D-Glucose

UV-method

for the determination of D-glucose in foodstuffs and other materials

Cat. No. 10 716 251 035

Test-Combination for 3 × 45 determinations

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For *in vitro* use only

Store at 2-8°C

For recommendations for methods and standardized procedures see references (A 2, B 2, C 2, D 2)

Principle (Ref. A 1)

D-Glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1).

(1) D-Glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).

(2) G-6-P + $NADP^+$ $\xrightarrow{G6P\text{-}DH}$ D-gluconate-6-phosphate + NADPH + H^+

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

The Test-Combination contains

1. Three bottles 1, each with approx. 7.2 g powder mixture, consisting of: triethanolamine buffer, pH approx. 7.6; NADP, approx. 110 mg; ATP, approx. 260 mg; magnesium sulfate
2. Three bottles 2, each with approx. 1.1 ml suspension, consisting of: hexokinase, approx. 320 U; glucose-6-phosphate dehydrogenase, approx. 160 U
3. Bottle 3 with D-glucose assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Dissolve contents of one bottle 1 with 45 ml redist. water.
2. Use contents of one bottle 2 undiluted.

Stability of reagents

The contents of the bottles 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 the bottles 2 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: 3.020 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 1-100 µg of D-glucose/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
Mix** and read absorbances of the solutions (A_1) after approx. 3 min. Start reaction by addition of:		
suspension 2	0.020 ml	0.020 ml
Mix**, wait until the reaction has stopped (approx. 10-15 min) and read the absorbances of the solutions (A_2). If the reaction has not stopped after 15 min, continue to read the absorbances at 2 min intervals until the absorbance increases constantly for 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)

*** Creep reactions occur very occasionally. They are mostly brought about by the contents of the sample solutions, such as enzymes or coloring agents. These interfering substances may be removed during sample preparation.

If the absorbance A_2 increases constantly, extrapolate the absorbance to the time of the addition of suspension 2 (HK/G6P-DH).
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.

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

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 "Sensitiviy and detection limit", pt. 3).

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-glucose:

$$c = \frac{3.020 \times 180.16}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-glucose}} = \frac{5.441}{\varepsilon} \times \Delta A_{D\text{-Glucose}} \text{ [g D-glucose/l sample solution]}$$

If the sample has been diluted during 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.

$$Content_{D\text{-glucose}} = \frac{c_{D\text{-glucose}} \text{ [g/l sample solution]}}{weight_{sample} \text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

1. Instructions for performance of assay

The amount of D-glucose present in the assay has to be between 2 µg and 100 µg (measurement at 365 nm) or 1 µg and 50 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a D-glucose concentration between 0.15 and 1.0 g/l or 0.08 and 0.5 g/l, respectively.

Dilution table

Estimated amount of D-glucose per liter, measurement at 340 or 334 nm	Estimated amount of D-glucose per liter, measurement at 365 nm	Dilution with water	Dilution factor F
< 0.5 g	< 1.0 g	-	1
0.5-5.0 g	1.0-10.0 g	1 + 9	10
5.0-50 g	10.0-100 g	1 + 99	100
> 50 g	> 100 g	1 + 999	1000

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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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 dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. The volume of water added must then be reduced so as 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. Specificity (Ref. A 1)

The method is specific for D-glucose.

In the analysis of commercial water-free D-glucose (molecular weight 180.16) and D-glucose monohydrate (molecular weight 198.17), results of $< 100\%$ have to be expected because the materials absorb moisture.

3. Sensitivity and detection limit

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 nm of a D-glucose concentration of 0.2 mg/l sample solution (if v = 0.100 ml, this corresponds to 4 mg/l sample solution).

The detection limit of 0.4 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.

4. Linearity

Linearity of the determination exists from approx. 1 µg D-glucose/assay (0.4 mg D-glucose/sample solution; sample volume v = 2.000 ml) to 100 µg D-glucose/assay (1 g D-glucose/sample solution; sample volume v = 0.100 ml).

5. 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-glucose concentration of approx. 4-8 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 0.04-0.08 g/100 g can be expected.)

The following data have been published in the literature:

D-glucose in blood: CV = 1.2 % (Ref. A 1.1)
CV = 1.8 % (Ref. A 1.2)

Diet bier:
D-glucose:

x = 1.0 g/100 ml	r = 0.030 g/100 ml	$s_{(r)} = \pm$ 0.011 g/100 ml
	R = 0.122 g/100 ml	$s_{(R)} = \pm$ 0.043 g/100 ml

For further data see references (Ref. A 2.4)

Fruit juice:
D-glucose:
$r = 0.42 + 0.027 \times (c_{\text{D-glucose}}$ in g/l) g/l
$R = 1.00 + 0.042 \times (c_{\text{D-glucose}}$ in g/l) g/l
D-fructose:
$r = 0.15 + 0.033 \times (c_{\text{D-fructose}}$ in g/l) g/l
$R = 1.05 + 0.045 \times (c_{\text{D-fructose}}$ in g/l) g/l
For further data see references (Ref. B 2.9)

Wine:
$r = 0.056 \times x_i$
$R = 0.12 + 0.076\ x_i$
x_i = D-glucose resp. D-fructose content in g/l (Ref. B2.17, 2.18)

Liquid whole egg:
D-glucose:

x = 0.44 g/100 g	r = 0.073 g/100 g	$s_{(r)} = \pm$ 0.026 g/100 g
	R = 0.106 g/100 g	$s_{(R)} = \pm$ 0.037 g/100 g

D-fructose:

x = 6.72 g/100 g	r = 0.587 g/100 g	$s_{(r)} = \pm$ 0.207 g/100 g
	R = 0.748 g/100 g	$s_{(R)} = \pm$ 0.264 g/100 g

sucrose:

x = 43.32 g/100 g	r = 1.722 g/100 g	$s_{(r)} = \pm$ 1.033 g/100 g
	R = 4.268 g/100 g	$s_{(R)} = \pm$ 1.501 g/100 g

For further data see references (Ref. C 2.4)

6. Recognizing interference during the assay procedure

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

6.2 On completion of the reaction, the determination can be restarted by adding D-glucose (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.

6.3 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.

6.4 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.

6.5 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.

7. Reagent hazard

The reagents used in the determination of D-glucose 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.

8. 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 approx. pH 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;
Measure **"colored" samples** (if necessary adjusted to approx. pH 8) against a sample blank (= buffer or redist. water + sample), adjust the photometer to 0.000 with the blank in the beam;
Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyvinylpolypyrrolidone (PVPP) or with polyamide, e.g. 1 g/100 ml;
Crush or homogenize **solid or semi-solid samples**, extract with water or dissolve in water and filter if necessary; resp. remove turbidities or dyestuffs by Carrez clarification;
Deproteinize **samples containing protein** with Carrez reagents;
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; alternatively clarify with Carrez-solutions after the extraction with hot water.

Carrez clarification:
Pipette the liquid sample into a 100 ml volumetric flask which contains approx. 60 ml redist. water, or weigh sufficient quantity of the sample into a 100 ml volumetric flask and add approx. 60 ml redist. water. Subsequently, carefully add 5 ml Carrez-I-solution (potassium hexacyanoferrate(II) (ferrocyanide), 85 mM = 3.60 g $K_4[Fe(CN)_6] \times 3\ H_2O$/100 ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g $ZnSO_4 \times 7\ H_2O$/100 ml). Adjust to pH 7.5-8.5 with sodium hydroxide (0.1 M; e.g. 10 ml). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

Samples containing protein should only be deproteinized with perchloric acid or with trichloroacetic acid in the absence of sucrose and maltose as these disaccharides are fully or partially hydrolized with the release of D-glucose. The Carrez clarification is recommended for normal use.

9. Application examples

Determination of D-glucose in milk
Pipette 20 ml milk into a 100 ml volumetric flask, add the following solutions and mix after each addition: 10 ml Carrez-I-solution (see pt. 8), 10 ml Carrez-II-solution (see pt. 8) and 20 ml sodium hydroxide (0.1 M). Fill up to the mark with redist. water, mix and filter. Use 1.000 ml or 2.000 ml, respectively, of the filtrate for the assay.

Determination of carbohydrates in diet beer for diabetics (total D-glucose after acid hydrolysis) (Ref. A 2.4)
Shake 300 to 500 ml of beer in order to remove carbon dioxide and filter through fluted filter paper. Pipette 50 ml filtrate into a 250 ml flask, add 10 ml HCl (25%; d = 1.125) and 50 ml redist water, boil for 3 h under reflux in a bath with boiling water. Cool down to 20-25°C, adjust the pH 6-7 by the addition of NaOH (5 M), transfer into a 250 ml volumetric flask, fill up to the mark with redist. water, mix and filter. Use the 1:10 (1+9) with redist. water diluted filtrate for the assay.

Determination of D-glucose in baking agents, spices and salt mixtures
Accurately weigh the homogenized sample into a volumetric flask and dissolve with water, resp. extract; if necessary, incubate for 30 min at 60-70°C. Remove turbities by means of Carrez reagents. Use the sample solution, after dilution according to the dilution table, for the assay.

10. Further applications

The method may also be used in the examination of cosmetics (Ref. B 3.10), pharmaceuticals (Ref. B 3.6), paper (Ref. D 2.2), and tobacco (Ref. C 3.7) and in research when analyzing biological samples. For sampling, treatment and stability of the sample see Ref. A 1.1, A 1.2.

Determination of D-glucose 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, but only by absence of disaccharides, or with Carrez-solutions. See the above-mentioned examples.
Homogenize gelatinous agar media with water and treat further as described.

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For further information see instructions for

Test-Combination D-Glucose/D-Fructose	**Cat. No. 10 139 106 035**
Test-Combination Maltose/Sucrose/ D-Glucose	**Cat. No. 11 113 950 035**
Test-Combination Sucrose/D-Glucose	**Cat. No. 10 139 041 035**
Test-Combination Sucrose/D-Glucose/ D-Fructose	**Cat. No. 10 716 260 035**
Test-Combination D-Sorbitol/Xylitol	**Cat. No. 10 670 057 035**
Test-Combination Starch	**Cat. No. 10 207 748 035**

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D-Glucose assay control solution (Bottle 3)

Concentration*: see bottle label

D-Glucose assay control solution is a stabilized aqueous solution of D-glucose. It serves as assay control solution for the enzymatic analysis of D-glucose in foodstuffs and other materials.

Application:

1. Addition of D-glucose assay control solution to the assay mixture:
Instead of sample solution the assay control solution is used for the assay.

2. Restart of the reaction, quantitatively:
After completion of the reaction with sample solution and measuring of A_2, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 15 min.). Calculate the concentration from the difference of (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 addition of the assay control solution, the result differs insignificantly from the data stated on the bottle label.

* Stated as anhydrous D-glucose

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
Mix, and read absorbances of the solutions (A_1) after approx. 3 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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