Megazyme

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GLYCEROL GK

(Glucokinase Format)

ASSAY PROCEDURE

K-GCROLGK 04/18

(*70 Manual Assays per Kit) or
(600 Auto-Analyser Assays per Kit) or
(700 Microplate Assays per Kit)

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

INTRODUCTION:

In the food industry, glycerol is an important moistening agent for baked goods. It is also added to candies and icings to prevent crystallisation 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 product quality. The smoothness of lotions, creams and toothpaste is due to the incorporation of glycerol. Due to its hygroscopic properties, glycerol is sprayed on pre-processed tobacco to prevent crumbling. This kit benefits from the presence of ADP-glucokinase which permits the detection and measurement of glycerol from an increase in absorbance.

PRINCIPLE:

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

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

D-Glucose is phosphorylated by the enzyme ADP-glucokinase (ADP-GK) and adenosine-5'-diphosphate (ADP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-monophosphate (AMP) (2).

$$\text{(2) ADP + D-glucose} \xrightarrow{\text{(ADP-GK)}} \text{glucose-6-phosphate + AMP}$$

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

$$\text{(3) Glucose-6-phosphate + } NAD^+ \xrightarrow{\text{(G6P-DH)}} \text{6-phosphogluconate + NADH}$$

The amount of NADH formed in this reaction is stoichiometric with the amount of glycerol. It is the NADH which is measured by the increase 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 0.186 mg/L of sample solution

at the maximum sample volume of 2.00 mL. The detection limit is 0.373 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 1.6 mL.

The assay is linear over the range of 1.0 to 35 µg 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 1.6 mL, this corresponds to a glycerol concentration of approx. 0.093 to 0.186 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 glycerol has been completed within the time specified in the assay 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 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 70 assays in manual format (or 600 assays in auto-analyser format or 700 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (20 mL, pH 7.4).
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+, ATP and D-glucose.
Freeze dried powder.
Stable for > 5 years below -10°C.

Bottle 3: ADP-glucokinase plus glucose-6-phosphate dehydrogenase suspension, 1.5 mL.
Stable for > 2 years at 4°C.

Bottle 4: Glycerokinase suspension (1.5 mL).
Stable for > 2 years at 4°C.

Bottle 5: Glycerol standard solution (5 mL, 0.20 mg/mL) in 0.02% (w/v) sodium azide.
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 one of bottle 2 in 7.5 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). Do not dissolve the contents of the second bottle until required.

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.**
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 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. Glass test tubes (round bottomed; 16 x 100 mm).
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 0.2 mL aliquots of NAD^+ (solution 2)].
   - with 25 mL Combitip® (to dispense 1.5 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) and GF/A glass fibre filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.04 mL
Sample solution: 0.8-35 µg of glycerol 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)	1.60 mL	1.50 mL
sample	-	0.10 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 (NAD^+/ATP/D-Glucose)	0.20 mL	0.20 mL
suspension 3 (ADP-GK/G6P-DH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:		
suspension 4 (GK)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 7 min at 25°C or approx. 4 min at 40°C).		

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

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 $\Delta A_{glycerol}$.

The value of $\Delta A_{glycerol}$ 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:

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

where:

V = final volume [mL]
MW = molecular weight of glycerol [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for glycerol:

$$c = \frac{2.04 \times 92.1}{6300 \times 1.0 \times 0.10} \times \Delta A_{glycerol} \quad [g/L]$$

$$= 0.2982 \times \Delta A_{glycerol} \quad [g/L]$$

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

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

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).

B. AUTO-ANALYSER ASSAY PROCEDURE:

This kit is suitable for the preparation of 132 mL of reagent (equivalent to 600 reactions of 0.230 mL). Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
bottle 1 (buffer) bottle 3 (ADP-GK/G6P-DH) bottle 4 (GK) distilled water	2.5 mL 0.25 mL (swirl to mix before use) 0.25 mL (swirl to mix before use) 17.0 mL
Total volume	20 mL

Preparation of R2:

Component	Volume
bottle 2 (NAD^+/ATP/Glucose)	2.0 mL (after adding 7.5 mL of distilled water to bottle 2)
Total volume	2.0 mL

METHOD:

R1:	0.20 mL
Sample:	~ 0.010 mL
R2:	0.020 mL
Reaction time:	~ 7 min at 25°C or ~ 4 min at 37°C
Wavelength:	340 nm
Prepared reaction stability:	> 14 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 20 µg/mL glycerol in final reaction mixture (equivalent to 460 mg/L of sample)

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for glycerol 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 glycerol **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: 0.204 mL
Linearity: 0.1-3.5 µg of glycerol per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.160 mL	0.150 mL	0.150 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.020 mL	0.020 mL	0.020 mL
solution 2 (NAD^+/ATP/D-Glucose)	0.020 mL	0.020 mL	0.020 mL
suspension 3 (ADP-GK/G6P-DH)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 4 min (at completion of the pre-reaction). Start the reactions by addition of:			
solution 4 (GK)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 7 min). If the reaction has not stopped after 3 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 solution 4.

CALCULATION (Microplate Assay Procedure):

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

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

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 1.0 and 30 µg. The sample solution must therefore be diluted sufficiently to yield a concentration between 0.01 and 0.30 g/L.

Dilution Table

Estimated concentration of glycerol (g/L)	Dilution with water	Dilution factor (F)
< 0.30	No dilution required	1
0.30-3.0	1 + 9	10
3.0-30	1 + 99	100
> 30	1 + 999	1000

If the value of $\Delta A_{glycerol}$ 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 1.60 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) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (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. 7.4 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing significant quantities of carbon dioxide, such as beer, 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: an additional sample blank, i.e. sample with no GK, 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 at 60°C. Adjust to room temperature 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, page 8). *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 stirring with a glass rod, 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 0.35 g/L (see dilution table, page 8). 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 decolourising as follows: adjust 25 mL of liquid sample to approx. pH 7.4 with 1 M NaOH and increase the volume to 50 mL with distilled water. Add 0.5 g of PVPP, stir for 5 min and filter through Whatman No. 1 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 approx. 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. *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, not supplied) 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 distilled 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.*

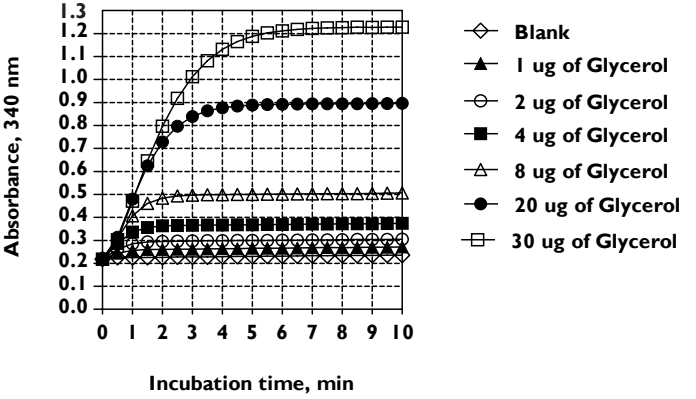

Figure 1. Increase in absorbance at 340 nm on incubation of 0-30 µg of glycerol with glycerol kinase, ADP-glucokinase and glucose-6-phosphate dehydrogenase in the presence of NAD^+ at 25°C using 1 cm path-length cuvettes (Manual Format; page 4).

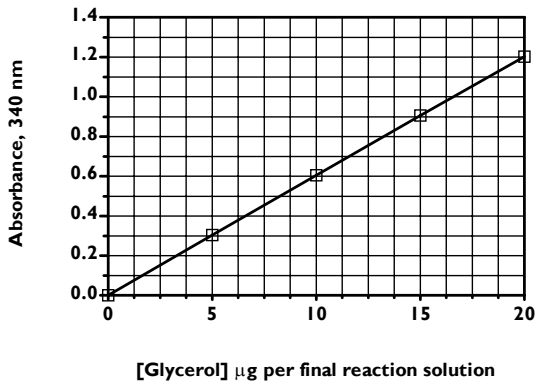

Figure 2. Calibration curve showing the linearity of reagent from **K-GCROLGK**. The reactions used to generate this calibration curve were performed at 37°C for 10 min and absorbance values were corrected for a 1 cm path-length (Auto-Analyser Format; page 6).

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