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 × *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.

(**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 flask*s.—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 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

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.

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 $1500 \times g$ 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 $1500 \times g$ 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 $1500 \times g$ for 10 min. For test samples containing <10% RS, directly centrifuge tubes at $1500 \times g$ 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.

Reference: *J. AOAC Int*. **85**, 1103(2002).