

# TOTAL STARCH ( $\alpha$ -AMYLASE / AMYLOGLUCOSIDASE)

## ASSAY PROTOCOL

**SKU: 700004351**  
**K-TSTA-100A**

02/24

100 Assays per Kit

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



## INTRODUCTION:

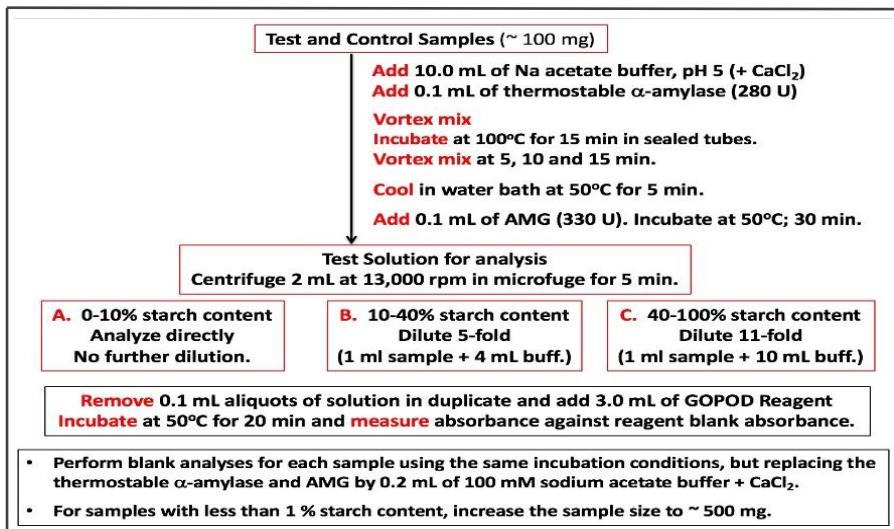
Starch is the major source of energy in the human diet. It is contained in many staple foods such as cereals, legumes, root vegetables and fruits. Widely used foods that contain starch are bread, pasta and breakfast cereals.<sup>1</sup> Starch also comprises a significant portion of many foods and feeds for animals and finds numerous industrial applications in both its native and chemically modified forms. Starch broadly comprises two components, amylose which is predominantly a 1,4- $\alpha$ -linked D-glucan containing very few 1,6- $\alpha$ -linked branch points; and amylopectin, which has a highly branched structure with a high proportion of 1,6- $\alpha$ -linked 1,4- $\alpha$ -D-glucan chains. Other polysaccharides with very similar structures are glycogen from animal tissues, phytglycogen from plant sources and some  $\alpha$ -glucans from fungi. These are all hydrolysed by the digestive enzymes in the human and animal small intestine and thus behave identically from a nutritional standpoint. For this reason, the Laboratory Methods and Services Committee of the Association of American Feed Control Officials (AAFCO)<sup>2</sup> with the involvement of industry and researchers developed a definition for “Dietary Starch”, namely “an  $\alpha$ -linked-glucose carbohydrate of or derived from plants, animals and microbes from which glucose is released after gelatinisation through the use of purified  $\alpha$ -amylases and amyloglucosidases (AMG) that are specifically active only on  $\alpha$ -(1-4) and  $\alpha$ -(1-6) linkages. Its concentration in feed is determined by enzymatically converting the  $\alpha$ -linked-glucose carbohydrate to glucose and then measuring the liberated glucose. This definition would encompass plant starch, glycogen, maltodextrins, and maltose/ isomaltose”.<sup>3</sup>

Historically, a range of methods have been employed for the measurement of starch, but in recent decades, the method of choice involves gelatinisation of the starch at elevated temperatures in the presence of a heat stable  $\alpha$ -amylase to produce a range of linear and branched dextrins, which are subsequently quantitatively hydrolysed to glucose with AMG.<sup>4-11</sup> Released glucose is measured with a glucose oxidase/peroxidase (GOPOD) reagent or employing hexokinase with glucose 6-phosphate dehydrogenase in the presence of ATP and NADP<sup>+</sup>. The use of heat stable  $\alpha$ -amylase with AMG in the measurement of starch was first introduced by Baur and Alexander,<sup>4</sup> and was streamlined by McCleary *et al*<sup>10</sup> and subjected to a combined AOAC International/AACC International/ICC interlaboratory evaluation. Following this successful study, the method was adopted as AOAC Method 996.11, AACC International Method 76-13.01 and ICC Method 168.

In a recently published method for the measurement of dietary starches in animal feeds and pet foods (AOAC Method 2014.10),<sup>3,11</sup> incubation was performed at 100°C for 1 h with a heat stable  $\alpha$ -amylase, at pH 5.0. The author reported that this modification gave higher starch values than was obtained with AOAC Method 996.11, and proposed that this was due to the isomerisation of reducing-end D-glucosyl residues in maltodextrins to D-fructose and thus loss of “starch” under the pH/temperature conditions employed with AOAC Method 996.11 and similar methods. The author also reported a non-linear colour response in the measurement of glucose with GOPOD reagent<sup>12</sup> and suggested that all previous

starch methods are “quasi-empirical”. On the basis of the claims by Hall,<sup>3,11,12</sup> we decided to re-evaluate starch analysis methods with the aim of better understanding each step. More specifically, starch liquefaction with  $\alpha$ -amylase, hydrolysis of starch dextrans and sucrose by AMG, maltulose production during starch hydrolysis, and measurement of glucose with GOPOD reagent, have been studied in detail.<sup>13</sup> The outcome of this study demonstrated that AOAC Method 996.11, as described previously, is an accurate and reliable method for measurement of total starch in a broad range of products, including animal feeds and pet foods (**Table 3**), beans and grains (**Table 4**), breakfast cereals (**Table 5**) and vegetables (**Table 6**).

Following this research, a slight modification of AOAC Method 996.11 (employing a thermostable  $\alpha$ -amylase which is active and stable at pH 5) was made to give an even more user-friendly method for measurement of starch. This method is the Rapid Total Starch (RTS) method (outlined in **Figure 1**) and forms the basis for method (a) on page 8 of this assay protocol.



**Figure 1.** A graphic overview of ‘The Rapid Total Starch (RTS) Method’ [method (a)]

The RTS method 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  $\alpha$ -amylase. Samples containing high levels of resistant starch (e.g. high amylose maize starch) require pre-dissolution in cold 1.7 M NaOH or hot DMSO. For samples containing only soluble starch or maltodextrins, incubation with thermostable  $\alpha$ -amylase is not required.

## PRINCIPLE:

Thermostable  $\alpha$ -amylase hydrolyses starch into soluble, branched and unbranched maltodextrins (1).

$\alpha$ -amylase, pH 5.0 or 7.0, 100°C



Where necessary, resistant starch in the sample is pre-dissolved by stirring the sample with cold 1.7 M NaOH, followed by neutralisation with sodium acetate buffer and hydrolysis with  $\alpha$ -amylase (2). Alternatively, dissolution in DMSO at 100°C is effective.

NaOH treatment, neutralisation +  $\alpha$ -amylase



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

AMG

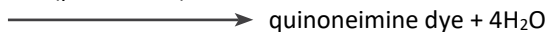


D-Glucose is oxidised to D-gluconate with the release of equimolar amounts of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) which is quantitatively measured in a colourimetric reaction employing peroxidase and the production of a quinoneimine dye (4, 5).

(glucose oxidase)



(peroxidase)



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  $\alpha$ -glucans (including starch, glycogen, phytyglycogen and non-resistant maltodextrins).

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to  $\sim 0.09$  g/100 g total starch "as is" using a sample weight of 100 mg and extract volume of 10.2 mL as per the recommended Total Starch (RTS) Method. The detection limit is  $\sim 0.18$  g/100 g total starch "as is", which is derived from an

absorbance difference of 0.020 with a sample weight of 100 mg and extract volume of 10.2 mL.

The assay is linear over the range of 4 to 100 µg of D-glucose per assay.

#### **INTERFERENCE:**

If the conversion of D-glucose has been completed within the time specified in the assay (approx. 20 min), it can be generally concluded that no interference has occurred.

#### **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 of total starch are available from Neogen. The kits contain the full assay method plus:

**Bottle 1:     **Thermostable α-amylase**** (10 mL, 3,000 U/mL on Ceralpha reagent\* at pH 6.5 and 40°C or 2,500 U/mL on Ceralpha reagent\* at pH 5.0 and 40°C). Store at 4°C or alternatively below -10°C. See individual label for expiry date.

**Bottle 2:     **Amyloglucosidase**** (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. Store at 4°C or alternatively below -10°C. See individual label for expiry date.

\*Full assay procedure is available at "[www.megazyme.com](http://www.megazyme.com)".

**Bottle 3:     **GOPOD Reagent Buffer.****  
Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.09% w/v). Store at 4°C. See individual label for expiry date.

**Bottle 4:     **GOPOD Reagent Enzymes.****  
Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder. Store at or below -10°C. See individual label for expiry date.

**Bottle 5:     **D-Glucose standard solution****  
(5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid. Store sealed at room temperature. See individual label for expiry date.

**Bottle 6: Standardised regular maize starch control.**

Starch content shown on vial label.

Store sealed at room temperature. See individual label for expiry date.

**A. PREPARATION OF REAGENT SOLUTIONS/ SUSPENSIONS:**

1. Use the contents of **bottle 1** as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser with a 5.0 mL tip (to dispense 0.1 mL aliquots).

**NOTE:** If the sample is to be analysed according to AOAC Official Method 996.11 [method (c)], the enzyme should be diluted 30-fold in **buffer [B(e)]** [MOPS buffer (50 mM, pH 7.0) plus calcium chloride (5 mM)] (see page 6 for preparation) before use. This is '**diluted thermostable  $\alpha$ -amylase**'.

2. Use the contents of **bottle 2** as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser with a 5.0 mL tip (to dispense 0.1 mL aliquots).
3. Dilute the contents of the **GOPOD Reagent Buffer** bottle to 1 L with distilled water (this is **solution 1**). 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 0.09% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of the **GOPOD Reagent Enzyme** bottle in 20 mL of **solution 1** and quantitatively transfer this to the bottle containing the remainder of **solution 1**. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is **Glucose Determination Reagent (GOPOD Reagent)**.

Stable for  $\geq 1$  month at 4°C or  $\geq 12$  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 time. The absorbance of this solution should be less than 0.05 when read against distilled water.

- 5.&6.** Use the contents of **bottles 5 & 6** as supplied.

## **B. REAGENTS (not supplied):**

- a. ***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 at 4°C. This is **buffer [B(a)]**.
- b. ***Sodium acetate buffer (200 mM, pH 4.5) plus calcium chloride (5 mM).***— Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the pH to 4.5 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 60 mL is required). Adjust the volume to 1 L and store at 4°C. This is **buffer [B(b)]**.
- c. ***Sodium acetate buffer (600 mM, pH 3.8) plus calcium chloride (5 mM).***— Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 1600 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Add 1.48 g of calcium chloride dihydrate and dissolve. Adjust the volume to 2 L with distilled water and store at room temperature. This is **buffer [B(c)]**.
- d. ***Sodium hydroxide solution (1.7 M).***— Add 68 g NaOH to 900 mL of deionised water and dissolve by stirring. Adjust the volume to 1 L. Store in a sealed container at room temperature.
- e. ***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 procedures (c) and (d). 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 HCl (approx. 17 mL is required). Add 0.74 g of calcium chloride dihydrate and 0.2g of sodium azide and dissolve. Adjust the volume to 1 L and store at 4°C. This is **buffer [B(e)]**.
- f. ***Ethanol (~ 80% v/v) and IMS (~ 80% v/v).***  
Add 800 mL of either ethanol (95% v/v) or industrial methylated spirits (IMS, 95% v/v) to 200 mL of distilled water. Store in a 1 L Duran bottle at room temperature.

## **C. APPARATUS REQUIRED:**

- a. **Grinding mill.**— centrifugal, with 12-tooth rotor and 0.5 mm sieve, or a similar device.
- b. **Bench centrifuge.**— capable of holding 101 x 65 mm polypropylene tubes, with rating of approx. 3,250 rcf (~ 4,000 rpm), e.g. Sigma Laboratory Centrifuges 4-15 No.10730.
- c. **Microfuge centrifuge.**— capable of 13,000 rpm.
- d. **Spectrophotometer.**— capable of operating at 510 nm,(10 mm path length).
- e. **Analytical balance.**— 0.1 mg readability, accuracy and precision.

- f. **Thermostatted water bath.**— set at 50°C.
- g. **Boiling water bath.**— with tube rack.
- h. **Magnetic stirrer.**— e.g. IKA KMO 2™ basic stirrer.
- i. **Magnetic stirring bars.**— Fisherbrand™ PTFE Stir Bars 12 x 6 mm ridged.
- j. **Vortex mixer.**— e.g. Daihan Scientific VM10.
- k. **Pipettors.**— capable of delivering 100 µL or 1.0 mL, e.g. Gilson Pipetman®, with disposable tips.
- l. **Positive displacement pipetter.**— e.g. Brand HandyStep® S
  - with 25 mL Brand PD-Tip® (to dispense 0.5-2.5 mL aliquots).
  - with 5 mL Brand PD-Tip® (to dispense 0.1 mL of α-amylase or AMG solution).
- m. **Dispensers.**— to dispense 4 mL and 10 mL of 100 mM sodium acetate buffer (pH 5.0), e.g. Brand® Bottle-top dispensette® S Digital, 2.5-25 mL, cat. no. 4600351.
- n. **Disposable polypropylene tube.**— 13 mL, 101 x 16.5 mm (e.g. Sarstedt cat no. 60.541.685, Sarstedt Ltd., Drinagh, Co. Wexford, Ireland).
- o. **Disposable 2.0 mL polypropylene microfuge tubes.**— e.g. Sarstedt cat. no. 72.691, Sarstedt Ltd., Drinagh, Co. Wexford, Ireland.
- p. **Glass test tubes.**— 16 x 100 mm, 14 mL capacity.
- q. **Digestion tubes.**— Corning® culture tubes (16 x 120 mm; Fisher cat. no. 14-933C) with screw caps.
- r. **Plastic “lunch box”.**— large enough to hold test-tube rack and serve as an ice-water bath (see **Figure 2**, page 17).

#### **D. CONTROLS AND PRECAUTIONS:**

- a. 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 (see **Figure 3**, page 19).
- b. With each set of determinations, reagent blanks and glucose controls (100 µg, quadruplicate) should be included. The glucose- GOPOD standard curve is perfectly linear (**Figure 4**, page 19).
  - i) The reagent blank consists of 0.1 mL distilled water plus 3.0 mL of **GOPOD Reagent**.
  - ii) The glucose control consists of 0.1 mL **bottle 5** (glucose standard solution) plus 3.0 mL of **GOPOD Reagent**.
- c. With each set of determinations, a standard flour or starch sample should be included.



## E. ANALYSIS OF SAMPLES - EXAMPLE METHODS:

### (a) The Rapid Total Starch (RTS) Method. Determination of starch in cereal and food products not containing resistant starch (Recommended Procedure; all incubations at pH 5.0).

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Accurately weigh ~ 100 mg of test sample, in duplicate (one as a sample blank) into Corning culture tubes (16 x 120 mm) [C(q)]. Record the exact weight. Tap the tube so that sample drops to the bottom of the tube.
3. To both tubes add 10 mL of **buffer [B(a)]** [Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)] using a bottle top dispenser [C(m)] to increase throughput. Stir the tubes vigorously on a vortex mixer for 5 sec.
4. To one of the tubes (sample tube) add 0.1 mL of **bottle 1** (thermostable  $\alpha$ -amylase) [A(1)] using a HandyStep® dispenser [C(l)] with 5 mL tip. To the second tube (sample blank) add 0.1 mL of **buffer [B(a)]** [Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)].
5. Vortex the tubes for 3 sec, cap the tubes loosely and immediately transfer them to a boiling water bath and start the timer. After approx. 2 min, tighten the caps and mix the tube contents vigorously on a vortex mixer. After a further 5 and 10 min, vortex the tube contents again for 5 sec and return the tubes to the boiling water bath. After 15 min (from addition of  $\alpha$ -amylase), remove tubes from the boiling water bath and mix the contents vigorously for 5 sec on a vortex mixer. Place the tubes in a water bath at 50°C and allow them to equilibrate to temperature over 5 min.
6. To one of the tubes (the sample tube), add 0.1 mL of **bottle 2** (Amyloglucosidase) [A(2)] using a HandyStep® dispenser with 5 mL tip and vortex for 3 sec. To the second tube (the sample blank) add 0.1 mL of **buffer [B(a)]** [Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)]. Incubate the tubes at 50°C for 30 min with no further mixing.
7. Remove the tubes from the water bath and allow them to cool to room temperature for 10 min. Invert the tubes a few times to ensure condensed water on the inside of the lid is mixed with liquid in the tube.
8. Transfer 2.0 mL of each solution (sample and sample blank) to microfuge tubes [C(o)] and centrifuge the tubes at 13,000 rpm for 5 min (Retain the remaining 8.2 mL of incubation solution and refer to the NOTE below). Using a Gilson Pipetman dispenser, accurately transfer a 1.0 mL aliquot of the supernatants to 12 x 120 mm tubes containing 4 mL of **buffer [B(a)]** [Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)] and mix the contents.
9. Accurately transfer duplicate 0.1 mL aliquots of each sample to the bottoms

of 16 x 120 mm glass test tubes. Also transfer a single 0.1 mL aliquot of sample blanks to a 16 x 120 mm glass test tube.

10. Add 3.0 mL of **GOPOD reagent** and incubate the solutions at 50°C for 20 min and measure absorbance against the reagent blank at 510 nm.

**Concurrently incubate:**

**Glucose controls:** 0.1 mL of **bottle 5** (glucose standard solution, 1.0 mg/mL) plus 3.0 mL of **GOPOD reagent**, in quadruplicate.

**Reagent Blank:** 0.1 mL of distilled water with 3.0 mL of **GOPOD reagent** in duplicate.

11. Calculate starch content (see Section F, page 14).

**NOTE:** For this extraction protocol, the final **Extract Volume (EV) = 10.2**

**NOTE 1:** If the GOPOD absorbance values for samples are less than 0.100, analyse the centrifuged sample solution (step 8) without further dilution. If absorbance values are greater than 1.0, then add 1.0 mL of the centrifuged sample solution to 10.0 mL of **buffer [B(a)]** [Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)], mix well and remove 0.1 mL aliquots for analysis using **GOPOD reagent**. For samples containing less than 1% (w/w) starch content, increase sample size to 500 mg and analyse 0.1 mL of undiluted sample solution. **Dilution (D) = 1, 5 or 11**

**NOTE 2:** When fibrous samples such as grasses and silage are being analysed, grind the sample using a Nutri Bullet PRO 900 blender (See **K-TSTA** video). Grind for approx. 60 sec (until the sample is homogeneous).

**b) Determination of total starch content of samples containing resistant starch (RTS-NaOH Procedure - Recommended).**

1. Accurately weigh 100 mg of test sample, in duplicate, into Corning culture tubes (16 x 120 mm) **[C(q)]**. Record the exact weight. Tap the tubes so that sample falls to the bottom of the tubes.
2. Add 0.2 mL of 80% v/v aqueous ethanol and stir the tubes on a vortex mixer to completely wet and disperse the sample (this step is very important in aiding the complete dissolution of samples with a high starch content).
3. Add 2 mL of cold 1.7 M sodium hydroxide solution **[B(d)]** using a HandyStep® dispenser with 25 mL tip and stir the tubes contents on a vortex mixer for 15 sec. Place the tubes in a rack in an ice-water bath over a magnetic stirrer and stir for 15 min (**Figure 2**, page 17). During this time, intermittently stir the contents of the tube vigorously on a vortex mixer 2-3 times. Ensure that there are no lumps in the sample slurry.
4. Add 8 mL of **buffer [B(c)]** [Sodium acetate buffer (600 mM, pH 3.8) plus

calcium chloride (5 mM)] using a bottle top dispenser [*C(m)*] to increase throughput. Stir the tubes on a vortex mixer. Ensure that that the pH is ~ 5.0.

5. Immediately add 0.1 mL of **bottle 1** (thermostable  $\alpha$ -amylase) [*A(1)*] using a HandyStep® dispenser with a 5 mL tip to one of the tubes (sample tube). Then add 0.1 mL of **bottle 2** (Amyloglucosidase) [*A(2)*] using a Handy Step® dispenser with a 5 mL tip to the same tube. To the second tube (sample blank), add 0.2 mL of **buffer [B(a)]** [Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM)]. Cap both tubes and vortex the contents for 3 sec.
6. Incubate the tubes at 50°C for 30 min.
7. Remove the tubes from the water bath and allow them to cool to room temperature. Invert the tubes a few times to ensure condensed water on the inside of the lid is mixed with liquid in the tube.
8. Proceed to step 8 of method **(a)** on page 8.

**NOTE:** For this extraction protocol, the final extraction and dilution values are:

**Extract volume (EV) = 10.4**

**Dilution (D) = 1, 5 or 11**

- c) **AOAC Official Method 996.11- Determination of starch in cereal and food products not containing resistant starch, 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 Corning culture tubes (16 x 120 mm) [*C(q)*]. 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) [*B(f)*] to wet the sample and aid dispersion. Stir the tube on a vortex mixer.
  4. Immediately add 3 mL of **diluted thermostable  $\alpha$ -amylase** [*A(1) Note*] (see page 5 for preparation details). Incubate the tube in a boiling water bath for 6 min, vortexing vigorously after 2, 4 and 6 min (to ensure complete homogeneity).
  5. Place the tube in a bath at 50°C; add 4 mL of **buffer [B(b)]** [Sodium acetate buffer (200 mM, pH 4.5) plus calcium chloride (5 mM)] followed by 0.1 mL of **bottle 2** (Amyloglucosidase) [*A(2)*]. Stir the tube on a vortex mixer and incubate at 50°C for 30 min.
  6. Transfer the entire contents of the tube to a 100 mL volumetric flask (with funnel to assist). Use a wash bottle to rinse the tube contents thoroughly. Adjust to volume with **buffer [B(b)]** [Sodium acetate buffer (200 mM,

pH 4.5) plus calcium chloride (5 mM)] and mix the contents thoroughly.

7. Transfer 2.0 mL of each solution to a microfuge tube [*C(o)*] and centrifuge the tube at 13,000 rpm for 5 min.
8. Accurately transfer duplicate 0.1 mL aliquots of each sample to the bottoms of 16 x 120 mm glass test tubes.
9. Add 3.0 mL of **GOPOD reagent** and incubate the solutions at 50°C for 20 min and measure absorbance against the reagent blank at 510 nm.
10. Calculate starch content (see Section F, page 14).

**NOTE:** For this extraction protocol, the final extraction and dilution values are:

**Extract volume (EV) = 100** (see calculations on page 14)

**Dilution (D) = 1**

**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 Corning culture tubes (16 x 120 mm) [*C(q)*].
3. Wet with 0.2 mL of aqueous ethanol (80% v/v) [*B(f)*] 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. Add 3 mL of **diluted thermostable  $\alpha$ -amylase** [*A(1) Note*] to each tube **immediately** as it is being removed from the boiling water bath and mix the contents vigorously for 20 sec on a vortex mixer. Incubate the tube in a boiling water bath for 6 min, vortexing vigorously after 2, 4 and 6 min (to ensure complete homogeneity).
6. Proceed from step 5 of method (c) on page 11.

**NOTE:** For this extraction protocol, the final extraction and dilution values are:

**Extract volume (EV) = 100;**

**Dilution (D) = 1**

- e) **Suggested procedure for the determination of starch in samples which contain D-glucose and/or maltodextrins- removal of D-glucose and maltodextrins with alcohol washing.**

**[Note: solubility of maltodextrins in aqueous ethanol is critically dependent on the DP range of the maltodextrins, the final ethanol concentration and the temperature of the solution].**

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 100 mm; 14 mL capacity) [C(p)].
3. Add 5.0 mL of aqueous ethanol (80% v/v) [B(f)] 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 3,250 rcf (~ 4,000 rpm) on a bench centrifuge [C(b)]. Discard the supernatant.
5. Resuspend the pellet in 5 mL of 80% v/v aqueous ethanol [B(f)] and stir on a vortex mixer. Add another 5 mL of 80% v/v aqueous ethanol and mix by inversion. Centrifuge as in step 4 and carefully pour off the supernatant.
6. Proceed to step 4 of method (c) on page 11.

**Extract volume (EV) = 100; Dilution (D) = 1**

**Alternatively:**

If the sample contains resistant starch, proceed to step 4 of method (d) on page 11.

**Extract volume (EV) = 100; Dilution (D) = 1**

- f) **Determination of starch in samples in which the starch is present in a soluble or suspended form**

1. Mix the sample contents thoroughly by stirring on a vortex mixer or by inversion. If necessary, heat or homogenise the sample to obtain a homogeneous suspension.
2. Immediately transfer 5 mL aliquots in duplicate (sample and sample blank) into two Corning culture tubes (16 x 120 mm) [C(q)] using a positive displacement dispenser. To each tube add 5 mL of **buffer [B(b)]** [sodium acetate buffer (200 mM, pH 4.5) plus calcium chloride (5 mM)] using a bottle top dispenser [C(m)]. Stir the tubes vigorously on a vortex mixer for 5 sec.
3. Proceed according to method (a) from step 4, page 8 for sample and sample blank.
4. Calculate starch content (see section F, page 15 for liquid samples).

**Diluted Sample Volume (DSV) = 10.2 mL**

**Dilution (D) 1, 5 or 11; Sample Volume (SV) = 5 mL**

**g) Determination of the starch content of samples containing resistant starch in a suspended form.**

1. Mix the sample contents thoroughly by stirring on a vortex mixer or by inversion. If necessary, heat or homogenise the sample to obtain a homogeneous suspension.
2. Immediately transfer 2 mL aliquots in duplicate into two Corning culture tubes (16 x 120 mm) [C(q)] using a positive displacement dispenser. Add 2 mL of cold 1.7 M sodium hydroxide solution [B(d)] using a HandyStep® dispenser with 25 mL tip and stir the tube contents on a vortex mixer for 15 sec. Place the tube in a rack in an ice-water bath over a magnetic stirrer and stir for 15 min (Figure 2, page 17). During this time, also intermittently stir the contents of the tube vigorously on a vortex mixer 2-3 times. Ensure that there are no lumps in the sample slurry.
3. Proceed according to method (b) from step 4, page 10 for sample and sample blank.

**Diluted Sample Volume (DSV) = 12.2 mL**

**Dilution (D) x 1, 5 or 11; Sample Volume (SV) = 2 mL**

**h) Determination of enzyme resistant starch.**

Resistant starch is accurately measured using the Resistant Starch assay kit (**K-RSTAR**) or the Rapid Resistant Starch assay kit (**K-RAPRS**) supplied by Megazyme. Results obtained using either **K-RSTAR** or **K-RAPRS** closely simulate those obtained under *in vivo* conditions.<sup>14</sup> The Resistant Starch assay procedure (**K-RSTAR**) has been successfully subjected to interlaboratory evaluation (37 labs, 16 samples) to become AOAC Official Method 2002.02<sup>15</sup> and AACC Method 32-40.01.

## F. CALCULATIONS

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

### 1. Calculations for solid samples:

$$\begin{aligned}\text{Starch, \%} &= \Delta A \times F \times \frac{EV}{0.1} \times D \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \\ &= \Delta A \times F \times EV \times \frac{D}{W} \times 0.90\end{aligned}$$

#### where:

$\Delta A$  = absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against the reagent blank (only where a sample blank is determined).

$F$  = factor to convert absorbance values to  $\mu\text{g}$  glucose (100  $\mu\text{g}$  glucose divided by the GOPOD absorbance value obtained for 100  $\mu\text{g}$  of glucose).

$EV$  = sample extraction volume [10.2 mL for procedure **(a)**, 10.4 for procedure **(b)** and 100 for procedures **(c)** and **(d)**].

0.1 = volume of sample analysed.

$D$  = further dilution of sample solution (either undiluted, or diluted 5-fold or 11-fold) [see method **(a)** **NOTE 1** and **Figure 1**].

1/1000 = conversion from  $\mu\text{g}$  to mg.

100/W = conversion to 100 mg sample;  $W$  = sample weight in mg.

162/180 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch.

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

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

## 2. Calculations for liquid samples (mg/100 mL):

$$\begin{aligned} \text{Starch} &= \Delta A \times F \times \frac{\text{DSV}}{\text{SV}} \times \frac{100}{0.1} \times \frac{1}{1000} \times \frac{162}{180} \times D \\ &= \Delta A \times F \times \text{DSV/SV} \times 0.9 \end{aligned}$$

Starch (g/100 g of sample)

$$= \text{mg/100 mL} \times 100/\text{sample dry weight (mg/100 mL)}$$

### **where:**

- $\Delta A$  = Absorbance (reaction) read against the reagent blank minus the absorbance of the sample blank read against the reagent blank.
- F = factor to convert absorbance values to  $\mu\text{g}$  of D-glucose) (= 100  $\mu\text{g}$  of D-glucose divided by the GOPOD absorbance value for 100  $\mu\text{g}$  of D-glucose).
- DSV = Diluted Sample Volume (i.e. volume of sample after adding acetate buffer plus  $\alpha$ -amylase and AMG).
- SV = volume of sample taken for analysis (5 mL for example (f) and 2 mL for example (g)).
- 100 = conversion to 100 mL sample volume.
- 0.1 = volume of sample analysed.
- 1/1000 = conversion from  $\mu\text{g}$  to mg.
- 162/180 = adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).
- D = further dilution of the incubation mixture (if performed).



**Table 1.** Results of an interlaboratory evaluation of the total starch assay procedure [AOAC Official Method 996.11; methods (c) and (d)].<sup>10</sup>

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 <sup>G</sup>	1 <sup>C</sup>	1 <sup>C</sup>
Average %	50.7	68.1	44.0	86.3	78.0
S <sub>r</sub>	1.6	1.8	1.5	2.5	2.2
RSD <sub>r</sub>	3.1	2.7	3.4	2.9	2.9
r	4.4	5.2	4.2	7.0	6.3
S <sub>R</sub>	2.4	3.4	2.1	4.1	3.3
RSD <sub>R</sub>	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
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 <sup>a</sup>	31	31	31	31
Outliers	0	1 <sup>C</sup>	1 <sup>C</sup>	1 <sup>C</sup>	1 <sup>C</sup>
Average %	97.2	42.2	76.6	97.2	96.5
S <sub>r</sub>	3.2	1.6	3.0	2.0	3.0
RSD <sub>r</sub>	3.3	3.8	3.9	2.1	3.1
r	9.0	4.5	8.4	5.7	8.4
S <sub>R</sub>	3.7	2.1	3.7	2.8	4.4
RSD <sub>R</sub>	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	38.7-46.8	70.1-81.8	91.6-101.9	86.0-104.0

No. of Labs. = Number of laboratories included in calculations

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

S<sub>r</sub> = Repeatability standard deviation

RSD<sub>r</sub> = Repeatability relative standard deviation

r = Repeatability value (2.8 x S<sub>r</sub>)

S<sub>R</sub> = Reproducibility standard deviation

RSD<sub>R</sub> = Reproducibility relative standard deviation

R = Reproducibility value (2.8 x S<sub>r</sub>)

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

<sup>a</sup> = With these samples, only 26 sets of results were supplied due to a misinterpretation of instructions.

In **Table 1**, the statistical evaluation of the results obtained from an interlaboratory evaluation of AOAC method 996.11 is shown. Thirty-two laboratories (worldwide) were involved, and sixteen samples (eight blind duplicates) were analysed. On the basis of these results, the method was accepted as a standard method by AOAC Int., AACC Int. and ICC.



**Figure 2.** Arrangement of test tube racks with tubes in a rack in an ice-water bath over a magnetic stirrer for dissolution of resistant starch in 1.7 M NaOH; method (b).

**Table 2.** A comparison of methods for the measurement of total starch in

Starch type and lot number	Moisture content % w/w	Starch content <sup>a</sup> RTS procedure % w/w		Starch content <sup>a</sup> NaOH-RTS procedure % w/w	
		as is	dwb <sup>b</sup>	as is	dwb <sup>b</sup>
Corn Starch; High AMP Sigma S-9679	10.9	88.38	99.2	88.54	99.4
Potato Starch – Sigma S-4251	12.3	83.15	94.8	82.81	94.4
Rice Starch – Sigma S-7260	11.6	85.33	96.5	87.28	98.7
Wheat Starch (native) – Sigma S-512	12.9	87.05	99.9	88.78	101.9
Hi Maize 1043 – Batch 02161	10.0	60.08	66.8	87.23	96.9
Corn Starch – Sigma S-4126	11.4	85.95	97.0	87.86	99.2
ACS Soluble Starch – Lot 21101	12.8	86.34	99.0	85.57	98.1
Novelose 240 – Native High amylose	11.3	64.37	72.5	87.01	98.1
Hi Maize (HAMS) – Batch CO-343	11.3	74.46	83.94	85.87	96.80
Novelose 330 – Native High Amylose (Hylon VII) – Lot 50904	9.8	58.21	63.8	88.69	97.2
Amylose (Potato) – Sigma A-9262	10.7	73.45	82.25	86.51	96.9
Fibersol 2 – Lot 112181A (Matsutani)	10.5	63.78	71.2	88.90	99.3
Purified Actistar (Cerestar)	7.5	8.52	9.21	9.06	9.79
Regular Maize Starch – Lot 140801	7.0	88.36	95.0	90.83	97.7
Fiberite RW (pre-gelatinated phosphate crosslinked starch)	13.7	85.97	99.6	87.17	101.0
Fibersym RW (not pre-gelatinated phosphate crosslinked starch)	10.5	68.54	76.6	55.72	62.3
Green Banana –	9.9	69.85	77.5	67.59	75.0
High amylose maize starch – 60107	9.1	63.00	69.3	63.35	69.7
Chemically modified Starch – 21101b	11.2	59.87	67.4	85.61	96.4
	11.6	83.33	94.3	84.30	95.4

<sup>a</sup> all values are the mean of duplicate analyses.

<sup>b</sup> dry weight basis

“pure” starch samples.<sup>13</sup>

In **Table 2**, a comparison of values obtained using the RTS and NaOH- RTS methods for determination of total starch in “pure” starch samples is shown. For most samples, very similar values were obtained. However, quantitative measurement of starch in samples containing high amylose starches, requires pre-treatment with NaOH.

**Table 3.** Repeatability study. Total starch content of animal feeds and pet foods determined using the RTS method [procedure (a)].<sup>13</sup>

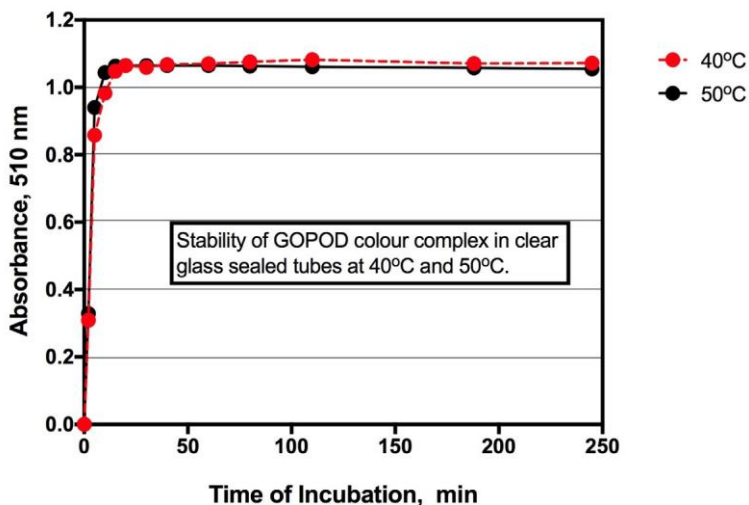
Sample	Total Starch, % (w/w), dwb <sup>a</sup> , Mean <sup>b</sup> ± 2 SD, (CV <sup>c</sup> %)				Interday mean, ± 2 SD, (CV <sup>c</sup> , %)
	Day 1	Day 2	Day 3	Day 4	
<b>Hen pellets</b>	<b>37 ± 0.5</b>	<b>36.1 ± 0.6</b>	<b>38 ± 0.9</b>	<b>38.7 ± 0</b>	<b>37.4 ± 2.1</b>
	0.65	0.82	1.17	0.00	2.80
<b>Soyabean meal</b>	<b>7.5 ± 0.3</b>	<b>7.5 ± 0.1</b>	<b>7.9 ± 0.7</b>	<b>7.7 ± 0.5</b>	<b>7.6 ± 0.5</b>
	1.79	0.38	4.41	2.95	3.01
<b>Alfalfa</b>	<b>0.3 ± 0</b>	<b>0.3 ± 0</b>	<b>0.3 ± 0.1</b>	<b>0.3 ± 0.1</b>	<b>0.3 ± 0</b>
	6.73	2.32	8.32	11.22	7.39
<b>Ulsakind horse feed</b>	<b>14.3 ± 0.4</b>	<b>13.7 ± 0.6</b>	<b>13 ± 1.2</b>	<b>13.9 ± 0.8</b>	<b>13.7 ± 1.2</b>
	1.39	2.16	4.74	2.75	4.33
<b>Freedom horse feed</b>	<b>9 ± 1.2</b>	<b>8.6 ± 0.2</b>	<b>9.2 ± 0.3</b>	<b>8.7 ± 1.2</b>	<b>8.9 ± 0.8</b>
	6.93	1.32	1.46	6.72	4.65
<b>Dairy feed complete</b>	<b>19.3 ± 0</b>	<b>19.3 ± 1.3</b>	<b>19.4 ± 0.9</b>	<b>19.8 ± 1.5</b>	<b>19.5 ± 0.9</b>
	0.11	3.40	2.44	3.68	2.38
<b>Dog adult kibble</b>	<b>40.6 ± 0.6</b>	<b>40.7 ± 0.4</b>	<b>41.4 ± 0.8</b>	<b>41.1 ± 0.7</b>	<b>40.9 ± 0.8</b>
	0.73	0.52	0.97	0.86	1.04
<b>Corn silage N16087</b>	<b>27.9 ± 0.5</b>	<b>29.4 ± 0.2</b>	<b>28.7 ± 1.2</b>	<b>28.4 ± 0.5</b>	<b>28.6 ± 1.3</b>
	0.94	0.41	2.10	0.82	2.25
<b>Flaked maize</b>	<b>74.9 ± 2</b>	<b>75.6 ± 1.8</b>	<b>76.8 ± 2.8</b>	<b>76.9 ± 1.7</b>	<b>76.1 ± 2.4</b>
	1.34	1.19	1.83	1.13	1.58
<b>Wet dog food</b>	<b>0.4 ± 0.1</b>	<b>0.4 ± 0.1</b>	<b>0.5 ± 0</b>	<b>0.4 ± 0</b>	<b>0.4 ± 0.1</b>
	12.12	11.16	0.00	1.79	12.28
<b>Regular maize starch</b>	<b>97.9 ± 4.4</b>	<b>99.1 ± 1.6</b>	<b>99.5 ± 1.9</b>	<b>99.6 ± 0.8</b>	<b>99.1 ± 2.4</b>
	2.27	0.83	0.94	0.43	1.22

<sup>a</sup> All results are presented as total starch on a dry weight basis.

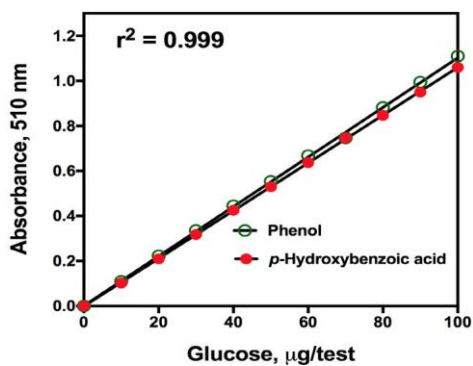
<sup>b</sup> On each day samples of each material were analysed in duplicate

<sup>c</sup> CV = Coefficient of Variation

In **Table 3**, repeatability data using the RTS method [procedure (a)] for a number of animal feeds and pet foods is shown. Highly repeatable data was obtained for a wide range of samples with starch contents ranging from 0.3 to 99% w/w (dry weight basis). This method gives very similar starch contents to those obtained with AOAC Method 996.11 but the assay is simpler to perform.



**Figure 3.** Time course of colour development (and colour stability) on incubation of 100 mg of glucose with 3.0 mL of **GOPOD reagent** at 40°C and 50°C. It can be seen that GOPOD-glucose colour development is complete within 15 min and the colour complex is stable for more than 100 min.<sup>13</sup>



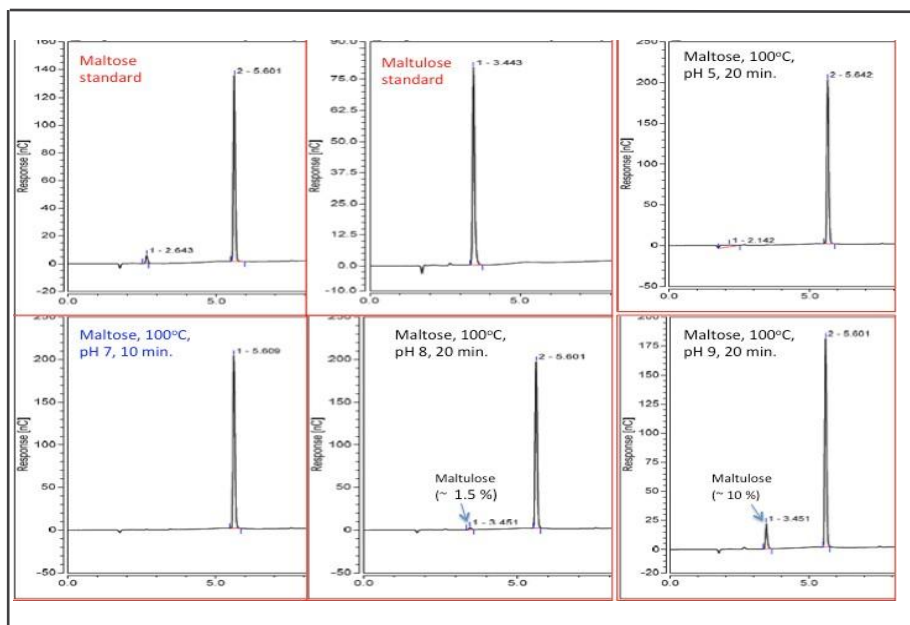
**Figure 4.** Linearity of glucose determination with **GOPOD reagents** containing either phenol or *p*-hydroxybenzoic acid as chromogen. The standard curve intersects the origin and is perfectly linear, with an  $r^2$  value of 0.999.<sup>13</sup>

**Table 4.** Total starch content of a range of grain and bean samples determined using the RTS Method [method (a)] and the NaOH- RTS Method [method (b)].<sup>13</sup>

Starch type and lot number	Moisture content % w/w	Starch content <sup>a</sup> RTS method [procedure (a)] % w/w		Starch content <sup>a</sup> NaOH-RTS method [procedure (b)] % w/w	
		"as is"	dwb <sup>b</sup>	"as is"	dwb <sup>b</sup>
Cannellini beans (Natures Gold)	12.07	34.2	38.9	34.9	39.7
Butter beans (Natures Gold)	11.22	31.7	35.6	33.2	37.3
Red kidney beans (Natures Gold)	12.02	31.3	35.6	32.4	36.8
Pinto beans (Natures Gold)	12.09	32.2	36.6	35.3	40.1
Haricot beans (Natures Gold)	13.40	33.4	38.6	34.5	39.8
Soya beans (Natures Gold)	8.70	0.9	1.0	0.7	0.8
Mung beans (Natures Gold)	11.80	38.9	44.1	40.8	46.3
Chick peas (Natures Gold)	10.27	39.7	44.2	41.9	46.7
Yellow split peas (Natures Gold)	12.36	44.7	51.0	46.6	53.2
Black eye beans (Natures Gold)	11.46	35.2	39.8	38.0	42.9
Lentils verts (Natures Gold)	10.64	33.0	36.9	33.3	37.2
Black baluga lentils (Natures Gold)	12.12	36.2	41.1	36.3	41.2
Quinoa (Natures Gold)	12.38	57.2	65.3	57.1	65.2
Spelt grain (Natures Gold)	12.67	59.8	68.5	59.2	67.8
Roasted buckwheat (Natures Gold)	4.94	72.3	76.1	70.6	74.3
Long grain brown rice (Natures Gold)	11.75	72.3	81.9	64.8	73.4
Oat groats (Natures Gold)	10.82	61.6	69.1	60.2	67.5
Millet (Natures Gold)	11.28	68.5	77.2	61.5	69.3
Wheat grain (Natures Gold)	11.24	57.1	64.3	57.2	64.4
Brown linseed (flaxseed)	4.38	1.0	1.0	0.4	0.4
Regular maize starch (control)	13.7	84.0	96.6	84.5	97.1

<sup>a</sup> all values are the mean of duplicate analyses.

<sup>b</sup> dry weight basis



**Figure 5.** Ion chromatography of maltose and maltulose standards stored at 100°C and pH 5, 7, 8 or 9 for 10 or 20 min.<sup>13</sup> At pH 7, the incubation was performed for just 10 min as this is in-line with the incubation time employed in AOAC Method 996.11.

**NOTE:** On incubation at 100°C, no maltulose is produced from maltose at pH values below 8.

**Table 5.** Total starch content of a selected range of breakfast cereals determined using the RTS Method [method (a)].<sup>13</sup>

Starch type and lot number	Moisture content % w/w	Free glucose % w/w	Starch plus free glucose % w/w, "as is"	Starch % w/w "as is"	Starch % w/w "dwb"
Kellogg® corn flakes	5.48	1.4	72.6	71.2	75.2
Kellogg® Special K Original	3.69	0.5	64.5	64.0	66.5
Nestle® Shreddies	4.04	1.0	56.7	55.7	58.1
Kellogg® All Bran Original	2.51	0.4	26.6	26.2	26.8
Kellogg® Frosties	4.05	1.0	50.8	49.8	51.8
Weetabix®	6.09	0.3	62.0	61.7	65.6
Macaroni pasta (Roma®)	8.57	0.04	69.1	69.1	75.5
Regular maize starch (control)	13.7	0.03	85.5	85.5	99.0

<sup>a</sup> all values are the mean of duplicate analyses.

**Table 6.** Total starch content of a selected range of vegetables determined using the RTS Method [method (a)].<sup>13</sup>

Sample	Starch content % w/w "dwb"	Moisture content, % w/w	Starch content % w/w of original material
Sweet potato	32.3	74.0	8.4
Potatoes (Rooster var.)	67.4	77.3	15.3
Cauliflower	0.1	90.6	0.01
Celery	0	95.0	0.0
Broccoli	0.1	88.5	0.01
Mushroom ( <i>A. bisporus</i> )	0.5	91.6	0.04
Red onions	0.6	87.7	0.07
Carrots	1.6	92.5	0.12
Swede	10.6	82.8	1.8
Red pepper	0.5	89.7	0.05
Ripe banana	18.9	74.1	4.9
Ripe mango	8.6	81.8	1.6

<sup>a</sup> all values are the mean of duplicate analyses.

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