

Megazyme

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DIGESTIBLE STARCH and RESISTANT STARCH ASSAY PROCEDURE

K-DSTRS 11/19

(40 Assays of each per Kit)



INTRODUCTION:

The terminology, rapidly digestible starch (RDS), slowly digestible starch (SDS) and amylase resistant starch (RS) were introduced by Englyst *et al.*¹ in 1992 to reflect the rate of starch digestion *in vivo*. Their work, and that of Wahlquist *et al.*,² Jenkins *et al.*³ and others showed that the physiological form of food and the nature of the starch are major determinants of the rate of digestion of the starch. Over this period of time it was also shown that “digestible” starch can be hydrolysed and absorbed at the same rate as simple sugars, but that some starch resists digestion in the human small intestine. Englyst *et al.*⁴ first observed amylase resistant starch (RS) in their research in developing a method for the measurement of non-starch polysaccharides (NSP). These authors decided that NSP was the best measure of dietary fiber (DF), so all starch was dissolved (using DMSO) and hydrolytically removed in the measurement of NSP.


More recently, Codex Alimentarius⁵ released a definition of dietary fiber which includes digestion resistant starch (RS) and the decision on inclusion of non-digestible oligosaccharides (NDO) was left to individual countries. Consequently, accurate measurement of RS is imperative in accurately measuring total dietary fiber (TDF). To this end, McCleary *et al.*^{6,7} developed an Integrated TDF procedure (INTDF; AOAC Method 2009.01 and 2011.25), which was recently updated to a Rapid Integrated TDF procedure (RINTDF; AOAC Method 2017.16)^{8,9} for the measurement of all dietary fiber components including RS and NDO. In the RINTDF procedure, the time of incubation with pancreatic α -amylase/amyloglucosidase (PAA/AMG) was set at 4 h, and the concentrations of both PAA and AMG were optimised to ensure that RS values obtained for a set of pure starch and starch containing food samples matched ileostomy data. Ultimately, the concentrations of both enzymes were saturating, in-line with work described by Englyst *et al.*¹ in their digestible starch methodology. The major difference is that in the RINTDF procedure, highly purified, ready to use enzymes are employed.

Literature indicates that the average time of residence of food in the human small intestine is 4 ± 1 h. Thus, this was chosen as the incubation time with PAA/AMG in the RINTDF procedure. In the Englyst *et al.*¹ procedure for digestible carbohydrates, samples are removed at 20 min for determination of rapidly digested starch (RDS) and at 120 min for slowly digested starch (SDS), and the remaining starch was defined as resistant starch (RS). Because the residence time of food in the small intestine is ~ 4 h, RS should be defined as that starch remaining after incubation for this period of time. We now introduce a new term, “total digestible starch (TDS)” to represent that starch which is hydrolysed during 4 h incubation. Thus, in the current assay protocol, an additional sample is removed from the incubation mixture at 4 h for measurement of TDS.

Resistant starch is the starch remaining after incubation of the sample with PAA/AMG for 4 h. In the current method, procedures are described for the measurement of RDS, SDS, TDS and RS. This method is applicable to all samples.

PRINCIPLE:

Pure starches, or starch-containing samples, are incubated with a mixture of pancreatic α -amylase and amyloglucosidase (PAA/AMG) in maleate buffer, pH 6.0, according to the procedure used for measurement of dietary fiber at 37°C for up to 4 h with continual stirring (**K-RINTDF**; AOAC Method 2017.16). Aliquots of the reaction solution are removed at 20 min (to measure RDS), 120 min (to measure SDS; SDS = starch value at 120 min - starch value at 20 min) and at 240 min (to measure TDS and RS). For RDS, SDS and TDS, 1.0 mL aliquots are removed while the suspension is stirring and transferred to 20 mL of 50 mM acetic acid (to terminate the reaction). These solutions are mixed thoroughly and 0.1 mL aliquots are incubated with 0.1 mL of AMG (100 U/mL) to hydrolyse remaining traces of maltose to glucose which is measured with GOPOD Reagent. To measure resistant starch (RS), a 4 mL aliquot is removed from the stirring solution after 240 min (4 h), added to an equal volume of IMS and mixed thoroughly. The sample is centrifuged and the pellet is washed with aqueous ethanol to remove free glucose and then suspended in sodium hydroxide to dissolve RS. The solution is neutralised and the starch hydrolysed to glucose with AMG and the glucose measured with GOPOD Reagent (Figure 1).



Wet sample (0.5 g) with 0.5 mL of ethanol and incubate in 20 mL of **sodium maleate buffer** (50 mM, pH 6.0) containing 2 mM CaCl_2 at 37°C with **2 KU PAA** and **0.85 KU AMG** and stir at 170 rpm.

Remove aliquots (**1.0 mL**) at various time intervals and add to **20.0 mL of 50 mM acetic acid** to terminate the reaction (21-fold dilution).

- 1.0 mL at 20 min – RDS
- 1.0 mL at 120 min – SDS
(SDS = value at 120 min – value at 20 min)
- 1.0 mL at 240 min – TDS (and Available Carbohydrates)
- 4.0 mL at 240 min + 4.0 mL ethanol – Resistant Starch

Remove aliquots (0.1 mL in duplicate) for incubation with AMG and analysis of glucose using GOPOD Reagent.

Figure 1. Procedures involved in the measurement of RDS, SDS, TDS and RS in starch and food samples according to Method 1.

APPLICABILITY AND ACCURACY:

The method is applicable to samples containing > 2% w/w digestible starch (DS) or resistant starch (RS). With such samples, standard errors of $\pm 5\%$ are achieved routinely. Higher errors are obtained for samples with DS or RS contents of < 2% w/w.

KITS:

Kits suitable for performing 160 assays (40 of each of RDS, SDS, TDS and RS or various other combinations) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: **Mixture of purified PAA (40 KU/g) and AMG (17 KU/g); 5.2 g.**
Stable for > 5 years; store sealed & dry below -10°C.

NOTE: For some individuals, these powdered enzymes are highly allergenic. Thus, they should be weighed and handled **ONLY** in a fume cupboard.

Bottle 2: **Amyloglucosidase** [8 mL, 3300 U/mL on soluble starch (or 200 U/mL on *p*-nitrophenyl β -maltoside)] at pH 4.5 and 40°C. AMG solution should be essentially free of detectable levels of free D-glucose. Stable for > 3 years at 4°C.

Bottle 3: **GOPOD Reagent Buffer** (50 mL, pH 7.4). *p*-hydroxybenzoic acid and sodium azide (0.09% w/v). Stable for > 3 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; stored sealed at room temperature.

Bottle 6: **Digestible/Resistant Starch Control** (~ 10 g). Digestible and resistant starch content shown on the label. Stable for > 5 years; stored sealed at room temperature.

A. PREPARATION OF REAGENT SOLUTIONS

I. **Stock PAA/AMG solution.**— PAA (4 KU/5 mL) plus AMG (1.7 KU/5 mL). Immediately before use, add 0.5 g of PAA/AMG powder mixture (**bottle 1**, page 3) to 25 mL of sodium

maleate buffer [B(a)] and stir on a magnetic stirrer for 5 min. Store on ice during use. Use within 4 h of preparation. **CAUTION:** If an analyst is allergic to powdered PAA and/or AMG, engage an analyst who is not allergic to prepare the powdered enzymes as an ammonium sulphate suspension as follows: gradually add 2.5 g of PAA/AMG powder mix (PAA 40 KU/g plus AMG 17 KU/g; **bottle 1**) to 35 mL of cold, distilled water in a 100 mL beaker on a magnetic stirrer in a fume cupboard and stir until the enzymes are completely dissolved (approx. 5 min). Add 17 g of granular ammonium sulphate and dissolve by stirring. Adjust the volume to 50 mL with ammonium sulphate solution [B(f)]. Stable for 3 months at 4°C.

- 2a. **(AMG)** Use the contents of **bottle 2** (amyloglucosidase) as supplied. Stable for > 3 years at 4°C.
- 2b. **(Dilute AMG).** Add 1 mL of the contents of **bottle 2** to 30 mL of 100 mM sodium acetate buffer (pH 4.5). Mix well and divide into ~ 10 mL aliquots in 13 mm polypropylene tubes [C(t)] and store below -10°C between use (AMG ~ 100 U/mL).
3. Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water. This is solution 3. Use immediately.
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 frozen it should be divided into ~ 250 mL aliquots and stored in polypropylene containers. Do not apply these stored solutions to more than one freeze-thaw cycle.

Freshly prepared reagent is either a light yellow or light pink colour. The solution will develop a stronger pink colour over 2-3 months at 4°C. The absorbance of this solution should be < 0.05 when read against distilled water.
5. Use the contents of bottle 5 as supplied. Stable for > 5 years; stored sealed at room temperature.
6. Use the contents of bottle 6 as supplied. Stable for > 5 years; stored sealed at room temperature.

B. REAGENTS (not supplied):

- a. **Sodium maleate buffer 50 mM, pH 6.0 plus 2 mM CaCl₂.**— Dissolve 11.6 g of maleic acid in 1600 mL of deionised water and adjust the pH to 6.0 with 4 M (160 g/L) NaOH solution. Add 0.6 g of calcium chloride dihydrate (CaCl₂·2H₂O), dissolve and adjust the volume to 2 L. Store in a well-sealed Duran[®] bottle and add two drops of toluene to prevent microbial infection. Stable for ~ 1 year at 4°C.
- b. **Acetic acid solution, 50 mM.**— Add 2.9 mL of glacial acetic acid (Sigma W200611-1KG-K; 1.05 g/mL) to a 1 L volumetric flask. Dilute to 1 L with deionised water and store in a Duran bottle. Stable for ~ 1 year at 4°C.
- c. **Sodium hydroxide solution, 1.7 M.**— Add 68 g of sodium hydroxide to 800 mL of distilled water in a fume cupboard and dissolve by stirring. Adjust the volume to 1 L with distilled water and store in a Duran[®] bottle at 4°C. Stable for > 4 years at 4°C.
- d. **Sodium acetate buffer, 1.0 M, pH 3.8 plus calcium chloride (5 mM).**— Add 57.0 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. Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 L with distilled water and store in a Duran[®] bottle. Stable for 12 months at room temperature.
- e. **Sodium acetate buffer, 100 mM, pH 4.5.**— Add 5.7 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 4.5 using 1 M sodium hydroxide. Adjust the volume to 1 litre with distilled water and store in a Duran[®] bottle. Stable for ~ 1 year at 4°C
- f. **Ammonium sulphate solution, 50% w/v.**— Add 50 g of ammonium sulphate to 80 mL of distilled water and dissolve by stirring. Adjust volume to 100 mL with distilled water. Store in a Duran bottle. Stable for > 2 years at room temperature.
- g. **Aqueous Ethanol, 50% v/v and Aqueous IMS 50% v/v.**— Add 500 mL of either ethanol (95% v/v) or industrial methylated spirits (IMS, 95% v/v) to 500 mL of distilled water. Store in a 1 L Duran bottle. Stable for > 4 years at room temperature.

C. APPARATUS REQUIRED:

- a. **Grinding mill.**— Centrifugal, with 12-tooth rotor and 0.5 mm sieve, or similar device. Alternatively, a cyclone mill can be used for small test laboratory samples provided the mill has sufficient air flow or other cooling to avoid overheating of samples.

- b. **Meat mincer.**— Hand operated or electric, fitted with a 4.5 mm screen.
- c. **Water bath.**— To accommodate a 2mag Mixdrive 15[®] submersible magnetic stirrer with an immersion heater (e.g. Lauda Alpha[®]) (Figure 2, page 11) (Megazyme cat. no. **D-TDFBTH**).
- d. **Submersible magnetic stirrer.**— 2mag Mixdrive 15[®] submersible magnetic stirrer.
- e. **Spectrophotometer.**— capable of operating at 510 nm, preferably fitted with flow-through cell (10 mm path length).
- f. **Analytical balance.**— 0.1 mg readability, accuracy and precision.
- g. **Bench centrifuge.**— Capable of holding 101 x 16.5 mm polypropylene tubes, with rating of approx. 3250 rcf (~ 4,000 rpm), e.g. Sigma Laboratory Centrifuges 4-15, No.10730.
- h. **Freeze-drier.**—Virtis Genesis[®] 25XL or similar. Biopharma Process Systems Biopharma House, Winchester, UK.
- i. **Microfuge centrifuge.**— Capable of 13,000 rpm.
- j. **Disposable 2.0 mL polypropylene microfuge tubes.**— e.g. Sarstedt, cat. no. 72.691 Sarstedt Ltd., Drinagh, Ireland.
- k. **pH meter.**— e.g. Seven Easy pH Mettler Toledo.
- l. **Vortex mixer.**— e.g. Daihan Scientific VM10.
- m. **Moisture analyser.**— e.g. OHAUS MB45.
- n. **Magnetic stirrer.**— e.g. IKA KMO 2 basic stirrer.
- o. **Magnetic stirring bars.**— e.g. Fisherbrand[™] PTFE Stir Bars 12 x 6 mm ridged, 20 x 6 mm ridged, 30 x 6 mm ridged and 15 x 5 mm non-ridged.
- p. **Digestion Bottles.**— 250 mL Fisherbrand[®] soda glass, wide mouth bottles with polyvinyl lined cap (cat. no. FB73219) (www.fisher.co.uk/supplies/fisherbrand.html).
- q. **Laboratory timer.**
- r. **Micro-pipettors.**— e.g. Gilson Pipetman[®] (100 µL). Woodside Industrial Estate, Dunstable, United Kingdom.
- s. **Positive displacement pipettor.**— e.g. Brand HandyStep[®]S
 - with 25 mL Brand PD-Tip[®] (to dispense 2.5 or 5 mL aliquots of PAA/AMG preparation, 4 mL aliquot of IMS or 50% IMS) and 10 mL of 50 mM acetic acid.
 - with 5.0 mL Brand PD-Tip[®] (to dispense 0.1 mL of AMG solution and 1.0 mL of sample solution).

- t. **Polypropylene tubes.**— Sarstedt polypropylene tube; 40 mL, 84 x 30 mm (cat no. 62.555) and Sarstedt polypropylene tube; 13 mL, 101 x 16.5 mm (cat no. 60.541.685).
- u. **Glass test tubes.**— 16 x 100 mm, 14 mL capacity.
- v. **Plastic “lunch box”.**— large enough to hold test-tube rack and serve as an ice-water bath.
- w. **Polypropylene sheet with precision cut holes.**— to hold and align 40 mL polypropylene tubes on the stirrer plate of the 2mag Mixdrive 15[®] submersible magnetic stirrer (Figure 2, page 11). Available from Megazyme (cat. no. **D-PPSTH**).

D. PREPARATION OF TEST SAMPLES:

Collect and prepare samples as intended to be eaten, *i.e.* baking mixes should be prepared and baked, pasta should be cooked etc. Defat per AOAC 985.29 if > 10% fat. For high moisture samples (> 25%) it is desirable to freeze dry. Grind ~ 50 g in a grinding mill [B(a)] to pass a 0.5 mm sieve. Transfer all material to a wide mouthed plastic jar, seal, and mix well by shaking and inversion. Store in the presence of a desiccant. Grind wet samples (e.g. wet pasta) in a meat mincer to yield an homogeneous paste. Remove a representative sample for analysis and record weight. Separately, determine the moisture content of the wet sample and allow for the liquid volume in the calculations.

E. ENZYME DIGESTION OF SAMPLES:

Method 1. Procedure for analysing ~ 0.5 g of sample.

- (a) **Accurately weigh** approx. 0.5 g sample, correct to the third decimal place, into a 30 x 84 mm (40 mL) polypropylene tube [C(t)]. Record the weight. Add a 20 x 6 mm stirrer bar [C(o)] to each tube.
- (b) **Addition of buffer.**— Wet the sample with 0.5 mL of 95% v/v EtOH (or IMS) and add 17.5 mL of maleate buffer [B(a)] to each tube. Cap the tubes and place them in the special polypropylene holder (Figure 2, page 11)(cat. no. **D-PPSTH**) on a 2mag Mixdrive 15[®] submersible magnetic stirrer [C(d)] in a water bath and allow the contents to equilibrate to 37°C over 5 min with stirring at 170 rpm.
- (c) **Incubation with PAA/AMG solution.**— Add 2.5 mL of PAA/AMG solution [A(1)], cap the tubes and incubate the reaction solutions at 37°C and at 170 rpm on the 2mag Mixdrive 15[®] submersible magnetic stirrer. **NOTE:** If using the (NH₄)₂SO₄ suspension of this enzyme preparation [A(1)], add 1 mL of enzyme suspension and 1.5 mL of sodium maleate buffer [B(a)].

Method 2. Procedure for analysing ~ 1.0 g of sample (using the incubation arrangement employed for AOAC Method 2017.16)

- (a) **Accurately weigh** approx. 1.0 g of sample, correct to the third decimal place, into a 250 mL Fisherbrand glass[®] bottle [C(p)]. Record the weight. Add a 30 x 6 mm stirrer bar [C(o)] to each bottle (Figure 3, page 12).
- (b) **Addition of buffer.**— Wet the sample with 1.0 mL of 95% v/v EtOH (or IMS) and add 35 mL of maleate buffer [B(a)] to each bottle. Cap the bottles and place them on a 2mag Mixdrive 15[®] submersible magnetic stirrer [C(d)] in a water bath [C(c)] and allow the contents to equilibrate to 37°C over 5 min with stirring at 170 rpm.
- (c) **Incubation with PAA/AMG solution.**— Add 5 mL of PAA/AMG solution [A(1)], cap the bottles and incubate the reaction solutions at 37°C and at 170 rpm on the 2mag Mixdrive 15[®] submersible magnetic stirrer. **NOTE:** If using the (NH₄)₂SO₄ suspension of this enzyme preparation [A(1)], add 2 mL of enzyme suspension and 3 mL of sodium maleate buffer [B(a)].

F. DETERMINATION OF DIGESTIBLE STARCH:

- (a) Carefully remove 1.0 mL of the stirring reaction solution at 20 min (for RDS determination), at 120 min (for SDS determination; SDS = digestible starch at 120 min - digestible starch at 20 min) and at 240 min (for TDS determination) using a positive displacement dispenser [C(s)]. Immediately add the 1.0 mL of sample solution to 20 mL of 50 mM acetic acid solution [B(b)], cap the tube and mix thoroughly, and store at room temperature or 4°C awaiting analysis.
- (b) Transfer 2 mL of each solution to 2.0 mL polypropylene microfuge tubes [C(j)] and centrifuge at 13,000 rpm for 5 min.
- (c) Transfer duplicate aliquots (0.1 mL) to the bottom of 16 x 100 mm glass test tubes [C(u)], add 0.1 mL of diluted AMG (100 U/mL) [A(2b)], mix well and incubate at 50°C for 30 min. Then add 3.0 mL of GOPOD Reagent [A(4)] and incubate at 50°C for 20 min. Measure the absorbance at 510 nm against a Reagent Blank.

Prepare reagent blank solutions by mixing 0.2 mL of 100 mM acetic acid (pH 4.5) [B(e)] with 3.0 mL of GOPOD reagent and **prepare D-glucose standards** (in quadruplicate) by mixing 0.1 mL of D-glucose (1 mg/mL) [A(5)] plus 0.1 mL of acetate buffer [B(e)] and 3.0 mL of GOPOD reagent [A(4)]. **Incubate these solutions** at 50°C for 20 min with the sample solutions.

- (d) Calculate RDS, SDS and TDS content as described in section H, or using the appropriate MegaCalc™ Excel® calculator.

G. DETERMINATION OF RESISTANT STARCH:

- (a) After 240 min (4 h), while the incubation solution (sample plus PAA/AMG) is stirring, remove 4.0 mL of the suspension with a positive displacement dispenser [C(s)] and transfer this to a 101 x 16.5 mm polypropylene tube [C(t)] containing 4.0 mL of 95% v/v ethanol or IMS. Cap the tube and mix the contents thoroughly by repeated inversion of the tube.
- (b) Centrifuge the tube at 4,000 rpm for 10 min in a bench centrifuge [C(g)] and carefully decant the supernatant solution immediately after the centrifuge has stopped. Re-suspend the pellet in 2 mL of 50% v/v aqueous ethanol [B(g)] by stirring on a vortex mixer. Then add another 6 mL of 50% v/v aqueous ethanol to the tube. Cap the tube and mix the contents on a vortex mixer. Centrifuge the tubes and recover the pellets. Ensure that all free liquid is removed by inverting the tubes on absorbent paper. Again, re-suspend the pellets in 50% v/v aqueous ethanol (2 + 6 mL) and centrifuge at 4,000 rpm for 10 min. Decant the supernatant solution, remove free liquid and cover the tubes with Parafilm® until RS determination is to be performed.
- (c) Add a magnetic stirrer bar (5 x 15 mm) [C(o)] and 2 mL of cold 1.7 M NaOH [B(c)] to each tube. Re-suspended the pellets (and dissolve the RS) by stirring the tube contents for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 4).
- (d) Add 8 mL of 1.0 M sodium acetate buffer (pH 3.8) [B(d)] to each tube with stirring on a magnetic stirrer. Immediately add 0.1 mL of AMG (3300 U/mL) [A(2a)], mix the contents well, place the tubes in a water bath at 50°C and incubate for 30 min with intermittent mixing on a vortex mixer.
- (e) For samples containing > 10% RS 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 the water wash bottle. Adjust volume to 100 mL with distilled water and mix well. Centrifuge an aliquot (2 mL) of the solution at 13,000 rpm for 5 min in a microfuge.
- (f) For samples containing < 10% RS content; centrifuge an aliquot (2 mL) of the solution at 13,000 rpm for 5 min (no dilution). For such samples, the final volume in the tube is approx.

10.3 mL (however, this volume will vary particularly if wet samples are analysed, and appropriate allowance for volume should be made in the calculations).

- (g) Transfer 0.1 mL aliquots (in duplicate) of either the centrifuged diluted [G(e)], or the undiluted [G(f)] supernatants into glass test tubes (16 x 100 mm), add 3.0 mL of GOPOD reagent and incubate at 50°C for 20 min. Measure absorbance at 510 nm against a reagent blank [3.0 mL of GOPOD Reagent plus 0.1 mL of 100 mM sodium acetate buffer (pH 4.5), 50°C for 20 min.]
- (h) Calculate RS content as described in section [H(b)], or using the appropriate MegaCalc™ Excel® calculator.

H. CALCULATIONS (digestible starch and resistant starch)

- (a) Calculate digestible starch (RDS, SDS & TDS) (% w/w, “as is” basis) in test samples as follows:

Methods 1 and 2 (pages 7 and 8).

Digestible Starch (RDS, SDS or TDS) (g/100 g sample):

$$= \Delta A \times F \times EV/W \times D/0.1 \times 100 \times 1/1,000,000 \times 162/180$$

$$= \Delta A \times F \times EV/W \times 0.0189$$

where:

- ΔA = absorbance (reaction) read against the reagent blank after 20 min (RDS); after 120 min-20 min (SDS); after 240 min (TDS).
- F = conversion from absorbance to μg (the absorbance obtained for 100 μg of D-glucose in the GOPOD reaction is determined) [F = 100 (μg of D-glucose) divided by the GOPOD absorbance for this 100 μg of D-glucose].
- EV = extraction vol. (mL) (**Method 1** = 20.5; **Method 2** = 41).
- W = “as is” weight of sample analysed in g; i.e. ~ 0.50 g (Method 1) or ~ 1.0 g (Method 2) (weighed accurately).
- D = dilution of sample (21; 1.0 mL of sample added to 20 mL of diluted acetic acid).
- 0.1 = volume of sample analysed.
- 100 = conversion to g/100 g.
- 1,000,000 = conversion from μg to g.
- 162/180 = factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch.

(b) Calculate resistant starch (RS) (% w/w, on an “as is” basis) in test samples as follows:

Resistant Starch (g/100g sample): (samples containing > 10% RS)

$$= \Delta A \times F \times EV/4 \times FV/0.1 \times 1/1,000,000 \times 100/W \times 162/180$$

$$= \Delta A \times F \times EV/W \times FV \times 0.000225$$

where:

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

F = conversion from absorbance to μg (the absorbance obtained for 100 μg of D-glucose in the GOPOD reaction is determined [$F = 100$ (μg of D-glucose) divided by the GOPOD absorbance for this 100 μg of D-glucose].

EV = extraction vol. (mL) (**Method 1** = 20.5; **Method 2** = 41).

4 = volume of solution taken from the reaction mixture for RS analysis.

FV/0.1 = 0.1 mL aliquots taken from final volume (FV; either 100 mL or 10.3 mL) for determination of glucose using GOPOD reagent.

1,000,000 = conversion from μg to g.

100/W = conversion to g/100g.

W = “as is” weight of sample analysed in g;
i.e. 0.50 g or ~ 1.0 g (weighed accurately).

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

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

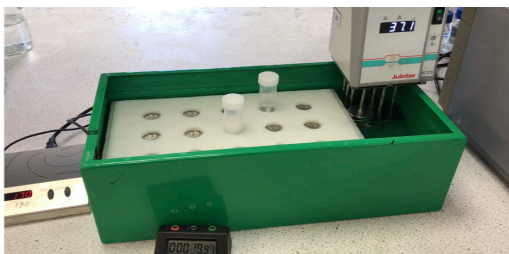


Figure 2. Incubation Method 1. Samples (~ 0.5 g) in 40 mL, 30 x 84 mm polypropylene tubes in a designed polypropylene tube holder (Megazyme cat. no. **D-PPSTH**) [C(w)] on a 2mag Mixdrive 15[®] submersible magnetic stirrer in a custom made water bath (Megazyme cat. no. **D-TDFBTH**). This arrangement allows stirring of 15 samples at controlled speed (170 rpm) and 37°C.

I. REFERENCES:

1. Englyst, H. N., Kingman, S. M. & Cummings, J. H. (1992). Classification and measurement of nutritionally important starch fractions. *Eur. J. Clin. Nutr.*, 46 (Suppl. 2), S33-S50.
2. Wahlquist, M. I., Wilmshurst, E. G., Murton, C. R. & Richardson, E. N. (1978). The effect of chain length on glucose absorption and the related metabolic response. *Am. J. Clin. Nutr.*, 31, 1998-2001.
3. Jenkins, D. J. A., Mayer, A., Jenkins, A. L., Wolever, T. M. S., Collier, G. R., Wesson, V. & Cuff, D. (1978). Simple and complex carbohydrates: lack of glycemic difference between glucose and glucose polymers. *J. Clin. Nutr. Gastroenterol.*, 2, 113-116.
4. Englyst, H., Wiggins, H. L. & Cummins, J. H. (1982). Determination of the non-starch polysaccharides in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyt.*, 107, 307-318.
5. Codex Alimentarius Commission. ALINORM 09/32/26 Published online at <http://www.codexalimentarius.net/web/archives.jsp>. FAO/WHO, Rome, Italy, 2009.
6. McCleary, B. V. (2007). An Integrated Procedure for the Measurement of Total Dietary Fibre (including resistant starch), non-digestible oligosaccharides and available carbohydrates. *Anal. Bioanal. Chem.*, 389, 291-308.
7. McCleary, B. V., DeVries, J. W., Rader, J. I., Cohen, G., Prosky, L, Mugford, D. C. & Okuma, K. (2012). Determination of insoluble, soluble, and total dietary fiber (CODEX definition) by enzymatic-gravimetric method and liquid chromatography: collaborative study. *J. AOAC Int.*, 95, 824-844.
8. McCleary, B. V., Sloane, N. & Draga, A. (2015). Determination of total dietary fibre and available carbohydrates: a rapid integrated procedure that simulates *in-vivo* digestion. *Starch*, 67, 860-883.
9. McCleary, B. V. (2018). Total dietary fiber (CODEX definition) in foods and food ingredients by a rapid enzymatic-gravimetric method and liquid chromatography: collaborative study, First Action 2017.16. *J. AOAC Int.*, 102, 196-207.

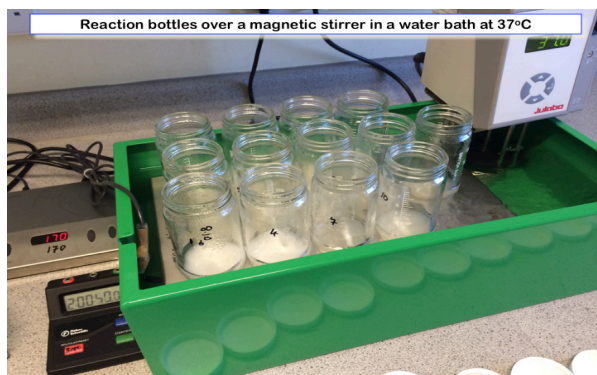


Figure 3. Incubation Method 2. Samples (~ 1.0 g) in Fisherbrand bottles on a 2mag Mixdrive 15[®] submersible magnetic stirrer in a custom made water bath (Megazyme cat. no. **D-TDFBTH**). This arrangement allows stirring of 15 samples at controlled speed (170 rpm) and 37°C.

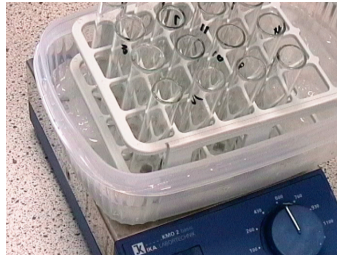


Figure 4. Arrangement of ice-water bath over a magnetic stirrer for dissolution of resistant starch in 1.7 M NaOH.

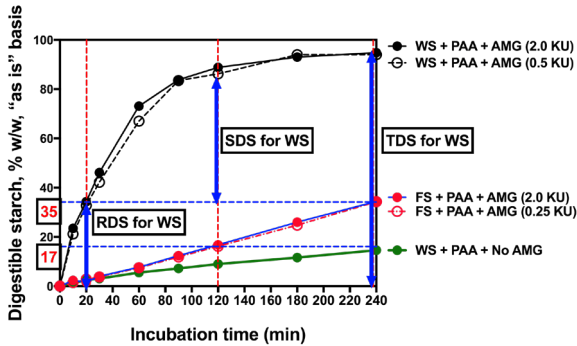


Figure 5. Hydrolysis of 1 g of wheat starch (WS) or Fibersym™ (FS; phosphate cross-linked starch; RS₄) by pancreatic α -amylase (2.0 KU) in the presence of amyloglucosidase (0.25 or 0.5 KU/assay). Samples removed at different time intervals, diluted, reactions terminated and D-glucose measured and digestible starch calculated.

Table 1. The repeatability (%RSD_r) for **Rapidly Digested Starch** measurement for samples analysed in duplicate on four separate days using the **DSTRS** assay procedure.

Sample	Rapidly Digested Starch, % (w/w) ^a , mean ^b ± 2 SD, (%RSD _r) ^c				Interday mean, ± 2 SD, (%RSD _r)
	Day 1	Day 2	Day 3	Day 4	
RMS	21 ± 1.4	19.9 ± 0.4	19.2 ± 1.3	19.7 ± 0.2	19.9 ± 1.6
	3.29	0.91	3.32	0.46	3.95
Hylon VII	7.4 ± 0.3	6.8 ± 0.5	6.5 ± 0.3	7 ± 0.4	6.9 ± 0.8
	2.35	3.61	2.60	2.78	5.52
UB Express Boiled Rice	58.9 ± 2.7	59.3 ± 3.9	57.3 ± 0.7	57.2 ± 2.6	58.2 ± 2.9
	2.29	3.32	0.60	2.24	2.48
ActiStar	21.2 ± 0.2	20.6 ± 0.9	20.3 ± 0.8	22.5 ± 0.7	21.2 ± 1.9
	0.43	2.11	1.91	1.65	4.38
Garden Peas	13 ± 0.1	12.7 ± 0.3	12.8 ± 0	12.7 ± 0.1	12.8 ± 0.4
	0.38	1.23	0.11	0.56	1.37
All Bran	23.6 ± 0.3	24 ± 0.3	24.1 ± 0.2	23.9 ± 0	23.9 ± 0.4
	0.56	0.62	0.44	0.03	0.83
Butter Beans	17.9 ± 0.5	18.5 ± 0.2	19.3 ± 1.8	19.1 ± 1.4	18.7 ± 1.4
	1.46	0.63	4.75	3.55	3.81

^a all results are presented as starch on an “as is” basis.

^b on each day, samples of each material were analysed in duplicate.

^c SD = standard deviation; RSD_r % = repeatability standard deviation.

Table 2. The repeatability (%RSD_r) for **Slowly Digested Starch** measurement for seven milled samples analysed in duplicate on four separate days using the **DSTRS** assay procedure.

Sample	Slowly Digested Starch, % (w/w) ^a , mean ^b ± 2 SD, (%RSD _r) ^c				Interday mean, ± 2 SD, (%RSD _r)
	Day 1	Day 2	Day 3	Day 4	
RMS	51.1 ± 2.5	48.4 ± 1.7	49.8 ± 1.2	47.3 ± 1.3	49.2 ± 3.3
	2.47	1.78	1.24	1.33	3.39
Hylon VII	14.3 ± 0.1	16.5 ± 0.2	16.5 ± 0.5	17.3 ± 0.1	16.2 ± 2.4
	0.31	0.75	1.62	0.42	7.42
UB Express Boiled Rice	13.6 ± 3.8	12.3 ± 1.4	11.7 ± 1.3	11.4 ± 0.4	12.2 ± 2.4
	13.85	5.61	5.43	1.69	9.77
ActiStar	5.3 ± 1.1	6 ± 0.2	5.7 ± 0	7 ± 0.2	6 ± 1.4
	10.44	1.78	0.22	1.62	11.63
Garden Peas	2.6 ± 0.3	2.7 ± 0.6	2.6 ± 0.1	2.5 ± 0.1	2.6 ± 0.3
	4.97	11.28	2.44	1.15	5.97
All Bran	1.4 ± 1.1	1.2 ± 0.7	0.7 ± 0.3	0.6 ± 0.3	1 ± 0.9
	40.84	30.81	19.00	22.52	45.75
Butter Beans	14.1 ± 2	13.3 ± 0.4	12.7 ± 0.3	12 ± 1.8	13 ± 2
	7.21	1.62	1.35	7.44	7.55

^a all results are presented as starch on an “as is” basis.

^b on each day, samples of each material were analysed in duplicate.

^c SD = standard deviation; RSD_r % = repeatability standard deviation.

The **Slowly Digested Starch** content of the samples tested covered a working range of 1.0 to 49.2% (w/w). The repeatability (%RSD_r) across this sample data set was excellent, less than or equal to 11.63% for samples containing > 1% SDS (**NOTE:** The very high value for All Bran[®] is a consequence of the very low absolute starch value for this fraction).

Table 3. The repeatability (%RSD_r) for **Total Digestible Starch** measurement for seven milled samples analysed in duplicate on four separate days using the **DSTRS** assay procedure.

Sample	Total Digested Starch, % (w/w) ^a , mean ^b ± 2 SD, (%RSD _r) ^c				Interday mean, ± 2 SD, (%RSD _r)
	Day 1	Day 2	Day 3	Day 4	
RMS	82.1 ± 0.4	79 ± 0.8	79.5 ± 0.4	79.8 ± 1.7	80.1 ± 2.7
	0.25	0.54	0.23	1.07	1.67
Hylon VII	33.2 ± 0.9	36.5 ± 1.7	35.7 ± 0.2	38.2 ± 0.6	35.9 ± 3.9
	1.34	2.39	0.32	0.84	5.47
UB Express Boiled Rice	72.5 ± 1.3	70.2 ± 0.9	70.8 ± 0.4	71.8 ± 0.1	71.3 ± 2
	0.93	0.66	0.29	0.06	1.40
ActiStar	34.6 ± 0.4	34.1 ± 4.1	33.1 ± 0.8	35.6 ± 2.3	34.4 ± 2.7
	0.55	6.05	1.21	3.21	3.89
Garden Peas	16.9 ± 0	16.6 ± 0.4	16.3 ± 0.5	16.4 ± 0.1	16.5 ± 0.6
	0.04	1.11	1.61	0.17	1.77
All Bran	24.9 ± 1.5	25.2 ± 0.2	25.1 ± 0.1	25.1 ± 0.2	25.1 ± 0.6
	2.93	0.32	0.22	0.45	1.27
Butter Beans	34.4 ± 0.6	34.5 ± 0.2	34.7 ± 0	34.9 ± 1.8	34.6 ± 0.8
	0.87	0.28	0.05	2.54	1.20

^a all results are presented as starch on an “as is” basis.

^b on each day, samples of each material were analysed in duplicate.

^c SD = standard deviation; RSD_r % = repeatability standard deviation.

Table 4. The repeatability (%RSD_r) for **Resistant Starch** measurement for seven milled samples analysed in duplicate on four separate days using the **DSTRS** assay procedure.

Sample	Resistant Starch, % (w/w) ^a , mean ^b ± 2 SD, (%RSD _r %)				Interday mean, ± 2 SD, (%RSD _r)
	Day 1	Day 2	Day 3	Day 4	
RMS	1.9 ± 0.1	2 ± 0.1	1.9 ± 0	2 ± 0.1	2 ± 0.1
	1.52	2.77	0.07	2.77	2.47
Hylon VII	48.2 ± 0.1	47.7 ± 1.9	47.1 ± 0.2	46.6 ± 0.6	47.4 ± 1.5
	0.14	1.95	0.17	0.60	1.60
UB Express Boiled Rice	2.5 ± 0.4	2.6 ± 0.3	2.6 ± 0.2	2.4 ± 0.2	2.5 ± 0.3
	8.06	5.22	4.58	3.28	6.33
ActiStar	52 ± 0.1	52.3 ± 0.3	51 ± 0.1	51.8 ± 0.9	51.8 ± 1.1
	0.07	0.25	0.13	0.88	1.05
Garden Peas	7.9 ± 0.4	7.9 ± 0.3	7.6 ± 0.7	8 ± 0.6	7.9 ± 0.5
	2.36	1.69	4.55	3.42	3.09
All Bran	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
	4.36	0.95	0.14	3.69	3.96
Butter Beans	3.5 ± 0.1	3.4 ± 0.1	3.5 ± 0.2	3.3 ± 0	3.4 ± 0.2
	1.43	1.16	2.24	0.38	3.22

^a all results are presented as starch on an “as is” basis.

^b on each day, samples of each material were analysed in duplicate.

^c SD = standard deviation; RSD_r % = repeatability standard deviation.

The Resistant Starch content of the samples tested covered a working range of 0.3 to 51.8% (w/w). The repeatability (%RSD_r) across this sample data set was excellent, less than or equal to 6.33% for all samples.



Megazyme

**Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.**

**Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com**

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