Sucrose/D-Glucose/ D-Fructose

UV method

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

Cat. Nr. 10 716 260 035
Test-Combination for 22 assays each

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)

The D-glucose concentration is determined before and after the enzymatic hydrolysis of sucrose; D-fructose is determined subsequently to the determination of D-glucose.

Determination of D-glucose before inversion:
At pH 7.6, the enzyme hexokinase (HK) catalyzes the phosphorylation of D–glucose by 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 glucose-6-phosphate dehydrogenase (G6P-DH), the D–glucose-6-phosphate (G-6-P) formed is specifically 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 NADPH formed in this reaction is stoichiometric to the amount of D–glucose and is measured by means of its light absorbance at 334, 340 or 365 nm.

Determination of D-fructose:
Hexokinase also catalyzes the phosphorylation of D-fructose to D-fructose-6-phosphate (F-6-P) with the aid of ATP (3).

(3) D-Fructose + ATP $\xrightarrow{HK}$ F-6-P + ADP

On completion of the reaction (3) F-6-P is converted by phosphoglucose isomerase (PGI) to G-6-P (4).

(4) F-6-P $\xrightarrow{PGI}$ G-6-P

G-6-P reacts again with NADP with formation of D-gluconate-6-phosphate and NADPH (2). The amount of NADPH formed now is stoichiometric to the amount of D-fructose.

Enzymatic inversion:
At pH 4.6, sucrose is hydrolyzed by the enzyme β-fructosidase (invertase) to D-glucose and D-fructose (5).

(5) Sucrose + H_2O $\xrightarrow{\beta\text{-fructosidase}}$ D-glucose + D-fructose

The determination of D-glucose after inversion (total D-glucose) is carried out according to the principle outlined above.

The sucrose content is calculated from the difference of the D-glucose concentrations before and after enzymatic inversion.

The Test-Combination contains

1. Bottle 1 with approx. 0.5 g lyophilizate, consisting of:
   citrate buffer, pH approx. 4.6; β-fructosidase, approx. 720 U
2. Bottle 2 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
3. Bottle 3 with approx. 1.1 ml suspension, consisting of:
   hexokinase, approx. 320 U; glucose-6-phosphate dehydrogenase, approx. 160 U
4. Bottle 4 with approx. 0.6 ml phosphoglucose isomerase suspension, approx. 420 U
5. Bottle 5 with sucrose assay control material for assay control purposes (measurement of the assay control material is not necessary for calculating the results.) Expiry date: see pack label
6. Bottle 6 with D-glucose assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) The assay control solution does not contain sucrose and D-fructose because of their insufficient stability in aqueous solutions. Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Dissolve contents of bottle 1 with 10 ml redist. water.
2. Dissolve contents of bottle 2 with 45 ml redist. water.
3. Use contents of bottle 3 undiluted.
4. Use contents of bottle 4 undiluted.

Stability of reagents

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

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 (3.040 ml, determination of D-fructose)
Read against air (without a cuvette in the light path) or against water
Sample solution: 4-150 µg sucrose + D-glucose + D-fructose/assay[3] (in 0.100-1.800 resp. 2.000 ml sample volume)

Pipette into cuvettes	Blank sucrose sample	Sucrose sample	Blank D-glucose/ D-fructose sample	D-Glucose/ D-fructose sample
solution 1*	0.200 ml	0.200 ml	-	-
sample solution**	-	0.100 ml	-	0.100 ml
Mix*, incubate for 15 min at 20-25°C or for 5 min at 37°C (before pipetting, warm up solution 1 to 37°C). Addition of:				
solution 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml
redist. water	1.800 ml	1.700 ml	2.000 ml	1.900 ml
Mix***, read absorbances of the solutions after approx. 3 min (A_1). Start reaction by addition of:				
suspension 3	0.020 ml	0.020 ml	0.020 ml	0.020 ml
Mix***, wait for completion of the reaction (approx. 10-15 min) and read 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 over 2 min. Addition of:				
suspension 4	-	-	0.020 ml	0.020 ml
Mix***, read absorbances of the solutions after 10-15 min (A_3).				

* Pipette solution 1 and sample solution each, onto the bottom of the cuvette and mix by gentle swirling. When using a plastic spatula, remove it from the cuvette only directly before measuring absorbance A_1.
** 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 absorbances A_2 to the time of the addition of suspension 3 (HK/G6P-DH).

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

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

The difference between $\Delta A_{total\ D\text{-}glucose}$ (from the sucrose sample) and $\Delta A_{D\text{-}glucose}$ (from the D-glucose sample) yields $\Delta A_{sucrose}$.

It follows for the determination of D-fructose:

Determine the absorbance differences (A_3-A_2) for both, blank and sample (D-glucose/D-fructose sample). Subtract the absorbance difference of the blank from the absorbance difference of the sample. This results in $\Delta A_{D\text{-}fructose}$.

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).

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 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

According to the general equation for calculating the concentrations:

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

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 sucrose:

$$c = \frac{3.020 \times 342.3}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{sucrose} = \frac{10.34}{\varepsilon} \times \Delta A_{sucrose}$$

[g sucrose/l sample solution]

for D-glucose:

$$c = \frac{3.020 \times 180.16}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-glucose}} = \frac{5.441}{\varepsilon} \times \Delta A_{glucose}$$

[g D-glucose/l sample solution]

for D-fructose:

$$c = \frac{3.040 \times 180.16}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-fructose}} = \frac{5.477}{\varepsilon} \times \Delta AE_{fructose}$$

[g D-fructose/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:

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

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

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

1. Instructions for performance of assay

The amount of sucrose + D-glucose + D-fructose present in the assay has to be between 8 µg and 150 µg (measurement at 365 nm) or 4 µg and 80 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a sucrose + D-glucose + D-fructose concentration between 0.10 and 1.5 g/l or 0.05 and 0.8 g/l, respectively.

Dilution table

Estimated amount of sucrose + D-glucose + D-fructose per liter measurements at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.8 g	< 1.5 g	-	1
0.8-8.0 g	1.5-15.0 g	1 + 9	10
8.0-80 g	15.0-150 g	1 + 99	100
> 80 g	> 150 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 dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml (D-glucose and D-fructose sample), or up to 1.800 ml (sucrose sample). 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.

If the estimated amount of sucrose is below 0.2 g/l, the incubation time stated in the assay scheme, when sucrose is splitted by β-fructosidase, may be reduced from 15 min to 5 min.

2. Technical information

If the ratio D-glucose to sucrose (D-glucose to D-fructose) in the sample is higher than e.g. 10:1, the precision of the sucrose and D-fructose determination is impaired. In this case, as much as possible of the D-glucose should be removed by means of glucose oxidase in the presence of oxygen from the air. (For details see pt. 11: Determination of sucrose, D-glucose and D-fructose in honey).

3. Specificity

β-Fructosidase hydrolyzes the β-fructosidic bonds in sucrose and other glycosides. If the sample only contains sucrose it will be measured specifically via D-glucose. Even in the presence of fructosanes, sucrose can be measured specifically if after enzymatic hydrolysis with β-fructosidase, D–glucose and D-fructose are determined and the ratio of these monosaccharides is 1:1. If the D-fructose part dominates the sample contains 2 β–fructosanes.

The measuring of the D-glucose and D-fructose is specific.

In the analysis of commercial sucrose results of 100% have to be expected. In the analysis of commercial water-free D-glucose (molecular weight 180.16), D-glucose monohydrate (molecular weight 198,17) and of D-fructose results of < 100% have to be expected because the materials absorb moisture. (Commercial D-fructose may also contain D-glucose.)

4. Sensitivity and detection limit

The smallest differentiating absorbance for the procedure in the determination of D-glucose or D-fructose 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 or D-fructose 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 D-glucose or D-fructose/l is derived from the absorbance difference of 0.010 (as measured at 340 nm) and a maximum sample volume v = 2.000 ml.

The smallest differentiating absorbance for the procedure in the determination of sucrose (in the presence of D-glucose in the sample) is 0.010 absorbance units. This corresponds to a maximum sample volume v = 1.800 ml and measurement at 340 nm of a sucrose concentration of 1 mg/l sample solution (if v = 0.100 ml, this corresponds to 15 mg/l sample solution).

The detection limit of 2 mg sucrose/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume v = 1.800 ml.

5. Linearity

Linearity of the determination exists from 4 µg sucrose + D-glucose + D–fructose/assay (2 mg sucrose + D-glucose + D-fructose/l sample solution; sample volume v = 1.800 ml) to 150 µg sucrose + D-glucose + D–fructose/assay (1.5 g sucrose + D-glucose + D-fructose/l sample solution; sample volume v = 0.100 ml).

6. Precision

In a double determination of D-glucose or D-fructose 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 or D-fructose 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.)

In a double determination of sucrose using one sample solution, a difference of 0.010 to 0.015 absorbance units may occur in the presence of D-glucose in the sample. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a sucrose concentration of approx. 15-25 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 0.15-0.25 g/100 g can be expected.)

The following data have been published in the literature:

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 = 43.32g/100g	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. A 2.4)

Fruit juice:

Sucrose:	r = 1.9 + 0.033 × ($c_{sucrose}$ in g/l)	g/l	
	R = 3.3 + 0.061 × ($c_{sucrose}$ in g/l)	g/l	(Ref. B 2.6)
D-Glucose:	r = 0.42 + 0.027 × ($c_{D\text{-glucose}}$ in g/l)	g/l	
	R = 1.0 + 0.042 × ($c_{D\text{-glucose}}$ in g/l)	g/l	
D-Fructose:	r = 0.15 + 0.033 × ($c_{D\text{-fructose}}$ in g/l)	g/l	
	R = 1.05 + 0.045 × ($c_{D\text{-fructose}}$ in g/l)	g/l	(Ref. C 2.9)

Wine:

r = 0.056 × x_i
R = 0.12 + 0.076 x_i
x_i = D-glucose- resp. D-fructose content in g/l (Reg. C 2.17, 2.18)

7. Recognizing interference during the assay procedure

7.1 If the conversion of D-glucose and D-fructose 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-glucose or D-fructose (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.

The reaction cannot be restarted with sucrose as, subsequent to altering the reaction conditions from pH 4.6 to pH 7.6 ("change of the buffer"), sucrose is no longer cleaved.

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.

The use of "single" and "double" sample volumes in double determinations is the simplest method of carrying out a control assay in the determination of sucrose.

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 sucrose, D-glucose and D–fructose are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulacion 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 (D-glucose, D-fructose), resp. 1.800 ml (sucrose);
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 (determination of D-glucose and D-fructose);
Adjust **acid and weakly colored samples** to approx. pH 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min (determination of D-glucose and D-fructose);
Measure **"colored" samples** (if necessary adjusted to pH 8) against 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 reagents 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 [$Fe(CN)_6$] × 3 H_2O/100 ml) and 5 ml Carrez-II-solution (zinc sulfate, 250 mM = 7.20 g $ZnSO_4$ × 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 not be deproteinized with perchloric acid or with trichloroacetic acid in the presence 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.

10. Application examples

Determination of sucrose, D-glucose and D-fructose in fruit juices and similar beverages
Filter turbid juices (alternatively clarify with Carrez reagents) and dilute sufficiently to yield a sucrose + D-glucose + D-fructose concentration of approx. 0.1-1.5 g/l. The diluted sample solution can also be used for the assay if it is colored. Only strongly colored juices which are used undiluted for the assay because of their low sucrose + D-glucose + D-fructose content are to be decolorized. In that case proceed as follows:

Mix 10 ml of juice and approx. 0.1 g of polyamide powder or polyvinylpolypyrrolidone, stir for 1 min and filter. Use the clear, slightly colored solution for the assay.

Determination of sucrose, D-glucose and D-fructose in beer
To remove the carbonic acid, stir approx. 5-10 ml of beer in a beaker for approx. 30s with a glass rod or filter through a fluted filter paper. The largely CO_2-free sample can be used undiluted for the assay.

Determination of sucrose, D-glucose and D-fructose in sweetened condensed milk
Accurately weigh approx. 1 g of sample into a 100 ml volumetric flask, add approx. 60 ml water and incubate for 15 min at approx. 70°C; shake from time to time. For clarification, add 5 ml Carrez-I-solution (3.60 g potassium hexacyanoferrate(II), $K_4[Fe(CN)_6]$ x 3 H_2O/100 ml), 5 ml Carrez-II-solution (7.20 g zinc sulfate, $ZnSO_4$ x H_2O/100 ml) and 10 ml NaOH (0.1 M), mix after each addition, adjust to room temperature and fill up to the mark with water, mix and filter. Use the clear, possibly slightly opalescent solution diluted according to the dilution table for the assay.

Determination of sucrose, D-glucose and D-fructose in jam and ice cream
Homogenize approx. 10 g sample in a mixer. Accurately weigh approx. 0.5 g of the homogenized sample into a 100 ml volumetric flask, mix with water, dilute to the mark and filter. Discard the first 5 ml of the filtrate. Use the clear filtrate diluted according to the dilution table, if necessary, for the assay.

Determination of sucrose, D-glucose and D-fructose in potatoes
Homogenize 50 g peeled potatoes with 50 ml water in a homogenizer for 3 min. Transfer quantitatively into a 250 ml beaker. Fill up to approx. 150 ml with water. Add successively 5 ml Carrez-I-solution (preparation see pt. 9) and 5 ml Carrez-II-solution (preparation see pt. 9) mix after each addition. Adjust to pH 7.0 to 7.5 (pH-meter) with sodium hydroxide (0.1 M). Transfer quantitatively into a 250 ml volumetric flask, rinse with water, add 0.3 ml n-octanol and shake until the foam has disappeared. Fill up to 250 ml with water, mix and filter.
Use the light yellow, occasionally yellow-green solution with v = 0.100 ml or 0.200 ml, if necessary, immediately for the assay.

Determination of sucrose, D-glucose and D-fructose in tobacco (Ref. A 3.7)
Accurately weigh approx. 0.3 g dried, finely ground and sieved tobacco leaves (grain size approx. 0.2 mm) into a 100 ml volumetric flask, add approx. 70 ml water and stir for 1 h (magnetic stirrer). Fill up to the mark with water, mix and filter.
In a 25 ml volumetric flask add successively 1.25 ml Carrez-I-solution and 1.25 ml Carrez-II-solution (preparation see pt. 9) to 10 ml of the filtrate, mix, and subsequently add 2.5 ml sodium hydroxide (0.1 M) and mix again. Fill up to the mark with water, mix and filter. Use the clear solution diluted, if necessary, for the assay.

11. Special sample preparation for the determination of sucrose and D-fructose in the presence of excess D-glucose

Determination of sucrose, D-glucose and D-fructose in honey
Thoroughly stir the honey with a spatula. Take approx. 10 g of the viscous (or crystalline) honey, heat in a beaker for 15 min at approx. 60°C, and stir occasionally with a spatula (there is no need to heat liquid honey). Allow to cool. Accurately weigh approx. 1 g of the liquid sample into a 100 ml volumetric flask. Dissolve first with only a small portion of water, and then fill up to the mark.

a) Determination of D-glucose and D-fructose
Dilute the 1% honey solution 1:10 (1 + 9) and use for the assay.

b) Determination of sucrose
If the estimated sucrose content in the honey lies between 5 and 10%, dilute the 1% solution 1:3 (1 + 2) and use for the assay.

If the estimated sucrose content in the honey lies between 0.5 and 5%, as much as possible of the excess of D-glucose should be removed before sucrose is determined, otherwise the precision of the sucrose determination will be impaired. D-Glucose is oxidized to D-Gluconate in the presence of glucose oxidase (GOD) and oxygen from the air:

$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{D-gluconate} + H_2O_2$$

The hydrogen peroxide is destroyed by catalase:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

Reagents
Glucose oxidase (GOD) from Aspergillus niger, 200 U/mg (25°C; D-glucose as substrate), amylase and β-fructosidase < 0.01 % each
Catalase
Triethanolamine hydrochloride
$MgSO_4 \times 7\ H_2O$
NaOH, 4 M

Preparation of solutions for 10 determinations
Enzyme solution:
Dissolve 5 mg (≙ approx. 1000 U) GOD with 0.750 ml redist. water, add 325 KU catalase (from bovine liver, 25°C; H_2O_2 as substrate), and mix.

Buffer solution:
Dissolve 5.6 g triethanolamine hydrochloride and 0.1 g $MgSO_4 \times 7\ H_2O$ in 80 ml redist. water, adjust to pH 7.6 with sodium hydroxide (4 M), and fill up to 100 ml with redist. water .

Stability of solutions
The enzyme solution must be prepared freshly daily.
The buffer solution is stable for 4 weeks at 2-8°C.

Procedure for D-glucose oxidation

Pipette into a 10 ml volumetric flask	
buffer solution sample solution (up to approx. 0.5% D-glucose) enzyme solution	2.000 ml 5.000 ml 0.100 ml
Pass a current of air (O_2) through the mixture for 1 h; during the oxidation process **check the pH with indicator paper and, if necessary, neutralize the formed acid with NaOH.**	

To inactivate the enzymes GOD and catalase, place the volumetric flask in a boiling water-bath for 15 min, allow to cool, and dilute to the mark with water. Mix and filter, if necessary. Use 0.500 ml of the clear solution for the determination of sucrose. Determine the residual D-glucose in a parallel assay and subtract as usual.

12. Further applications

The method may also be used in the examination of pharmaceuticals, paper (Ref. B 2.2) and in research when analyzing biological samples.

Determination of sucrose, D-glucose and D-fructose in fermentation samples and cell culture media
Place the sample (after centrifugation, if necessary) in a waterbath 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 Carrez reagents. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

D-Glucose assay control solution (Bottle 6)

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 determination of D-glucose in foodstuffs and other materials.

Application:

1. *Addition of D-glucose assay control solution to the assay mixture D-glucose/D-fructose sample:*
   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_3, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_4 after the end of the reaction (approx. 15 min). Calculate the concentration from the difference of (A_4-A_3) 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.
3. *Internal standard:*
   The assay control solution can be used as an internal standard in order to check the determination of D-glucose 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 2	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:

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

Note:
An assay control solution of sucrose and D-fructose is not contained in the Test-Combination because an aqueous solution of sucrose and D-fructose is not stable enough.

* Stated as anhydrous D-glucose

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Note

for Test-Combination Sucrose/D-Glucose/D-Fructose

Sucrose is supplied in this pack as assay control material (see Bottle 5). It may be used for the preparation of an assay control solution (concentration e.g. 1 g/l) which is pipetted (v = 0.100 ml) instead of the sample according to the pipetting scheme.

Furthermore, the sucrose may also be used for performing the Swiss Sucrose Test in order to check performance of the assay.

The Swiss Sucrose Test
An assay control solution is prepared and the concentration is measured enzymatically. The results are used for the evaluation of accuracy and precision.

Reagents
Prepare solutions according to the instructions in the Test-Combination.

Sample solution (assay control solution)
Weigh 1.6 g of sucrose (accuracy 0.1 mg) and dissolve with redist. water in a 1 liter volumetric flask, fill up to the mark and mix thoroughly.

Procedure
For details of performing the assays and calculating the results see instructions in the Test-Combination.

Run 2 blank and 6 sample assays.

Pipetting scheme

Pipette into cuvettes	Blanks		Samples					
	blank 1	blank 2	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6
solution 1*	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml	0.200ml
sample solution*	-	-	0.100ml	0.100ml	0.100ml	0.100ml	0.100ml	0.100ml
Mix**, incubate for 15 min at 20-25C°. Mix in:								
solution 2	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml	1.000ml
redist. water	1.800ml	1.800ml	1.700ml	1.700ml	1.700ml	1.700ml	1.700ml	1.700ml
Mix, read absorbances of the solutions after approx. 3 min (A_1). Start reaction by addition of:								
suspension 3	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml	0.020ml
Mix, incubate for 15 min at 20-25C°. Read the absorbances of the solutions (A_2).								
* Pipette the solution onto the bottom of the cuvettes. ** Mix by gentle shaking the cuvettes. If a mixing spatula is used, remove the spatula from the cuvette before reading A_1, not earlier.								
Readings								
A_1:								
A_2:								
$A_2 - A_1$:								
Mean of the blanks' absorbance differences								
$(A_2 - A_1)_{sample} - (A_2 - A_1)_{mean\ of\ the\ blanks} = \Delta A$:								

Calculation
Calculate the absorbance differences (A_2-A_1) for each blank and sample assay. Subtract the mean absorbance difference of the blanks from the absorbance differences of the samples. It follows:

$\Delta A_{sample\ 1, 2, \ldots . 6}$

Calculate the concentration of sucrose in g/l:

$$c = \frac{V \times MG}{\varepsilon \times d \times v \times 1000} \times \Delta A_{sample\ 1, 2, \ldots . , 6}$$

$$c = \frac{3.020 \times 342.3}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta E_{sample\ 1, 2, \ldots . , 6}$$

$$c = \frac{10.34}{\varepsilon} \times \Delta E_{sample\ 1, 2, \ldots . , 6}$$

$c_{sucrose}$ (g/l):						

From the 6 results $c_1, c_2, \ldots . , c_6$ calculate the mean $\bar{c}$ and the standard deviation s_c:

mean $c_{sucrose}$ ($\bar{c}$): _____ g/l

standard deviation s_c: _____ g/l

Calculation of the mean yield $\bar{Y}$ and its standard deviation s_A:

$$\bar{Y} = \frac{(\bar{c})\ [g/l] \times 100}{\text{weighed sucrose } [g/l]} = \frac{\_\_\_\_ \times 100}{\_\_\_\_} = \_\_\_\_ \text{ g/100 g}$$

$$s_Y = \frac{s_c\ [g/l] \times 100}{\text{weighed sucrose } [g/l]} = \frac{\_\_\_\_ \times 100}{\_\_\_\_} = \_\_\_\_ \text{ g/100 g}$$

Evaluation of the standard deviation
Standard deviation $s_Y \leqq 0.79$ g/100g:
The precision of the determination is ideal.

Standard deviation $s_Y > 0.79$ g/100g:
The standard deviation is too high. This may result either from the use of unsuitable equipment (photometer, cuvettes, pipettes) or from their wrong handling. Something should be done to overcome these difficulties (e.g. control of photometer, cuvettes and pipettes).

Evaluation of yield
Deviation of the mean yield $\bar{Y}$ from the theoretical yield ($\triangleq$ 100 g/100 g) = ΔY

$\Delta Y = |\ 100 - \bar{Y}\ | \leqq 0.42$ g/100 g:
The accuracy of the determination is ideal.

$\Delta Y = |\ 100 - \bar{Y}\ | = 0.43$ to 1.42 g/100 g:
Systematic errors are evident. This has to be accepted because they lie within the specifications of most photometers.

$\Delta Y = |\ 100 - \bar{Y}\ | > 1.42$ g/100 g:
The deviation of the mean yield from the theoretical yield is too high. The reason is also either the use of unsuitable equipment (balance, photometer, cuvettes, pipettes) or due to their wrong handling. Something should be done to overcome these difficulties (e.g. control of balance, photometer, cuvettes and pipettes).

References

1 Walter, E. (1980) Zur Prüfung der Laborausrüstung für enzymatische Bestimmungen, alimenta **19,** 159-164

2 Schweizerisches Lebensmittelbuch/Swiss Food Manual (1981), 5. Auflage, Kapitel 61: Enzymatische Bestimmungen, Enzymatic determinations, Allgemeiner Teil, General part, pp. 17-18 (Elaborated from the members of the 26th Sub-Commission, edited by the "Eidgenössische Drucksachen- und Materialzentrale", CH-Bern)

Also available:

Test-Combination D-Glucose	**Cat.No. 10 716 251 035**
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