Megazyme

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D-GLUCONIC ACID/ D-GLUCONO-δ-LACTONE

ASSAY PROCEDURE

K-GATE 04/20

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

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

INTRODUCTION:

D-Gluconic acid is found in many foodstuffs and beverages, such as wine, soft drinks, vinegar, meat, fruit juice, dairy products, rice and honey. This non-volatile organic acid imparts a sour, but refreshing, taste and, given its occurrence in many natural foods and in human metabolism, has been granted GRAS status. The ratios of ethanol and glycerol to D-gluconic acid are a quality indicator in the wine industry. *Botrytis* infected grapes exhibit increased levels of this acid, that can reach 1-2 g/L. There are many other applications of D-gluconic acid, including dietary supplements, detergents and in the pickling of foods. D-Glucono-δ-lactone is found in association with D-gluconic acid, e.g. in wine, and is also widely used in the food industry. Foodstuffs containing D-glucono-δ-lactone include bean curd, yogurt, cottage cheese, bread, confectionery and meat.

PRINCIPLE:

D-Gluconic acid (D-gluconate) is phosphorylated to D-gluconate-6-phosphate by adenosine-5'-triphosphate (ATP) and the enzyme gluconate kinase (GCK) with the formation of adenosine-5'-diphosphate (ADP) (1).

$$\text{(1)}\quad \text{D-Gluconate} + \text{ATP} \xrightarrow{\text{(GCK)}} \text{D-gluconate-6-P} + \text{ADP}$$

In the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$), D-gluconate-6-phosphate is oxidatively decarboxylated by 6-phosphogluconate dehydrogenase (6-PGDH) to ribulose-5-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).

$$\text{(2)}\quad \text{D-gluconate-6-P} + NADP^+ \xrightarrow{\text{(6-PGDH)}} \text{ribulose-5-P} + \text{NADPH} + CO_2 + H^+$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-gluconic acid. It is the NADPH which is measured by the increase in absorbance at 340 nm.

D-Glucono-δ-lactone is determined by the same principle after alkaline hydrolysis (3).

$$\text{(3)}\quad \text{D-Glucono-}\delta\text{-lactone} + H_2O \xrightarrow{\text{(pH 11)}} \text{D-gluconate}$$

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for D-gluconic acid.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.198 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.792 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 0.8 to 50 µg of D-gluconic acid 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-gluconic acid concentration of approx. 0.198 to 0.396 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.

Analysis of commercial D-gluconic acid salts (D-gluconates) should yield results of ~ 100%. However, if the salts contain some free D-gluconic acid, then values of > 100% will be obtained (if the calculations use the molecular weights of the respective D-gluconic acid salts).

INTERFERENCE:

If the conversion of D-gluconic acid has been completed within the time specified in the assay (approx. 6 min), it can generally be concluded that no interference has occurred. However, this can be further checked by adding D-gluconic acid (approx. 25 µg in 0.1 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-gluconic acid 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 60 assays in manual format (or 600 assays in auto-analyser format or 600 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (12.5 mL, pH 7.6) plus sodium azide (0.02% w/v)as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: $NADP^+$ plus ATP.
Stable for > 5 years below -10°C.

Bottle 3: 6-Phosphogluconate dehydrogenase suspension (1.25 mL).
Stable for > 2 years at 4°C.

Bottle 4: Gluconate kinase suspension (1.25 mL).
Stable for > 2 years at 4°C.

Bottle 5: Sodium D-gluconate (~ 2 g).
Stable for > 5 years; store sealed 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 bottle 2 in 12.5 mL of distilled water. **Stable for ~ 4 weeks 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 bottles to mix contents before use.**
Stable for > 2 years at 4°C.

5. Accurately weigh approx. 279 mg sodium D-gluconate (MW = 216.13) to the nearest 0.1 mg into a 1 L volumetric flask. Fill to the mark with distilled water and mix thoroughly (this corresponds to approx. 0.25 g D-gluconic acid/L). Store 10 mL aliquots of this solution below -10°C.
Stable for > 2 years below -10°C.

NOTE: The D-gluconic acid 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 D-gluconic acid is determined directly from the extinction coefficient of NADPH (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, 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 buffer (bottle 1) and $NADP^+$/ATP solutions].
   - 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.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.54 mL
Sample solution: 0.8-50 µg of D-gluconic acid per cuvette (in 0.1-2.0 mL sample volume)

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

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample*	-	0.10 mL
solution 1 (buffer)	0.20 mL	0.20 mL
solution 2 ($NADP^+$/ATP)	0.20 mL	0.20 mL
suspension 3 (6-PGDH)	0.02 mL	0.02 mL
Mix**, read the absorbances of the solutions (A_1) after approx. 5 min and start the reactions by addition of:		
suspension 4 (GCK)	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min).		

* rinse the dispensing pipette tip with sample solution before dispensing the sample solution.

** 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 $\Delta A_{D\text{-gluconic acid}}$.

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

The concentration of D-gluconic acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{D\text{-gluconic acid}} \quad [g/L]$$

where:

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

It follows for D-gluconic acid:

$$c = \frac{2.54 \times 196.1}{6300 \times 1.0 \times 0.1} \times \Delta A_{\text{D-gluconic acid}} \quad [g/L]$$

$$= 0.7906 \times \Delta A_{\text{D-gluconic acid}} \quad [g/L]$$

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 D-gluconic acid

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

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

Determination of D-glucono-δ-lactone:

Adjust the pH of sample solution to approx. 11 with 2 M NaOH and incubate at approx. 25°C for 5-10 min. Monitor the pH of the solution with pH test-strips and adjust if necessary. Use an aliquot of this solution in the assay. D-Glucono-δ-lactone is determined together with "free" D-gluconic acid and is calculated as total D-gluconic acid.

It follows for D-glucono-δ-lactone:

$$c = \frac{2.54 \times 178.1}{6300 \times 1.0 \times 0.1} \times \Delta A_{\text{total D-gluconic acid}} \quad [g/L]$$

$$= 0.7181 \times \Delta A_{\text{total D-gluconic acid}} \quad [g/L]$$

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for D-gluconic acid 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-gluconic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
bottle 1 (buffer) bottle 2 ($NADP^+$) bottle 3 (6-GPDH) distilled water	2.4 mL 2.4 mL (after adding 12.5 mL of H_2O to bottle 2) 0.24 mL 21 mL
Total volume	26.04 mL

Preparation of R2:

Component	Volume
bottle 4 (GCK) distilled water	0.24 mL 3 mL
Total volume	3.24 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 6 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.5 g/L of D-gluconic acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-gluconic acid 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-gluconic acid **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: 0.254 mL
Linearity: 0.1-5 µg of D-gluconic acid 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)	0.020 mL	0.020 mL	0.020 mL
solution 2 ($NADP^+$/ATP)	0.020 mL	0.020 mL	0.020 mL
suspension 3 (6-PGDH)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:			
suspension 4 (GCK)	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. 6 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).

CALCULATION (Microplate Assay Procedure):

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

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 D-gluconic acid and hydrolysed D-glucono-δ-lactone present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.8 and 50 µg. The sample solution must therefore be diluted sufficiently to yield a D-gluconic acid concentration between 0.08 and 0.5 g/L.

Dilution Table

Estimated concentration of D-gluconic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.5	No dilution required	1
0.5-5.0	1 + 9	10
5.0-50	1 + 99	100
> 50	1 + 999	1000

If the value of $\Delta A_{\text{D-gluconic acid}}$ 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) {$K_4[Fe(CN)_6].3H_2O$} (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. 7.6 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: a sample blank, i.e. sample with no GCK, 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. 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 by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, use Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-gluconic acid in wine.

For hydrolysis of D-glucono-δ-lactone, adjust the pH of the wine to approx. 11.0 with 2 M NaOH and incubate at room temperature for 5-10 min. Monitor the pH and adjust if necessary. Dilute the sample according to the dilution table. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(b) Determination of D-gluconic acid or D-glucono-δ-lactone in meat products.

Accurately weigh approx. 5 g of representative homogenised sample into a 100 mL Duran® bottle and add 20 mL of 1 M perchloric acid. Homogenise with an Ultra-turrax® or Polytron® homogeniser (or equivalent) on a medium setting for 2 min at room temperature. Wash the homogeniser head with water and adjust the total volume to approx. 60 mL. Adjust the pH to approx. 11.0 with 2 M KOH with stirring on a magnetic stirrer and monitor the pH with a pH meter. Quantitatively transfer the solution to a 100 mL volumetric flask and adjust to volume with distilled water. The fatty layer must be above the mark and the aqueous layer at the mark. To aid separation of the fat and precipitation of potassium perchlorate, store the solution in a refrigerator, or on ice, for 20 min. Filter the solution. Discard the first few mL and use the clear or slightly turbid solution in the assay. *Typically, no dilution is required and a sample volume of 0.5 mL is satisfactory.*

REFERENCE:

Moellering, H. & Bergmeyer, H. U. (1988). D-Gluconate (D-Glucono-δ-lactone) and D-Gluconate 6-Phosphate. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 220-227, VCH Publishers (UK) Ltd., Cambridge, UK.

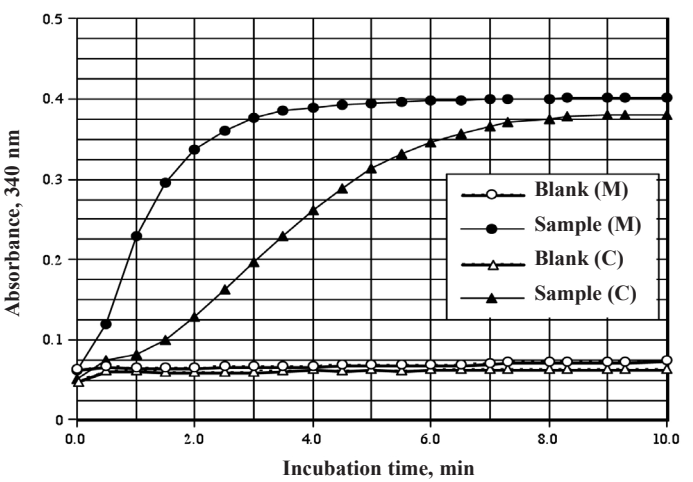

Figure 1. Increase in absorbance at 340 nm on incubation of 25 µg of D-gluconate with gluconate kinase and 6-phosphogluconate dehydrogenase in the presence of $NADP^+$. (M), Megazyme kit; (C) competitor kit.

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