Starch

UV-method

for the determination of native starch[1] and of partially hydrolized starch in foodstuffs and other materials

Cat. No. 10 207 748 035

Test-Combination for 27 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 (2)

Principle (Ref. 1)

Starch is hydrolyzed to D-glucose at pH 4.6 in the presence of the enzyme amyloglucosidase (AGS) (1).

$$\text{(1) Starch} + \text{(n-1) } H_2O \xrightarrow{\text{AGS}} \text{n D-glucose}$$

The D-glucose formed is determined with hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH) at pH 7.6. D-Glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) by adenosine-5'-triphosphate (ATP) in the presence of hexokinase with the simultaneous formation of ADP (2).

$$\text{(2) D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

In the presence of G6P-DH, D-glucose-6-phosphate is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (3).

$$\text{(3) G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The amount of NADPH formed in the above reaction is stoichiometric to the amount of D-glucose formed by hydrolysis of starch. NADPH is determined by means of its light absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 100 mg lyophilizate, consisting of:
   citrate buffer, pH approx. 4.6; amyloglucosidase, approx. 84 U
2. Bottle 2 with approx. 5 g powder mixture, consisting of:
   triethanolamine buffer, pH approx. 7.6; NADP, approx. 75 mg; ATP, approx. 190 mg; magnesium sulfate
3. Bottle 3 with approx. 0.7 ml enzyme suspension, consisting of:
   hexokinase, approx. 200 U; glucose-6-phosphate dehydrogenase, approx. 100 U
4. Flasche 4 with starch assay control material for assay control purposes (for the preparation of the assay control material for analysis, see pt. 8.1: weigh approx. 50-100 mg/100 ml. The measurement of the assay control material is not necessary for calculating the results. Expiry date: see pack label)

Preparation of solutions

1. Dissolve contents of bottle 1 with 6.0 ml redist. water.
2. Dissolve contents of bottle 2 with 27 ml redist. water.
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 6 weeks at 2-8°C, or for 3 months at −15 to −25°C. Bring solution 1 to 20-25°C before use.
The contents of bottle 2 are stable at 2-8°C (see pack label).
Solution 2 is stable for 4 weeks at 2-8°C, or for 2 months at −15 to −25°C. Bring solution 2 to 20-25°C before use.
The contents of bottle 3 are stable at 2-8°C (see pack label).

Procedure

Wavelength[2]:	340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[3]:	1.00 cm light path
Temperature:	55-60°C (incubation); 20-25°C (measurement)
Final volume:	2.320 ml
Read against air	(without a cuvette in the light path) or against water
Sample solution:	1.2-70 µg starch/assay[4] (in 0.100-1.000 ml sample volume, or in 0.100-0.200 ml when analyzing DMSO-containing solutions)

If the sample contains free D-glucose which has to be determined in analysis, a separate determination of D-glucose is necessary e.g. after the extraction of the sample with water. In the presence of maltose and its homologues as well as of an excess of other oligosaccharides, the alcohol extraction is recommended for the determination of starch (see instructions pt. 8 and 9).

Pipette into cuvettes	Reagent blank	Sample	Sample blank[5]
solution 1*	0.200 ml	0.200 ml	-
sample solution**	-	0.100 ml	0.100 ml
redist. water	0.100 ml	-	-
Mix*, incubate for 15 min at 55-60°C (water-bath); stopper cuvettes with the lid or with Parafilm. Addition of:			
solution 2	1.000 ml	1.000 ml	1.000 ml
redist. water	1.000 ml	1.000 ml	1.200 ml
Mix***. After approx. 3 min read absorbances of the solutions (A_1). Start reaction by addition of:			
suspension 3	0.020 ml	0.020 ml	0.020 ml
Mix***, after completion of the reaction (ca. 10-15 min) read absorbances of the solutions (A_2). If the reaction has not stopped after 15 min, read absorbances in 2 min intervals until the absorbance increases constantly over 2 min.			

* Pipette solution 1, the sample solution and redist. water, 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 absorbances A_2 increases constantly, extrapolate the absorbances to the time of addition of suspension 3 (HK/G6P-DH).

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

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

For sample blank see pt. 8.1 and 9

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.3).

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]
(for starch: $MW_{\text{D-glucose}} - MW_{\text{water}} = 162.1$)

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}]

1 Modified kinds of starch (phosphorylized or oxidized ones) do not react.
2 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.
3 If desired, disposable cuvettes may be used instead of glass cuvettes.
4 See instructions for performance of assay.
5 See instructions for sample preparation, pts. 8 and 9

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It follows for starch:

$$c = \frac{2.320 \times 162.1}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{3.761}{\varepsilon} \times \Delta A \text{ [g starch/l sample solution]}$$

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:

$$\text{content}_{\text{starch}} = \frac{c_{\text{starch}} \text{ [g/l sample solution]}}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100 \text{ [g/100 g]}$$

1. Instructions for performance of assay

The starch content present in the assay has to be between 2.5 µg and 70 µg (measurement at 365 nm) or 1.2 µg and 40 µg (measurement at 340, 334 nm), respectively. In order to get a sufficient absorbance difference, the sample solution is diluted to yield a starch concentration between 0.06 and 0.7 g/l, or 0.03 and 0.4 g/l, respectively.

Dilution table

Estimated amount of starch per liter measurement at		Dilution with water	Dilution factor F
340 or 334 nm	365 nm		
< 0.4 g	< 0.7 g	-	1
0.4-4.0 g	0.7-7.0 g	1 + 9	10
4.0-40 g	7.0-70 g	1 + 99	100
> 40 g	> 70 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 1.000 ml (solution in water). 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 sample volume v must be taken into account in the calculation.

Note: *After solubilization of starch with DMSO and HCl (see pt. 8.1), immediately use 0.100 ml (maximum 0.200 ml) sample solution undiluted for the assay.*

2. Specificity (Ref. 1)

Amyloglucosidase hydrolyzes α-1,4-and α-1,6-glucan linkages in polysaccarides, like e.g. amylose, amylopectin, starch, dextrin, glycogen and also of glucosyl-oligosaccharides (maltose, maltotriose etc.).

Enzymatically, it is not possible to differentiate between "high polymer" and "low polymer" starch. This can be done, however, by washing the sample with an ethanol/water mixture in the course of sample preparation: starch is insoluble in ethanol/water (the ratios used depend on the method employed) whereas oligosaccharides are soluble. The solubility of oligosaccharides decreases with the increase in concentration of ethanol.

The determination of D-glucose is specific.

In the analysis of "pure starch", results of approx. 99% (calculated on a dry mass basis) have to be expected.

3. Sensitivity and detection limit (Ref. 1.3)

The smallest differentiating absorbance for the procedure is 0.010 absorbance units. This corresponds to a maximum sample volume v = 1.000 ml and measurement at 340 nm of a starch concentration of 0.6 mg/l sample solution (if v = 0.100 ml, this corresponds to 6 mg/l sample solution).

The detection limit of 1.2 mg/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume v = 1.000 ml.

Note: *The maximum sample volume after sample preparation with DMSO and HCl is 0.200 ml (see pt. 8.1).*

4. Linearity

Linearity of the determination exists from 1.2 µg starch/assay (1.2 mg starch/l sample solution; sample volume v = 1.000 ml) to 70 µg starch/assay (0.7 g starch/l sample solution; sample volume v = 0.100 ml).

Note: *The maximum sample volume after sample preparation with DMSO and HCl is 0.200 ml (see pt. 8.1).*

5. Precision

In a double determination using one sample solution, a difference of 0.010 to 0.015 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to a starch concentration of approx. 6-10 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 0.06-0.1 g/100 g can be expected.)

The following data have been published in the literature:

Starch:

21.4 µg/assay	n = 18	CV = 1.60 %	
53.5 µg/assay	n = 18	CV = 0.51 %	
85.6 µg/assay	n = 16	CV = 1.73 %	(Ref. 1.2)

Boiled finely minced pork sausage:

x = 1.3 g/100 g	r = 0.170 g/100 g	$s_{(r)}$ = ± 0.060 g/100 g	
	R = 0.217 g/100 g	$s_{(R)}$ = ± 0.077 g/100 g	

Rusks for children:

x = 43.5 g/100 g	r = 2.33 g/100 g	$s_{(r)}$ = ± 0.82 g/100 g	
	R = 8.42 g/100 g	$s_{(R)}$ = ± 2.97 g/100 g	

For further data see references (Ref. 2.6)

6. Recognizing interference during the assay procedure

6.1 If the conversion of D-glucose (after hydrolysis of starch) 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.

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

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 enzymatic determination of starch (after solubilization with DMSO/HCl or by autoclaving, resp. after acid or alkaline hydrolysis) 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. Information on sample preparation

8.1 Solubilization of starch with dimethylsulfoxide (DMSO) and HCl

(Starch products, flours, pastries, meat-balls and other meat products, milk products, margarine, animal feed, etc.)

Starch or starch-containing products have to be pretreated before the determination in order to convert the starch into a soluble form. Because of the simple handling, solubilization with dimethylsulfoxide (DMSO) is recommended:

Homogenize sample in a powder mill or homogenizer, and pass solid samples through a sieve of 0.2 mm pore diameter. Accurately weigh 100 mg to 1 g of the homogenized sample (containing up to 70 mg starch) into a 100 ml Erlenmeyer flask, add 20 ml dimethylsulfoxide and 5 ml hydrochloric acid (8 M). Concerning fat containing samples, it is recommended to add first the dimethylsulfoxide whereas fat-free samples or samples with a low fat content are to be treated first with hydrochloric acid. Close the Erlen-

meyer flask e.g. with Parafilm and incubate for 30 min (in some cases for 60 min, e.g. if bread crumbs have been used for the preparation of meat balls) at 60°C in a water-bath (shaking water-bath or heatable magnetic stirrer; care should be taken that no clotting of the sample occurs; if necessary, crush with a glass rod).

Cool quickly to 20-25°C, add approx. 50 ml water, and adjust to pH 4-5 with sodium hydroxide (5 M) under vigorous shaking (check with pH meter). Transfer to a 100 ml volumetric flask, rinse with water, fill up to the mark with water and filter the solution (after washing the filter paper with boiling redist. water) if necessary. As an alternative to filtration, let the volumetric flask stand for a few minutes and take the sample solution from the top of the solution by means of a piston type pipette. Centrifugation cannot be recommended because of the precipitation of starch, thus obtaining too low results.

Use 0.100 ml (maximum 0.200 ml) undiluted for the assay (sample and sample blank), immediately.

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

Under the aforementioned conditions, no D-glucose will be released from starch.

Note: *Sucrose is quantitatively hydrolyzed with the formation of D-glucose and D-fructose, lactose and maltose are partially hydrolyzed (approx. 10%) with the formation of the monosaccharides under the conditions of solubilization of starch by means of DMSO and hydrochlorid acid. D-Fructose is decomposed up to approx. 60% (Ref. 1.2). Therefore, the absorbance difference of the sample blank only serves for the subtraction from the absorbance difference of the sample assay in the calculation of starch (and not for the calculation of the D-glucose and D-fructose content).*

For simplification of the sample preparation procedure with DMSO and HCl, it is recommended (Ref. 3.8) to incubate the sample with DMSO and HCl followed by the addition of 5 ml NaOH (8 M), transfer into a 100 ml volumetric flask, rinsing and filling up to the mark with citrate buffer (0.112 M; pH 4; prepared by mixing a solution of citric acid (0.112 M) and a solution of trisodium citrate (0.112 M)). (Adjustment of the pH is not necessary in this case.)

Note: *Dimethylsulfoxide, DMSO (which should not be confused with the highly toxic dimethylsulfate) is weakly hazardous to water. The LD50 oral rat is 14.5 g/kg. Contact with the skin and with the eyes has to be avoided.*

8.2 Alkaline and enzymatic hydrolysis of starch (Ref. 2.4, 3.4-3.6)

For the following procedure, starch means the sum of α-glucans which are insoluble in ethanol (40%; v/v) and which are hydrolyzed to D-glucose by means of the enzyme amyloglucosidase. Starch hydrolysis products are maltodextrins, D-glucose and starch syrup, as well as dextrins, which are soluble in ethanol (40% v/v) and which are also hydrolized by amyloglucosidase with the formation of D-glucose.

The sample material is washed with an ethanol/water mixture (40% ethanol; v/v) in order to remove the starch hydrolysis products; the residue is treated with sodium hydroxide followed by amyloglucosidase in order to solubilize and hydrolyze starch.

Reagents

Ethanol, 40%; (v/v)
Sodium hydroxide solution, 0.5 M
Amyloglucosidase (AGS) solution (10 mg lyophilizate, corresponding to 60 U, Cat. No. 11 202 332, resp. 11 202 367, are dissolved in 1 ml redist. water before use.)

Acetate buffer pH 4.6 (mix 70 ml NaOH (0.5 M) with 950 ml redist. water, adjust pH 4.6 with glacial (acetic) acid). (For the measurement of starch hydrolysis products only.)

Accurately weigh sample material containing up to 100 mg starch into a centrifuge tube, add 50 ml ethanol (40%; v/v) and stir for 20 min at 20-25°C. Let the magnetic stirrer in the tube and centrifuge for 5 min. Take the supernatant carefully by a Pasteur pipette and a water-jet vacuum pump and use it for the determination of the soluble starch hydrolysis products and of free D-glucose. Wash the residue at least twice with 25 ml ethanol (40%; v/v) again, and centrifuge. Combine the supernatants.

Add 50 ml NaOH (0.5 M) to the residue and stir for 30 min at 60°C in a water bath. Adjust the pH to 4.6 to 4.8 by the addition of glacial (acetic) acid (min 96%). Then add 1 ml AGS solution and stir for another 30 min. Cool down to 20-25°C, transfer the solution into a 100 ml volumetric flask, rinse with water, fill up to the mark, mix and filter if necessary.

Use the filtrate for the determination of D-glucose (see below) and calculate the result as starch.

For the determination of the starch hydrolysis products, evaporate the collected extracts (supernatants) with a flash evaporator until a slight opalescence occurs. Transfer the liquid into a 100 ml volumetric flask, rinse with acetate buffer (pH 4.6) and fill up to the mark.

Perform the determination of starch hydrolysis products as described under "Procedure": sample blank and sample. The calculation of the total starch hydrolysis products (without "free D-glucose") may be done as "starch".

The content of "free D-glucose" can be determined as described below (see also pt. 10).

Determination of D-Glucose[7]

Pipette into cuvettes	Blank	Sample
solution 2 from TC-Starch sample solution redist. water	1.000 ml - 2.000 ml	1.000 ml 0.100 ml 1.900 ml
Mix, read absorbances of the solutions (A_1) after approx. 3 min and start reaction by addition of:		
suspension 3 from TC-Starch	0.020 ml	0.020 ml
Mix, wait for the end 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 increase constantly over 2 min.		

If the absorbance A_2 increases constantly, extrapolate the absorbances to the time of addition of suspension 3 (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, thereby obtaining $\Delta A_{starch,\ resp.\ free\ D\text{-}glucose}$.

$$\Delta A_{starch,\ resp.\ free\ D\text{-}glucose} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$$

It follows for starch:

$$c = \frac{3.020 \times 162.1}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{starch} = \frac{4.895}{\varepsilon} \times \Delta A_{starch}\ [\text{g starch/l sample solution}]$$

for "free D-glucose":

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

8.3 Acid and enzymatic hydrolysis of starch (Ref. 2.3)

Reagents

Ethanol, 40%; (v/v), HCl, 32% (m/m), NaOH, 5 M

a) Dry samples (powders)

Accurately weigh sample material containing up to 70 mg starch into a centrifuge tube, wash 3 times with 15 ml ethanol (40%; v/v) each while stirring for 20 min at 20-25°C, centrifuge, remove the supernatants. Add 10 ml HCl (1 part HCl, 32%; m/m; diluted with 2 parts of redist. water) to the residue and stir for 60 min at 60°C in a water bath. Transfer the solution quantitatively into a 100 ml beaker, rinse with water and adjust the pH to approx. 4.5 by the addition of NaOH (5 M). Transfer the solution into a 100 ml volumetric flask, rinse with water, fill up to the mark, mix and filter if necessary.

b) Water-containing samples

Accurately weigh sample material containing up to 70 mg starch into a centrifuge tube. Add 10 ml ethanol (96%; v/v) while stirring, and centrifuge. Continue as described under pt. a. (including 3 times washing with 15 ml ethanol (40%; v/v).

Perform the determination od starch as described under "Procedure": reagent blank, sample.

$$\Delta A_{starch} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{reagent\ blank}$$

8.4 Autoclaving and enzymatic hydrolysis of starch

Reagents

Acetate buffer (2 M; pH 4.8: 120 ml glacial (acetic) acid and 164 g anhydrous sodium acetate are dissolved in redist. water to 1 l).
Amyloglucosidase (AGS) solution (dissolve 10 mg lyophilizate, corresponding 60 U, Cat. No. 1 202 332[6], resp. 1 202 367[6], in 1 ml redist. water before use.)

Samples which contain free D-glucose or oligo-glucosides are extracted with ethanol (40%; v/v) as described under pt. 8.2 and 8.3.

Accurately weigh sample material containing up to 70 mg starch into a 100 ml Erlenmeyer flask, add 25 ml redist. water, heat with a Bunsen burner and boil for 3 min. Cover the Erlenmeyer flask with aluminum foil and autoclave at 130 °C and 1.8 kg × cm^{-2} for 1 h. Cool to 20-25°C, add 2.5 ml acetate

6 Available from Roche Applied Science
7 The determination of D-glucose can also be done with Test-Combination D-Glucose, Cat. No. 10 71625 035

buffer, 22.5 ml redist. water and 5 ml AGS solution. Incubate in a water-bath while stirring for 2 h at 60°C. Transfer the solution into a 100 ml volumetric flask, rinse with water, fill up to the mark, mix and filter if necessary.

Perform the determination of starch as described under "Procedure": reagent blank, sample.

$$\Delta A_{starch} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{reagent\ blank}$$

9. Presence of mono- or oligosaccharides

Sample solutions from samples containing free D-glucose or sucrose (hydrolyzed by the DMSO/HCl pretreatment; see pt. 8.1) must be determined in a second assay **without** incubation with amyloglucosidase **(sample blank and sample)**.

Subtract the absorbance difference of the sample blank from the absorbance difference of the sample.

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

The performance of a reagent blank is not necessary.

In the presence of maltose or its homologues, the alcohol extraction has to be carried out, because maltose is partially hydrolized under the conditions of DMSO/HCl solubilization of starch and is also cleaved by amyloglucosidase.

Accurately weigh approx. 0.1 - 1 g of the homogenized and sieved sample containing up to 70 mg starch into a 100 ml centrifuge beaker (the water content of the sample is to be determined and should not exceed 20%, otherwise the sample has to be dried before). Wash the sample three times with 10 ml ethanol (40%; v/v) each and centrifuge. Filter the supernatant and discard the filtrate. Combine precipitate in the centrifuge beaker with the precipitate in the filter and transfer with 4 portions of 5 ml dimethylsulfoxide each into a 100 ml Erlenmeyer flask, add 5 ml HCl (8 M), and continue working as stated above (see pt. 8.1).

In this case, the determination of the sample blank can be neglected, but the reagent blank has to be determined.

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

10. Determination of starch-partial hydrolysates

a) Dextrins in beer

When determining dextrins in beer, the solubilization with dimethylsulfoxide is not necessary. The sample can be used directly for the assay with a volume of 0.100 ml (up to 1.000 ml, if necessary): sample blank and sample assay.

The dextrin concentration is calculated as starch after subtraction of possibly present free D-glucose (= sample blank).

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

$$\Delta A_{free\ D\text{-}glucose} = (A_2\text{-}A_1)_{sample\ blank} - (A_2\text{-}A_1)_{reagent\ blank}$$

b) Solutions containing starch partial hydrolysates

Follow the instructions given for the determination of dextrins in beer. Dilute the sample according to the dilution table in order to get a solution containing oligoglucosides up to 0.4 g/l for the measurement at 340 and Hg 334 nm (resp. 0.7 g/l for the measurement at Hg 365 nm) for sample assay and sample blank and in the case of the deternmination of D-glucose a solution containing D-glucose up to 0.4 g/l for the measurement at 340 and Hg 334 nm (resp. 0.7 g/l for the measurement at Hg 365 nm) for reagent blank and sample blank.

c) "Glucose syrup" in fruit juices

Normally, fruit juices contain a high excess of D-glucose in comparison with the concentration of glucose syrup (starch). The determination of starch is carried out directly with the sample solution according to "Procedure" (= starch sample) without preceding solubilization with dimethylsulfoxide.
The hydrolysis of the starch sample is carried out with amyloglucosidase for 30 min at 20-25°C (sample assay). In another assay, D-glucose is determined **without** amyloglucosidase (D-glucose sample, corresponds to the sample blank assay when starch is determined).

$$\Delta A_{glucose\ syrup^*} = (A_2\text{-}A_1)_{starch\ sample} - (A_2\text{-}A_1)_{sample\ blank}$$

If the ratio of D-glucose to glucose syrup is higher than e.g. 10:1, the precision of the determination of glucose syrup is impaired. To attain a higher precision of the measurement, D-glucose present in the sample solution must be removed as much as possible before the determination of glucose syrup.

In the presence of the enzymes glucose oxidase (GOD) and oxygen from the air, D-glucose is oxidized to D-gluconate:

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

The hydrogen peroxide is removed by catalase:

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

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 $MgSO_4 \times 7\ H_2O$ with 80 ml water, adjust to pH 7.6 with NaOH (4 M) and fill up to 100 ml with redist. water.

Stability of solutions

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

Performance of D-glucose oxidation

Pipette into 10 ml volumetric flask	
buffer solution sample solution (with approx. 1% D-glucose) enzyme solution	2.000 ml 5.000 ml 0.100 ml
Pass a current of air (O_2) through the mixture for 1h; 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 fill up to the mark with water. Mix and filter, if necessary. Use the clear solution for the determination of the glucose syrup and the remaining D-glucose.

d) "Starch syrup" in jam with a high sucrose content

For the determination of starch syrup besides an extremely high content of sucrose it is recommended to prepare the homogenized sample according to the conditions of the solubilization with dimethylsulfoxide (see pt. 8.1).

Hereby, the sucrose in the sample is completely hydrolyzed to D-glucose and D-fructose. The excess of D-glucose formed when using this method of sample preparation should be removed as stated under pt. 10b as to attain a higher precision of the measurement.

Use up to 5 g of the sample (jam) for the assay.

Perform the determination as described under "Procedure": sample assay and sample blank assay.

$$\Delta A_{starch\ syrup} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{sample\ blank}$$

11. Determination of modified starch

Modified starch, e.g. phosphorylated or also oxidized starch, is only determinable in a limited amount. Amyloglucosidase hydrolyzes the modified starch up to a modification grade of 1% (if 1% of the D-glucose contained in the starch molecule is modified). The released D-glucose can be determined according to the usual method, whereas the modified D-glucose does not react.

Modified starch with a higher grade of modification is no more recognized by amyloglucosidase as a cleavable substrate.

12. Further applications

The method may also be used in the examination of paper (Ref. 2.2.) and in research when analyzing biological samples. For details of sampling, treatment and stability of the sample, see Ref. 1.1 and 1.4.

* Glucose syrup, calculated as starch

References

1.1 Keppler, D. & Decker, K. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., Hrsg.) 3. Aufl., Bd. 2, S. 1171-1176, Verlag Chemie, Weinheim and (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd ed., vol. 3, pp. 1127-1131, Verlag Chemie, Weinheim/Academic Press, Inc., New York and London

1.2 Beutler, H.-O. (1978) Enzymatische Bestimmung von Stärke in Lebensmitteln mit Hilfe der Hexokinase-Methode, Starch/Stärke **30,** 309-312

1.3 Beutler, H.-O. (1984) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 3rd ed., vol. VI, pp. 2-10, Verlag Chemie, Weinheim, Deerfield Beach/Florida, Basel

1.4 Keppler, D. & Decker, K. (1984) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 3rd ed., vol. VI, pp. 11-18, Verlag Chemie, Weinheim, Deerfield Beach/Florida, Basel

2.1 Nederlandse Norm NEN 3574 (Dezember 1974) Onderzoekingsmethoden voor veevoeders; Bepaling van het gehalte aan zetmeel met behulp van enzymatische hydrolyse (Test methods for feeding stuffs; Determination of the starch content by enzymatic hydrolysis)

2.2 Untersuchung von Papieren, Kartons und Pappen für Lebensmittelverpackungen (gem. Empfehlung XXXVI der Kunststoffkommission des Bundesgesundheitsamtes) Kapitel 8 (Methoden), Pkt. 3.4.1 (März 1979)

2.3 Gombocz, E., Hellwig, E., Vojir, F. & Petuely, F. (1981) Deutsche Lebensmittel-Rundschau **77,** S. 3 (D-Glucose), Oligosaccharide (11-12) und 12-13 (Stärke)

2.4 Schweizerisches Lebensmittelbuch, Kapitel 22 (Diätetische Lebensmittel)/6.1: Bestimmung der Stärke und Stärkeabbauprodukte (1982), Kapitel 12 (Fleischextrakte, Bouillonpräparate, Sulzen)/11 (1981), Kapitel 13 (Würzen, Suppen, Saucen)/10 (1981)

2.5 Brautechnische Analysenmethoden, Band III, S. 593-596 (1982), Methodensammlung der Mitteleuropäischen Brautechnischen Analysenkommission (MEBAK), herausgegeben von F. Drawert im Selbstverlag der MEBAK, Freising

2.6 Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG; Untersuchung von Lebensmitteln: Bestimmung von Stärke in Fleischerzeugnissen, 07.00-25 (Mai 1983); Bestimmung von Stärke in Wurstwaren, 08.00-26 (Mai 1983); Bestimmung von Stärke in teiladaptierter Säuglingsnahrung auf Milchbasis, 48.01-5 (Mai 1985); Bestimmung von Stärke in Kinder-Zwieback und Zwiebackmehl, 48.02.07-3 (Mai 1985)

2.7 Deutsche Norm DIN 54604 (Jan. 1988) Prüfung von Papier und Pappe, Bestimmung des Gehaltes an Stärke

2.8 Niederlande: Warenwet, Uitvoeringsvoorschriften (C II-6) Regeling Onderzoekingsmethoden voor brood; Methode 15: De Bepaling van Zetmeel (Oktober 1986); Dit voorschrift beschrijft een methode voor de bepaling van zetmeel in de gedroogde kruim van gluten- of alcuronaat brood

2.9 Code of Practice for Evaluation of Fruit and Vegetable Juices (1996) edited by Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community (A.I.J.N.)

3.1 Menger, A. (1974) Anwendung enzymatischer Sorbit-, Zucker- und Stärkebestimmungsmethoden zur Lösung analytischer Probleme bei Brot und Backwaren, Getreide Mehl Brot **28,** 36-38

3.2 Meuser, F. (1972) Beckman-Report 1/72

3.3 Meuser, F. & Rajani, Ch. (1976) Stärkebestimmung in Futtermitteln, Getreide Mehl Brot **30,** 173-177

3.4 Ettel, W. (1980) Enzymatische Stärkebestimmung, Mitt. Gebiete Lebensm.Hyg. **71,** 45

3.5 Ettel, W. (1981) Eine neue enzymatische Stärkebestimmung für Lebensmittel, alimenta **20,** 7-11

3.6 Walter, E. (1984) Ringversuche zur enzymatischen Stärkebestimmung, Z.Lebensm. Unters.Forsch. **179,** 210-217

3.7 Bandion, F., Wurzinger, A. & Neumann, K. (1986) Zum Nachweis bestimmter Stärkederivate in Wein, Mitt. Klosterneuburg **36,** 207-209

3.8 Lukaszewska, A. & Gorin, N. (1988) Simplification of the Boehringer Mannheim Enzymic Analysis of Starch in the Methanol-Insoluble Residue of Freeze-dried Corollas from Cut "Sonia" Roses, Gartenbauwissenschaft **53,** 49-51

For further information see instructions for

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

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