OIV-MA-AS311-02 Glucose and fructose

Type II method

1. Definition

Glucose and fructose may be determined individually by an enzymatic method, with the sole aim of calculating the glucose/fructose ratio.

2. Principle

Glucose and fructose are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalyzed by hexokinase (HK), to produce glucose-6□phosphate (G6P) and fructose-6□phosphate (F6P):

$$glucose + ATP \overset{HK}{\leftrightarrow} G6P + ADP$$

$$fructose + ATP \overset{HK}{\leftrightarrow} F6P + ADP$$

G6PD
H The glucose-6□phosphate is first oxidized to gluconate-6□phosphate by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6□phosphate dehydrogenase (G6PDH). The quantity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced corresponds to that of glucose-6□phosphate and thus to that of glucose.

$$G6P + NADP^{+} \overset{G6PDH}{\longleftrightarrow} gluconate - 6 - phosphate + NADPH + H^{+}$$

The reduced nicotinamide adenine dinucleotide phosphate is determined from its absorption at 340 nm.

At the end of this reaction, the fructose-6□phosphate is converted into glucose-6□phosphate by the action of phosphoglucose isomerase (PGI):

$$F6P \overset{PGI}{\leftrightarrow} G6P$$

The glucose-6□phosphate again reacts with the nicotinamide adenine dinucleotide phosphate to give gluconate-6□phosphate and reduced nicotinamide adenine dinucleotide phosphate, and the latter is then determined.

3. Apparatus

A spectrophotometer enabling measurements to be made at 340 nm, the wavelength at which absorption by NADPH is at a maximum. Absolute measurements are involved (i.e. calibration plots are not used but standardization is made using the extinction coefficient of NADPH), so that the wavelength scales of, and absorbance values obtained from, the apparatus must be checked.

If not available, a spectrophotometer using a source with a discontinuous spectrum that enables measurements to be made at 334 nm or at 365 nm may be used.

Glass cells with optical path lengths of 1 cm or single-use cells.

Pipettes for use with enzymatic test solutions, 0.02, 0.05, 0.1, 0.2 mL.

4. Reagents

Solution 1: buffer solution (0.3 M triethanolamine, pH 7.6, 0.004 M Mg^{2+}): dissolve 11.2g triethanolamine hydrochloride, $(CH_2CH_2OH)_3$N.HCl, and 0.2 g magnesium sulfate, $MgSO_4\,7H_2O$, in 150 mL of double-distilled water, add about 4 mL 5 M sodium hydroxide solution to obtain a pH value of 7.6 and make up to 200 mL.

This buffer solution may be kept for four weeks at approx. + 4°C.

Solution 2: nicotinamide adenine dinucleotide phosphate solution (about 0.0115 M): dissolve 50 mg disodium nicotinamide adenine dinucleotide phosphate in 5 mL of double-distilled water.

This solution may be kept for four weeks at approx. +4°C.

Solution 3: adenosine-5'-triphosphate solution (approx. 0.081 M): dissolve 250 mg disodium adenosine-5'-triphosphate and 250 mg sodium hydrogen carbonate, NaHCO3, in 5 mL of double-distilled water.

This solution may be kept for four weeks at approx. +4°C.

Solution 4: hexokinase/glucose-6-phosphate-dehydrogenase: mix 0.5 mL hexokinase (2 mg of protein/mL or 280 U/mL with 0.5 mL glucose-6-phosphate-dehydrogenase (1 mg of protein/mL).

This mixture may be kept for a year at approx. +4°C.

Solution 5: phosphoglucose-isomerase (2 mg of protein/mL or 700 U/mL). The suspension is used undiluted.

This may be kept for a year at approx. +4°C.

Note: All solutions used above are available commercially.

5. Procedure

5.1. Preparation of sample

Depending on the estimated amount of glucose + fructose per liter (g/L) dilute the sample as follows:

Measurement at 340 and 344 nm (g/L)	Measurement at 365 nm (g/L)	Dilution with water	Dilution factor F
up to 0.4	0.8	-	-
up to 4.0	8.0	1 + 9	10
up to 10.0	20.0	1 + 24	25
up to 20.0	40.0	1 + 49	50
up to 40.0	80.0	1 + 99	100
Above 40.0	80.0	1 + 999	1000

5.2. Determination

With the spectrophotometer adjusted to the 340 nm wavelength, make measurements using air (no cell in the optical path) or water as reference.

Temperature between 20 and 25°C.

Into two cells with 1 cm optical paths, place the following:

	Reference cell	Sample cell
Solution 1 (taken to 20°C)	2.50 mL	2.50 mL
Solution 2		0.10 mL
Solution 3		0.10 mL
Sample to be measured		0.20 mL
Double -distilled water	0.20 mL	

Mix, and after three minutes read the absorbance of the solutions (A_1). Start the reaction by adding:

Solution 4	0.02 mL	0.02 mL

Mix, read the absorbance after 15 minutes and after two more minutes check that the reaction has stopped (A_2).

Add immediately:

Solution 5	0.02 mL	0.02 mL

Mix; read the absorbance after 10 minutes and after two more minutes check that the reaction has stopped (A_3).

Calculate the differences in the absorbance between the reference cell and sample cells.:

$A_2 - A_1$ corresponds to glucose, $A_3 - A_2$ corresponds to fructose,

Calculate the differences in absorbance for the reference cells (□AT) and the sample cell (□AD) and then obtain:

- for glucose: $\Delta A_G = \Delta A_D - \Delta A_T$
- for fructose: $\Delta A_F = \Delta A_D - \Delta A_T$

Note: The time needed for the completion of enzyme activity may vary from one batch to another. The above value is given only for guidance and it is recommended that it be determined for each batch.

5.3. *Expression of results*

5.3.1. Calculation

The general formula for calculating the concentrations is:

$$C = \frac{V \times MV}{\varepsilon \times d \times v \times 1000} \Delta A \, (\frac{g}{L})$$

where:

V = volume of the test solution (mL)

v = volume of the sample (mL)

MW = molecular mass of the substance to be determined

d = optical path in the cell (cm)

□ = absorption coefficient of NADPH at 340 nm = 6.3

(mmole-1 x *l* □ cm-1)

V = 2.92 mL for the determination of glucose

V = 2.94 mL for the determination of fructose

v = 20 mL

PM = 180

$d = 1$

so that:

For glucose : C(g/L) = 0.417 ▫ ΔA_G

For fructose: C(g/L) = 0.420 ▫ ΔA_F

If the sample was diluted during its preparation, multiply the result by the dilution factor F.

Note: If the measurements are made at 334 or 365 nm, then the following expressions are obtained:

- measurement at 334 nm: ε= 6.2 (mmole -1 ▯ absorbance ▯ cm-1)
- for glucose : C(g/L) = 0.425 ▯ ΔA_G
- for fructose: C(g/L) = 0.428 ▯ ΔA_F
- measurement at 365 nm: ε = 3.4 (mmole-1 ▯ absorbance ▯ cm-1)
- for glucose: C(g/L) = 0.773 ▯ ΔA_G
- for fructose: C(g/L) = 0.778 ▯ ΔA_F

5.3.2. Repeatability (*r*):

$r = 0.056\ xi$

xi = the concentration of glucose or fructose in g/L

5.3.3. Reproducibility (R):

$R = 0.12 + 0.076\ xi$

x_i = the concentration of glucose or fructose in g/L

Bibliography

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