FOOD COMPOSITION AND ADDITIVES

Measurement of Resistant Starch by Enzymatic Digestion in Starch and Selected Plant Materials: Collaborative Study

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Interlaboratory performance statistics was determined for a method developed to measure the resistant starch (RS) content of selected plant food products and a range of commercial starch samples. Food materials examined contained RS (cooked kidney beans, green banana, and corn flakes) and commercial starches, most of which naturally contain, or were processed to yield, elevated RS levels. The method evaluated was optimized to yield RS values in agreement with those reported for in vivo studies. Thirty-seven laboratories tested 8 pairs of blind duplicate starch or plant material samples with RS values between 0.6 (regular maize starch) and 64% (fresh weight basis). For matrixes excluding regular maize starch, repeatability relative standard deviation (RSD_r) values ranged from 1.97 to 4.2%, and reproducibility relative standard deviation (RSD_R) values ranged from 4.58 to 10.9%. The range of applicability of the test is 2–64% RS. The method is not suitable for products with <1% RS (e.g., regular maize starch; 0.6% RS). For such products, RSD_r and RSD_R values are unacceptably high.

By definition, resistant starch (RS) is that portion of 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 considered to be one of the components that make up total dietary fiber (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 nonstarch polysaccharides (1). This work was extended by Berry (2), who developed a procedure for the measurement of RS incorporating the α-amylase/pullulanase treatment used by Englyst et al. (1), but omitting the initial heating step at 100°C to more closely mimic physiological conditions. Under these conditions, the measured RS content of samples was much higher. This finding was subsequently confirmed by Englyst and Cummins (3–5) through studies with healthy ileostomy subjects.

By the early 1990s the physiological significance of RS was fully realized. Several new and modified methods were developed during the European Research Program EURESTA [European FLAIR–Concerted Action No. 11 (Cost 911); 6, 7]. The Champ (7) method modified the method of Berry (2) and gave a direct measurement of RS. Basically, test portion size was increased from 10 to 100 mg, the matrix was digested with pancreatic α-amylase only [not pancreatic α-amylase plus pullulanase, as used by Englyst et al. (1) and Berry (2)], and incubations were performed at pH 6.9 [pH 5.2 was used by Englyst et al. (1) and Berry (2)]. RS determinations were performed directly on the pellet.

Muir and O'Dea (8) developed a procedure in which foods were chewed by human volunteers, treated with pepsin and then with a mixture of pancreatic α-amylase and amyloglucosidase (AMG) in a shaking water bath at pH 5.0 and 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, dimethyl sulfoxide (DMSO), and thermostable α-amylase treatments.

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

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In developing the current modified method for the measurement of RS, our aim was to provide a robust and reliable method which reflected in vivo conditions and yielded values that were physiologically significant (13). To do this, we studied the effect of concentration of pancreatic α-amylase, the pH of incubation, the importance of maltose inhibition of α-amylase, and the need, or otherwise, of amyloglucosidase or protease inclusion, the effect of shaking and stirring on the determined values, and problems in recovering and analyzing the RS containing pellet (14). In the current study, the performance of this method for the measurement of RS was evaluated.

Collaborative Study

Eight coded homogeneous test samples containing RS, including pure starch, cereal, fruit, and beans were thoroughly mixed and provided as 16 blind duplicates. Four test samples with stated contents of RS, namely, high amylose maize starches (HAMS; Penford Australasia Ltd., Lane Cove, NSW, Australia), high amylose maize starch (Hylon VII®; National Starch and Chemical Co., Bridgewater, NJ), regular maize starch (RMS; Penford Australasia Ltd.), and kidney beans (Batchelors Ltd., Cabra West, Dublin, Ireland), were also provided to familiarize analysts with the method. The kidney beans were drained, freeze-dried, and milled to pass a 1.0 mm screen. To help decide whether to dilute the incubation solutions before glucose determination, laboratories were advised which products contained more or less than 10% RS. Collaborators were requested to perform single determinations on each material by the enclosed method, but duplicate glucose determinations on the extracts. They were requested to provide data on an "as is" basis (i.e., not to dry the test samples before analysis). Results were evaluated according to AOAC guidelines (15). Outlier results identified by the Cochran test for extremes of repeatability and the Grubbs test for extremes of reproducibility were identified and omitted from calculations. The within (s_r) and between (s_R) laboratory standard deviations, repeatability (r) and reproducibility (R) as $2.8 \times s_r$ and $2.8 \times s_R$, respectively, and relative standard deviations (RSD_r and RSD_R) from s_r and s_R as percentages of mean values, were determined by using the AOAC statistics package.

Full details of the unknown test samples sent to collaborators are as follows:

(*1*) Commercially available Kellogg corn flakes (Tesco Supermarket, Greystones, Ireland) were freeze-dried and milled to pass a 1.0 mm screen of a Retsch centrifugal mill (samples B/C).

(*2*) Canned kidney beans (Chivers Ireland Ltd., Coolock, Dublin) were drained and then freeze-dried and milled to pass a 1.0 mm screen (samples D/F).

(*3*) Green bananas (Tesco Supermarket) were crushed and then freeze-dried and milled to pass a 1.0 mm screen (samples K/N).

(*4*) Regular maize starch (Penford Australasia Ltd.; samples A/E).

(*5*) Hylon VII, a native high amylose maize starch (National Starch and Chemical Co.; samples M/O).

(*6*) CrystaLean®, a retrograded, high amylose maize starch (Opta Food Ingredients, Inc., Bedford, MA; samples H/J).

(*7*) Actistar®, an enzyme-modified cassava starch (Cerestar, Vilvoorde, Belgium; samples G/L).

(*8*) Native potato starch (Avebe, Foxhol, The Netherlands; samples I/P).

AOAC Official Method 2002.02
Resistant Starch in Starch and Plant Materials

Enzymatic Digestion
First Action 2002

[Applicable to plant and starch materials containing resistant starch (RS) contents ranging from 2.0 to 64% on an "as is" basis.]

See Table **2002.02** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Nonresistant starch is solubilized and hydrolyzed to glucose by the combined action of pancreatic α-amylase and amyloglucosidase (AMG) for 16 h at 37°C. The reaction is terminated by addition of ethanol or industrial methylated spirits (IMS) and RS is recovered as a pellet by centrifugation. RS in the pellet is dissolved in 2M KOH by vigorously stirring in an ice–water bath. 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), which is a measure of RS content. Nonresistant starch (solubilized starch) is determined by pooling the original supernatant and the washings and measuring the glucose content with GOPOD.

B. Apparatus

(**a**) *Grinding mill*.—Centrifugal, with 12-tooth rotor and 1.0 mm sieve, or similar device. Alternatively, a cyclone mill can be used for small test samples.

(**b**) *Meat mincer*.—Hand-operated or electric, fitted with 4 mm screen.

(**c**) *Bench centrifuge*.—Holding 16 × 100 mm glass test tubes, operating at ca $1500 \times g$.

(**d**) *Shaking water bath*.—Grant OLS 200 [Grant Instruments (Cambridge) Ltd., Royston Hertfordshire SG8 6GB, UK, Tel.: +44 (0) 1763 260811; Fax: +44 (0) 1763 262410; E-mail: paulp@grantinst.co.uk], or equivalent. Set in linear motion at 100 rpm on the dial (equivalent to a shake speed of 200 strokes/min), a stroke length of 35 mm, and 37°C.

(**e**) *Water bath*.—Maintaining 50 ± 0.1°C.

(**f**) *Vortex mixer*

(**g**) *Magnetic stirrer*

(**h**) *Magnetic stirrer bars*.—5 × 15 mm.

(**i**) *pH Meter*

(**j**) *Stop-clock timer*.—Digital.

(**k**) *Analytical balance*.—Weighing to 0.1 mg.

(**l**) *Spectrophotometer*.—Operating at 510 nm, preferably fitted with flow-through 10 mm path length cell.

Table 2002.02. Interlaboratory study results for measurement of resistant starch by enzymatic digestion in starch samples and selected plant materials

Sample	Mean RS[a], %	No. of labs[b,c]	s_r	s_R	RSD_r, %	RSD_R, %	r[d]	R[e]	HORRAT
Hylon VII (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 (retrograded HAMS)	39.04	34(3)	0.77	2.00	1.97	5.13	2.15	5.61	2.23
ActiStar (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 lyophilized basis).
[b,c] b = Number of collaborating laboratories (number of outlier laboratories).
[d] $r = 2.8 \times s_r$.
[e] $R = 2.8 \times s_R$.
[f] High amylose maize starch.

(**m**) *Pipets*.—Delivering 100 μL; with disposable tips. Alternatively, use motorized hand-held dispenser.

(**n**) *Pipetter*.—Delivering 2.0, 3.0, and 4.0 mL.

(**o**) *Culture tubes*.—Corning, glass screw-cap, 16 × 125 mm.

(**p**) *Glass test tubes*.—16 × 100 mm, 14 mL.

(**q**) *Test tube racks*.—Holding 16 × 100 mm tubes.

(**r**) *Thermometer*.—37 ± 0.1 and 50 ± 0.1°C.

(**s**) *Volumetric flasks*.—100, 200, and 500 mL; 1 and 2 L.

C. Reagents

(**a**) *Sodium maleate buffer*.—100mM, pH 6.0. Dissolve 23.2 g maleic acid in 1600 mL water and adjust pH to 6.0 with 4M (160 g/L) NaOH solution. Add 0.6 g $CaCl_2 \cdot 2H_2O$ and 0.4 g sodium azide, and adjust volume to 2 L. Solution is stable at 4°C for 12 months.

(**b**) *Sodium acetate buffer*.—1.2M, pH 3.8. Add 70 mL glacial acetic acid to 800 mL water and adjust to pH 3.8 with 4M NaOH solution. Adjust volume to 1 L with water. Solution is stable at room temperature for 12 months.

(**c**) *Sodium acetate buffer*.—100mM, pH 4.5. Pipette 5.8 mL glacial acetic acid to 900 mL water and adjust to pH 4.5 with 4M NaOH solution. Adjust volume to 1 L with water. Solution is stable at 4°C for 2 months.

(**d**) *Potassium hydroxide solution*.—2M. Add 11.2 g KOH to 150 mL water and dissolve by stirring. Adjust volume to 200 mL with water. Stable at room temperature for at least 12 months.

(**e**) *Aqueous ethanol or IMS*.—Approximately 50% (v/v). Dilute 500 mL ethanol (95 or 99%) or IMS (denatured ethanol; ca 95% ethanol plus 5% methanol) to 1 L with water. Stable at room temperature for at least 12 months.

(**f**) *Stock amyloglucosidase (AMG) stock solution*.—3300 units (U)/mL in 50% glycerol. Use directly without dilution. Solution is viscous; dispense from positive displacement dispenser. AMG solution is stable for up to 5 years when stored at 4°C. (*Note*: One unit enzyme activity is amount of enzyme required to release 1 μmol glucose from soluble starch per minute at 40°C and pH 4.5.) AMG solution should be devoid of detectable levels of free glucose.

(**g**) *AMG solution*.—300 U/mL. Dilute 2 mL concentrated AMG solution, (**f**), to 22 mL with 100mM sodium maleate buffer (pH 6.0), (**a**). Divide into 5 mL aliquots and store frozen in polypropylene containers between use. Stable to repeated freeze–thaw cycles for > 5 years at –20°C.

(**h**) *Pancreatic α-amylase suspension*.—10 mg (30 U/mL) plus AMG (3 U/mL). Immediately before use, suspend 1 g pancreatic α-amylase in 100 mL sodium maleate buffer, (**a**), and stir for 5 min. Add 1 mL AMG solution (300 U/mL), (**g**), and mix well. Centrifuge at >1500 × *g* for 10 min, and carefully decant the supernatant. Use this solution on the day of preparation.

(**i**) *GOPOD–aminoantipyrine buffer mixture*.—Mixture of glucose oxidase, >12 000 U/L; peroxidase, > 650 U/L; and 4-aminoantipyrine, 0.4mM. Prepare buffer concentrate by dissolving 136 g KH_2PO_4, 42 g NaOH, and 30 g 4-hydroxybenzoic acid in 900 mL water. Adjust to pH 7.4 with either 2M HCl or 2M NaOH. Dilute solution to 1 L, add 1 g sodium azide, and mix well until dissolved. Buffer concentrate is stable for up to 3 years at 4°C.

To prepare GOPOD–aminoantipyrine buffer mixture, dilute 50 mL buffer concentrate to 1.0 L. Use part of diluted buffer to dissolve entire contents of vial containing freeze-dried GOPOD–aminoantipyrine mixture. Transfer contents of vial to 1 L volumetric flask containing diluted buffer, and adjust to volume (GOPOD). Reagent is stable 2–3 months when stored at 4°C and 2–3 years when stored at –20°C. Check color formation and stability of GOPOD–aminoantipyrine buffer mixture by incubating (in duplicate) 3.0 mL GOPOD–aminoantipyrine buffer mixture with certified glucose standard (100 μg dried crystalline glucose in 0.2 mL 0.2% sodium benzoate solution). After 15, 20, 30, and 60 min incubation, read absorbance, A, of solution at 510 nm. Maximum color should

be reached within 20 min, and color should be stable for at least 60 min at 50°C after maximum color is achieved.

(**j**) *Glucose standard solution.*—1 mg/mL. Dissolve 1.00 g anhydrous, analytical reagent grade crystalline D-glucose (99.5%) in 900 mL of 0.2% benzoic acid solution in water. Adjust volume to 1 L in volumetric flask and store in well-sealed glass container. Stable at room temperature >5 years.

Items (**f**) and (**h**)–(**j**) are supplied in the Resistant Starch Assay Kit available from Megazyme International Ireland Ltd. (Bray Business Park, Bray, County Wicklow, Ireland), but preparations of reagents and buffers which meet these criteria may also be used.

D. Preparation of Test Samples

Grind ca 50 g test sample of grain or lyophilized plant material in grinding mill, **B**(**a**), to pass 1.0 mm sieve. Transfer all material to wide-mouthed plastic jar and mix well by shaking and inversion. Grinding is not required with industrial starch preparations supplied as a fine powder.

E. Measurement of Resistant Starch

(**a**) *Hydrolysis of nonresistant starch.*—Accurately weigh 100 ± 5 mg test portion directly into each screw-cap tube, **B**(**o**), and gently tap the tube to ensure that material falls to the bottom. Add 4.0 mL pancreatic α-amylase (10 mg/mL) containing AMG (3 U/mL), **C**(**h**), to each tube. Tightly cap the tubes, mix on a vortex mixer, and attach them horizontally, under water, in a shaking water bath, **B**(**d**), aligned in the direction of motion. Incubate at 37°C with continuous shaking (200 strokes/min for 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.)

Remove tubes from water bath and remove excess water on tubes with paper towel. Remove tube caps and add 4.0 mL IMS (99%, v/v) or ethanol (95–99%). Mix tube contents vigorously on vortex mixer. Centrifuge tubes at ca 1500 × *g* for 10 min (noncapped). Carefully decant supernatants and resuspend pellets in 2 mL 50% IMS, **C**(**e**), with vigorous mixing on vortex mixer, **B**(**f**). Add additional 6 mL 50% IMS, **C**(**e**), mix tubes, and centrifuge again at 1500 × *g* for 10 min. Repeat this suspension and centrifugation step once more. Carefully decant supernatants and invert tubes on absorbent paper to drain excess liquid.

(**b**) *Measurement of RS.*—Add magnetic stirrer bar (5 × 15 mm) and 2 mL 2M KOH, **C**(**d**), to each tube and resuspend the pellets. Dissolve RS by stirring for ca 20 min in an ice–water bath over a magnetic stirrer (do not mix on a vortex mixer as this may cause the starch to emulsify). In this step, ensure that tube contents are being vigorously stirred when KOH solution is added to avoid formation of a lump of starch material which would be difficult to dissolve.

Add 8 mL 1.2M sodium acetate buffer (pH 3.8), **C**(**b**), to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL AMG (3300 U/mL), **C**(**f**), mix well on magnetic stirrer, and then place tubes in a water bath at 50°C. Incubate tubes for 30 min with intermittent mixing on a vortex mixer.

For test samples containing >10% RS, quantitatively transfer contents of tube to 100 mL volumetric flask using water wash bottle. Use external magnet to retain stirrer bar in the tube while washing the solution from the tube with a water wash bottle. Adjust to 100 mL with water. Centrifuge an aliquot of the solution at 1500 × *g* for 10 min. For test samples containing <10% RS, directly centrifuge tubes at 1500 × *g* for 10 min without dilution. For such products, the final volume in the tube is 10.3 ± 0.05 mL.

Transfer 0.1 mL aliquots (in duplicate) of either diluted or undiluted supernatants into glass test tubes (16 × 100 mm), **B**(**p**), add 3.0 mL GOPOD reagent, **C**(**i**), mix tube contents on vortex mixer, and incubate at 50°C for 20 min. Prepare reagent blank solutions by mixing 0.1 mL 0.1M sodium acetate buffer (pH 4.5), **C**(**c**), and 3.0 mL GOPOD reagent. Prepare glucose standards (in quadruplicate) by mixing 0.1 mL glucose (1 mg/mL), **C**(**j**), and 3.0 mL GOPOD reagent, **C**(**i**). Incubate at 50°C for 20 min, cool, and set spectrophotometer to 0 with the reagent blank. Measure the absorbance of each solution at 510 nm against the reagent blank. Average duplicate absorbance values. The GOPOD color response with glucose is linear over the absorbance range 0.0–1.5 absorbance units.

F. Calculations

Calculate RS (%, "as is" basis) in test samples as follows:

(*1*) *For products containing >10% RS.*—

$$\text{RS (g/100 g sample)} = \Delta A \times F \times (100/0.1) \times (1/1000) \times (100/W) \times (162/180) = \Delta A \times F/W \times 90$$

(*2*) *For products containing <10% RS.*—

$$\text{RS (g/100 g sample)} = \Delta A \times F \times (10.3/0.1) \times (1/1000) \times (100/W) \times (162/180) = \Delta A \times F/W \times 9.27$$

where ΔA = averaged absorbance (reaction) read against the reagent blank; F = conversion factor from absorbance to micrograms [the absorbance obtained for 100 μg glucose in the GOPOD reaction is determined and F = 100 (micrograms of glucose divided by the GOPOD absorbance for this 100 μg glucose]; 100/0.1 = volume adjustment (0.1 mL taken from 100 mL); 1/1000 = conversion from micrograms to milligrams; W = "as is" weight of test portion analyzed; 100/W = factor to present starch as a percentage of test portion weight; 162/180 = factor to convert from free glucose, as determined, to anhydro-glucose as occurs in starch; 10.3/0.1 = volume adjustment (0.1 mL taken from 10.3 mL) for test portion containing 0–10% RS where the incubation solution is not diluted and the final volume is 10.3 ± 0.05 mL.

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Results and Discussion

To simulate food movement in the small intestine, we recommended the use of a shaking water bath set to linear motion for the initial incubation step with pancreatic α-amylase plus

Table 1. Collaborative results for determination of resistant starch (% w/w "as is" basis) in starch and plant samples (Part 1)[a]

	Products							
	Regular maize starch		Corn flakes		Kidney beans		Actistar (RS)	
Lab No.	A	E	B	C	D	F	G	L
1	0.54	0.63	2.33	2.34	4.67	4.77	48.46	48.42
2	0.85	1.07	2.36	2.29	4.81	4.58	48.55	51.18
3	0.66	0.57	2.12	2.09	4.47	4.59	48.82	47.99
4	1.13	0.68	2.26	2.42	4.81	4.75	52.03	49.85
5	0.20	0.25	2.32	2.29	4.63	4.81	50.01	48.27
6	0.89	0.80	2.22	2.21	4.88	4.85	46.52	48.25
7	0.65	0.79	2.14	2.15	4.61	4.56	48.95	47.42
8	0.58	0.72	2.51	2.38	4.85	4.93	53.88	53.81
9	0.87	1.07	2.34[b]	1.68[b]	4.93	5.00	52.04	52.62
10	0.62	0.45	2.42	2.32	4.83	4.53	51.11	48.44
11	0.33	0.62	1.73[b]	2.19[b]	4.65	4.7	48.09	48.78
12	0.41	0.50	2.23	2.15	4.60	4.51	49.43	50.56
13	1.0	0.93	2.52	2.47	5.33[c]	5.92[c]	52.70	51.53
14	1.5	1.51	2.22	2.19	4.62	4.54	44.33	46.59
15	0.33	0.23	1.65	1.73	4.48	4.49	48.61	49.47
16	0.82	1.36	2.40	2.47	4.69	4.38	49.96	46.4
17	0.36	0.52	2.38	2.31	4.75	4.66	48.53	47.99
18	0.95	0.80	2.46	2.47	4.93	4.90	51.36	49.62
19	0.63	0.61	2.16	2.24	4.45	4.65	47.81	49.04
20	0.41	0.45	2.33	2.30	5.00	4.81	37.95[b]	44.56[b]
21	0.42	0.44	2.20	2.20	4.62	4.79	47.44	47.24
22	0.68	0.85	2.16	2.30	4.51	4.12	43.9	46.41
23	0.62	0.73	2.31	2.26	4.93	4.97	50.12	51.68
24	0.11	0.13	1.29[c]	1.19[c]	3.43[c]	3.44[c]	41.73	42.69
25	0.93	1.18	2.48	2.32	4.65	4.84	52.19	50.82
26	0.78	0.87	1.86	1.56	4.95	4.55	47.91	46.82
27	0.54	0.61	2.24	2.24	4.71	4.74	48.22	48.37
28	0.72	0.71	2.43	2.33	4.55	4.57	49.85	49.91
29	0.40	0.43	2.10	2.11	4.41	4.46	47.02	47.07
30	1.83[b]	2.74[b]	2.30	2.30	4.71	4.74	48.33	48.72
31	0.54	0.69	2.13	2.12	4.20	4.25	42.5	43.6
32	1.05	0.63	2.27	2.26	4.56	4.63	49.18	48.11
33	0.59	0.61	2.48	2.21	4.85	4.8	47.01	49.88
34	0.31	0.21	1.58	1.59	4.11	4.22	43.04	40.9
35	0.84	0.81	1.84	1.62	4.61	4.83	43.37	43.27
36	0.25	0.50	1.75	1.90	4.95	4.85	48.55	45.77
37	0.72	1.07	2.02	2.02	4.42	4.33	48.42	48.41
No. labs	36		34		35		36	
No. outliers	1		3		2		1	
Mean	0.67		2.2		4.66		48.28	
s_r	0.14		0.08		0.11		1.12	
s_R	0.30		0.24		0.21		2.81	
r	0.38		0.21		0.32		3.14	
R	0.84		0.67		0.60		7.87	
RSD_r	21.4		3.43		2.42		2.32	
RSD_R	44.8		10.9		4.58		5.83	
HORRAT	10.5		3.08		1.44		2.61	

[a] Results represent values uncorrected for moisture content. Repeatability and reproducibility data are based on averages of duplicate GOPOD determinations on hydrolyzates of each pair.

[b] Cochran outlier.

[c] Grubbs outlier.

Table 2. Collaborative results for determination of resistant starch (% w/w "as is" basis) in starch and plant samples (Part 2)[a]

	Products							
	CrystaLean (retrograded HAMS)		Native potato starch		Green banana		Hylon VII (HAMS)	
Lab No.	H	J	I	P	K	N	M	O
1	37.67	38.00	64.63	63.26	42.12	42.49	46.10	45.17
2	40.11	40.11	66.58	67.55	46.99	47.20	47.65	47.68
3	40.00	39.64	60.78	54.49	47.77	48.95	48.56	48.43
4	39.47	38.65	66.75	67.44	43.63	42.10	49.50	50.05
5	39.60	39.40	65.97	60.60	45.79	46.11	46.93	46.37
6	38.25	37.65	64.48	60.82	40.67	43.84	44.76	45.34
7	38.20	38.22	63.93	60.87	45.53	44.57	44.52	46.2
8	43.64	43.38	69.81	65.89	49.60	50.60	52.94	52.69
9	42.111	43.28	70.16	69.07	46.63	47.67	52.24	50.86
10	41.88	41.64	65.08	63.60	45.00	44.60	47.17	47.13
11	39.19	36.07	62.38	65.24	43.39	43.69	43.60	44.30
12	40.81	40.85	67.19	66.63	47.51	45.38	49.85	47.88
13	39.85	39.57	66.4	66.13	50.70	47.74	50.10	47.80
14	38.13	38.96	62.25	60.74	44.30	44.73	48.56	44.85
15	39.15	38.79	62.55	62.91	45.66	47.28	47.43	48.25
16	37.82	37.64	76.48	64.75	39.38	38.18	52.00	49.72
17	38.1	40.00	58.57	60.94	47.22	43.58	45.75	45.16
18	39.84	40.21	66.56	62.11	44.10	44.68	47.12	54.32
19	36.72	37.53	61.42	62.74	45.51	43.42	50.91	44.83
20	39.2	37.46	57.19	64.79	42.27	43.20	45.10	43.88
21	37.84	37.54	64.11	62.94	44.54	44.01	42.52	43.03
22	36.54	36.41	45.15[b]	53.92[b]	35.52	37.09	41.27	41.56
23	40.84	42.49	64.75[c]	48.12[c]	45.95	44.22	47.43	54.57
24	31.72[d]	32.40[d]	62.44	62.32	39.23	41.25	35.70	40.83
25	41.46	42.17	70.22	62.92	49.84	49.26	51.51	51.47
26	34.47	36.65	63.06	61.66	43.64	40.44	41.60	40.34
27	38.21	38.46	61.61	61.47	39.57	40.86	44.03	46.20
28	39.14	39.61	62.99	62.73	40.79	39.38	47.50	48.36
29	37.11	37.36	58.82	57.49	43.87	42.91	45.15	43.7
30	38.67	39.81	55.55	63.10	41.38	44.19	46.54	47.49
31	34.78	34.83	60.59	61.51	43.67	40.42	48.04	46.13
32	39.37	38.91	62.89	61.98	41.07	42.33	43.82	45.63
33	42.74	40.03	61.76	65.00	46.03[c]	35.27[c]	45.27	47.98
34	31.38[b]	30.72[b]	69.00	65.93	42.61	38.10	34.60	39.95
35	36.62[c]	29.73[c]	58.03	56.24	33.66	32.13	38.46	41.44
36	38.18	38.57	61.22	58.34	45.10	41.19	45.56	44.09
37	38.63	37.32	65.53	66.31	42.73	41.42	46.51	45.72
No. labs	34		35		36		37	
No. outliers	3		2		1		0	
Mean	39.04		63.39		43.56		46.29	
s_r	0.77		2.66		1.39		1.91	
s_R	2.00		3.77		3.69		3.87	
r	2.15		7.45		3.88		5.34	
R	5.61		10.54		10.34		10.84	
RSD_r	1.97		4.20		3.18		4.12	
RSD_R	5.13		5.94		8.47		8.37	
HORRAT	2.23		2.77		3.74		3.72	

[a] Results represent values uncorrected for moisture content. Repeatability and reproducibility data are based on averages of duplicate GOPOD determinations on hydrolyzates of each pair.

[b] Grubbs outlier.

[c] Cochran outlier.

[d] Double Grubbs outlier.

AMG. Most laboratories conformed to this, but about 8 only had access to baths with rotary motion. The results obtained with this arrangement were in agreement with those obtained with the recommended linear motion. One laboratory (Laboratory 32) performed this initial incubation in a temperature-controlled, air-heated, shaking cabinet. These results also agreed with the average values obtained. Before proceeding with the unknown samples, each laboratory analyzed the 4 test samples, and, in all cases, values were within 5% of the stated value.

Collaborators' data were evaluated statistically according to AOAC protocols using AOAC-supplied software. Of the 296 pairs of assay results reported, 12 were statistical outliers, according to AOAC protocols (15; Tables 1 and 2). Three outlier results were obtained for Laboratory 24, and single outliers by Laboratories 9, 11, 13, 20, 22, 23, 30, and 33–35. Cochran (repeatability) outliers were reported by Laboratories 30 (samples A/E); 9 and 11 (samples B/C); 20 (samples G/L); 35 (samples H/J); 23 (samples I/P); and 33 (samples K/N). Grubbs (reproducibility) outliers were reported by Laboratories 24 (samples B/C); 13 and 24 (samples D/F); 34 (samples H/J); and 22 (samples I/P). A double Grubbs outlier was reported by Laboratory 24 (samples H/J). No statistically significant outliers were found in results for samples M/O (Hylon VII, native high amylose maize starch).

For all samples (except regular maize starch), RSD_r values were between 1.97 and 4.2%. RSD_R values ranged from 4.58 to 10.9%. For regular maize starch, RSD_r and RSD_R values are very high because the amount of RS is so low. The most difficult sample to analyze was native potato starch, because of the high RS content and thus the difficulty in redissolving the RS pellet. Two outliers were identified: Laboratories 23 (Cochran repeatability) and 22 (Grubbs reproducibility).

More relative variability was observed with products containing low RS, e.g., regular maize starch, corn flakes, and beans, possibly because they also contain high levels of nonresistant starch. For kidney beans, with an RS content of about 4.6% ("as is" basis), the RSD_r (2.42%) and RSD_R (4.58%) values were acceptable. With corn flakes, containing approximately 2.2% RS ("as is" basis), the RSD_r was acceptable (3.43%), but the RSD_R value (10.9%) was higher than that for beans. Regular maize starch was included in this study mainly to confirm that the α-amylase/AMG incubation step proceeded correctly for each of the collaborators. The RS content of regular maize starch is <1% (a mean value of 0.64% was obtained in this study). RSD_r and RSD_R values are very high, but the range of values obtained for RS content of the material showed that in the incubation step, hydrolysis of the starch was between 98.5 and 99.8%, i.e., the range of RS values was between 0.2 and 1.5%. Clearly, there are problems in getting accurate RS values for products that contain a high total starch content and with <1% RS. The HORRAT values range from 1.44 to 10.5, which are high. The HORRAT acceptability is 0.5–2.0. However, considering the complexity of the analyte and the matrix, this variability is acceptable for the intended use of this method.

Results reported by collaborators were on an "as is" basis. Table 3 shows the average RS values along with values on a "dry weight" basis. Moisture contents were determined in a single laboratory using AOAC Method **925.10** (16). Kidney beans, corn flakes, and green bananas were freeze-dried with shelf temperature increased to 50°C on the third day of drying. This explains the low moisture contents of these products.

Collaborators' Comments

Of the 37 collaborators, 12 reported problems in handling samples I and/or P (native potato starch). This problem is associated with the high RS content of this material, and problems in redissolving the RS pellet in KOH. Without exception, this problem, as experienced by collaborators, occurred because a vortex mixer was used rather than the recommended magnetic stirrer under an ice–water bath. Eleven of these laboratories reanalyzed all of the high RS products (test samples G–P) exactly according to the stated procedure, and in all cases the values improved significantly. Some collaborators suggested the

Table 3. Resistant starch content of products used in interlaboratory study

Sample	Resistant starch ("as is" basis)[a]	Moisture content, %[b]	Resistant starch (dry weight basis), % (w/w)	Resistant starch as a percentage of total starch, % (w/w)
Regular maize starch	0.67	13.6	0.78	0.80
Corn flakes (freeze-dried)[c]	2.20	2.5	2.26	3.45
Kidney beans (freeze-dried)[c]	4.66	2.0	4.76	12.82
Actistar (enzyme treated cassava starch)	48.3	8.7	52.9	53.9
CrystaLean (retrograded high amylose maize starch)	39.0	7.0	41.9	43.0
Native potato starch	63.4	12.3	72.3	73.8
Green banana[c]	43.6	1.0	44.0	72.7
Hylon VII (native high amylose maize starch)	46.4	12.5	53.0	54.1

[a] Average of values obtained from interlaboratory study.

[b] Moisture contents determined at Megazyme using AOAC Method **925.10.**

[c] Samples were freeze-dried for 3 days, with sample chamber temperature increased to 50°C after 2 days.

use of polypropylene tubes for the initial incubation step with α-amylase/AMG for 2 reasons. First, a few collaborators experienced some slight leakage from an odd tube during the 16 h incubation step; second, there were a few cases of tubes breaking when the caps were put on, or during centrifugation. Initially, we also experienced each of these problems on a rare occasion. We tried polypropylene tubes, but experienced more leakage problems with them than with the Corning culture tubes. Other brands of polypropylene tubes need to be evaluated. A few collaborators found that on centrifugation of the tubes during the alcohol washing steps, some pellets were not very firm, requiring extra careful handling during the decantation steps. This problem was resolved by increasing the centrifugal force to about $1500 \times g$. We have successfully used a centrifugal force as low as $1000 \times g$, but in such cases, it is important to decant the supernatant as soon as the centrifuge stops.

Recommendations

The Study Directors recommend that the method for determination of RS in starch and selected plant materials be adopted First Action as an approved new method.

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