

TOTAL STARCH HK ASSAY PROTOCOL

K-TSHK

02/22

100 Assays per Kit



A Modification of:
AOAC Method 996.11
AACC Method 76.13.01
ICC Standard Method No. 168



Megazyme

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 produced 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.¹⁰ Consequently, we have updated our total starch methodology to incorporate such an enzyme.¹¹ 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), which is resistant to hydrolysis by amyloglucosidase and α -amylase. The method has been further modified by adjusting the D-glucose determination to a hexokinase/glucose-6-phosphate dehydrogenase/NADP⁺ based format.

The Megazyme total starch analysis procedure (**K-TSHK**) allows the measurement of total starch in most 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

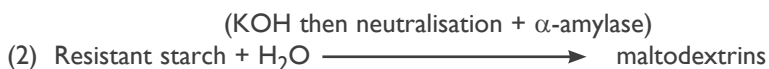
of thermostable α -amylase. Samples containing high levels of resistant starch (e.g. high amylose maize starch) require pre-dissolution in cold 2 M KOH and samples high in D-glucose and maltodextrins are washed with aqueous ethanol before analysis. For samples containing only soluble starch or maltodextrins, cooking with thermostable α -amylase is not required.

PRINCIPLE:

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



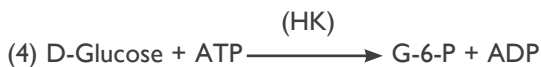
Where necessary, resistant starch in the sample is pre-dissolved by stirring the sample with 2 M KOH at approx. 4°C, followed by neutralisation with sodium acetate buffer and hydrolysis with α -amylase (2).



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



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



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



The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose. It is the NADPH which is measured by the increase in absorbance at 340 nm (Figure 1, page 14).

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 0.324 mg of D-glucose (or 0.292 mg starch)/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.649 mg D-glucose (or 0.584 mg starch)/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 4 to 80 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 2.00 mL, this corresponds to a D-glucose concentration of approx. 0.162 to 0.324 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. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-glucose (approx. 40 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 100 assays are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1:** Thermostable α -amylase [12 mL, 8,300 U/mL on soluble starch (or 3,000 U/mL on Ceralpha reagent)¹² at pH 5.0 and 40°C]. Stable for > 3 years at 4°C.
- Bottle 2:** Amyloglucosidase [10 mL, 3,300 U/mL on soluble starch (or 200 U/mL on *p*-nitrophenyl β -maltoside)¹³] at pH 4.5 and 40°C. Stable for > 3 years at 4°C.
- Bottle 3:** Buffer (15 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
- Bottle 4:** NADP⁺ plus ATP. Stable for > 5 years below -10°C.
- Bottle 5:** Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.25 mL. Stable for > 2 years at 4°C.
- Bottle 6:** D-Glucose standard solution (5 mL, 1.0 mg/mL). Stable for > 5 years; store sealed at room temperature.
- Bottle 7:** Standardised regular maize starch control. Starch content shown on vial label. Stable for > 5 years; store sealed at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dilute 1.0 mL of the contents of bottle 1 to 30 mL with Reagent I (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.
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 Multipette[®] with 5.0 mL Combitip[®] (to dispense 0.1 mL aliquots). Stable for > 3 years at 4°C.
3. Use the contents of bottle 3 as supplied. Stable for > 2 years at 4°C.

4. Dissolve the contents of bottle 4 in 12 mL of distilled water. **Stable for ~ 4 weeks at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).
5. Use the contents of bottle 5 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.
6. Use the contents of bottle 6 as supplied.
Stable for > 5 years; store sealed at room temperature.
7. Use the contents of bottle 7 as supplied.
Stable for > 5 years; store sealed at room temperature.

REAGENTS (NOT SUPPLIED):

1. 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 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 30 mL is required). Stable for approx. 2 months at 4°C.

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 > 6 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. Reagent 2: Sodium acetate buffer (1.2 M, pH 3.8).

Add 68.6 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. 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.

4. **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 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. **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 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 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman[®] (20 μ L and 100 μ L).
4. Positive displacement pipettor, e.g. Eppendorf Multipette[®]
 - with 5.0 mL Combitip[®] (to dispense 0.1 mL aliquots of amyloglucosidase solution, imidazole buffer and NADP⁺/ATP solution).
 - with 25 mL Combitip[®] (to dispense 2.0 mL aliquots of distilled water).
 - with 50 mL Combitip[®] (to dispense 3.0 mL aliquots of bacterial α -amylase solution).
5. Bench centrifuge (required speed 3,000 rpm; approx. 2,000 g).
6. Analytical balance.
7. Spectrophotometer set at 340 nm.
8. Vortex mixer (e.g. IKA[®] Yellowline Test Tube Shaker TTS2).
9. Thermostated water bath set at 40°C.
10. Boiling water bath with tube rack.
11. Stop clock.
12. Whatman No. 1 (9 cm) filter papers.

PROCEDURE:

Wavelength:	340 nm
Cuvette:	1 cm light path (glass or plastic)
Temperature:	~ 25°C
Final volume:	2.27 mL
Sample solution:	4-80 µg of D-glucose per cuvette (in 0.05-2.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.05 mL	2.00 mL
sample	-	0.05 mL
solution 3 (buffer)	0.10 mL	0.10 mL
solution 4 (NADP ⁺ /ATP)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A ₁) after approx. 3 min and start the reactions by addition of:		
suspension 5 (HK/G-6-PDH)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A ₂) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®

** if the absorbance continues to increase, this may be due to effects of colour compounds or enzymes in the sample. These interfering substances may be removed during sample preparation.

CONTROL:

With each set of determinations, a standard flour or starch sample should be included.

CALCULATION:

Determine the absorbance difference (A₂-A₁) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining ΔA_{D-glucose}. The value of ΔA_{D-glucose} should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of starch can be calculated as follows:

$$c = \frac{V \times MW}{\epsilon \times d \times v} \times \frac{162}{180} \times \Delta A_{D\text{-glucose}} \quad [\text{g/L}]$$

where:

- V = final volume [mL]
MW = molecular weight of D-glucose [g/mol]
 ϵ = extinction coefficient of NADPH at 340 nm
= 6300 [$\text{l} \times \text{mol}^{-1} \times \text{cm}^{-1}$]
d = light path [cm]
v = sample volume [mL]
162/180 = Adjustment from free D-glucose to anhydro D-glucose
(as occurs in starch).

It follows for starch:

$$c = \frac{2.27 \times 180.16}{6300 \times 1.0 \times 0.05} \times \frac{162}{180} \times \Delta A_{\text{D-glucose}} \quad [\text{g/L}]$$
$$= 1.1685 \times \Delta A_{\text{D-glucose}} \quad [\text{g/L}]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of starch:

$$= \frac{c_{\text{starch}} \text{ [g/L sample solution]} \times 100}{\text{weight}_{\text{sample}} \text{ [g/L sample solution]}} \quad [\text{g/100 g}]$$

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

SAMPLE PREPARATION:

Sample dilution.

The amount of D-glucose present in the cuvette (i.e. in the 0.05 mL of sample being analysed) should range between 4 and 80 μg . The sample solution must therefore be diluted sufficiently to yield a D-glucose concentration between 0.08 and 1.6 g/L.

Dilution Table

Estimated concentration of D-glucose (g/L)	Dilution with water	Dilution factor (F)
< 1.6	No dilution required	1
1.6-16	1 + 9	10
16-160	1 + 99	100
> 160	1 + 999	1000

If the value of $\Delta A_{D\text{-glucose}}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.05 mL and using the new sample volume in the equation.

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.

5. Place the tube in a bath at 50°C. Add 0.1 mL of the contents of bottle 2 (amylglucosidase, 20 U). 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. Centrifuge an aliquot of this solution at 3,000 rpm for 10 min. Use the clear, undiluted filtrate for the assay. Typically, no further dilution is required and a sample volume of 0.05-0.10 mL is satisfactory.

ALTERNATIVELY, at Step 6, if the starch content is unknown and is thought to be less than 10% w/w, adjust the volume to 10 mL (10 g) with distilled water (taking note of the original weight of sample plus tube) 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. Typically, a sample volume of 0.05-0.10 mL is satisfactory.

For samples containing 10-100% starch, dilute 1.0 mL of this solution to 10 mL with distilled water before analysis. Typically, a sample volume of 0.05-0.10 mL is satisfactory.

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 4 mL of Reagent 5 (200 mM sodium acetate buffer, 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 product to pass a 0.5 mm screen.
2. Add flour 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 Reagent 3 (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 2, page 15).

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 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 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. Proceed from **step 6 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. Use the clear, undiluted filtrate for the assay. Typically, no further dilution is required and a sample volume of 0.1-0.2 mL is satisfactory.

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 water, taking account of the original weight of the tube.
5. Add 0.1 mL of AMG (bottle 2) diluted 50-fold in Reagent 1 (i.e. 6.6 U of AMG on starch) and incubate in a water bath at 50°C for 30 min.
6. If the solution is turbid, centrifuge the tube at 2,000 g for 10 min in a bench centrifuge (or at 12,000 rpm in a Microfuge). Use the clear filtrate for the assay. Typically, either no dilution, or a dilution of 10-fold is required and a sample volume of 0.05-0.1 mL is satisfactory.

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).

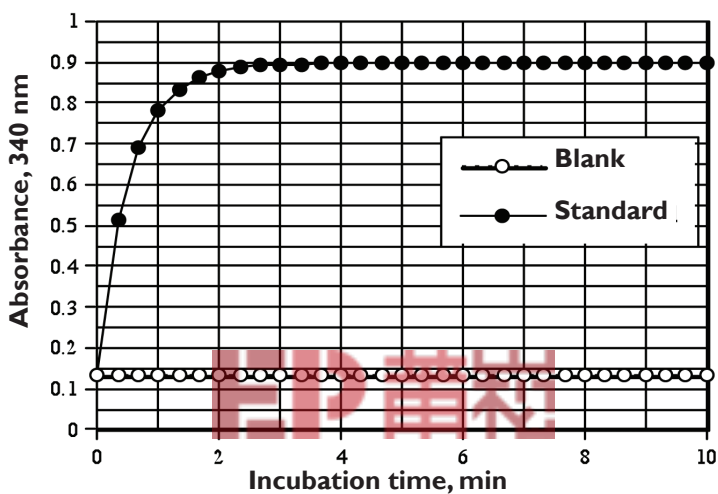


Figure 1. Increase in absorbance at 340 nm on incubation of 50 μg of D-glucose with hexokinase and glucose 6-phosphate dehydrogenase in the presence of NADP^+ and ATP.

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Figure 2. Arrangement of ice-water bath over a magnetic stirrer for treatment of samples with 2 M KOH and dissolution of RS.

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, D-glucose was determined with hexokinase/glucose-6-phosphate dehydrogenase.

Sample	Total starch (as is basis) ^a	
	AOAC Method 996.11	Current Method (pH 5, HK)
Regular maize starch	85.7	86.7
Wheat flour	68.4	69.6
High maize starch ^b	77.8	77.3
ACS soluble starch	83.5	83.4
Novelose 240 ^b	66.3	66.3
Hylon VII ^b	76.0	77.5
Amylose (potato) ^b	64.5	64.5
Oat bran	34.7	34.6
Chicken feed	9.3	9.4

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

^b high amylose starches.

Table 2. Results of an interlaboratory evaluation of the total starch assay procedure (AOAC Official Method 996.11; examples “b” and modification with DMSO). [Note: glucose assays were performed with GOPOD reagent]

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 2. (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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