Megazyme

RESISTANT STARCH

ASSAY PROCEDURE

RSTAR 11/02

AOAC Method 2002.02
AACC Method 32-40

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 recognized by Englyst *et al*. in 1982 during their research on the measurement of non-starch polysaccharides (1). This work was extended by Berry (2) 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 realized. Several new/modified methods were developed during the European Research Program EURESTA (6, 7). The Champ (7) method was based on modifications to the method of Berry (2) and gave a direct measurement of resistant starch. 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 Englyst (1) and Berry (2)], and, incubations were performed at pH 6.9 [pH 5.2 was used by Englyst (1) and Berry(2)]. RS determinations were performed directly on the pellet. Muir and O'Dea (8) 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 hr. 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). To do this, we (13) 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 analyzing 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 hour period. The procedure has been subjected to interlaboratory evaluation (see Table 2) under the auspices of AOAC International and AACC (14) and accepted by both associations.

References:

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APPLICABILITY AND ACCURACY:

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

KITS:

Kits contain sufficient reagent for performing 100 assays, i.e. they contain the full assay method plus:

1. Pancreatic α-amylase (Pancreatin, 10g, 3 Ceralpha Units/mg)
   Store dry at 4°C. Stable for >3 years.

2. Amyloglucosidase (12 mL, 3300 Units/mL)
   Store at 4°C. Stable for >3 years.

3. Glucose Determination Reagent (GOPOD) (for 1 litre)
   Reagent concentrations after dissolution in buffer:

Glucose oxidase	>12,000 U/litre
Peroxidase	>650 U/litre
4-Aminoantipyrine	0.4 mM

   Stable at -20°C for > 3 years.

4. Glucose Reagent Buffer (concentrate) (50 mL).
   Stable at 4°C for > 3 years (buffer salts may crystallise).

   Dilute the entire contents to 1 litre with distilled water and use to dissolve the Glucose Determination Reagent (GOPOD). Divide this reagent (GOPOD Reagent) into aliquots of desired volume for storage.

Stability:	2-3 months at 4°C
	> 2 years at -20°C.

5. Glucose Standard Solution (1 mg/mL in 0.2% benzoic acid).
   Stable at room temperature for > 5 years.

6. Resistant Starch Control (with stated level of RS).
   Store dry at room temperature. Stable for > 5 years.

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

Measurement of Resistant Starch in Pure Starches and Plant and Food Materials.

A. Principle

Samples are incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase (AMG) for 16 hr at 37°C, during which time non-resistant starch is solubilised and hydrolyzed to 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 neutralized with acetate buffer and the starch is quantitatively hydrolyzed to glucose with AMG. 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) can be determined by pooling the original supernatant and the washings, adjusting the volume to 100 mL and measuring glucose content with GOPOD.

B. Apparatus

(a) Grinding mill. - Centrifugal, equipped with 12-tooth rotor and a 1.0 mm sieve, or similar device. Alternatively, cyclone mill can be used for small samples.
(b) Meat mincer. - Hand operated or electric, fitted with a 4.5 mm screen.
(c) Bench centrifuge. - Capable of holding 16 x 120 mm glass test tubes, with rating of ca 1500 *g* (~ 3,000 rpm).
(d) Shaking water bath (Grant OLS 200) (Grant Instruments Cambridge Ltd.) (or similar) set in linear motion at 100 revolutions per minute on the dial (equivalent to a shake speed of 200 strokes/min), a stroke length of 35mm and 37°C.
(e) Water bath. - Capable of maintaining 50 + 0.1°C.
(f) Vortex mixer.
(g) Magnetic stirrer.
(h) Magnetic stirrer bars. – 5 x 15 mm.
(i) pH Meter.
(j) Stop-clock timer (digital).
(k) Analytical balance (correct to 0.1 milligram).
(l) Spectrophotometer. - capable of operating at 510 nm, preferably fitted with flow-through cell (10 mm path length).
(m) Pipettor. - capable of delivering 100 µL; with disposable tips. Alternatively, motorised hand-held dispenser can be used.

(n) Positive displacement pipetter. - Equipped with 50 mL tips capable of delivering 2.0 mL, 3.0 mL and 4.0 mL.
(o) 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
(p) Glass test tubes. - 16 x 100 mm, 14 mL capacity.
(q) Plastic "lunch box", large enough to hold test-tube rack and serve as an ice-water bath (see Figure 1).
(r) Thermometer. - Capable of reading 37 + 0.1^{o}C and 50 + 0.1^{o}C.
(s) Volumetric flasks. - 100 ml, 200 ml, 500 ml, 1 L and 2 L capacity.

C. Reagents

All reagents should be analytical purity grade.

(a) *Sodium maleate buffer. - 0.1 M, pH 6.0.* Dissolve 23.2 g maleic acid in 1600 mL of distilled water and adjust the pH to 6.0 with 4M (160 g/litre) sodium hydroxide. Add 0.6 g of calcium chloride ($CaCl_2.2H_2O$) and 0.4 g of sodium azide and adjust the volume to 2 L. Stable at 4°C for 12 months.

(b) *Sodium acetate buffer. – 1.2 M, pH 3.8.* Add 69.6 mL of glacial acetic acid to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1 litre with distilled water. Stable at room temperature for 12 months.

(c) *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 litre with distilled water. Stable at 4°C for 2 months.

(d) *Potassium Hydroxide. - 2 M.* Add 112.2 g KOH to 900 mL of deionised water and dissolve by stirring. Adjust volume to 1 litre.

(e) *Aqueous IMS. – About 50% v/v.* Dilute 500 mL of ethanol (95% or 99%) or industrial methylated spirits (IMS; denatured ethanol; ~ 95% ethanol plus 5% methanol) to 1 L with H_2O. Store in a well sealed bottle. Stable at room temperature for > 12 months.

(f) *Stock Amyloglucosidase (AMG) solution. - 12 mL, 3300 U/mL* in 50% glycerol. Solution is viscous; for dispensing use positive displacement dispenser. AMG solution is stable for up to 5 years when stored at 4°C. (***Note***: One unit [**U**] of enzyme activity is amount of enzyme required to release 1 micromole of glucose from soluble starch per minute at 40°C and pH 4.5). AMG solution should be free of detectable levels of free glucose.

(g) *Dilute Amyloglucosidase solution. – 300 U/mL.* Dilute 2 mL of concentrated AMG solution [**C**(f)] to 22 ml with 0.1 M sodium maleate buffer (pH 6.0)[**C**(a)]. Divide into 5 mL aliquots and store frozen in polypropylene containers between use. Stable to repeated freeze-thaw cycles, and for > 5 years at -20°C.

(h) *Pancreatic α-amylase (10 mg/mL) plus AMG (3 U/mL). –* Immediately before use, suspend 1 gram of pancreatic α-amylase in 100 mL of sodium maleate buffer [**C**(a)] and stir for 5 min. Add 1.0 mL of AMG (300 U/mL) [**C**(g)] and mix well Centrifuge at > 1,500 *g* for 10 min, and carefully decant the supernatant. This solution should be used on the day of preparation.

(i) *Glucose oxidase-peroxidase-aminoantipyrine reagent (GOPOD).* Prepare glucose oxidase-peroxidase-aminoantipyrine reagent (GOPOD) mixture by diluting 50 mL of buffer concentrate to 1.0 L. Use part of this diluted buffer to dissolve the entire contents of the vial containing freeze-dried glucose oxidase-peroxidase-aminoantipyrine mixture. Quantitatively transfer the contents of the vial to 1 L volumetric flask containing diluted buffer. This mixture contains;

glucose oxidase	> 12000 U/L;
peroxidase	> 650 U/L; and
4-aminoantipyrine	0.4 mM.

Reagent is stable 2-3 months when stored at 4°C and > 2 years when stored at -20°C.

Preparation of additional buffer concentrate.
Dissolve 136 g potassium dihydrogen orthophosphate, 42 g sodium hydroxide and 30 g 4-hydroxybenzoic acid in 900 mL of distilled water. Adjust to pH 7.4 with either 2 M HCl or 2 M NaOH. Dilute the solution to 1 litre and add 0.4 g sodium azide and mix well until dissolved. Buffer concentrate is stable for up to 3 years at 4°C.

Check color formation and stability of glucose oxidase-peroxidase-aminoantipyrine buffer mixture by incubating (in duplicate) 3.0 mL glucose oxidase-peroxidase-aminoantipyrine reagent mixture (GOPOD) with 0.1 mL glucose standard (1 mg/mL in 0.2% benzoic acid solution) plus 0.1 mL of 0.1 M sodium acetate buffer [**C**(c)]. Maximum color formation should be achieved within 20 min and should be stable for > 60 min.

Items (f), (h) and (i) are supplied in the RS Assay Kit.

D. Preparation of Test Samples.

Grind an approximately 50 g sample of grain or lyophilised plant or food product in grinding mill ***B*(a)** 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.

E. Measurement of Resistant Starch

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

i. Accurately weigh a 100 $\pm$ 5 mg sample directly into each screw cap tube (Corning culture tube; 16 x 125 mm [***B***(o)]) and gently tap the tube to ensure that the sample falls to the bottom.

ii. Add 4.0 ml of pancreatic α-amylase (10 mg/ml) containing AMG (3 U/ml [***C***(h)] to each tube.

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

iii. Tightly cap the tubes, mix them on a vortex mixer and attach them horizontally in a shaking water bath [***B***(d)], aligned in the direction of motion (see Figures 2 and 3).

iv. Incubate tubes at 37°C with continuous shaking (200 strokes/min for **exactly 16 hr**. (**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%) or IMS (99%) 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 supernatants and re-suspend the pellets in 2 ml of 50% ethanol or IMS [***C***(e)] 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.

ix. Decant the supernatants and **repeat** this suspension and centrifugation step once more.

x. 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) [***B***(h)] and 2 mL of 2 M KOH [***C***(d)] to each tube and re-suspended the pellets (and dissolve the RS) by stirring for approx. 20 min in an ice/water bath [***B***(q)] over a magnetic stirrer.

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) [***C***(b)] to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL of AMG (3300 U/ml)[***C***(f)], 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 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 to 100 mL with water and mix well. Centrifuge an aliquot of the solution at 1,500 *g* for 10 min.

v. ***For samples containing < 10% RS content;*** directly centrifuge the tubes at 1,500 *g* for 10 min (no dilution). For such samples, the final volume in the tube is approximately 10.3 ml (however, this volume will vary particularly if wet samples are analyzed, and appropriate allowance for volume should be made in the calculations).

vi. Transfer 0.1 mL aliquots (in duplicate) of either the diluted or undiluted supernatants into glass test tubes (16 x 100 mm), treat with 3.0 mL of GOPOD reagent [***C***(i)] 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 0.1 M sodium acetate buffer (pH 4.5)[***C***(c)] and 3.0 mL of GOPOD reagent. **Prepare glucose standards** (in quadruplicate) by mixing 0.1 mL of glucose (1 mg/ml) and 3.0 mL of GOPOD reagent.

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

i. Combine the supernatant solutions obtained on centrifugation of the initial incubation [***E***(a)vii] with the supernatants obtained from the subsequent two 50% ethanol washings [***E***(a)ix and x] and adjust the volume to 100 mL in a volumetric flask. **Mix well.**

ii. Incubate 0.1 mL aliquots of this solution (in duplicate) with 3.0 mL of GOPOD reagent [***C***(i)] for 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.

G. Calculations

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

Resistant Starch (g/100g sample)(samples containing > 10% RS):
= $\Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180$
= $\Delta E \times F/W \times 90$.

Resistant Starch (g/100g sample)(samples containing < 10% RS):
= $\Delta E \times F \times 10.3/0.1 \times 1/1000 \times 100/W \times 162/180$
= $\Delta E \times F/W \times 9.27$.

Non-Resistant (Solubilised) Starch (g/100g sample):
= $\Delta E \times F \times 100/0.1 \times 1/1000 \times 100/W \times 162/180$
= $\Delta E \times F/W \times 90$.

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 glucose in the GOPOD reaction is determined and F = 100 (µg of glucose) divided by the GOPOD absorbance for this100 µg of 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 analyzed
= "as is" weight x (100-moisture content)/100].

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

162/180 = factor to convert from free glucose, as determined, to anhydro-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 hould be allowed for in the calculations.**

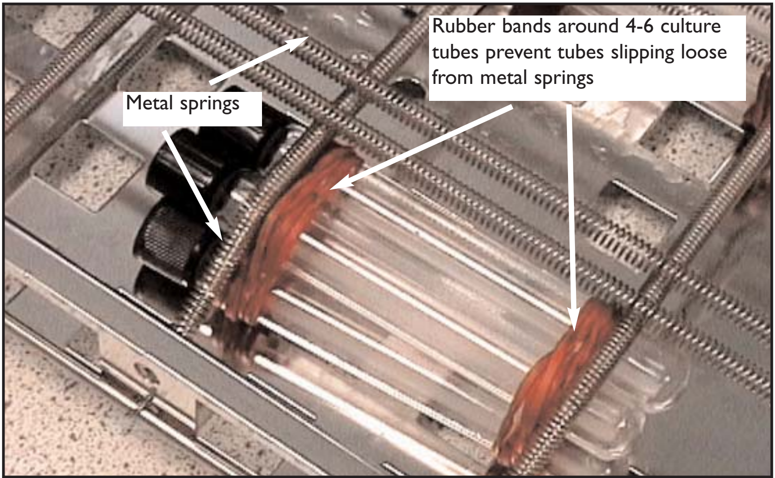

Figure 2. Attachment of Corning culture tubes to shaking tray in Grant shaking water bath (close view).

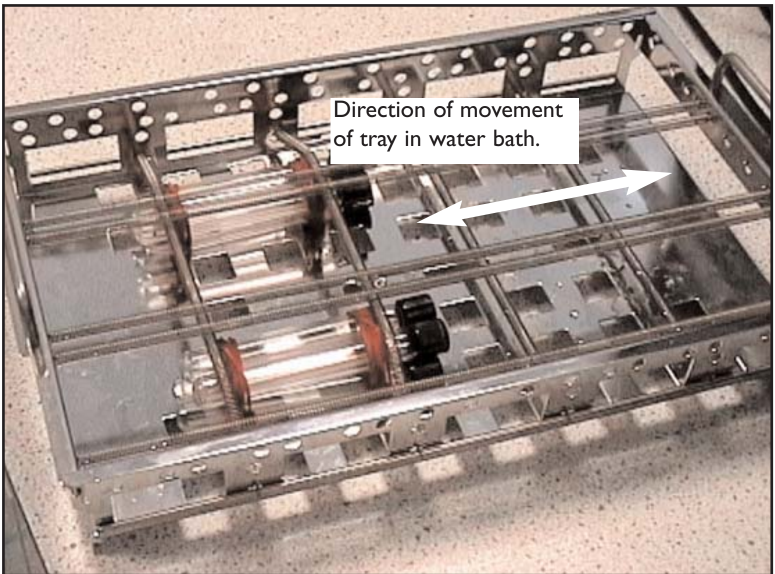

Figure 3. Attachment of Corning culture tubes to shaking tray in Grant shaking water bath.

NOTE: These pictures can be seen more clearly at **www.megazyme.com**/purchase products/Diagnostic kits/ Resistant Starch/booklet.

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

Source of starch	RS (*in vitro* method/results)[a]					RS
	Englyst	Faisant	Champ	McCleary	Goni[b]	(*in vivo*)
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[R], 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(c) b=number of collaborating labs (number of outlier labs)
d $r = 2.8 \times S_r$.
e $R = 2.8 \times S_R$.
f High amylose maize starch

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 hr, 37oC.

Sample	Weigh (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							

Glucose/100mg =……………………………………; Average =……………..; F = ……….;

Starch, % = ΔE x F x 100/0.1 x 1/1000 x 100/W x 162/180 = ΔE x F/W x 90 (for samples with > 10% RS).
= ΔE x F x 10.3/0.1 x 1/1000 x 100/W x 162/180 = ΔE x F/W x 9.27 (for samples with < 10% RS).

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