Method OIV-MA-AS312-05 Type IV method

Glycerol

(Resolution Oeno 377/2009)

1 Principle

The glycerokinase (GK) catalyses the phosphorylation of glycerol to glycerol-3-phosphate by adenosine-5'-triphosphate (ATP) (1):

(1) $$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate + ADP}$$

The adenosine-5'-diphosphate (ADP) is then converted into ATP by phosphoenol-pyruvate (PEP) in presence of pyruvate-kinase (PK) with pyruvate (2) formation:

(2) $$\text{ADP + PEP} \xrightarrow{\text{PK}} \text{ATP + pyruvate}$$

Pyruvate is converted into lactate by reduced nicotinamide-adenine dinucleotide NADH) in presence of lactate-dehydrogenase (LDH) (3):

(3) $$\text{pyruvate + NADH + H}^+ \xrightarrow{\text{LDH}} \text{lactate + NAD}^+$$

The quantity of NAD^+ formed during the reaction is proportional to the quantity of glycerol. The NADH oxidization is measured by the decrease of its extinction at wavelengths of 334 nm, 340 nm or 365 nm.

2. Apparatus

2.1 Spectrophotometer enabling measurements to be made at 340 nm, at which absorption by NADH is at a maximum.

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

2.2 Glass cells of 1 cm optical path length or single-use cells.

2.3 Micropipettes enabling the selection of volumes from 0.02 to 2 mL.

3. Reagents

3.1 Buffer solution (0.75 M glycylglycine, Mg^{2+} 10^{-3} M, pH = 7.4)

Dissolve 10.0 g of glycylglycine and 0.25 g of magnesium sulfate ($MgSO_4 \cdot 7 H_2O$) in about 80 mL of double distilled water, add about 2.4 mL of 5 M sodium hydroxide solution to obtain a pH of 7.4 and make up to 100 mL. This buffer solution may be kept for 3 months at + 4°C.

3.2 (NADH $8.2.10^{-3}$ M, ATP 33.10^{-3} M, PEP 46.10^{-3} M)

Dissolve:

42 mg of nicotinamide-adenine-dinucleotide reduced - Na_2
120 mg of adenosine-5'-triphosphate, Na_2H_2
60 mg of phosphoenol pyruvate, Na and
300 mg of sodium bicarbonate ($NaHCO_3$)
in 6 mL of double distilled water.
This may be kept for 2-3 days at + 4°C.

3.3 Pyruvate-kinase/lactate-dehydrogenase (PK/LDH)

(PK 3 mg /mL, LDH 1 mg /mL)
Use the suspension without diluting it.
This may be kept for a year at about + 4°C.

3.4 Glycerokinase (GK 1 mg/mL)

The suspension may be kept for a year at about + 4°C.

Note: All reagents needed for the above are available commercially.

4. Preparation of sample

The determination of glycerol is generally made directly on the wine, which is diluted with double distilled water so that the resulting glycerol concentration is between 30 and 500 mg/L. Wine diluted 2 /100 is usually sufficient.

5. Procedure

With spectrophotometer adjusted to 340 nm wavelength the absorbance measurements are made in the glass cells with optical path length of 1 cm, with air as a reference.

Into cells with 1 cm optical paths place the following:

	Reference cell	Sample cell
Solution 3.1	1.00 Ml	1.00 mL
Solution 3.2	0.10 mL	0.10 mL
Sample to be measured	- 0.10 mL	
Water	2.00 mL	1.90 mL
Suspension 3.3	0.01 mL	0.01 mL

Mix, and after about 5 min, read the absorbances (A_1). Start the reaction by adding:

Suspension 3.4 …….	0.01 mL	0.01 mL

Mix, wait until the end of the reaction (5 to 10 min), read the absorbance of the solutions (A_2). Read the absorbance after 10 min and check every 2 min until the absorbance is constant for 2 min.
Calculate the differences in the absorbance:

$$A_2 - A_1$$

for the reference and sample cells.

Calculate the differences in absorbance between the reference cell (ΔA_T) and the sample cell (ΔA_D) using the equation:

$$\Delta A = \Delta A_D - \Delta A_T$$

6. Expression of results

6.1 *Calculation*

The general formula for calculating the concentration is:

$$C = \frac{V \ x \ PM}{\varepsilon \ x \ d \ x \ v \ x \ 1000} X \Delta A$$

V = volume of the test in mL (3.12 mL)
v = volume of the sample mL (0.1 mL)
PM = molecular weight of the substance to be determined (glycerol = 92.1)
d = optical path length of the cell (1 cm)
ε = absorption coefficient of NADH at 340 nm

$$\varepsilon = 6.3 \ (mmol^{-1} \times l \times cm^{-1})$$

When using the amounts given in brackets this reduces to:

$$C = 0.456 \times \Delta A \times F$$

F = dilution factor

Note:
— Measurement at 334 nm, ε = 6.2 ($mmol^{-1}$ x *l* x cm^{-1})

$$C = 0.463 \times \Delta A \times F$$

— Measurement at 365 nm, ε = 3.4 ($mmol^{-1}$ x *l* x cm^{-1})

$$C = 0.845 \times \Delta A \times F$$

BIBLIOGRAPHY

BOERHINGER, Mannheim, *Methods of Enzymatic and chemical analysis*, documentation technique.