

Megazyme

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RESISTANT STARCH

ASSAY PROCEDURE

K-RSTAR 08/18

(100 Assays per Kit)

AOAC Method 2002.02
AACC Method 32-40.01
Codex Type II Method



INTRODUCTION:

By definition, resistant starch (RS) is that portion of the starch that is not broken down by human enzymes in the small intestine. It enters the large intestine where it is partially or wholly fermented. RS is generally considered to be one of the components that make up total dietary fibre (TDF).

The presence of a starch fraction resistant to enzymic hydrolysis was first recognised by Englyst *et al.* in 1982 during their research on the measurement of non-starch polysaccharides.¹ This work was extended by Berry² who developed a procedure for the measurement of RS incorporating the α -amylase/pullulanase treatment employed by Englyst *et al.*,¹ but omitting the initial heating step at 100°C, so as to more closely mimic physiological conditions. Under these conditions, the measured resistant starch contents of samples were much higher. This finding was subsequently confirmed by Englyst *et al.*³⁻⁵ through studies with healthy ileostomy subjects.

By the early 1990's the physiological significance of RS was fully realised. Several new/modified methods were developed during the European Research Program EURESTA.^{6, 7} The Champ⁷ method was based on modifications to the method of Berry² and gave a direct measurement of RS. Basically, sample size was increased from 10 mg to 100 mg, the sample was digested with pancreatic α -amylase only (not pancreatic α -amylase plus pullulanase, as used by Englyst¹ and Berry²) and incubations were performed at pH 6.9 (pH 5.2 was used by Englyst¹ and Berry²). RS determinations were performed directly on the pellet. Muir and O'Dea⁸ developed a procedure in which samples were chewed, treated with pepsin and then with a mixture of pancreatic α -amylase and amyloglucosidase in a shaking water bath at pH 5.0, 37°C for 15 h. The residual pellet (containing RS) was recovered by centrifugation, washed with acetate buffer by centrifugation and the RS was digested by a combination of heat, DMSO and thermostable α -amylase treatments.

More recently, these methods have been modified by Fausant *et al.*,⁹ Goni *et al.*,¹⁰ Akerberg *et al.*¹¹ and Champ *et al.*¹² These modifications included changes in enzyme concentrations employed, types of enzymes used (all used pancreatic α -amylase, but pullulanase was removed and, in some cases, replaced by amyloglucosidase), sample pre-treatment (chewing), pH of incubation and the addition (or not) of ethanol after the α -amylase incubation step. All of these modifications will have some effect on the determined level of RS.

In developing the current modified method for the measurement of RS, our aim was to provide a robust and reliable method which (as much as feasible) reflected *in vivo* conditions, and which yielded values that were physiologically significant (see Table 1, page 12). To do this, we¹³ studied the effect of concentration of pancreatic α -amylase, the pH of the incubation, the importance of maltose inhibition of α -amylase and the need, or otherwise, of amyloglucosidase inclusion, the effect of shaking and stirring on the determined values, and problems in recovering and analysing the resistant starch containing pellet.

The method that we developed, as described in this booklet, allows the measurement of resistant starch, solubilised starch and total starch content of samples. Twenty four samples can be analysed within a 24 h period. The procedure has been subjected to interlaboratory evaluation (see Table 2, page 13) under the auspices of AOAC International and AACC International¹⁴ and accepted by both associations (AOAC Official Method 2002.02; AACC Method 32-40.01).

PRINCIPLE OF THE CURRENT METHOD:

Samples are incubated in a shaking water bath with pancreatic α -amylase and amyloglucosidase (AMG) for 16 h at 37°C, during which time non-resistant starch is solubilised and hydrolysed to D-glucose by the combined action of the two enzymes. The reaction is terminated by the addition of an equal volume of ethanol or industrial methylated spirits (IMS, denatured ethanol) and the RS is recovered as a pellet on centrifugation. This is then washed twice by suspension in aqueous IMS or ethanol (50% v/v), followed by centrifugation. Free liquid is removed by decantation. RS in the pellet is dissolved in 2 M KOH by vigorously stirring in an ice-water bath over a magnetic stirrer. This solution is neutralised with acetate buffer and the starch is quantitatively hydrolysed to glucose with AMG. D-Glucose is measured with glucose oxidase/peroxidase reagent (GOPOD) and this is a measure of the RS content of the sample. Non-resistant starch (solubilised starch) is determined by pooling the original supernatant and the washings, adjusting the volume to 100 mL and measuring D-glucose content with GOPOD.

APPLICABILITY AND ACCURACY:

The method is applicable to samples containing more than 2% w/w RS. With such samples, standard errors of $\pm 5\%$ are achieved routinely. Higher errors are obtained for samples with RS contents < 2% w/w.

KITS:

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

Bottle 1: **Amyloglucosidase** [12 mL, 3,300 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.

*Full assay procedure is available at “www.megazyme.com” - Product Code: **R-AMGR3**.

Bottle 2: **Pancreatic α -amylase** (Pancreatin, 10 g, 3 Ceralpha Units/mg).
Stable for > 3 years below -10°C.

Bottle 3: **GOPOD Reagent Buffer.** Buffer (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.095% w/v).
Stable for > 4 years at 4°C.

Bottle 4: **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine.
Freeze-dried powder.
Stable for > 5 years below -10°C.

Bottle 5: **D-Glucose standard solution** (5 mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years at room temperature.

Bottle 6: **Resistant starch control.** Resistant starch content shown on the label.
Stable for > 5 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

I. Use the contents of bottle 1 (AMG; Solution 1) as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser, e.g. Eppendorf Multipipette® with 5.0 mL Combitip® (to dispense 0.1 mL aliquots).
Stable for > 3 years at 4°C.

Dilute AMG (300 U/mL). Dilute 2 mL of concentrated AMG solution (bottle 1) to 22 mL with 0.1 M sodium maleate buffer (0.1 M, pH 6.0; Reagent 1; not supplied). Divide into 5 mL aliquots and store frozen in polypropylene containers between use. Stable to repeated freeze/thaw cycles and for 5 years below -10°C.

2. **Immediately before use**, suspend 1 g of the contents of bottle 2 (pancreatic α -amylase) in 100 mL of sodium maleate buffer (100 mM, pH 6.0; Reagent 1; not supplied) and stir for 5 min. Add 1.0 mL of **Dilute AMG (300 U/mL)** and mix well. Centrifuge at $> 1,500$ g for 10 min and carefully decant the supernatant solution. Use this solution (Solution 2) on the day of preparation.
3. Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water (this is solution 3). Use immediately.

NOTE:

1. On storage, salt crystals may form in the concentrated buffer. These must be completely dissolved when this buffer is diluted to 1 L with distilled water.
 2. This buffer contains 0.095% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.
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 $2-5^{\circ}\text{C}$ or > 12 months below -10°C .

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

When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink colour over 2-3 months at 4°C . The absorbance of this solution should be less than 0.05 when read against distilled water.
- 5 & 6. Use the contents of bottles 5 and 6 as supplied. Stable for > 5 years at room temperature.

REAGENTS (NOT SUPPLIED):

Reagents should be analytical purity grade.

1. **Sodium maleate buffer** (100 mM, pH 6.0) plus 5 mM calcium chloride dihydrate and sodium azide (0.02% w/v).

Dissolve 23.2 g of maleic acid (Sigma cat. no. M0375) in 1600 mL of distilled water and adjust the pH to 6.0 with 4 M (160 g/L) sodium hydroxide. Add 1.47 g of calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and 0.4 g of sodium azide and dissolve. Adjust the volume to 2 L.
Stable for 12 months at 4°C.

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

Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1 L with distilled water.
Stable for 12 months at room temperature.

3. **Sodium acetate buffer** (100 mM, pH 4.5).

Add 5.8 mL of glacial acetic acid to 900 mL of distilled water and adjust to pH 4.5 using 4 M sodium hydroxide. Adjust the volume to 1 L with distilled water.
Stable for 2 months at 4°C.

4. **Potassium hydroxide solution** (2 M).

Add 112.2 g KOH to 900 mL of deionised water and dissolve by stirring. Adjust volume to 1 L. Store in a sealed container.
Stable for > 2 years at room temperature.

5. **Aqueous ethanol (or IMS)** (approx. 50% v/v).

Add 500 mL of ethanol (95% v/v or 99% v/v) or industrial methylated spirits (IMS; denatured ethanol; ~ 95% v/v ethanol plus 5% v/v methanol) to 500 mL of H_2O . Store in a well-sealed bottle.
Stable for > 2 years at room temperature.

NOTE:

A set of control samples containing RS levels from 0.6 to 78% w/w is available from Megazyme International Ireland (Cat. no. K-RSTCL).

EQUIPMENT (RECOMMENDED):

1. Grinding mill - Centrifugal, equipped with 12-tooth rotor and a 1.0 mm sieve, or similar device. Alternatively, a cyclone mill can be used for small samples.
2. Meat mincer - Hand operated or electric, fitted with a 4.5 mm screen.
3. Bench centrifuge - Capable of holding 16 x 120 mm glass test tubes, with rating of approx. 1,500 g (~ 3,000 rpm).
4. Shaking water bath (Grant OLS 200) (Grant Instruments Cambridge Ltd.) (or similar) set in linear motion at 100 revolutions per min on the dial (equivalent to a shake speed of 200 strokes/min), a stroke length of 35 mm and 37°C.
5. Water bath - Capable of maintaining 50 +/- 0.1°C.
6. Vortex mixer.
7. Magnetic stirrer.
8. Magnetic stirrer bars – 5 x 15 mm.
9. pH Meter.
10. Stop-watch/timer (digital).
11. Analytical balance (correct to 0.1 mg).
12. Spectrophotometer - capable of operating at 510 nm, preferably fitted with flow-through cell (10 mm path length).
13. Pipettor - capable of delivering 100 µL; with disposable tips. Alternatively, motorised hand-held dispenser can be used.
14. Positive displacement pipettor - Equipped with 50 mL tips capable of delivering 2.0 mL, 3.0 mL and 4.0 mL.
15. Corning® Culture Tubes - screw cap, 16 x 125 mm [Fisher Scientific Cat No. TKV-173-030B (tubes); TKV-178-020V (caps)], Fisher Scientific, interact@fisher.co.uk.
16. Glass test tubes - 16 x 100 mm, 14 mL capacity.
17. Plastic “lunch box”, large enough to hold test-tube rack and serve as an ice-water bath (see Figure 1, page 9).
18. Thermometer - Capable of reading 37 +/- 0.1°C and 50 +/- 0.1°C.
19. Volumetric flasks - 100 mL, 200 mL, 500 mL, 1 L and 2 L capacity.

SAMPLE PREPARATION:

Grind approx. 50 g of sample of grain or lyophilised plant or food product in grinding mill to pass a 1.0 mm sieve. Transfer all material to a wide-mouthed plastic jar and mix well by shaking and inversion. Industrial starch preparations are usually supplied as a fine powder, so grinding is not required. Mince fresh samples (e.g. canned beans, banana, potatoes) in a hand operated or electric meat mincer to pass an ~ 4.5 mm screen. Determine moisture content of dry samples by AOAC Method 925.10 (15) and of fresh samples by lyophilisation followed by oven drying according to AOAC Method 925.10.

ASSAY PROCEDURE:

(a) Hydrolysis and solubilisation of non-resistant starch.

- i. Accurately weigh a 100 ± 5 mg sample directly into each screw cap tube (Corning® culture tube; 16 x 125 mm) and gently tap the tube to ensure that the sample falls to the bottom.

NOTE: For wet samples such as minced canned beans or food product, the sample size is approx. 0.5 g (weighed accurately). With such materials, the moisture content is usually 60-80%.

- ii. Add 4.0 mL of pancreatic α -amylase (10 mg/mL) containing AMG (3 U/mL) (Solution 2) to each tube.
- iii. Tightly cap the tubes, mix them on a vortex mixer and attach them horizontally in a shaking water bath, aligned in the direction of motion (see Figures 2 and 3, pages 11 and 12).
- iv. Incubate tubes at 37°C with continuous shaking (200 strokes/min) for **exactly 16 h** (**Note:** for linear motion, a setting of 100 on the water bath is equivalent to 200 strokes/min; 100 forward and 100 reverse).
- v. Remove the tubes from the water bath and remove excess surface water with paper towel. Remove the tube caps and treat the contents with 4.0 mL of ethanol (99% v/v) or IMS (99% v/v) with vigorous stirring on a vortex mixer.
- vi. Centrifuge the tubes at 1,500 g (approx. 3,000 rpm) for 10 min (non-capped).
- vii. Carefully decant the supernatants and re-suspend the pellets in 2 mL of 50% ethanol or 50% IMS with vigorous stirring on a vortex mixer. Add a further 6 mL of 50% IMS, mix the tubes and centrifuge again at 1,500 g for 10 min.

- viii. Decant the supernatants and **repeat** this suspension and centrifugation step once more.
- ix. Carefully decant the supernatants and invert the tubes on absorbent paper to drain excess liquid.

(b) Measurement of Resistant Starch.

- i. Add a magnetic stirrer bar (5 x 15 mm) and 2 mL of 2 M KOH to each tube and re-suspend the pellets (and dissolve the RS) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 1, page 9).

NOTE:

1. Do not mix on a vortex mixer as this may cause the starch to emulsify.
 2. Ensure that the tube contents are vigorously stirring as the KOH solution is added. This will avoid the formation of a lump of starch material that will then be difficult to dissolve.
- ii. Add 8 mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL of AMG (solution 1; 3,300 U/mL), mix well and place the tubes in a water bath at 50°C.
 - iii. Incubate the tubes for 30 min with intermittent mixing on a vortex mixer.
 - iv. **For samples containing > 10% RS;** 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 to 100 mL with distilled water and mix well. Centrifuge an aliquot of the solution at 1,500 g for 10 min.
 - v. **For samples containing < 10% RS;** directly centrifuge the tubes at 1,500 g for 10 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).
 - vi. Transfer 0.1 mL aliquots (in duplicate) of either the diluted (step iv) or the undiluted (step v) supernatants into glass test tubes (16 x 100 mm), add 3.0 mL of GOPOD reagent (solution 4) and incubate at 50°C for 20 min.

- vii. Measure the absorbance of each solution at 510 nm against the reagent blank.

Prepare reagent blank solutions by mixing 0.1 mL of 100 mM sodium acetate buffer (pH 4.5) and 3.0 mL of GOPOD reagent.

Prepare D-glucose standards (in quadruplicate) by mixing 0.1 mL of D-glucose (1 mg/mL) and 3.0 mL of GOPOD reagent.

(c) Measurement of Non-Resistant (Solubilised) Starch.

- i. Combine the supernatant solutions obtained on centrifugation of the initial incubation [(a)vii, page 7] with the supernatants obtained from the subsequent two 50% ethanol washings [(a)viii and (a)ix, page 8] and adjust the volume to 100 mL with 100 mM sodium acetate buffer (pH 4.5) in a volumetric flask. **Mix well.**
- ii. Incubate 0.1 mL aliquots of this solution (in duplicate) with 10 μ L of **dilute AMG solution** (300 U/mL) in 100 mM sodium maleate buffer (pH 6.0) for 20 min at 50°C. Add 3.0 mL of GOPOD reagent (Solution 4) and incubate the tubes for a further 20 min at 50°C.
- iii. Measure the absorbance at 510 nm against a reagent blank.
- iv. Calculate the content of non-resistant (solubilised) starch.

Total starch content is the sum of resistant starch and non-resistant (solubilised) starch.

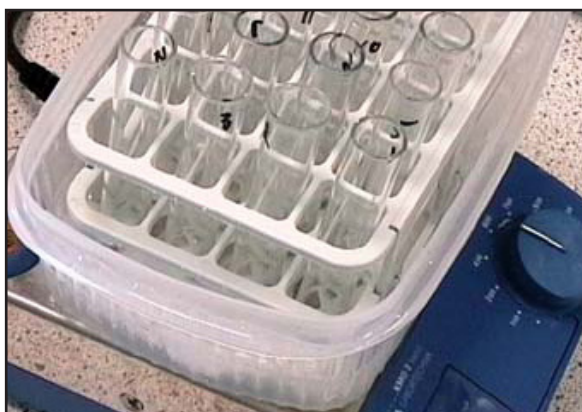


Figure 1. Arrangement of ice-water bath over a magnetic stirrer for treatment of samples with 2 M KOH and dissolution of RS.

CALCULATIONS:

Calculate resistant starch, non-resistant (solubilised) starch and total starch content (% , on a dry weight basis) in test samples as follows:

$$\begin{aligned} \text{Resistant Starch (g/100 g sample)} & (\text{samples containing } > 10\% \text{ RS}): \\ & = \Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180 \\ & = \Delta E \times F/W \times 90 \end{aligned}$$

$$\begin{aligned} \text{Resistant Starch (g/100 g sample)} & (\text{samples containing } < 10\% \text{ RS}): \\ & = \Delta E \times F \times 10.3/0.1 \times 1/1000 \times 100/W \times 162/180 \\ & = \Delta E \times F/W \times 9.27 \end{aligned}$$

$$\begin{aligned} \text{Non-Resistant (Solubilised) Starch (g/100 g sample):} \\ & = \Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180 \\ & = \Delta E \times F/W \times 90 \end{aligned}$$

Total Starch = Resistant Starch + Non-Resistant Starch

where:

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

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

$100/0.1$ = volume correction (0.1 mL taken from 100 mL).

$1/1000$ = conversion from micrograms to milligrams.

W = dry weight of sample analysed
= "as is" weight $\times [(100 - \text{moisture content})/100]$.

$100/W$ = factor to present RS as a percentage of sample weight.

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

$10.3/0.1$ = volume correction (0.1 mL taken from 10.3 mL) for samples containing 0-10% RS where the incubation solution is not diluted and the final volume is ~ 10.3 mL.

When wet samples are analysed, this volume will be larger and this should be allowed for in the calculations.

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).

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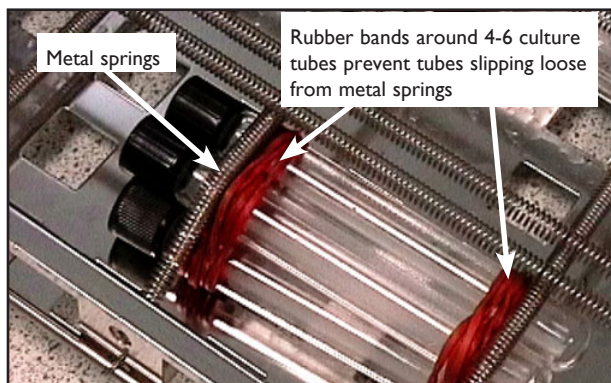


Figure 2. Attachment of Corning® culture tubes to shaking tray in Grant shaking water bath (close view).

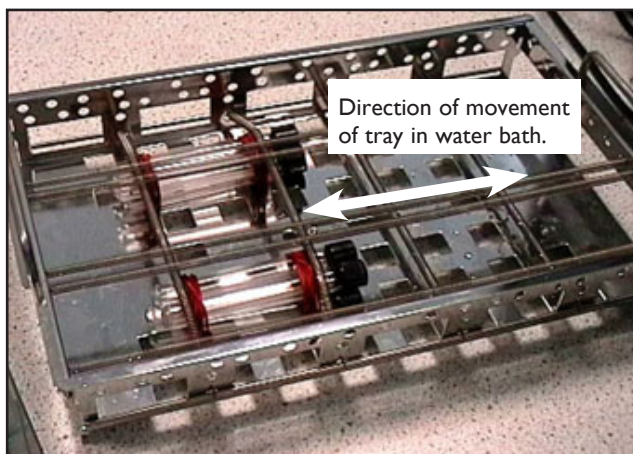


Figure 3. Attachment of Corning® culture tubes to shaking tray in Grant shaking water bath.

Table 1. Comparison of RS values obtained using several *in vitro* analytical methods to *in vivo* results.

Source of starch	RS (<i>in vitro</i> method/results) ^a					RS (<i>in vivo</i>)
	Englyst	Faisant	Champ	McCleary	Goni ^b	
Potato starch (native)	66.5	83.0	77.7	77.0	-	78.8
Amylomaize starch (native)	71.4	72.2	52.8	51.7	-	50.3
Amylomaize starch (retrograded)	30.5	36.4	29.6	42.0	37.8 ^b	30.1
Bean flakes	10.6	12.4	11.2	14.3	15.3 ^c	9-10.9
Corn flakes	3.9	4.9	4.3	4.0	4.7 ^c	3.1-5.0
Canned beans	17.1	-	17.1	16.5	-	16.5
ActiStar ^d	63 ^d	-	57 ^d	58.0	57 ^d	54 ^d

^a Values are presented as a percentage of the total starch content of the sample. All data except that of McCleary, Goni *et al.* (10) and values for ActiStar[®], are from Champ *et al.* (16).

^b From Goni *et al.* (10).

^c From Goni *et al.* (10), calculating RS as a percentage of total starch, assuming a starch content for bean flakes of 40%, and for corn flakes of 70% (based on in house results for similar materials).

^d Results kindly provided by Bernd Kettlitz, Cerestar, Vilvoorde, Belgium, except for values by McCleary, which were produced in-house. The "Englyst" data was produced by Englyst Carbohydrate Services; "Champ" data at INRA, Nantes, and "Goni" data at Cerestar, Vilvoorde.

Table 2. Method performance for measurement of resistant starch by enzymic digestion in starch samples and selected plant materials (AOAC/AACC interlaboratory study results).

Sample	Mean RS ^a , %	No. of labs ^{b(c)}	S _r	S _R	RSD _r %	RSD _R %	r ^d	R ^e	Horrat
Hylon VII ^R (HAMS) ^f	46.29	37(0)	1.91	3.87	4.12	8.37	5.34	10.84	3.72
Green banana	43.56	36(1)	1.39	3.69	3.18	8.47	3.88	10.34	3.74
Native potato starch	63.39	35(2)	2.66	3.77	4.20	5.94	7.45	10.54	2.77
CrystaLean ^R , (Retrograded HAMS)	39.04	34(3)	0.77	2.00	1.97	5.13	2.15	5.61	2.23
ActiStar ^R , RS	48.28	36(1)	1.12	2.81	2.32	5.83	3.14	7.87	2.61
Kidney beans (canned)	4.66	35(2)	0.11	0.21	2.42	4.58	0.32	0.60	1.44
Corn flakes	2.20	34(3)	0.08	0.24	3.43	10.9	0.21	0.67	3.08

a Calculated on "as is" basis ("as is" for banana, kidney beans and corn flakes means on a lyophilised basis)

b = number of collaborating labs (number of outlier labs)

c $r = 2.8 \times S_r$

d $R = 2.8 \times S_R$

e High amylose maize starch

f

DETERMINATION OF RESISTANT STARCH

Date.....

(100 ± 5 mg sample; pancreatic α-amylase (4 mL, 10 mg/mL, pH 6.0) containing AMG (3 U/mL); shaking (200 strokes/min; 35 mm stroke length); 16 h, 37°C.

Sample	Weight (mg)	Moisture content (%)	Corrected weight (mg)	Final vol. (mL)	Absorbance values (510 nm)	Average absorbance (510 nm)	Resistant Starch (% w/w)
1							
2							
3							
4							
5							
6							
7							
8							

D-Glucose/100 µg =; Average =; F =

Starch, % = $\Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180 = \Delta E \times F/W \times 90$ (for samples with > 10% RS).
 = $\Delta E \times F \times 10.3/0.1 \times 1/1000 \times 100/W \times 162/180 = \Delta E \times F/W \times 9.27$ (for samples with < 10% RS).



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