SUCROSE / D-FRUCTOSE / D-GLUCOSE

PRODUCT INSTRUCTIONS

SKU: 700004342
K-SUFRG

01/25

100 Manual Assays per Kit

Megazyme by NEOGEN

INTRODUCTION:

Sucrose, D-glucose and D-fructose are found in most plant and food products. In plant materials, D-glucose and D-fructose occur both as free sugars and as the disaccharide sucrose. They are also found in a range of oligosaccharides (galactosyl-sucrose oligosaccharides and fructo-oligosaccharides) and in polysaccharides such as fructans (inulins), starch, 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. In the wine industry, the addition of sucrose is only permitted in a few situations, for example in the production of champagne.

PRINCIPLE:[1-3]

The D-glucose concentration is determined before and after hydrolysis of sucrose by β-fructosidase (invertase). The D-fructose content of the sample is determined subsequent to the determination of D-glucose, after isomerisation by phosphoglucose isomerase (PGI).

D-Glucose determination:

At pH 7.6, hexokinase (HK) catalyses the phosphorylation of D-glucose by adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1).

$$\text{(1)}\quad \text{D-Glucose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{G-6-P} + \text{ADP}$$

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) (2).

$$\text{(2)}\quad \text{G-6-P} + NADP^+ \xrightarrow{\text{(G6P-DH)}} \text{gluconate-6-phosphate} + \text{NADPH} + H^+$$

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

D-Fructose determination:

Hexokinase also catalyses the phosphorylation of D-fructose to fructose-6-phosphate (F-6-P) by adenosine-5'-triphosphate (ATP) (3).

$$\text{(3)}\quad \text{D-Fructose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{F-6-P} + \text{ADP}$$

The F-6-P is subsequently converted to G-6-P by PGI (4).

(4) F-6-P $\xleftrightarrow{\text{(PGI)}}$ G-6-P

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.

Hydrolysis of sucrose:

At pH 4.6, sucrose is hydrolysed by β-fructosidase to D-glucose and D-fructose.

(5) Sucrose + H_2O $\xrightarrow{\text{(β-fructosidase)}}$ D-glucose + D-fructose

The D-glucose in the sample following hydrolysis of sucrose (total D-glucose) is determined as described above.

The sucrose content is calculated from the difference in D-glucose concentrations before and after hydrolysis by β-fructosidase.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assays are specific for D-glucose and D-fructose. Since β-fructosidase also hydrolyses low molecular weight fructans[4] (e.g. kestose) this 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. This can be checked by measurement of D-fructose in the "sucrose sample" subsequent to the determination of total D-glucose. Sufficient PGI is provided in the kit to allow for this further analysis if desired.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.69 mg/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 1.38 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, D-fructose or 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. 0.35 to 0.69 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.

Analysis of commercial sucrose should yield recoveries of ~ 100%. However, values of less than 100% will be obtained for D-glucose monohydrate and D-fructose due to moisture absorption by these compounds.

To confirm that sucrose is completely hydrolysed by the β-fructosidase, perform the incubation for the recommended time and for twice the recommended time. The final determined values for D-glucose and D-fructose should be the same.

INTERFERENCE:

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 and sucrose determination is impaired. If required contact your local Neogen representative for assistance in troubleshooting this sample type.

If the conversion of D-glucose or D-fructose has been completed within the times 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. 20 µg of each in 0.1 mL), but not sucrose, 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 sucrose, D-glucose or D-fructose to the sample in the initial extraction steps.

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 both the Megazyme® and Neogen® websites.

KITS:

Kits suitable for performing 100 assays of sucrose, D-glucose and D-fructose are available from Neogen. The kits contain the full assay method plus:

Bottle 1: Buffer 1 (25 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative.
Store at 4°C. See individual label for expiry date.

Bottle 2: $NADP^+$ plus ATP.
Store below -10°C. See individual label for expiry date.

Bottle 3: Hexokinase plus glucose-6-phosphate dehydrogenase suspension, (4.1 mL).
Store at 4°C. See individual label for expiry date.

Bottle 4: Phosphoglucose isomerase suspension (2.25 mL).
Store at 4°C. See individual label for expiry date.

Bottle 5: D-Glucose plus D-fructose standard solution (5 mL, 0.2 mg/mL of each sugar).
Store at 4°C. See individual label for expiry date.

Bottle 6: β-Fructosidase (pH 4.6), lyophilised powder.
Store below -10°C. See individual label for expiry date.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of **bottle 1** as supplied.

2. Dissolve the contents of **bottle 2** in 22 mL of distilled water. Stable for 4 weeks at 4°C or for 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes). This is **solution 1** ($NADP^+$/ATP solution).

3 & 4. Use the contents of **bottles 3** and **4** as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper.
Subsequently, store the bottles in an upright position. **Swirl the bottles to mix contents before use.**

5. Use the contents of **bottle 5** as supplied.

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 7).

6. Dissolve the contents of **bottle 6** in 20 mL of distilled water. Divide into aliquots of approx. 5 mL. Store below -10°C in polypropylene tubes between use and keep cool during use if possible. This is **solution 2** (β-Fructosidase solution). Stable for 2 years below -10°C.

NOTE: A sucrose solution can be used to confirm the effectiveness of the β-fructosidase hydrolysis reaction. With commercially available crystalline sucrose, recoveries of 100% should be achieved. Prepare the required solution as follows: Accurately weigh 0.50 g of crystalline sucrose into a 1 L volumetric flask and dissolve in distilled water. Adjust to the mark with distilled water. Store in appropriately sized aliquots below -10°C. Keep cool during use if possible.

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 0.1 mL aliquots of **bottle 1** 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.
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: 2.42 mL (D-glucose)
2.44 mL (D-fructose)
Sample solution: 4-80 µg of sucrose + D-glucose + D-fructose 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 sample	Sucrose sample	Blank D-glucose/ D-fructose sample	D-Glucose/ D-fructose sample
solution 2 (β-Fructosidase solution)	0.20 mL -	0.20 mL 0.10 mL	- -	- 0.10 mL
Mix** and incubate for 5 min (NOTE: before pipetting **solution 2**, prewarm to 25°C). **Then add:**				
distilled water (at ~ 25°C) **bottle 1** (buffer) **solution 1** ($NADP^+$/ATP solution)	2.00 mL 0.10 mL 0.10 mL	1.90 mL 0.10 mL 0.10 mL	2.20 mL 0.10 mL 0.10 mL	2.10 mL 0.10 mL 0.10 mL
Mix** and read absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
bottle 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***. **Then add:**				
bottle 4 (PGI)	-	-	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_3) after approx. 10 min.				

* pipette both **solution 2** 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.

CALCULATION:

NOTE: These calculations can be simplified by using the ***Mega-Calc™***, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

Determine the absorbance differences (A_2-A_1) and (A_3-A_2) for both blanks and samples, and calculate values of $\Delta A_{D\text{-glucose}}$, $\Delta A_{sucrose}$ and $\Delta A_{D\text{-fructose}}$ as described below:

Determination of free D-glucose:

$\mathbf{\Delta A_{D\text{-glucose}}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$ (from the D-glucose/ D-fructose sample).

If the sample has been diluted during preparation, $\mathbf{\Delta A_{D\text{-glucose}}}$ must be multiplied by the dilution factor, F.

Determination of sucrose:

Calculate $\mathbf{\Delta A_{total\ D\text{-glucose}}}$ (from the sucrose sample).

$\mathbf{\Delta A_{total\ D\text{-glucose}}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$ (from the sucrose sample).

If the sample has been diluted during preparation, $\mathbf{\Delta A_{total\ D\text{-glucose}}}$ must be multiplied by the dilution factor, F.

The difference between $\mathbf{\Delta A_{total\ D\text{-glucose}}}$ (from the sucrose sample) and $\mathbf{\Delta A_{D\text{-glucose}}}$ (from the D-glucose/D-fructose sample) yields $\mathbf{\Delta A_{sucrose}}$.

$\mathbf{\Delta A_{sucrose}} = \mathbf{\Delta A_{total\ D\text{-glucose}}} - \mathbf{\Delta A_{D\text{-glucose}}}$

Determination of free D-fructose:

$\mathbf{\Delta A_{D\text{-fructose}}} = (A_3\text{-}A_2)_{sample} - (A_3\text{-}A_2)_{blank}$ (from the D-glucose/ D-fructose sample).

If the sample has been diluted during preparation, $\mathbf{\Delta A_{D\text{-fructose}}}$ must be multiplied by the dilution factor, F.

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

The concentration of D-glucose, sucrose 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 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]

It follows for D-glucose:

$$c = \frac{2.42 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

$$= 0.6920 \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

for sucrose:

$$c = \frac{2.42 \times 342.3}{6300 \times 1.0 \times 0.1} \times \Delta A_{sucrose} \quad [g/L]$$

$$= 1.315 \times \Delta A_{sucrose} \quad [g/L]$$

for D-fructose:

$$c = \frac{2.44 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-fructose}} \quad [g/L]$$

$$= 0.6978 \times \Delta A_{D\text{-fructose}} \quad [g/L]$$

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

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

Content of sucrose

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

Content of D-fructose

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

SAMPLE PREPARATION:

1. Sample dilution.

The amount of sugar (D-glucose plus D-fructose plus sucrose) present in the cuvette (i.e. in the 0.1 mL of 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 plus sucrose (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 $\Delta A_{D\text{-glucose}}$, $\Delta A_{sucrose}$ or $\Delta A_{D\text{-fructose}}$ 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 **bottle 6** components in the reaction is 2.20 mL, and using the new sample volume in the equation.

NOTE: The below are suggested sample preparation examples only. Users should perform in-house matrix validation work prior to routine use. This process will highlight any problematic matrices encountered.

2. Sample clarification.

Samples containing high amounts of protein or fats may cause interference in target analyte determination and require sample clarification prior to analysis using Carrez Clarification reagents. These reagents are available to purchase separately from Neogen in the Carrez Clarification Kit (**K-CARREZ**). The **K-CARREZ** reagents should be diluted prior to use as described on page 2 of the **K-CARREZ** assay protocol and the assay procedure for clarification followed as described on page 3. The clarified sample solution prepared using the **K-CARREZ** assay kit can then be analysed for D-glucose/D-fructose/sucrose content as described in the assay procedure.

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 > 0.1 mL of an acidic sample is to be used undiluted (such as wine or 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 a significant amount of carbon dioxide,

such as beer, 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, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman™ No. 1 filter paper.

(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 using **K-CARREZ**.

(h) Samples containing protein: deproteinise samples containing protein using **K-CARREZ**.

SUGGESTED SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-glucose, D-fructose and sucrose in fruit juices and similar beverages.

Filter turbid juices or clarify using **K-CARREZ**. Dilute to give a sucrose plus D-glucose plus D-fructose concentration of approx. 0.1-1.5 g/L. If the solution is slightly coloured, it can be assayed directly. If the solution is strongly coloured, add 0.2 g of (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper. Use the clear, slightly coloured solution directly for assay. *Typically, for apple and orange juice, a dilution of 1:100 and sample volume of 0.1 mL are satisfactory.*

(b) Determination of D-glucose, D-fructose and sucrose in beer.

Remove carbon dioxide by stirring approx. 5-10 mL of the beer for 1 min or filter through a fluted filter paper. Use the clear, slightly coloured solution directly for assay. *Typically, no dilution will be required and a sample volume of 0.1-0.2 mL is satisfactory.*

(c) Determination of D-glucose, D-fructose and sucrose in sweetened condensed milk.

Clarify using **K-CARREZ** according to the assay protocol. Use the clear, possibly slightly opalescent, solution, diluted according to the dilution table, for the assay. *Typically, a dilution of 1:5 and a sample volume of 0.1 mL are satisfactory.*

(d) Determination of D-glucose, D-fructose and sucrose in jam.

Homogenise approx. 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. *Typically, no dilution will be*

required and a sample volume of 0.1-0.2 mL is satisfactory.

(e) Determination of D-glucose, D-fructose and sucrose in ice cream.
Homogenise approx. 10 g of sample in a mixer. Clarify using **K-CARREZ** according to the assay protocol. *Typically, a dilution of 1:5 and a sample volume of 0.1-0.2 mL are satisfactory.*

(f) Determination of D-glucose, D-fructose and sucrose 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. Accurately weigh approx. 1 g of the liquid sample into a 100 mL volumetric flask. Dissolve first with only a small volume of distilled water and then fill up to the mark.
- ***Determination of D-glucose and D-fructose:*** dilute the 1% (w/v) honey solution 1:10 (1+9) and use 0.1 mL for the assay.
- ***Determination of sucrose:*** if the estimated sucrose content of the honey lies between 5 and 10% (w/v), dilute the 1% (w/v) solution 1:3 (1+2) and use 0.1 mL for the assay. If the estimated sucrose content in the honey lies between 0.5 and 5% (w/v), as much as possible of the excess D-glucose should be removed before sucrose is determined, otherwise the precision of the sucrose determination will be impaired. If required contact your local Neogen representative for assistance in troubleshooting this sample type.

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