

Megazyme

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D-FRUCTOSE and D-GLUCOSE

ASSAY PROCEDURE

K-FRUGL 04/18

(*110 Assays per Kit) or
(1100 Auto-Analyser Assays per Kit) or
(1100 Microplate Assays per Kit)

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

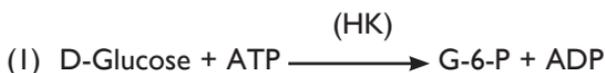


INTRODUCTION:

D-Glucose and D-fructose are found in most plant products. 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 D-glucose and D-fructose content, often referred to as total reducing sugars, is one of the most important quality parameters and is monitored at each stage of the wine making process. These sugars can be measured either independently (see page 4, "A") or, if more appropriate, simultaneously (see page 7, "B"). The procedure for auto-analyser applications is described on page 9 ("C"), and the microplate procedures for independent or simultaneous determinations are described on pages 10 and 11 ("D" and "E"), respectively.

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 simultaneous formation of adenosine-5'-diphosphate (ADP) (1), (2).

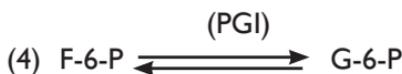


In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate (NADP^+) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (3).



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).



The G-6-P formed 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 0.332 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.663 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-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 2.00 mL, this corresponds to a D-glucose concentration of approx. 0.166 to 0.332 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 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 plus D-fructose standard (20 µg of each sugar in 0.10 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 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 110 assays in manual format (or 1100 assays in auto-analyser format or 1100 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (15 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

- Bottle 2:** NADP⁺, ATP and PVP.
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:** Phosphoglucose isomerase suspension (2.25 mL).
Stable for > 2 years at 4°C.
- Bottle 5:** D-Glucose plus D-fructose standard solution (5 mL, 0.2 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.
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 & 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 bottle to mix contents before use.**
Stable for > 2 years at 4°C.
5. Use the contents of bottle 5 as supplied.
Stable for > 2 years 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 and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette[®]
 - with 5.0 mL Combipip[®] (to dispense 0.1 mL aliquots of buffer and NADP⁺/ATP solution).
 - with 25 mL Combipip[®] (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. Whatman No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE; D-FRUCTOSE AND D-GLUCOSE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.32 mL (D-glucose)
 2.34 mL (D-fructose)
Sample solution: 4-80 µg of D-glucose plus D-fructose 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)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer)	0.10 mL	0.10 mL
solution 2 (NADP ⁺ /ATP)	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
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:		
suspension 4 (PGI)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A ₃) at the end of the reaction (approx. 8-10 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.

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:

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

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 [$\text{l} \times \text{mol}^{-1} \times \text{cm}^{-1}$]

d = light path [cm]

v = sample volume [mL]

It follows for D-glucose:

$$\begin{aligned} c &= \frac{2.32 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-glucose}} \quad [\text{g/L}] \\ &= 0.6634 \times \Delta A_{D\text{-glucose}} \quad [\text{g/L}] \end{aligned}$$

for D-fructose:

$$\begin{aligned} c &= \frac{2.34 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-fructose}} \quad [\text{g/L}] \\ &= 0.6692 \times \Delta A_{D\text{-fructose}} \quad [\text{g/L}] \end{aligned}$$

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

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

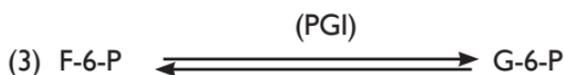
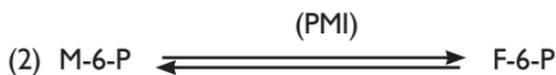
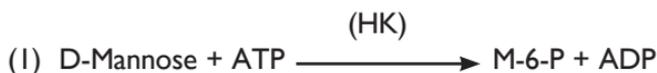
Content of D-fructose

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

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

DETERMINATION OF D-MANNOSE:

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



Since phosphomannose isomerase (PMI) acts slowly in imidazole buffer (as used in the assay format described above) we recommend the use of TEA buffer, as described in the Megazyme “D-Mannose/D-Fructose/D-Glucose Assay Kit” booklet (see **K-MANGL** at www.megazyme.com).

B. MANUAL ASSAY PROCEDURE; TOTAL REDUCING SUGARS:

In the wine industry the sum of D-glucose plus D-fructose is a key quality parameter, as this represents the amount of sugar that is available to the yeast for conversion into ethanol. In the vast majority of cases it is unnecessary to differentiate between these monosaccharides, allowing them to be quantified together using a more convenient and rapid assay format, as follows:

Additional preparation step:

Gently shake bottles 3 and 4 to remove any enzyme that may have settled on the rubber stoppers. Using a pipette, transfer the entire contents of bottle 4 (PGL) into bottle 3 (HK/G6P-DH). After replacing the rubber stopper, mix the enzymes by gentle swirling. This HK/G6P-DH/PGL mixture is now ready for use. *After performing this step, D-glucose and D-fructose cannot be measured individually with this kit reagent mixture.*

PROCEDURE:

Wavelength:	340 nm
Cuvette:	1 cm light path (glass or plastic)
Temperature:	~ 25°C
Final volume:	2.34 mL (D-glucose plus D-fructose)
Sample solution:	4-80 µg of D-glucose plus D-fructose 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)	2.10 mL	2.00 mL
sample solution	-	0.10 mL
solution 1 (buffer)	0.10 mL	0.10 mL
solution 2 (NADP ⁺ /ATP)	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/PGL)	0.04 mL	0.04 mL
Mix* and read the absorbances of the solutions (A_{total}) at the end of the reaction (approx. 10 min). If the reaction has not stopped after 10 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.

CALCULATION:

Determine the absorbance difference ($A_{total} - A_1$) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining

$$\Delta A_{D\text{-glucose} + D\text{-fructose}}$$

The value of $\Delta A_{D\text{-glucose} + D\text{-fructose}}$ 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 + D-fructose:

$$c = \frac{2.34 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-glucose} + D\text{-fructose}} \quad [g/L]$$

$$= 0.6692 \times \Delta A_{D\text{-glucose} + D\text{-fructose}} \quad [g/L]$$

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

C. AUTO-ANALYSER ASSAY PROCEDURE; TOTAL REDUCING SUGARS:

This kit is suitable for the preparation of 254.1 mL of reagent (equivalent to 1155 reactions of 0.222 mL). Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
bottle 1 (buffer)	1.0 mL
bottle 2 (NADP ⁺ /ATP)	1.0 mL (after adding 12 mL of H ₂ O to bottle 2)
bottle 4 (PGI)	0.2 mL (swirl to mix before use)
PVP (10 g/L)	1.0 mL (or replace with H ₂ O)
H ₂ O	18.0 mL
Total volume	21.2 mL

Preparation of R2:

Component	Volume
bottle 3 (HK/G6P-DH)	0.2 mL (swirl to mix before use)
H ₂ O	1.9 mL
Total volume	2.1 mL

EXAMPLE METHOD:

R1: 0.200 mL

Sample: ~ 0.002 mL

R2: 0.020 mL

Reaction time: 10 min at 25°C or 5 min at 37°C

Wavelength: 340 nm

Prepared reagent stability: > 7 days when refrigerated

Calculation: endpoint

Reaction direction: increase

Linearity: up to 108 µg/mL of D-glucose + D-fructose in final reaction mixture

D. MICROPLATE ASSAY PROCEDURE; RESIDUAL SUGARS

NOTES:

1. The Microplate Assay Procedure for D-glucose/D-fructose can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of D-glucose/D-fructose **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength:	340 nm
Microplate:	96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature:	~ 25°C
Final volume:	0.234 mL
Linearity:	0.1-8 µg of D-glucose/D-fructose per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.010 mL	0.010 mL	0.010 mL
solution 2 (NADP ⁺ /ATP)	0.010 mL	0.010 mL	0.010 mL
Mix* and read the absorbances of the solutions (A ₁) after approx. 3 min (at completion of the pre-reaction). Start the reactions by addition of:			
suspension 3+4 (HK/G6P-DH/PGI)	0.004 mL	0.004 mL	0.004 mL
Mix* and read the absorbances of the solutions (A ₂) at the end of the reaction (approx. 10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbances increase constantly over 2 min**.			

* for example using microplate shaker, shake function on a microplate reader, or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this “creep” rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 3+4.

CALCULATION (Microplate Assay Procedure):

$$\text{g/L} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{g/L standard} \times F$$

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

E. MICROPLATE ASSAY PROCEDURE; D-GLUCOSE AND D-FRUCTOSE (Sequential)

NOTE:

The Microplate Assay Procedure for sequential determination of D-glucose/D-fructose can only be performed using a microplate reader with the capability of converting absorbance values to a 1 cm pathlength.

Wavelength:	340 nm
Microplate:	96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature:	~ 25°C
Final volume:	0.234 mL
Linearity:	0.1-8 µg of D-glucose/D-fructose per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.010 mL	0.010 mL	0.010 mL
solution 2 (NADP ⁺ /ATP)	0.010 mL	0.010 mL	0.010 mL

Mix* and read the absorbances of the solutions (A_1) after approx. 3 min (at completion of the pre-reaction). Start the reactions by addition of:

suspension 3 (HK/G6P-DH)	0.002 mL	0.002 mL	0.002 mL
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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 increase constantly over 2 min**.

suspension 4 (PGI)	0.002 mL	0.002 mL	0.002 mL
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Mix* and read the absorbances of the solutions (A_3) at the end of the reaction (approx. 8-10 min).

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this “creep” rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 3.

CALCULATION (Microplate Assay Procedure):

Once converted to a 1 cm pathlength the absorbance values can be applied to the calculation used for the Manual Assay Procedure (page 5) or analysed using the Megazyme **Mega-Calc**TM.

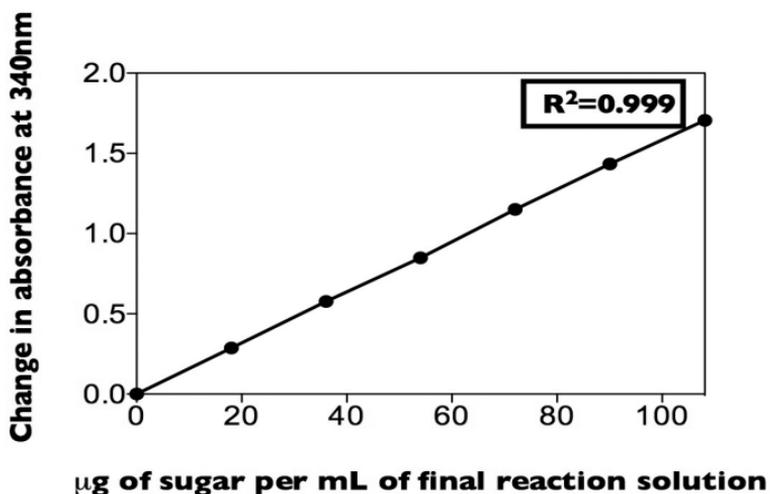


Figure 1. Calibration curve showing the linearity of reagent prepared from **K-FRUGL**. The reactions used to generate this calibration curve were performed at 25°C for 10 min, using 4.6 mm path-length cuvettes.

SAMPLE PREPARATION (Manual Formats A and B):

1. Sample dilution.

The amount of sugar (D-glucose plus D-fructose) 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 (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 $\Delta A_{D\text{-glucose}}$ 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 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 (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 15).

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6] \cdot 3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4 \cdot 7H_2O$) (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 > 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 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 room temperature and fill the volumetric flask to the mark with distilled 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 grapes.

Add 200 mL of distilled water to a 500 mL measuring cylinder. Add a representative number of grapes to raise the volume to just over 400 mL. Add additional distilled water in a volume equivalent to the volume above 400 mL (e.g. if the measured volume is 420 mL, add another 20 mL of distilled water). This gives a 2-fold dilution of the volume of the grapes. Transfer the water plus grapes to a blender (e.g. kitchen blender) and homogenise for approx. 3 min. Filter an aliquot through a Whatman No. 1 filter sheet (15 cm diameter). Discard the first few mL and collect the next approx. 20-30 mL. Dilute the filtrate as required. *Typically, a further dilution of 1:1000 and a sample volume of 0.1 mL are satisfactory (i.e. a total sample dilution of 2000-fold).*

(b) Determination of D-glucose and D-fructose in grape juice.

This can generally be performed without any sample treatment (except dilution according to the dilution table). *Typically, a dilution of 1:2000 and a sample volume of 0.1 mL are satisfactory.*

(c) Determination of D-glucose and D-fructose in red and white wine.

This can generally be performed without any sample treatment (except dilution according to the dilution table). Should a red wine require decolourisation, proceed as described for “strongly coloured samples” on page 13 [General considerations (e)]. *Typically, a dilution of 1:10 and a sample volume of 0.1 mL are satisfactory.*

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

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. Use the clear, undiluted filtrate for the assay. *Typically, no further dilution is required and a sample volume of 0.1-2.0 mL is satisfactory.*

(e) 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 distilled 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 solution (100 mM). Fill up to the mark with distilled water, mix and filter. Use the clear, possibly slightly opalescent solution for the assay, with dilution according to the dilution table. *Typically, no further dilution is required and a sample volume of 0.1-2.0 mL is satisfactory.*

(f) 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 distilled water. Mix, filter, and use the clear solution for assay, with dilution according to the dilution table if necessary. *Typically, no further dilution is required and a sample volume of 0.1-2.0 mL is satisfactory.*

(g) 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 distilled water, and then dilute to the mark and mix. *Typically, a dilution of 1:10 and a sample volume of 0.1 mL is satisfactory.*

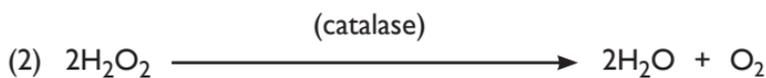
SPECIAL SAMPLE PREPARATION FOR THE DETERMINATION OF D-FRUCTOSE IN THE PRESENCE OF EXCESS D-GLUCOSE:

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 (I).



The hydrogen peroxide (H_2O_2) is decomposed by catalase (2).



Reagents.

1. Sodium phosphate buffer (300 mM, pH 7.6) plus 5 mM MgCl_2 .

Add 53.4 g of disodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) to 900 mL of distilled water and dissolve by stirring. Add 0.47 g of MgCl_2 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 on extended storage, 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 . 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 Sodium phosphate buffer (pH 7.6 + MgCl_2)	5.0 mL
sample solution (up to approx. 5 mg/mL D-glucose)	5.0 mL
enzyme solution	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 2). While this oxidation reaction 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 sodium phosphate buffer used).

After the reaction, to inactivate the glucose oxidase plus catalase, incubate the volumetric flask in a boiling water bath for 10 min, allow 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.



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