 5 as supplied.
Stable for > 2 years at room temperature or at 4°C.

> **NOTE:** The D-glucose plus D-fructose 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 and D-fructose are determined directly from the extinction coefficient of NADPH (page 5).

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 1000 µL).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
   - with 12.5 mL Combitip® (to dispense 1.0 mL aliquots of imidazole buffer and 1.5 mL aliquots of distilled water).
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of $NADP^+$/ATP solution).

5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA YellowLab Test Tube Shaker TTS).
8. Stop clock.
9. Whatman No.1 (9 cm) filter papers.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.72 mL (D-glucose)
2.74 mL (D-fructose)

Read against air (without a cuvette in the light path) or against water
Sample solution: 4-80 µg of D-glucose plus D-fructose per cuvette
(in 0.10-1.60 mL sample volume)

<table>
<tr><th>Pipette into cuvettes</th><th>Blank</th><th>Sample</th></tr>
<tr><td>solution 1 (imidazole buffer)
solution 2 ($NADP^+$/ATP)
sample solution
distilled water</td><td>1.00 mL
0.10 mL
-
1.60 mL</td><td>1.00 mL
0.10 mL
0.10 mL
1.50 mL</td></tr>
<tr><td colspan="3">Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:</td></tr>
<tr><td>suspension 3 (HK/G-6-PDH)</td><td>0.02 mL</td><td>0.02 mL</td></tr>
<tr><td colspan="3">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:</td></tr>
<tr><td>suspension 4 (PGI)</td><td>0.02 mL</td><td>0.02 mL</td></tr>
<tr><td colspan="3">Mix*, read absorbances of the solutions (A_3) after 8-10 min .</td></tr>
</table>

* 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.

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 $\Delta A_{D\text{-fructose}}$.

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

The concentration of D-glucose and D-fructose 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 or D-fructose [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:

$$c = \frac{2.72 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

$$= 0.7778 \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

for D-fructose:

$$c = \frac{2.74 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{D\text{-fructose}} \quad [g/L]$$

$$= 0.7836 \times \Delta A_{D\text{-fructose}} \quad [g/L]$$

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

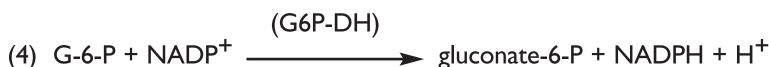
$$= \frac{c_{D\text{-glucose}} \text{ [g/L sample solution]}}{\text{weight}_{sample} \text{ [g/L sample solution]}} \times 100 \quad \text{[g/100 g]}$$

Content of D-fructose

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

DETERMINATION OF D-MANNOSE:

D-Mannose can be determined according to the following reactions:



Since phosphomannose isomerase (PMI) acts slowly in imidazole buffer (as used in the assay format described above) we recommend the use of glycylglycine buffer, as described in the Megazyme "D-Mannose and D-Glucose Assay Kit" booklet (see www.megazyme.com).

SAMPLE PREPARATION:

1. Sample dilution.

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

If the absorbance difference measured (ΔA) 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 1.60 mL, making sure that the sum of the sample and distilled water components in the reaction is 1.60 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 (e.g. 10-fold higher), then the precision of the D-fructose determination is 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).

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 litre 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: a 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 EXAMPLES:

(a) Determination of D-glucose and D-fructose in preserves, and other vegetable and fruit products.

Homogenise about 10 g of jam in a mixer. Accurately weigh approx. 0.5 g of the sample into a 100 mL volumetric flask, mix with 50 mL of distilled water to dissolve, make up to the mark, mix and filter. Discard the first 5 mL of the filtrate. Use the clear, undiluted filtrate for the assay (0.1-1.6 mL).

(b) Determination of D-glucose and D-fructose in desserts and ice-cream.
Weigh approx. 1 g of sample accurately into a 100 mL volumetric flask, add 60 mL of water and incubate for 15 min at approx. 50°C, with shaking from time to time. For protein precipitation, add the following solutions and mix after each addition: 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH (100 mM). Fill up to the mark with distilled water, mix and filter. Use the clear, possibly slightly opalescent solution for the assay, dilute according to the dilution table.

(c) Determination of D-glucose and D-fructose in solid foodstuffs.
Mill plant materials to pass a 0.5 mm screen. Homogenise solid foodstuffs such as bread, pastries, chocolate confectionery or candy in a mixer, meat grinder or mortar. Weigh out a representative sample and extract with water (heated to 60°C, if necessary). Quantitatively transfer to a volumetric flask and dilute to the mark with water. Mix, filter, dilute according to the dilution table, and use the clear solution for assay.

(d) Determination of D-glucose and D-fructose in honey.
Stir honey thoroughly with a spatula. Transfer approx. 5-10 g of viscous or crystalline honey to a beaker and heat for 5 min at approx. 60°C, stirring occasionally with a spatula (there is no need to stir liquid honey). Allow to cool. Pour approx. 1 g of the liquid sample, accurately weighed, into a 100 mL volumetric flask, dissolve at first with only a small volume of water, and then dilute to the mark and mix. Prepare a 1:10 (1 + 9) dilution of the 1 % (w/v) honey solution. Use 0.1 mL per assay.

SPECIAL SAMPLE PREPARATION FOR THE DETERMINATION OF SUCROSE AND D-FRUCTOSE 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).

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

The hydrogen peroxide is decomposed by catalase (2).

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

Reagents.

1. 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.

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

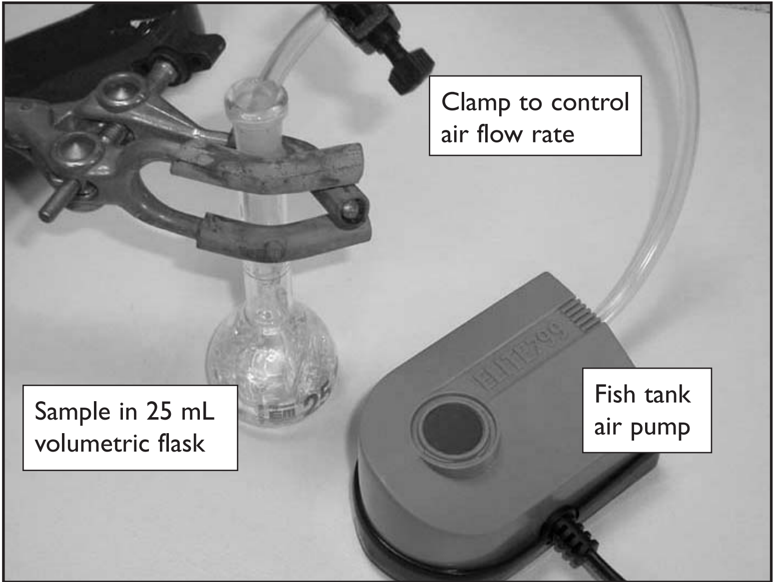
Add 53.4 g of di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) to 900 mL of distilled water and dissolve by stirring. Add 1.11 g of $MgCl_2.7H_2O$ and dissolve. Adjust the pH to 7.6 with 1 M NaOH (40 g/L) and adjust the volume to 1 L with distilled water. Store at 4°C in a well sealed Duran®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

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.



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