

# Megazyme

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## GLYCEROL

### ASSAY PROCEDURE

K-GCROL 08/04

(50 Assays per Kit)

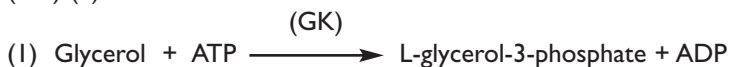


## INTRODUCTION:

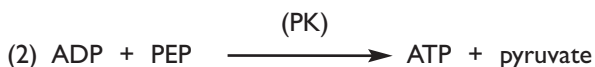
In the food industry, glycerol is an important moistening agent for baked goods. It is also added to candies and icings to prevent crystallization and as a solvent for food colours and carrier for extracts and flavouring agents. As a product of fermentation, glycerol is monitored in the beer and wine industries, where it occurs at concentrations of approx. 1 % (v/v), and is an indicator of quality. The smoothness of lotions, creams and toothpaste is due to the presence of glycerol. Due to its humectant properties, glycerol is sprayed on pre-processed tobacco to prevent crumbling.

## PRINCIPLE:

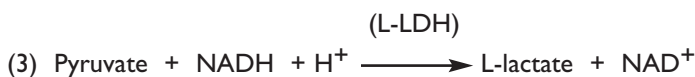
Glycerol is phosphorylated by adenosine-5'-triphosphate (ATP) to L-glycerol-3-phosphate in the reaction catalysed by glycerokinase (GK) (1).



The adenosine-5'-diphosphate (ADP) formed in the reaction is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase (PK) into ATP with the formation of pyruvate (2).



In the presence of the enzyme L-lactate dehydrogenase (L-LDH) pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the production  $\text{NAD}^+$  (3).



The amount of  $\text{NAD}^+$  formed in the above reaction pathway is stoichiometric with the amount of glycerol. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

## SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for glycerol. In the analysis of pure glycerol (free of water), results of approx. 100 % can be expected.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.19 mg/L sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.37 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 2.00 mL.

The assay is linear over the range of 1 to 40  $\mu\text{g}$  of glycerol 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 glycerol concentration of approx. 0.09 to 0.19 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:

The slow hydrolysis of ATP and PEP as well as the air oxidation of NADH results in a slow creep reaction which can be accounted for by extrapolation. Extrapolation is not necessary if the absorbances of the blank and sample are measured immediately one after the other.

If the conversion of glycerol has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding glycerol (approx. 20 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease 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 glycerol to the sample in the initial extraction steps.

### SAFETY:

The reagents used in the determination of glycerol are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

### KITS:

Kits suitable for performing 50 determinations are available from Megazyme. The kits contain the full assay method plus:

**Bottle 1:** Tris/HCl buffer (20 mL, 1.0 M, pH 7.4) containing magnesium chloride (30 mM) and sodium azide (0.02 % w/v) as a preservative.  
Stable for > 2 years at 4°C.

**Bottle 2: (x2)** NADH (13.5 mg) plus ATP (40 mg) and PEP (20 mg).  
Stable for > 2 years at -20°C.

**Bottle 3:** Pyruvate kinase (600 U/mL) plus L-Lactate dehydrogenase (550 U/mL) suspension, 1.1 mL.  
Stable for > 2 years at 4°C.

**Bottle 4:** Glycerokinase suspension (1.1 mL, 85 U/mL).  
Stable for > 2 years at 4°C.

**Bottle 5:** Glycerol standard solution (5 mL, 0.2 mg/mL).  
Stable for > 2 years at room temperature.

### 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 one of bottle 2 in 5.5 mL of distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and one ice during use. **Do not** dissolve the contents of the second bottle until required. Once dissolved, the reagent is stable for > 12 months at -20°C.
- 3 & 4 Use the contents of bottles 3 and 4 as supplied.  
Stable for > 12 months at 4°C.
5. Use the contents of bottles 5 as supplied.  
Stable for > 12 months at 4°C.

**NOTE:** The glycerol 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 concentration of glycerol is determined directly from the extinction coefficient of NADH (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 200 µL).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
  - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of Tris/HCl buffer and 0.2 mL aliquots of NADH/ATP/PEP 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 (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No.1 (9 cm) filter papers.

## PROCEDURE:

<b>Wavelength:</b>	340 nm
<b>Cuvette:</b>	1 cm light path (glass or plastic)
<b>Temperature:</b>	~ 25°C
<b>Final volume:</b>	2.54 mL
<b>Sample solution:</b>	1-40 µg of glycerol per cuvette (in 0.10-2.0 mL sample volume)

**Read against air** (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water	2.10 mL	2.00 mL
sample solution	-	0.10 mL
solution 1 (Tris/HCl buffer)	0.20 mL	0.20 mL
solution 2 (NADH/ATP/PEP)	0.20 mL	0.20 mL
suspension 3 (PK/L-LDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions ( $A_1$ ) after approx. 6 min (at completion of the pre-reaction**). Start the reaction by addition of:		
suspension 4 (GK)	0.02 mL	0.02 mL
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.		

\* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

\*\* it is necessary to wait for completion of the pre-reaction after the addition of suspension 3 (PK/L-LDH).

## CALCULATION:

Determine the absorbance difference ( $A_1 - A_2$ ) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining  $\Delta A_{\text{glycerol}}$ .

The value of  $\Delta A_{\text{glycerol}}$  should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of glycerol can be calculated as follows:

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

### where:

V = final volume [mL]

MW = molecular weight of glycerol [g/mol]

$\epsilon$  = extinction coefficient of NADH 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 glycerol:

$$c = \frac{2.54 \times 92.1}{6300 \times 1 \times 0.10} \times \Delta A_{\text{glycerol}} \quad [\text{g/L}]$$

$$= 0.3713 \times \Delta A_{\text{glycerol}} \quad [\text{g/L}]$$

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 glycerol

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

## SAMPLE PREPARATION:

### 1. Sample dilution.

The amount of glycerol present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 1 and 40  $\mu\text{g}$ . The sample solution must therefore be diluted sufficiently to yield a concentration between 0.04 and 0.4 g/L.

#### Dilution Table

Estimated concentration of glycerol (g/L)	Dilution with water	Dilution factor (F)
< 0.4	No dilution required	1
0.4-4.0	1 + 9	10
4.0-40	1 + 99	100
> 40	1 + 999	1000

If the value of  $\Delta A_{\text{glycerol}}$  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.

### 2. Sample clarification.

#### a. Solutions:

**Carrez I solution.** Dissolve 3.60 g of potassium hexacyanoferrate (II)  $\{\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}\}$  (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 ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) (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 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 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. 7.4 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. 7.4 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 GK, 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 activated carbon. 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 glycerol in wine.**

In general, the glycerol concentration of white and red wine can be determined without any sample treatment (except dilution according to the dilution table). *Typically, a dilution of 1:20 and sample volume of 0.1 mL are satisfactory.*

### **(b) Determination of glycerol in beer.**

After removal of carbon dioxide by increasing the pH of the solution to approx. 7.4 with 2 M NaOH and gentle stirring, dilute the sample according to the dilution table and analyse. *Typically, a dilution of 1:5 and sample volume of 0.1 mL are satisfactory.*

### **(c) Determination of glycerol in fruit juice, concentrates and related beverages.**

Dilute the sample to yield a glycerol concentration of less than 0.4 g/L (see dilution table). Clear, neutral solutions can generally be determined without any sample treatment (except dilution). Turbid liquids generally only require filtering before the dilution step. Coloured solutions are usually suitable for analysis after dilution to an

appropriate glycerol concentration. However, if coloured solutions require analysis undiluted, they may need decolorising as follows: 25 mL of liquid sample is adjusted to approx. pH 7.4 with 1 M NaOH and the volume increased to 50 mL with distilled water. Add 3 g of activated charcoal, stir for 2 min and filter through Whatman GF/A glass fibre filter paper. Use the clear, slightly coloured filtrate directly in the assay. *Typically, no further dilution is required and a sample volume of 1.0 mL is satisfactory.*

#### **(d) Determination of glycerol in tobacco products.**

Grind the sample to a particle size of 0.2 mm. Accurately weigh approx. 1 g into a 100 mL volumetric flask. After addition of approx. 60 mL of distilled water, stir the contents vigorously (magnetic stirrer) for approx. 1 h at 20-25°C. Remove the magnetic stirrer bar and fill up to the mark with distilled water. Mix, filter and pipette 25 mL of the filtrate into a 50 mL volumetric flask. Successively add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM), with mixing after each addition. Fill to the mark with distilled water, mix and filter through Whatman GF/A glass fibre filter paper. Use the filtrate for the assay. *Typically, no further dilution is required and a sample volume of 1.0 mL is satisfactory.*

#### **(e) Determination of glycerol in soap.**

Accurately weigh approx. 1 g of grated soap into a beaker, add approx. 50 mL of 0.1 M HCl and stir vigorously on a hot-plate magnetic stirrer until boiling. Transfer the aqueous phase with a pipette into a 100 mL volumetric flask. Repeat the extraction with 30 mL of 0.1 M HCl. Bring the temperature of the flask to 20-25°C and fill to the mark with distilled water. Place the volumetric flask in an ice-water bath or refrigerator for 15 min and filter an aliquot of the solution through Whatman GF/A glass fibre filter paper. Take 25 mL of filtrate, add 2 mL of 2 M Tris/HCl buffer (pH 7.4) and adjust the pH to approx. 7.4 with 1 M NaOH. Adjust the volume to 50 mL and use the filtrate (either undiluted, or diluted according to the dilution table) for the assay. *Typically, no further dilution is required and a sample volume of 1.0 mL is satisfactory.*

#### **(f) Determination of glycerol in toothpaste.**

Accurately weigh approx. 1 g of toothpaste into 70 mL of water, stir at 70°C for 30 min and then clarify by centrifugation (~ 3,000 g for 10 min). Wash the pellet twice by resuspending in 50 mL of water followed by mixing and centrifugation. Make the volume up to 250 mL and filter if necessary. *Typically, a dilution of 1:3 and sample volume of 0.1 mL are satisfactory.*







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