D-Gluconic acid/ D-Glucono-δ-lactone

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

UV-method

for the determination of D-gluconic acid and D-glucono-δ-lactone in foodstuffs and other materials

Cat. No. 0 428 191

Test-Combination for approx. 25 determinations

For use in *in vitro* only

Store at 2-8°C

For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

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

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

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

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

The amount of NADPH formed in the above reaction is stoichiometric to the amount of D-gluconate. The increase in NADPH is measured by means of its light absorbance at 340, 334 or 365 nm.

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

(3) D-Glucono-δ-lactone + H_2O $\xrightarrow{\text{pH 10}}$ D-gluconate

If there is no alkaline hydrolysis of D-glucono-δ-lactone during sample preparation (see below), D-glucono-δ-lactone reacts under assay conditions within approx. 50 min.

The Test-Combination contains

1. Bottle 1 with approx. 4.5 g of powder mixture, consisting of: triethanolamine buffer, pH approx. 7,6; NADP, approx. 60 mg; ATP, approx. 150 mg; magnesium sulfate
2. Bottle 2 with approx. 0.5 ml 6-PGDH suspension, approx. 110 U
3. Bottle 3 with approx. 0.5 ml gluconate kinase suspension, approx. 13 U

Preparation of solutions

1. Dissolve content of bottle 1 with 27 ml redist. water.
2. Use contents of bottle 2 undiluted.
3. Use contents of bottle 3 undiluted.

Stability of reagents

The contents of bottle 1 are stable at 2-8°C (see pack label).
Solution 1 is stable for 4 weeks at 2-8°C and for 2 months at −15 to −25°C.
Bring solution 1 to 20-25°C before use.
The contents of bottle 2 and 3 are stable at 2-8°C (see pack label).

Procedure

Wavelength[1]: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.040 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 1-120 µg D-gluconic acid and hydrolyzed D-glucono-δ-lactone/assay[3] (in 0.100-2.000 ml sample volume)

Pipette into cuvettes	Blank	Sample
solution 1	1.000 ml	1.000 ml
sample solution*	-	0.100 ml
redist. water	2.000 ml	1.900 ml
suspension 2	0.020 ml	0.020 ml
Mix**, and read the absorbances of the solutions (A_1) after approx. 5 min. Start reaction by addition of:		
suspension 3	0.020 ml	0.020 ml
Mix**, wait for completion of the reaction (approx. 20 min) and read the absorbances of the solutions (A_2). If the reaction has not stopped after 20 min, continue to read the absorbances at 2 min intervals until the absorbance increases constantly over 2 min.		

* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.
** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

If the absorbance A_2 increases constantly, extrapolate the absorbance to the time of the addition of suspension 3 (gluconate kinase).

Determine the absorbance differences (A_2-A_1) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt. 4).

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = extinction coefficient of NADPH at:
340 nm = 6.3 [l × $mmol^{-1}$ × cm^{-1}]
Hg 365 nm = 3.5 [l × $mmol^{-1}$ × cm^{-1}]
Hg 334 nm = 6.18 [l × $mmol^{-1}$ × cm^{-1}]

It follows for D-gluconic acid:

$$c = \frac{3.040 \times 196.1}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{5.961}{\varepsilon} \times \Delta A \text{ [g D-gluconic acid/l sample solution]}$$

If the sample has been diluted on preparation, the result must be multiplied by the dilution factor F.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

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

Determination of D-glucono-δ-lactone

Adjust sample solution to pH 10 - 11 with potassium hydroxide (2 M) and incubate for 5-10 min at 20-25°C. Check pH value of the solution and correct, if necessary. Use the solution for the assay (as for D-gluconic acid).

D-Glucono-δ-lactone is determined together with the original D-gluconic acid and is calculated as total D-gluconic acid.

1 The absorption maximum of NADPH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm **or** 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 See instructions for performance of assay

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Calculation of D-glucono-δ-lactone (GdL)

$$c = \frac{3.040 \times 178.1}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{GdL} = \frac{5.414}{\varepsilon} \times \Delta A_{GdL}$$ [D-glucono-δ-lactone/l sample solution]

The differentiation between D-gluconic acid and D-glucono-δ-lactone is not possible.

1. Instructions for performance of assay

The amount of D-gluconic acid and hydrolyzed D-glucono-δ-lactone present in the assay has to be between 2 µg and 120 µg (measurement at 365 nm) or 1 µg and 60 µg (measurement at 340 or 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a D-gluconic acid and hydrolyzed D-glucono-δ-lactone concentration between 0.2 and 1.2 g/l or 0.1 and 0.6 g/l, respectively.

Dilution table

Estimated amount of D-gluconic acid and hydrolyzed D-glucono-δ-lactone per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.6 g	< 1.2 g	-	1
0.6-6.0 g	1.2-12.0 g	1 + 9	10
6.0-60 g	12.0-120 g	1 + 99	100
> 60 g	> 120 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. <0.100), the sample solution should be prepared again (weigh out more sample or use a lower dilution) *or* the sample volume pipetted into the cuvette can be increased up to 2.000 ml. Adjust sample solution to pH 7.5-8.0, if necessary. The volume of water added must then be reduced to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Technical information

In carrying out the calculation, a clear indication should be given as to whether the results are to be given as D-gluconic acid (molar mass 196.1 g/mol) or as D-gluconate (molar mass 195.1 g/mol). (In enzymatic determinations, the D-gluconate ion is measured.)

3. Specificity (Ref. 1)

The method is specific for D-gluconic acid.

In the analysis of commercial D-gluconic acid salts (D-gluconates), results of >100% have to be expected if the substances (salts) contain free D-gluconic acid and the results are calculated with the molecular weight of the respective D-gluconic acid salt.

4. Sensitivity and detection limit (Ref. 1.2)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml and measurement at 340 of a D-gluconic acid concentration of 0.25 mg/l sample solution (if v = 0.100 ml, this corresponds to 5mg/l sample solution).

The detection limit of 0.5 mg/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume v = 2.000 ml.

5. Linearity

Linearity of the determination exists from 1 µg D-gluconic acid/assay (0.5 mg D-gluconic acid/l sample solution; sample volume v = 2.000 ml) to 120 µg D-gluconic acid/assay (1.2 g D-gluconic acid/l sample solution; sample volume v = 0.100 ml).

6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a D-gluconic acid concentration of approx. 5-10 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.05-0.1 g/100 g can be expected.)

The following data have been published in the literature:

CV = 2 % n = 12 sausage (Ref. 1.2)

Boiled finely minced pork sausage:

x = 0.1 g/100 g r = 0.012 g/100 g $s_{(r)} = \pm$ 0.004 g/100 g
R = 0.014 g/100 g $s_{(R)} = \pm$ 0.005 g/100 g

Milk

x = 0.362 g/100 g r = 0.02 g/100 g R = 0.08 g/100 g

Feta cheese:

x = 2.57 g/100 g r = 0.15 g/100 g R = 0.26 g/100 g (Ref. 2.3)

7. Recognizing interference during the assay

7.1 If the conversion of D-gluconic acid has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

7.2 On completion of the reaction, the determination can be restarted by adding D-gluconic acid salts (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

7.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.
When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

7.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

7.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

8. Reagent hazard

The reagents used in the determination of D-gluconic acid are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

9. General information on sample preparation

In carrying out the assay:
Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml;
Filter **turbid solutions**;
Degas **samples containing carbon dioxide** (e.g. by filtration);
Adjust **acid samples** to approx. pH 8 by adding sodium or potassium hydroxide solution;
Adjust **acid and weakly colored samples** to pH 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;
Measure **"colored" samples** (if necessary adjusted to pH 8) against a sample blank (= buffer or redist. water + sample), adjust the photometer to 0.000 with the blank in the beam;
Crush or homogenize **solid or semi-solid samples,** extract with water or dissolve in water and filter if necessary;
Deproteinize **samples containing protein** with percloric acid;
Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter.

10. Application examples

Determination of D-gluconic acid in wine (Ref. 2.2, 3.1-3.7)
For hydrolysis of D-glucono-δ-lactone adjust wine to pH 10-11 with KOH (2 M). Incubate for 5-10 min at 20-25°C. Check pH value and correct, if necessary. Hereby D-glucono-δ-lactone present in wine is converted into D-gluconic acid. Dilute with water (see dilution table) and use the solution for the assay. D-Glucono-δ-lactone is determined together with D-gluconic acid and is calculated as total D-gluconic acid.

Determination of D-gluconic acid or D-glucono-δ-lactone, respectively, in meat products (Ref. 2.1, 2.3)
Accurately weigh approx. 5 g of homogenized sample into a homogenizer beaker, add approx. 20 ml perchloric acid (1 M) and homogenize for 10 min. Transfer the content quantitatively into a beaker with approx. 40 ml water. Adjust to pH 10-11 with potassium hydroxide (2 M) while stirring (magnetic stirrer). Transfer the content quantitatively into a 100 ml volumetric flask with

water and fill up to the mark, whereby care must be taken that the fatty layer is above the mark and the aqueous layer is **at** the mark. Shake the mixture. For separation of fat and for precipitation of the potassium perchlorate place the mixture for 20 min in a refrigerator. Filter, and discard the first few ml of the filtrate. Use the clear, possibly slightly turbid solution for the assay.
For calculation of the amount of D-gluconic acid or D-glucono-δ-lactone, respectively, take the volume displacement factor 0.98 into account. Volume displacement is caused by insoluble ingredients of the sample.

Determination of D-glucono-δ-lactone (GdL) in meat additives
Accurately weigh approx. 2 g of meat additives into a 250 ml beaker and mix with approx. 70 ml water. Adjust to pH 10 with potassium hydroxide (2 M) while stirring (magnetic stirrer). After 10 min transfer the solution into a 100 ml volumetric flask. Fill up to the mark with water. After mixing, filter through a filter paper moistened with the solution. Use the almost clear solution for the assay, diluted according to the dilution table, if necessary.

11. Further applications

The method may also be used in research when analyzing pharmaceuticals (Ref. 3.8) and biological samples. Details for sampling, treatment and stability of the sample see Ref. 1.

Determination of D-gluconic acid in fermentation samples and cell culture media

Place the sample (after centrifugation, if necessary) in a water-bath at 80°C for 15 min to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with perchloric acid (see the above-mentioned examples).
Homogenize gelatinous agar media with water and treat as described above.

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D-Gluconic acid assay control solution

The assay control solution serves as a control for the enzymatic determination of D-gluconic acid in foodstuffs and other materials.

Reagents

Sodium D-gluconate, AR grade (M = 218.13 g/mol)
Potassium D-gluconate, AR grade (M = 234.25 g/mol)

Preparation of the assay control solution

Accurately weigh approx. 67 mg sodium D-gluconate, resp. 72 mg potassium D-gluconate to the nearest 0.1 mg into a 100 ml volumetric flask, fill up to the mark with redist, water and mix thoroughly (corresponds approx. 0.6 g D-gluconic acid/l).

Prepare assay control solution freshly before use. The assay control solution may be frozen in portions.

Application:

1. Addition of D-gluconic acid assay control solution to the assay mixture:
Instead of sample solution the assay control solution is used for the assay. (The measurement of the assay control solution is not necessary for calculating the results.)

2. Restart of the reaction, quantitatively:
After completion of the reaction with sample solution and measuring A_2, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 30 min). Calculate the concentration from the difference (A_3-A_2) according to the general equation for calculating the concentration. The altered total volume must be taken into account.

Because of the dilution of the assay mixture by the addition of the assay control solution, the result differs insignificantly from the result got according to pt. 1.

3. Internal standard:
The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
solution 1	1.000 ml	1.000 ml	1.000 ml	1.000 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml
suspension 2	0.020 ml	0.020 ml	0.020 ml	0.020 ml
Mix, and read absorbances of the solutions (A_1) after approx. 5 min. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.				

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$

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