DIGESTIBLE AND RESISTANT STARCH

ASSAY PROTOCOL

K-DSTRS

07/23

(40 Assays per Kit)

Play Training Video

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 Alimentarius[5] 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], AOAC Method 2022.01[10]) 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 the 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 2017.16/2022.01). 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 is hydrolysed to glucose with AMG and the glucose is measured with **GOPOD Reagent** (See **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 ± 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. 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.
Store sealed and dry at -10°C. See individual label for expiry date.
NOTE: Consult the SDS on appropriate handling of this product.

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.
Store at 4°C. See individual label for expiry date.

Bottle 3: **GOPOD Reagent 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 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.
Stored sealed at room temperature. See individual label for expiry date.

Bottle 6: **Digestible/Resistant Starch Control** (~ 10 g). Digestible and resistant starch content shown on the label.
Stored sealed at room temperature. See individual label for expiry date.

A. PREPARATION OF REAGENT SOLUTIONS

1. ***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**) to 25 mL of **buffer *[B(a)]*** (sodium maleate buffer 50 mM, pH 6.0 plus 2 mM $CaCl_2$, see page 4 for preparation) and stir on a magnetic stirrer for 5 min. Store on ice during use. Use within 4 h of preparation. This is **solution 1** (Stock PAA/AMG solution).

   NOTE: An ammonium sulphate suspension can be prepared as follows: gradually add 2.5 g of **bottle 1** (PAA 40 KU/g plus AMG 17 KU/g) 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)]* (see page 5 for preparation).
   Stable for ≥ 3 months at 4°C.

2a. **AMG.** Use the contents of **bottle 2** (amyloglucosidase) as supplied.

2b. **Dilute AMG.** Add 1 mL of the contents of **bottle 2** to 30 mL of **buffer *[B(e)]*** (100 mM sodium acetate buffer pH 4.5, see page 5 for preparation). Mix well and divide into ~ 10 mL aliquots in 13 mm polypropylene tubes *[C(t)]* and store below -10°C between use. This is **solution 2** (dilute AMG (~110 U/mL)).

3. Dilute the contents of the **GOPOD Reagent Buffer** bottle to 1 L with distilled water. This is **solution 3**. Use immediately.

4. Dissolve the contents of the **GOPOD Reagent Enzymes** bottle 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 ≥ 1 month at 4°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. Upon storage at 4°C the **GOPOD reagent** may develop a stronger pink colour. The absorbance of this solution should be < 0.05 when read against distilled water.

5. Use the contents of **bottle 5** as supplied.

6. Use the contents of **bottle 6** as supplied.

B. REAGENTS (not supplied):

a. ***Sodium maleate buffer 50 mM, pH 6.0 plus 2 mM $CaCl_2$.***— 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 ($CaCl_2.2H_2O$), dissolve and adjust the volume to 2 L. Store in a well-sealed Duran® bottle at 4°C. This is **buffer *[B(a)]***.

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 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.

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 at 4°C. This is **buffer *[B(d)]***.

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 L with distilled water and store in a Duran® bottle at 4°C. This is **buffer *[B(e)]***.

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 at 4°C.

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 a1 L Duran bottle 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®). (A setup as shown in **Figure 2**, page 11 is optimal).

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 Centrifuge 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 and15 x 5 mm non-ridged.

p. ***Digestion Bottles.***— 250 mL Fisherbrand® soda glass, wide mouth bottles with polyvinyl lined cap (cat. no. FB73219)

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 **solution 1** (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 **solution 2** 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. ***Optional: 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 12).

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 a 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 **buffer [*B(a)*]** (sodium maleate buffer 50 mM, pH 6.0 plus 2 mM $CaCl_2$) to each tube. Cap the tubes and place on a 2mag Mixdrive 15® submersible magnetic stirrer *[C(d)]* in a water bath and allow the contents to equilibrate to 37°C for 5 min with stirring at 170 rpm (see **Figure 3**, page 11).

(c) ***Incubation with PAA/AMG solution.***— Add 2.5 mL of **solution 1** (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 $(NH_4)_2SO_4$ suspension of this enzyme as prepared in *[A(1)]*, add 1 mL of enzyme suspension and 1.5 mL of **buffer [*B(a)*]** (sodium maleate buffer 50 mM, pH 6.0 plus 2 mM $CaCl_2$).

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.

(b) ***Addition of buffer*.**— Wet the sample with 1.0 mL of 95% v/v EtOH (or IMS) and add 35 mL of **buffer [*B(a)*]** (Sodium maleate buffer 50 mM, pH 6.0 plus 2 mM $CaCl_2$) 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 for 5 min with stirring at 170 rpm (see **Figure 3**, page 11).

(c) ***Incubation with PAA/AMG solution.***— Add 5 mL of **solution 1** (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 $(NH_4)_2SO_4$ suspension of this enzyme as prepared in *[A(1)]*, add 2 mL of enzyme suspension and 3 mL of **buffer *[B(a)]*** (Sodium maleate buffer 50 mM, pH 6.0 plus 2 mM $CaCl_2$).

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 **solution 2** (dilute AMG (110 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 of the sample and glucose standards (prepared as below) at 510 nm against the reagent blank.

Prepare reagent blank solutions by mixing 0.2 mL of **buffer *[B(e)]*** (sodium acetate buffer, 100 mM, pH 4.5) with 3.0 mL of **GOPOD reagent** and **prepare D-glucose standards** (in quadruplicate) by mixing 0.1 mL of **bottle 5** (D-glucose, 1 mg/mL) *[A(5)]* plus 0.1 mL **buffer *[B(e)]*** (sodium acetate buffer, 100 mM, pH 4.5) with 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 **buffer *[B(d)]*** (1.0 M sodium acetate buffer, pH 3.8) to each tube while stirring on a magnetic stirrer. Immediately add 0.1 mL of **bottle 2** (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.

(f) 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.

(g) 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).

(h) 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 0.1 mL of **buffer *[B(e)]*** *(*100 mM sodium acetate buffer, pH 4.5) and 3.0 mL **GOPOD reagent**. Incubate at 50°C for 20 min. Measure the absorbance of the sample and glucose standards (prepared as below) at 510 nm against the reagent blank.

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

(i) Calculate RS content as described in section *[H(b)],* or by using the appropriate MegaCalc™ Excel® calculator.

H. CALCULATIONS (digestible starch and resistant starch)

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

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

Methods 1 and 2 (page 7).

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 = For RDS (ΔA^{RDS}) absorbance reaction read against the reaction blank after 20 min. For SDS (ΔA^{SDS}) absorbance reaction read against the reaction blank after 120 min minus ΔA^{RDS}. For TDS (ΔA^{TDS}) absorbance reaction read against the reaction blank after 240 min.

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/100 g sample):

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

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

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/100 g.

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.

APPENDIX:

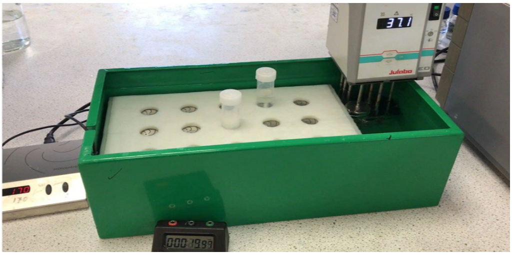

Figure 2. Incubation Method 1. Samples (~ 0.5 g) in 40 mL, 30 x 84 mm polypropylene tubes in a 2mag Mixdrive 15® submersible magnetic stirrer in a water bath. This arrangement allows stirring of 15 samples at a controlled speed (170 rpm) and 37°C.

Figure 3. Incubation Method 2. Samples (~ 1.0 g) in Fisherbrand bottles on a 2mag Mixdrive 15® submersible magnetic stirrer in a water bath. This arrangement allows stirring of 15 samples at a 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.

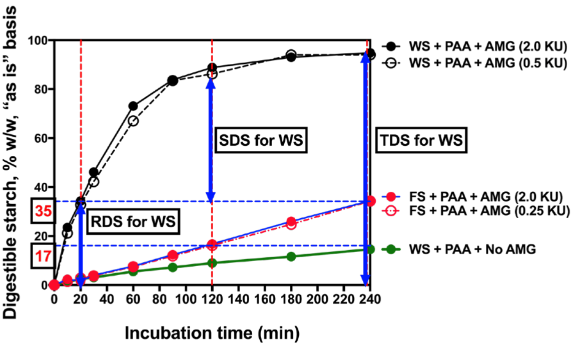

Figure 5. Hydrolysis of 1 g of wheat starch (WS) or Fibersym™ (FS; phosphate cross-linked starch; RS_4) by pancreatic α-amylase (2.0 KU) in the presence of amyloglucosidase (0.25 or 0.5 KU/assay). Samples removed at different time intervals, diluted, reactions terminated, and D-glucose measured and digestible 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; RSDr % = 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$_r$[c] %)				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.

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