Total Starch, colorimetric method

Catalogue number: AK00291, 100 tests

Application

This simple, accurate and convenient colorimetric method is used for the determination of total starch in cereal, flours and other food and feed products.

Introduction

Starch determination is essential in several food and feed industries. Enzymatic methods are commonly used to determine starch. However, procedures differ in pre-treatment steps, starch gelatinisation, liquefaction and dextrinisation, hydrolysis of dextrins to glucose and glucose measurement.

This simple, nonetheless quantitative and reliable, procedure for the measurement of total starch is based on the use of thermostable α-amylase and amyloglucosidase. This method has been adopted by AOAC (Official Method 996.11) and AACC (Method 76.13). The use of a thermostable α-amylase that is active and stable at low pH allows both the thermostable α-amylase and amyloglucosidase incubation steps to be performed at the same pH (pH 5.0), which simplifies the assay.

Principles

<table>
<thead>
<tr>
<th>Starch granules + H₂O</th>
<th>α-amylase</th>
<th>maltodextrins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltodextrins + H₂O</td>
<td>AMG</td>
<td>D-glucose</td>
</tr>
<tr>
<td>D-Glucose + O₂ + H₂O</td>
<td>GOx</td>
<td>D-glucanate + H₂O₂</td>
</tr>
<tr>
<td>2 H₂O₂ + p-hydroxybenzoic acid + 4-aminoantipirine</td>
<td>POD</td>
<td>Quinoneimine dye + 4 H₂O</td>
</tr>
</tbody>
</table>

Thermostable α-amylase hydrolyses starch into soluble branched and unbranched maltodextrins. Amyloglucosidase (AMG) hydrolyses maltodextrins to D-glucose. D-glucose obtained is determined directly with GODPOD Reagent by conversion to a red coloured quinoneimine dye compound through the combined action of glucose oxidase and peroxidase.

Specificity

The assay is specific for α-glucans (including starch, glycogen, phytoglycogen and non-resistant maltodextrins).

Sensitivity and detection limit

The sensitivity of the assay is based on 0.010 AU. This corresponds to a D-glucose concentration of 1.0 mg (or 0.9 mg starch)/L sample solution for a maximum sample volume of 1.00 mL. The detection limit of 2.0 mg (or 1.8 mg starch)/L is derived from the absorbance difference of 0.020 and a maximum sample volume of 1.00 mL. If the GODPOD absorbance is above 1.1 AU, dilute the sample and repeat the analysis.

Linearity and precision

Linearity of the determination exists from 5 to 100 μg D-glucose per assay. In a double assay using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of 1.00 mL, this corresponds to a D-glucose concentration of approx. 0.5 to 1.0 mg/L. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be anticipated.

Kit composition

Suspension 1: Thermostable α-amylase (12 mL, 8300U/mL) in 25% glycerol Stable for 3 years at 4°C.

Dilute 1.0 mL of the contents of bottle 1 to 30 mL with Reagent 1 (100 mM sodium acetate buffer, pH 5.0; not supplied). Divide into appropriately sized aliquots and store in PP tubes at -20°C between use and keep cool during use if possible. Stable for > 3 years at -20°C.

Note: If the sample is to be analyzed following Assay II (AOAC Official method 996.11), the enzyme must be diluted in Reagent 4 (50 mM MOPS, pH 7.0)

Suspension 2: Amyloglucosidase (10 mL, 3300U/mL) in 50% glycerol Stable for 3 years at 4°C.

Use as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser.
Solution 3. GOD-POD reagent buffer (30 mL, pH 7.4), p-hydroxybenzoic acid and sodium azide (0.64% w/v) as a preservative. Stable for 3 years at 4 °C.

Dilute the contents of the bottle to 1.0 L with distilled water and use immediately.

Note: On storage, salt crystals may form in the concentrated buffer. These must be completely dissolved when this buffer is diluted to 1 L with distilled water.

Mixture 4. GOD-POD reagent enzymes. Freeze-dried powder of glucose oxidase (GOD), peroxidase (POD) and 4-aminoantipyrine. Stable for 5 years at -20 °C.

Dissolve the contents of one bottle in approx. 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Cover this bottle with aluminum foil to protect the enclosed reagent from light. Stable for 3 months at 2-5 °C or 12 months at -20 °C.

Solution 5. D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.02% benzoic acid. Stable for 2 years at room temperature.

Powder 6. Control regular maize starch. Starch content shown on vial label. Stable for > 5 years at room temperature.

Safety

Reagents that are used in the determination of starch are not hazardous materials (see Hazardous Substances Regulations). However, the concentrated buffer contains sodium azide as a preservative. The general safety measures that apply to all chemical substances should be followed. For more information regarding the safe usage of this kit please refer to the MSDS available at www.nzytech.com.

Buffers preparation (not supplied)

Reagent 1. Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)

Add 5.8 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 5.0 Stable for approx. 2 months at 4°C.

Add 0.74 g of calcium chloride dihydrate and dissolve. Add 0.2 g sodium azide. Adjust the volume to 1 L and store the buffer at 4°C. Stable for approx. 2 years at room temperature.

Warning: Sodium azide should not be added until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.

Reagent 2. Sodium acetate buffer (1.2 M, pH 3.8)

Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8. Adjust the volume to 1 L with distilled water. Stable for 12 months at room temperature.

Reagent 3. Potassium hydroxide solution (2 M)

Add 112.2 g KOH to 900 mL of deionised water and dissolve by stirring. Adjust volume to 1 L. Store in a sealed container. Stable for > 2 years at room temperature.

Reagent 4. MOPS buffer (50 mM, pH 7.0) plus calcium chloride (5 mM) and sodium azide (0.02% w/v).

Optional: Only required if samples are analysed according to Assay II.

Dissolve 11.55 g of MOPS sodium salt in 900 mL of distilled water and adjust the pH to pH 7.0. Add 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide and dissolve. Adjust the volume to 1 L. Stable for 6 months at 4°C.

Reagent 5. Sodium acetate buffer (200 mM, pH 4.5) plus sodium azide (0.02% w/v).

Optional: Only required if samples are analysed according to Assay II.

Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.5. Add 0.2 g of sodium azide and dissolve. Adjust the volume to 1 L. Stable for 6 months at 4°C.

Warning: Sodium azide should not be added until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.

Precautions and controls

Include reagent blanks and D-glucose controls (100 µg, in quadruplicate) with each set of assays. Analyse at least one extract from the control maize starch with each set of assays.

The time of incubation with GOD-POD reagent is not critical, but should be at least 20 min. However, the time for maximum colour formation with 100 µg of D-glucose standard should be checked, for each new batch of GOD-POD reagent (usually 15 min). The colour formed through the reaction is stable at room temperature for at least 2 hours after development.

Procedure (endpoint analysis)

Wavelength: 510 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: 50 °C

Final volume: 3.10 mL

Sample solution: 10-100 µg of D-glucose per cuvette

Read against reagent blank.

<table>
<thead>
<tr>
<th>Pipette into cuvettes (mL)</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOD-POD reagent</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>D-Glucose standard</td>
<td>-</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
</tr>
<tr>
<td>Buffer or water</td>
<td>0.10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mix, incubate at 50 °C for 20 min and read absorbances at 510 nm against the reagent blank to obtain ΔA_sample and ΔA_zero glucose standard.

Mixtures can be obtained with a plastic spatula or by gentle inversion after sealing with a cuvette cap or Parafilm®.
Interferences

If the conversion of D-glucose completes within the time specified in the assay, we can be generally concluded that no interference has occurred. However, an internal standard should be included during sample analysis if the presence of interfering substances is suspected. A quantitative recovery of this standard should be expected. Identification of losses in sample handling and extraction may be identified by performing recovery experiments, i.e., by adding D-glucose to the sample in the initial extraction steps.

Calculation

Solid samples

The concentration of starch of solid samples can be calculated as follows:

\[
\text{Starch, } \% \text{ w/w} = \Delta A \times F \times \frac{FV}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \\
= \Delta A \times \frac{F}{W} \times FV \times 0.9
\]

Where,

\(\Delta A\) = sample GOD-POD absorbance read against the reagent blank

\(F\) = factor to convert from absorbance to \(\mu g\) of glucose

\(\frac{100 \ (\mu g \ of \ D-Glucose)}{\text{absorbance of } 100 \mu g \ of \ D-Glucose}\)

\(FV\) = final volume (i.e. Assay I and Assay II – 100 mL or 10 mL; Assay III – 100 mL or 10.4 mL; Assay IV and V – 100 mL or 10 mL; 0.1 = volume of sample analyzed

\(\frac{1}{1000}\) = conversion from \(\mu g\) to mg

\(\frac{100}{W}\) = factor to express starch as a percentage of sample weight

\(W\) = weight of the sample analyzed in mg (“as is”)

\(\frac{162}{180}\) = factor to convert free D-glucose, as determined, to anhydro-D-glucose, as occurs in \(\beta\)-glucans

Starch, % w/w (dry weight basis)

\[
\text{Starch, } \% \text{ w/w (dry weight basis)} = \beta\text{-Glucans, } \% \text{ w/w (“as is”) } \times \frac{100}{100 \text{– moisture content (w/w)}}
\]

Note:

Dry weight = fresh weight \(\times\) \(\frac{100 \text{– moisture content (w/w)}}{100}\)

Moisture content is usually determined by NIR reflectance. Alternatively this can be determined by recording weight loss on storage of flour samples (0.5 g) at 80\(^\circ\)C for 20 h. or until weight stabilization. The moisture content of cereal flour samples is regularly in the range of 10–14%.

Liquid samples

The concentration of starch of liquid samples are calculated as follows:

\[
\text{Starch, mg/100 mL} = \Delta A \times F \times \frac{100}{0.1} \times \frac{1}{1000} \times \frac{162}{180} \times 2 \times D \\
= \Delta A \times F \times D \times 1.8
\]

Where,

\(\Delta A\) = sample GOD-POD absorbance read against the reagent blank

\(F\) = factor to convert from absorbance to \(\mu g\) of glucose

\(\frac{100 \ (\mu g \ of \ D-Glucose)}{\text{absorbance of } 100 \mu g \ of \ D-Glucose}\)

\(0.1\) = volume of sample analyzed

\(\frac{1}{1000}\) = conversion from \(\mu g\) to mg

\(\frac{162}{180}\) = factor to convert free D-glucose, as determined, to anhydro-D-glucose, as occurs in \(\beta\)-glucans

\(D\) = dilution of the sample solution on incubation with AMG

General information on sample preparation

The amount of D-glucose present in the cuvette should range between 5 and 100 \(\mu g\). Thus, if a sample volume of 0.10 mL is used the sample solution must be diluted to yield a D-glucose concentration between 0.05 and 1.0 g/L.

Sample blanks can be determined using the Assay I procedure with the modifications that in Step 4, three (3) mL of distilled water is used and in Step 5, AMG is replaced by water. Instead, the need to perform sample blank analysis can be avoided by pre-extraction of samples with aqueous ethanol (80% v/v) [see Assay V].
Examples of sample preparation

I. Assay method for determination of starch in cereal and food products not containing resistant starch, D-glucose and/or maltodextrins
(Recommended Procedure; all incubations at pH 5.0)

1. Mill sample (cereal, plant or food product; approx. 50 g) to pass a 0.5 mm screen using a centrifugal mill.
2. Accurately weigh flour sample (~100 mg) to a glass centrifuge tube (17 mL capacity). Tap the tube to ensure that all sample falls to the bottom of the tube.
3. Add 0.2 mL of aqueous ethanol (80% v/v) to wet the sample and aid dispersion. Stir the tube on a vortex mixer.
4. Immediately add 3 mL of diluted thermostable α-amylase (Suspension 1 diluted 1:30 in Reagent 1; pH 5.0). Incubate the tube in a boiling water bath for 6 min (Stir the tube vigorously after 2, 4 and 6 min).

The tube must be stirred vigorously to ensure complete homogeneity of the slurry (removal of lumps). Also, stirring after 2 min prevents the possibility of some of the sample expelling from the top of the tube when the alcohol is evaporating.

5. Place the tube in a bath at 50°C. Add 0.1 mL of Suspension 2 (AMG). Stir the tube on a vortex mixer and incubate it at 50°C for 30 min.
6. Transfer the entire contents of the test tube to a 100 mL volumetric flask (with a funnel to assist transfer). Use a wash bottle to rinse the tube contents thoroughly. Adjust to volume with distilled water. Mix thoroughly.
7. Centrifuge an aliquot of this solution at 3,000 rpm for 10 min. Use the clear, undiluted filtrate for the assay, in duplicate.

Note:
As an alternative, at Step 6, if the starch content is unknown and is supposed to be less than 10% w/w, adjust the volume to 10 mL (10 g) with distilled water and then centrifuge the tubes at 3,000 rpm for 10 min. For samples containing 1-10% w/w starch content, use this solution directly for analysis. For samples containing 10-100% starch, dilute 1.0 mL of this solution to 10 mL with distilled water before analysis.

II. Assay method for determination of starch in cereal and food products not containing resistant starch, D-glucose and/or maltodextrins
(AOAC Official Method 996.11)

1. Mill sample (cereal, plant or food product; approx. 50 g) to pass a 0.5 mm screen using a centrifugal mill.
2. Accurately weigh flour sample (~100 mg) to a glass centrifuge tube (17 mL capacity). Tap the tube to ensure that all sample falls to the bottom of the tube.
3. Add 0.2 mL of aqueous ethanol (80% v/v) to wet the sample and aid dispersion. Stir the tube on a vortex mixer.
4. Immediately add 3 mL of thermostable α-amylase (Suspension 1 diluted 1:30 in Reagent 4; pH 7.0). Incubate the tube in a boiling water bath for 6 min (Stir the tube vigorously after 2, 4 and 6 min).

The tube must be stirred vigorously to ensure complete homogeneity of the slurry (removal of lumps). Also, stirring after 2 min prevents the possibility of some of the sample expelling from the top of the tube when the alcohol is evaporating. If PP tubes are used, increase the incubation time to 12 min, with stirring after 4, 8 and 12 min.

5. Place the tube in a bath at 50°C; add 3 mL of Reagent 5 (200 mM sodium acetate buffer, pH 4.5). Then add Suspension 2 (AMG). Stir the tube on a vortex mixer and incubate at 50°C for 30 min.
6. Proceed from Step 6 of Assay I.

III. Assay method for determination of total starch content of samples containing resistant starch, but no D-glucose and/or maltodextrins
(KOH Format - Recommended)

1. Mill sample (cereal, plant or food product; approx. 50 g) to pass a 0.5 mm screen using a centrifugal mill.
2. Accurately weigh flour sample (~100 mg) to a glass centrifuge tube (17 mL capacity). Tap the tube to ensure that all sample falls to the bottom of the tube.
3. Wet with 0.2 mL of aqueous ethanol (80% v/v) to aid dispersion and stir the tube on a vortex mixer.
4. Add a magnetic stirrer bar and 2 mL of Reagent 3 (2 M KOH) to each tube and re-suspend the pellets (and dissolve the resistant starch) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer.

Note:
- Do not mix on a vortex mixer to avoid the starch to emulsify.
- Make sure that the tube contents are vigorously stirred as the KOH solution is added. This will avoid the formation of a lump of starch material that will then be difficult to dissolve.

5. Add 8 mL of Reagent 2 (1.2 M sodium acetate buffer pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL of Suspension 1 (thermostable α-amylase) and 0.1 mL of Suspension 2 (AMG), mix well and place the tubes in a water bath at 50°C.
6. Incubate the tubes for 30 min with intermittent mixing on a vortex mixer.
7. For samples containing > 10% total starch: quantitatively transfer the contents of the tube to a 100 mL volumetric flask (using a water wash bottle). Adjust to 100 mL with distilled water and mix well. Centrifuge an aliquot of the solution at 3,000 rpm for 10 min.
8. For samples containing < 10% total starch: directly centrifuge the tubes at 3,000 rpm for 10 min (no dilution). Usually, the final volume in the tube is approx. 10.4 mL (however, this volume will vary particularly if wet samples are analysed, and appropriate allowance for volume should be made in the calculations).
9. Proceed from Step 6 of Assay I.
IV. Assay method for determination of total starch content of samples containing resistant starch but no D-glucose and/or maltodextrins (DMSO Format - AOAC Official Method 996.11)

1. Mill sample (cereal, plant or food product; approx. 50 g) to pass a 0.5 mm screen using a centrifugal mill.
2. Accurately weight flour sample (~100 mg) to a glass centrifuge tube (17 mL capacity). Tap the tube to ensure that all sample falls to the bottom of the tube.
3. Add 0.2 mL of aqueous ethanol (80% v/v) to wet the sample and aid dispersion. Stir the tube on a vortex mixer.
4. Immediately add 2 mL of dimethyl sulphoxide (DMSO) and stir the tube on a vortex mixer. Place the tube in a vigorously boiling water bath and remove after 5 min.
5. Proceed from Step 4 of Assay I.

V. Assay method for determination of starch in samples which also contain D-glucose and/or maltodextrins

1. Mill sample (cereal, plant or food product; approx. 50 g) to pass a 0.5 mm screen using a centrifugal mill.
2. Accurately weight flour sample (~100 mg) to a glass centrifuge tube (17 mL capacity). Tap the tube to ensure that all sample falls to the bottom of the tube.
3. Add 5.0 mL of aqueous ethanol (80% v/v), and incubate the tube at 80-85°C for 5 min. Mix the contents on a vortex stirrer and add another 5 mL of 80% v/v aqueous ethanol.
4. Centrifuge the tube for 10 min at 3,000 rpm on a bench centrifuge. Reject the supernatant.
5. Resuspend the pellet in 10 mL of 80% v/v aqueous ethanol and stir on a vortex mixer. Centrifuge as above and carefully pour off the supernatant.
6. Proceed from Step 4 of Assay I. Alternatively: Proceed from Step 4 of Assay III if the sample contains resistant starch.

VI. Assay method for determination of starch in samples in which the starch is present in a soluble form and D-glucose and maltodextrins are not present

1. Filter an aliquot of the sample solution through Whatman No. 1 filter paper (or Whatman GF/A Glass fibre filter paper if necessary). Use the clear filtrate for the assay.
2. Add 10 mL of this filtrate to a glass tube. Add 2 mL of Reagent 1 (100 mM acetate buffer, pH 5.0) plus 0.1 mL of Suspension 2 (AMG) diluted 50-fold in Reagent 1 and incubate in a water bath at 50°C for 30 min. Adjust volume to 20 mL (or 20 g) with distilled water.
3. Use the clear, undiluted filtrate for the assay.

VII. Assay method for determination of starch in samples in which the starch is present in a soluble form and D-glucose and maltodextrins are present

1. Filter an aliquot of the sample solution through Whatman No. 1 filter paper (or Whatman GF/A Glass fibre filter paper if necessary). Use the clear filtrate for the assay.
2. Add 2 mL of the solution to be analysed to a 17 mL glass test tube. Then add 8 mL of 95% v/v ethanol and mix vigorously on a vortex mixer. Allow to stand at room temperature for 30 min and centrifuge at 3,000 rpm for 10 min.
3. Reject the supernatant solution and redissolve the starch containing pellet in 1 mL of water. If needed, heat the tube and contents in a boiling water bath to aid redissolution. Adjust the volume to 3.9 mL (3.9 g) with Reagent 1 (100 mM acetate buffer, pH 5.0), taking account of the original weight of the tube.
4. If required, repeat the ethanol precipitation and centrifugation steps (e.g. for samples containing high levels of free D-glucose and/or maltodextrins). Discard the supernatant solution and redissolve the starch containing pellet in 1 mL of water. If necessary, heat the tube and contents in a boiling bath to aid redissolution. Adjust the volume to 3.9 mL (3.9 g) with water, taking account of the original weight of the tube.
5. Add 0.1 mL of Suspension 2 (AMG) diluted 50-fold in Reagent 1 and incubate in a water bath at 50°C for 30 min.
6. If the solution is turbid, centrifuge the tube at 12,000 rpm for 10 min. Use the clear filtrate for the assay. Usually, either no dilution, or a dilution of 10-fold might be required.

References


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Please enquire info@nzytech.com to obtain any additional information about this kit, including additional specific applications.

<table>
<thead>
<tr>
<th>Test</th>
<th>Criteria</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Performance</td>
<td>Reaction completed within time stated</td>
<td>Meets specification</td>
</tr>
<tr>
<td></td>
<td>Target value for recommended standard material +/- 10%</td>
<td>Meets specification</td>
</tr>
<tr>
<td>Blank reaction absorbance</td>
<td>+/- 10% of the blank value</td>
<td>Meets specification</td>
</tr>
</tbody>
</table>

Approved by: 
José Prates  
Senior Manager, Quality Systems