

Megazyme

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TOTAL STARCH

ASSAY PROCEDURE

(AMYLOGLUCOSIDASE/ α -AMYLASE METHOD)

K-TSTA-50A/K-TSTA-100A 03/18

(50/100 Assays per Kit)

AOAC Method 996.11
AACC Method 76-13.01
(and improvements)



INTRODUCTION:

Starch determination methods are broadly grouped into acid hydrolysis or enzymic procedures.¹ Acid hydrolysis procedures can only be applied to pure starch samples and thus have limited application. Enzymic procedures vary in pre-treatment steps,² starch gelatinisation, liquefaction and dextrinisation, hydrolysis of dextrans to glucose and glucose measurement.³ AACC Method 76-11⁴ specifies starch gelatinisation under aqueous conditions in an autoclave, followed by starch conversion to glucose with amyloglucosidase and glucose measurement. AACC Method 76-11 underestimates starch content in a range of samples and materials, including high amylose maize starches and many processed cereal products. Most methods in use today incorporate treatment with thermostable α -amylase either during or immediately following the starch gelatinisation step.^{5,6} For samples which are difficult to gelatinise (such as high amylose maize starch) solvents such as sodium hydroxide or dimethyl sulphoxide (DMSO)^{7,8} have been employed. In a procedure to ensure complete solubilisation of starch in dietary fibre determination, Englyst and Cummings (1988)⁷ included treatment with the starch debranching enzyme, pullulanase.

To satisfy the need for an extremely simple, yet quantitative and reliable, procedure for the measurement of total starch Megazyme produces, and offers, a total starch assay kit based on the use of thermostable α -amylase and amyloglucosidase (McCleary *et al.*⁹). This method has been adopted by AOAC (Official Method 996.11) and AACC (Method 76.13.01).

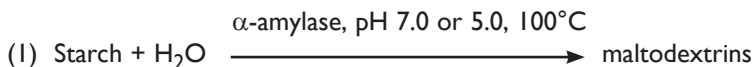
More recently, thermostable α -amylases that are active and stable at lower pH values have become available. Consequently, we have updated our total starch methodology to incorporate such an enzyme.¹⁰ The major advantage of this improvement is to allow both the thermostable α -amylase and amyloglucosidase incubation steps to be performed at the same pH (pH 5.0) which, in turn, simplifies the assay and minimises the possibility of production of maltulose (4- α -glucopyranosyl-D-fructose),¹¹ which is resistant to hydrolysis by amyloglucosidase and α -amylase.

The Megazyme total starch analysis procedure allows the measurement of total starch in a wide range of food, feed, plant and cereal products (natural or processed). For most samples (e.g. wheat flour), starch is completely solubilised on incubating the sample at approx. 100°C in the presence of thermostable α -amylase. Samples

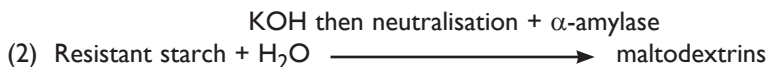
containing high levels of resistant starch (e.g. high amylose maize starch), require pre-dissolution in cold 2 M KOH or hot DMSO. For samples containing soluble starch or maltodextrins, cooking with thermostable α -amylase is not required.

PRINCIPLE:

Thermostable α -amylase hydrolyses starch into soluble branched and unbranched maltodextrins (1).



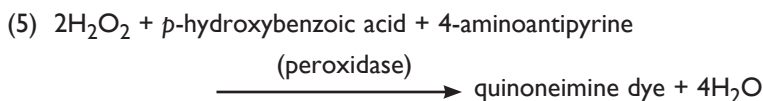
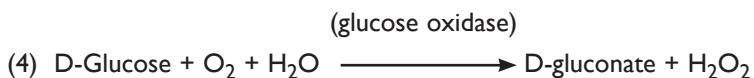
Where necessary, resistant starch in the sample is pre-dissolved by stirring the sample with 2 M KOH at approx. 4°C, followed by neutralisation with sodium acetate buffer and hydrolysis with α -amylase (2). Alternatively, dissolution in DMSO at 100°C is effective.



Amyloglucosidase (AMG) quantitatively hydrolyses maltodextrins to D-glucose (3).



D-Glucose is oxidised to D-gluconate with the release of one mole of hydrogen peroxide (H_2O_2) which is quantitatively measured in a colourimetric reaction employing peroxidase and the production of a quinoneimine dye.



Samples containing high levels of D-glucose and maltodextrins are washed with aqueous ethanol (80% v/v) before analysis.

Analysis of a single sample can be performed within 70 min. Twenty samples can be analysed within 2 h.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

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

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 1.0 mg of D-glucose (or 0.9 mg starch)/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 2.0 mg D-glucose (or 1.8 mg starch)/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 1.00 mL. If the GOPOD absorbance is above 1.1, dilute the sample and re-analyse.

The assay is linear over the range of 5 to 100 μ g of D-glucose per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 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 of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. 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 expected.

INTERFERENCE:

If the conversion of D-glucose has been completed within the time specified in the assay (approx. 15 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-glucose (approx. 50 μ g in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding D-glucose to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50/100 determinations of total starch are available from Megazyme. The kits contain the full assay method plus:

50 determinations assay kit (cat. no. **K-TSTA-50A**)

- Bottle 1:** Thermostable α -amylase (E-BSTAA, 5 mL, 3,000 U/mL on Ceralpha reagent* at pH 6.5 and 40°C or 1,600 U/mL on Ceralpha reagent at pH 5.0 and 40°C).
Stable for > 4 years at 4°C.
- Bottle 2:** Amyloglucosidase (**E-AMGDF**, 5 mL, 3,300 U/mL on soluble starch or 200 U/mL on *p*-nitrophenyl β -maltoside*) at pH 4.5 and 40°C.
Stable for > 3 years at 4°C.
- Bottle 3:** **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.095% w/v).
Stable for > 4 years at 4°C.
- Bottle 4:** **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder.
Stable for > 5 years below -10°C.
- Bottle 5:** D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years at room temperature.
- Bottle 6:** Standardised regular maize starch control. Starch content shown on vial label.
Stable for > 5 years at room temperature.

100 determinations assay kit (cat. no. **K-TSTA-100A**)

- Bottle 1:** Thermostable α -amylase (E-BSTAA, 10 mL, 3,000 U/mL on Ceralpha reagent* at pH 6.5 and 40°C or 1,600 U/mL on Ceralpha reagent at pH 5.0 and 40°C).
Stable for > 4 years at 4°C.
- Bottle 2:** Amyloglucosidase (**E-AMGDF**, 10 mL, 3,300 U/mL on soluble starch or 200 U/mL on *p*-nitrophenyl β -maltoside*) at pH 4.5 and 40°C.
Stable for > 3 years at 4°C.
- Bottle 3:** **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.095% w/v).
Stable for > 4 years at 4°C.

- Bottle 4:** **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder.
Stable for > 5 years below -10°C.
- Bottle 5:** D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years at room temperature.
- Bottle 6:** Standardised regular maize starch control. Starch content shown on vial label.
Stable for > 5 years at room temperature.

*Full assay procedure is available at “www.megazyme.com” - Product Codes: **K-CERA** (bottle 1) and **R-AMGR3** (bottle 2).

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

- Solution 1.** 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). Store the diluted enzyme frozen between use. Divide into appropriately sized aliquots and store in polypropylene tubes below -10°C between use and keep cool during use if possible.
Stable for > 3 years below -10°C.

NOTE:

If the sample is to be analysed according to AOAC Official Method 996.11 (example b), the enzyme is diluted in MOPS buffer (50 mM, pH 7.0; Reagent 4)

- Solution 2.** Use the contents of bottle 2 as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser, e.g. Eppendorf Multipipette® with 5.0 mL Combitip® (to dispense 0.1 mL aliquots).
Stable for > 3 years at 4°C.

- Solution 3.** Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water. Use immediately.

NOTE:

1. 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.
2. This buffer contains 0.095% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

Solution 4. Dissolve the contents of bottle 4 in 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is Glucose Determination Reagent (**GOPOD Reagent**). Stable for ~ 3 months at 2-5°C or > 12 months below -10°C.

If this reagent is to be stored in the frozen state, preferably it should be divided into aliquots. Do not freeze/thaw more than once.

When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink colour over 2-3 months at 4°C. The absorbance of this solution should be less than 0.05 when read against distilled water.

Solutions 5 & 6. Use the contents of bottles 5 and 6 as supplied. Stable for > 5 years at room temperature.

REAGENTS (NOT SUPPLIED):

- 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 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 30 mL is required).

Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 L and store the buffer at 4°C. Stable for approx. 2 months at 4°C.

The stability of this buffer can be increased by adding sodium azide (0.2 g of sodium azide/L buffer).

Stable for approx. 2 years at room temperature.

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

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

Add 68.7 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1 L with distilled water. Stable for 12 months at room temperature.

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.

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 example (b).

Dissolve 11.55 g of MOPS (sodium salt, Sigma cat. no. M9381) in 900 mL of distilled water and adjust the pH to pH 7.0 by the addition of 1 M (10% v/v) HCl (approx. 17 mL is required). 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.

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 example (b).

Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.5 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 60 mL is required). Add 0.2 g of sodium azide and dissolve. Adjust the volume to 1 L. Stable for 6 months at 4°C.

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

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 120 mm or 18 x 150 mm).
2. Micro-pipettors, 100 μL (e.g. Gilson Pipetman[®] or Rainin EDP-2[®] motorised dispenser).
3. Positive displacement pipettor, e.g. Eppendorf Multipette[®]
 - with 5.0 mL Combitip[®] (to dispense 0.1 mL aliquots of amyloglucosidase solution).
 - with 50 mL Combitip[®] (to dispense 3 mL aliquots of bacterial α -amylase solution).
4. Bench centrifuge (required speed 3,000 rpm; approx. 1,800 g).
5. Analytical balance.
6. Spectrophotometer set at 510 nm.
7. Vortex mixer (e.g. IKA[®] Yellowline Test Tube Shaker TTS2).
8. Thermostated water bath set at 50°C.
9. Boiling water bath with tube rack.
10. Stop clock.

CONTROLS AND PRECAUTIONS:

1. The time of incubation with GOPOD reagent is not critical but should be at least 20 min. The colour formed should be measured within 60 min.
2. With each set of determinations, reagent blanks and glucose controls (100 μg , quadruplicate) should be included.
 - a) The reagent blank consists of 0.1 mL distilled water + 3.0 mL GOPOD Reagent.
 - b) The glucose control consists of 0.1 mL glucose standard solution (100 $\mu\text{g}/0.1\text{ mL}$) + 3.0 mL GOPOD Reagent. The Factor "F" (pages 14 and 15) is calculated by dividing the amount of D-glucose analysed (100 μg) by the absorbance obtained for this amount of D-glucose in the standard assay (e.g. $100/1.038 = 96.339$). **The absorbance value will vary.**
3. With each set of determinations, a standard flour or starch sample should be included.
4. With each new batch of GOPOD Reagent, the time for maximum colour formation with 100 μg of glucose standard should be checked. This is usually approx. 15 min.

SAMPLE BLANKS:

Sample blanks can be determined using the Standard Assay Procedure [example (a)] with the modifications that in Step 4, three (3) mL of distilled water is used and in Step 5, amyloglucosidase is replaced by water. Alternatively, the need to perform sample blank analysis can be avoided by pre-extraction of samples with aqueous ethanol (80% v/v) [see example (e)].

SAMPLE PREPARATION EXAMPLES:

(a) 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 cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~ 100 mg; weighed accurately) to a glass test tube (16 x 120 mm). Tap the tube to ensure that all of the sample drops 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 (contents of bottle 1 diluted 1:30 in Reagent 1; 100 mM sodium acetate buffer, pH 5.0). Incubate the tube in a boiling water bath for 6 min (Stir the tube vigorously after 2, 4 and 6 min).

NOTE: In this step it is essential that the tube 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 polypropylene 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 0.1 mL of the contents of bottle 2 (amyloglucosidase, 330 U on starch). Stir the tube on a vortex mixer and incubate 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. Centrifuge an aliquot of this solution at 3,000 rpm for 10 min. Use the clear, undiluted filtrate for the assay.

ALTERNATIVELY, at Step 6, adjust the volume to 10 mL with distilled water and then centrifuge the tubes at 3,000 rpm for 10 min. For samples containing 1-10% starch content, this solution is used directly in Step 7. For samples containing 10-100% starch, an aliquot (1.0 mL) is diluted to 10 mL with distilled water before proceeding to Step 7.

7. Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).
8. Add 3.0 mL of GOPOD Reagent to each tube (including the D-glucose controls and reagent blanks) and incubate the tubes at 50°C for 20 min.
9. **D-Glucose controls** consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent. **Reagent Blank solutions** consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.
10. Read the absorbance for each sample, and the D-glucose control at 510 nm against the reagent blank.

(b) Determination of starch in cereal and food products not containing resistant starch, D-glucose and/or maltodextrins (AOAC Official Method 996.11).

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~ 100 mg; weighed accurately) to a glass test tube (16 x 120 mm). Tap the tube to ensure that all of the sample drops 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 (contents of bottle 1 diluted 1:30 in Reagent 4; 50 mM MOPS buffer, pH 7.0). Incubate the tube in a boiling water bath for 6 min (Stir the tube vigorously after 2, 4 and 6 min).

NOTE: In this step it is essential that the tube 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 polypropylene 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 sodium acetate buffer (4 mL, 200 mM, pH 4.5), followed by amyloglucosidase (0.1 mL, 20 U). Stir the tube on a vortex mixer and incubate at 50°C for 30 min.
6. Proceed according to **Step 6 of example (a)**.

(c) Determination of total starch content of samples containing resistant starch, but no D-glucose and/or maltodextrins (KOH Format - Recommended).

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~ 100 mg, weighed accurately) to a glass tube (16 x 120 mm).
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 (5 x 15 mm) and 2 mL of 2 M KOH to each tube and re-suspend the pellets (and dissolve the RS) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 1, page 19).

NOTE:

1. Do not mix on a vortex mixer as this may cause the starch to emulsify.
 2. Ensure that the tube contents are vigorously stirring 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 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL of thermostable α -amylase (bottle 1) and 0.1 mL of AMG (bottle 2), 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 content;** quantitatively transfer the contents of the tube to a 100 mL volumetric flask (using a water wash bottle). Use an external magnet to retain the stirrer bar in the tube while washing the solution from the tube with a water wash bottle. Adjust to 100 mL with distilled water and mix well. Centrifuge an aliquot of the solution at 1,800 g for 10 min.
 8. **For samples containing < 10% total starch content;** directly centrifuge the tubes at 1,800 g for 10 min (no dilution). For

such samples, 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 7 of example (a)**.

(d) 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 cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~ 100 mg, weighed accurately) to a glass tube (16 x 120 mm).
3. Wet with 0.2 mL of aqueous ethanol (80% v/v) to aid dispersion, and 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 examples (a) or (b)**.

(e) Determination of starch in samples which also contain D-glucose and/or maltodextrins.

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~ 100 mg, weighed accurately) to a glass centrifuge tube (16 x 120 mm; 17 mL capacity).
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 1,800 g (approx. 3,000 rpm) on a bench centrifuge. Discard 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 example (a) or (b)**.

Alternatively:

Proceed from **Step 4 of example (c)** if the sample contains resistant starch.

(f) 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 AMG (bottle 2) diluted 10-fold in Reagent 1 (i.e. 33 U of AMG on starch) and incubate in a water bath at 50°C for 30 min. Adjust volume to 20 mL (or 20 g) with distilled water.
3. Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).
4. Add 3.0 mL of GOPOD Reagent to each tube (including D-glucose controls and reagent blanks) and incubate the tubes at 50°C for 20 min.
5. **D-Glucose controls** consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent. **Reagent Blank solutions** consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.
6. Read the absorbance for each sample and the D-glucose control at 510 nm against the reagent blank.

(g) 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. To this, 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 1,800 g for 10 min.
3. Decant the supernatant solution and redissolve the starch containing pellet in 1 mL of water. If necessary, 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 necessary, repeat the ethanol precipitation and centrifugation steps (e.g. for samples containing high levels of free D-glucose and/or maltodextrins). Decant the supernatant solution and redissolve the starch containing pellet in 1 mL of water.

If necessary, heat the tube and contents at 100°C to aid redissolution. Adjust the volume to 3.9 mL (3.9 g) with Reagent I (100 mM acetate buffer, pH 5.0), taking account of the original weight of the tube.

5. Add 0.1 mL of AMG (bottle 2) diluted 50-fold in Reagent I (i.e. 6.6 U of AMG on starch) and incubate in a water bath at 50°C for 30 min.
6. Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).
7. Add 3.0 mL of GOPOD Reagent to each tube (including D-glucose controls and reagent blanks) and incubate the tubes at 50°C for 20 min.
8. **D-Glucose controls** consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent. **Reagent Blank solutions** consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.
9. Read the absorbance for each sample, and the D-glucose control at 510 nm against the reagent blank.

(h) Determination of enzyme resistant starch.

This can be accurately measured using the Resistant Starch assay kit (**K-RSTAR**) supplied by Megazyme. Results obtained closely simulate those obtained under *in vivo* conditions. Details of this method can be obtained from the Megazyme website (www.megazyme.com; **K-RSTAR**). This method has been successfully subjected to interlaboratory evaluation (37 labs, 16 samples) under the auspices of AOAC INTERNATIONAL (AOAC Official Method 2002.02)^{12, 13} and AACC International (Recommended Method 32-40.01).

CALCULATIONS (Solid samples):

$$\begin{aligned} \text{Starch, \%} &= \Delta A \times F \times \frac{FV}{0.1} \times \frac{I}{1000} \times \frac{100}{W} \times \frac{162}{180} \\ &= \Delta A \times \frac{F}{W} \times FV \times 0.9 \end{aligned}$$

where:

ΔA = absorbance (reaction) read against the reagent blank.

F = $\frac{100 \text{ } (\mu\text{g of D-glucose})}{\text{absorbance for 100 } \mu\text{g of glucose}}$ (conversion from absorbance to μg)

FV = final volume (i.e. equals 100 mL or 10 mL in examples (a) and (b); 100 mL or 10.4 mL in example (c); 100 mL or 10.0 mL in example (d); and 100 mL or 10 mL in example (e).

0.1 = volume of sample analysed.

$\frac{1}{1000}$ = conversion from μg to mg.

$\frac{100}{W}$ = factor to express "starch" as a percentage of flour weight.

W = the weight in milligrams ("as is" basis) of the flour analysed.

$\frac{162}{180}$ = adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).

Starch % w/w (dry wt. basis):

$$= \text{Starch \% w/w (as is)} \times \frac{100}{100 - \text{moisture content (\% w/w)}}$$

CALCULATIONS (Liquid samples; mg/100 mL):

$$\begin{aligned} \text{Starch} &= \Delta A \times F \times \frac{100}{0.1} \times \frac{1}{1000} \times \frac{162}{180} \times 2 \times D \\ &= \Delta A \times F \times D \times 1.8 \end{aligned}$$

where:

ΔA = absorbance (reaction) read against the reagent blank.

F = $\frac{100 \text{ } (\mu\text{g of D-glucose})}{\text{absorbance for 100 } \mu\text{g of glucose}}$ (conversion from absorbance to μg)

100 = conversion to 100 mL sample volume.

0.1 = volume of sample analysed.

$\frac{1}{1000}$ = conversion from μg to mg.

$\frac{162}{180}$ = adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).

2 = dilution of the sample solution on incubation with AMG.

D = further dilution of the incubation mixture (if required).

NOTE: These calculations can be simplified by using the Megazyme **Mega-Calc™**, for solid or liquid samples, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

REFERENCES:

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Table 1. Comparison of total starch values determined with AOAC Method 996.11 and the current method in which α -amylase and amyloglucosidase incubations were performed at pH 5.

Sample	Total starch (<i>as is basis</i>) ^a	
	AOAC Method 996.11	Current Method (pH 5)
Regular maize starch	85.0	84.8
Wheat flour	69.1	68.7
High amylose maize starch ^b	76.8	77.2
ACS soluble starch	83.5	83.4
Chemically modified starch	81.7	82.7
Oat bran	37.2	37.0

^a The average of duplicate analyses by two separate analysts.

^b high amylose starch (total starch value is underestimated).

Table 2. Comparison of total starch values determined with AOAC Method 996.11 (DMSO modification) and the current modification in which starch is dissolved in 2 M KOH, pH adjusted and α -amylase and amyloglucosidase incubations performed at pH 5.

Sample	Total starch (<i>as is basis</i>) ^a	
	AOAC Method 996.11	Current Method (KOH / pH 5)
Regular maize starch	84.3	85.2
High amylose maize starch ^b	83.2	83.4
Potato amylose ^b	86.1	86.7
Novelose 240 ^c	84.5	84.7
Hylon VII ^b	85.1	84.9

^a The average of duplicate analyses by two separate analysts.

^b native high amylose starch.

^c retrograded high amylose starch.

Table 3. Results of an interlaboratory evaluation of the total starch assay procedure (AOAC Official Method 996.11; examples “b” and modification with DMSO).

Sample	Chicken Feed Pellets	White Bread	Green Pea	High Amylose Maize Starch*	White Wheat Flour
Moisture %	11.4	10.7	12.4	13.4	12.8
No. of labs.	32	32	31	25	31
Outliers	0	0	1 ^G	1 ^C	1 ^C
Average %	50.7	68.1	44.0	86.3	78.0
S_r	1.6	1.8	1.5	2.5	2.2
RSD_r	3.1	2.7	3.4	2.9	2.9
r	4.4	5.2	4.2	7.0	6.3
S_R	2.4	3.4	2.1	4.1	3.3
RSD_R	4.6	5.0	4.8	4.8	4.2
R	6.6	9.5	6.0	11.6	9.2
HORRAT	2.1	2.4	2.1	2.8	2.0
Range	45.4-55.3	62.0-74.9	39.4-47.4	78.7-96.8	71.6-85.8

Table 3. (cont.)

Sample	Wheat Starch*	Oat Bran	Spaghetti	High Amylose Maize Starch DMSO procedure	Wheat Starch DMSO procedure
Moisture %	12.3	8.8	11.8	13.4	12.3
No. of labs.	26	31	31	31	31
Outliers	0	1 ^C	1 ^C	1 ^C	1 ^C
Average %	97.2	42.2	76.6	97.2	96.5
S_r	3.2	1.6	3.0	2.0	3.0
RSD_r	3.3	3.8	3.9	2.1	3.1
r	9.0	4.5	8.4	5.7	8.4
S_R	3.7	2.1	3.7	2.8	4.4
RSD_R	3.8	5.0	4.8	2.9	4.6
R	10.4	6.0	10.3	7.8	12.4
HORRAT	1.9	2.2	2.3	1.4	2.3
Range	91.8-105.0	38.7-46.8	70.1-81.8	91.6-101.9	86.0-104.0

Number of Labs. = Number of laboratories included in calculations

Outliers = Number of outlier laboratories, not included in calculations (C=Cochran, G=Grubbs outlier)

S_r = Repeatability standard deviation

RSD_r = Repeatability relative standard deviation

r = Repeatability value ($2.8 \times S_r$)

S_R = Reproducibility standard deviation

RSD_R = Reproducibility relative standard deviation

R = Reproducibility value ($2.8 \times S_r$)

HORRAT = Horwitz ratio, an indication of the precision of the method

* = With these samples, only 26 sets of results were supplied due to a misinterpretation of instructions

In this table the statistical evaluation of results from an interlaboratory evaluation of the methods is shown. Thirty-two laboratories (worldwide) were involved and sixteen samples (eight blind duplicates) were analysed.



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