32.2.10

AOAC Official Method 995.16
β-D-Glucan in Barley and Oats

Streamlined Enzymatic Method
First Action 1995
AACC–AOAC Method

[Applicable to the determination of β-D-glucan in flours from whole oat grains, milling fractions, and unsweetened cereal products and to flours of other cereal grains (e.g., barley and rye). Method is also applicable to measurement of β-D-glucan in cereal products containing high levels of glucose, after pre-extraction with aqueous ethanol.]

See Table **995.16** for the results of the interlaboratory study supporting the acceptance of the method.

A. Principle

Method is rapid procedure for direct, quantitative measurement of (1→3)(1→4)-β-D-glucan (β-D-glucan, mixed-linkage β-D-glucan) in flour slurries using highly purified lichenase and β-glucosidase. β-D-Glucan is specifically hydrolyzed by lichenase to oligosaccharides, which are then quantitatively cleaved to glucose by β-glucosidase. Glucose is measured using glucose oxidase–peroxidase–buffer mixture.

B. Apparatus

(**a**) *Grinding mill*.—Centrifugal, with 12-tooth rotor and 0.5 mm sieve, or similar device. Alternatively, cyclone mill can be used for small test laboratory samples.

(**b**) *Bench centrifuge*.—Holding 16 × 120 mm glass test tubes, operating to ca 1000 × *g*.

(**c**) *Water bath*.—Maintaining 50 ± 1°C.

(**d**) *Boiling water bath*.—Boiling H_2O at 95–100°C (e.g., fryer filled with water).

(**e**) *Vortex mixer*.

(**f**) *pH Meter*.

(**g**) *Stopclock timer (digital)*.

(**h**) *Top-loading balance*.

(**i**) *Analytical balance*.

(**j**) *Laboratory oven*.—With forced-convection for determining dry weight of test sample; maintaining 103 ± 1°C.

(**k**) *Spectrophotometer*.—With flow-through cell, operating at 510 nm.

(**l**) *Pipets*.—Accurately delivering 100 and 200 µL, with disposable tips. Alternatively, motorized hand-held dispenser can be used.

(**m**) *Positive displacement pipetter*.—With 5.0 mL tips accurately delivering 100 or 200 µL, and 50 mL tips delivering 4.0 or 5.0 mL.

(**n**) *Dispenser*.—For use with glucose oxidase–peroxidase–buffer mixture; 500 mL capacity, with adjustable volume 0–5.0 mL.

(**o**) *Glass test tubes*.—16 × 120 mm, 17 mL capacity, for centrifugation at ca 1000 × *g*.

(**p**) *Test tube racks*.— 48 place, holding 16 × 120 mm test tubes.

(**q**) *Thermometer*.—Reading 103 ± 1°C.

(**r**) *Filter paper*.—Fast, ashless.

C. Reagents

(**a**) *Lichenase solution*.—50 U/mL. Dilute 1 mL lichenase–$(NH_4)_2SO_4$ solution to 20.0 mL with 20mM phosphate buffer, (**e**). Divide enzyme solution into 5 mL aliquots, and store frozen in polypropylene containers to prevent microbial contamination. Undiluted, lichenase suspension in $(NH_4)_2SO_4$ is stable 6 years when stored at 4°C. [*Note*: One unit (U) of enzyme activity is the amount of enzyme required to release 1 µM glucose reducing sugar equivalents/min from barley β-glucan (10 mg/mL) at pH 6.5 and 40°C.]

(**b**) β-*Glucosidase solution*.—2 U/mL. Dilute 1 mL β-glucosidase–$(NH_4)_2SO_4$ solution to 20 mL with 50mM sodium acetate buffer, (**f**)(*2*). Divide enzyme solution into 5 mL aliquots, and store frozen in polypropylene containers to prevent microbial contamination. Undiluted, β-glucosidase suspension in $(NH_4)_2SO_4$ is stable 6 years when stored at 4°C.

[*Note:* Do not cross-contaminate lichenase and β-glucosidase solutions. To check purity of enzymes, perform steps **D**–**F** using corn starch as test material. B-D-Glucan content must be 0% (i.e., analyte absorbance = absorbance of reagent blank).]

Test each new lot of lichenase and β-glucosidase for activity using control flours, (**i**). Check purity of β-glucosidase by incubating enzyme with lichenase reaction mixture from step **E**(*6*) (0.1 mL, per standard assay procedure) for extended periods of time (i.e., up to several hours instead of recommended 10 min).

(**c**) *Glucose oxidase–peroxidase–buffer mixture*.—Mixture of glucose oxidase, >12000 U/L; peroxidase, >650 U/L; and 4-aminoantipyrine; 0.4 m*M* (81.3 mg/L).

Prepare buffer concentrate by dissolving 13.6 g KH_2PO_4, 4.2 g NaOH, and 3.0 g 4-hydroxybenzoic acid in 96 mL distilled H_2O. Adjust pH to 7.4 with either 2*M* HCl (16.7 mL/100 mL) or 2*M* NaOH (8.0 g/100 mL). Dilute solution to 100 mL, add 0.4 g sodium azide, and mix until dissolved. Buffer concentrate is stable up to 3 years at 4°C.

Table 995.16 Interlaboratory study results for detemination of β-D-glucan in oats by streamlined enzymatic method[a]

Sample	Mean, % dry basis	s_r	s_R	RSD_r, %	RSD_R, %	r[b]	R[c]
Oat flour	2.73	0.083	0.241	3.1	8.8	0.232	0.675
Oat bran	6.39	0.296	0.456	4.6	7.1	0.829	1.277
Rolled oats	4.27	0.283	0.315	6.6	7.4	0.792	0.882
Oat bran (breakfast cereal)	3.93	0.484	0.484	12.3	12.3	1.355	1.355
Instant oat bran	8.00	0.480	0.524	6.0	6.6	1.344	1.467

[a] Based on results from 8 laboratories; no outliers identified.

[b] $r = 2.8 \times s_r$.

[c] $R = 2.8 \times s_R$.

To prepare glucose oxidase–peroxidase–buffer mixture, dilute 50 mL buffer concentrate to 1.0 L and add entire content of vial containing freeze-dried glucose oxidase–peroxidase mixture, to obtain required concentration. Reagent is stable 2–3 months at 4°C and 2–3 years at –20°C. Color formed with glucose is stable several hours. (*Note*: Glucose oxidase must not be contaminated with β- and/or α-glucosidase and chromogen color complex must be stable at least 60 min.)

Check color formation and stability of glucose oxidase–peroxidase–buffer mixture by incubating (in duplicate) 3.0 mL glucose oxidase–peroxidase–buffer mixture with 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 formation should be achieved within 20 min, and color should be stable at least 60 min at 50°C.

(**d**) *Aqueous ethanol*.—About 50% (v/v). Dilute 95% ethanol (laboratory grade) with equal volume of distilled H_2O. Store in well-sealed bottle at room temperature.

(**e**) *Sodium phosphate buffer*.—20m*M*, pH 6.5. Dissolve 3.12 g $NH_2PO_4 \cdot 2H_2O$ in 900 mL H_2O and adjust pH to 6.5 with 100mM NaOH (4 g/L) solution. Buffer is stable 1 month at 4°C.

(**f**) *Sodium acetate buffer*.—(*1*) *200 mM, pH 4.0*.—Add 7.6 mL glacial acetic acid to 990 mL H_2O and then add and dissolve 4.8 g sodium acetate trihydrate. Check pH and adjust to pH 4.0, if necessary, with either 2M HCl or 2M NaOH. Adjust volume to 1 L. Buffer is stable at least 2 months when stored at 4°C. (*2*) *50mM, pH 4.0*.—Dilute 250 mL 200 mM acetate buffer to 1 L with distilled H_2O.

(**g**) *D-Glucose standard stock solution*.—1 mg/mL. Before preparing solution, dry powdered crystalline glucose (purity >97%) 16 h at 60°C under vacuum.

(**h**) *Corn starch*.—For confirmation of absence of starch-degrading enzyme activities in lichenase and β-glucosidase preparations.

(**i**) *Control flours*.—Containing known amount of β-D-glucan (low and high).

Items (**a**), (**b**), and (**i**) are supplied in Mixed-Linkage β-Glucan Assay kit, and item (**c**) is supplied in Glucose Assay kit. Item (**g**) is supplied in both test kits. Kits are available from Megazyme International Ireland Ltd, Bray Business Park, Bray County, Wicklow, Ireland.

D. Preparation of Test Sample, Standards, and Reagent Blank

(**a**) *Test sample*.—Grind ca 50 g laboratory sample in grinding mill to pass 0.5 mm sieve. Transfer all material (test sample) into wide-mouth plastic jar and mix well by shaking and inversion.

Determine H_2O content by drying 2–5 g ground test portion 16 h in laboratory oven at 103 ± 1°C. Use information on H_2O content in final calculations, **F**.

(**b**) *D-Glucose standard working solutions*.—50 and 100 µg. Add 50 and 100 µL D-glucose standard stock solution, **C**(**g**), to separate test tubes, and adjust volume in each tube to 200 µL with 50mM sodium acetate buffer, **C**(**f**)(*2*). Prepare solutions immediately before use.

(**c**) *Reagent blank*.—Transfer 0.2 mL 50mM sodium acetate buffer into test tubes.

E. Determination

Run D-glucose working standard solutions (in quadruplicate), reagent blank (in duplicate), and control flours with each set of tests. Use reagent blank to zero spectrophotometer.

[*Note*: Carefully follow steps (*1*)–(*3*) to obtain homogenous slurry.]

(*1*) Accurately weigh 80–100 mg ground test portion directly into glass test tube. Tap tube gently on laboratory bench to ensure that all particles drop to bottom of tube. (*Note*: When analyzing cereal products containing high levels of glucose, pre-extract 80–100 mg ground test portion 2×with 10 mL aqueous ethanol, **C**(**d**), at ca 80°C for 10 min. Centrifuge slurry at 1000×*g* and discard supernatant. Use sediment for analysis.)

(*2*) Add 0.2 mL 50% ethanol to tube and stir on Vortex mixer to ensure that test material is wet. Add 4.0 mL phosphate buffer, **C**(**e**), and mix vigorously on Vortex mixer to ensure complete dispersion.

(*3*) Immediately place test tube in vigorously boiling H_2O bath. Incubate 1 min, remove from H_2O bath, and mix vigorously on Vortex mixer. Return tube to boiling H_2O bath for additional 2 min and then mix vigorously on Vortex mixer. (*Note*: Part of solids will adhere to side of test tube; this will not affect analysis since tube content will be treated with enzyme in next step.)

(*4*) Place tubes in H_2O bath set at 50°C and equilibrate 5 min. Add 0.2 mL lichenase solution, **C**(**a**), into tube and mix on Vortex mixer. Mix tube contents until material adhering to side of tube is wetted by lichenase-containing slurry. Cap tube with marble and incubate 60 min at 50°C, vigorously stirring on Vortex mixer (ca 10 s) 3 or 4 times during incubation. (Alternatively, place magnetic stirrers in each tube and put test tube rack in Pyrex dish filled with H_2O and placed on hot-plate magnetic stirrer set to maintain bath at 50°C; or use stirring incubation bath.) (*Note*: It is not necessary to stir contents continuously throughout whole incubation; however, it can be performed as alternative to Vortex mixing, if appropriate equipment is available.)

(*5*) Add 5.0 mL 200mM sodium acetate buffer, **C**(**f**), to each tube and thoroughly mix on Vortex mixer.

(*6*) Cool tubes 5–10 min to room temperature and then centrifuge 10 min at ca 1000×*g*. [*Note*: Supernates may still be slightly translucent. This does not affect accuracy of assay since blank contains the same supernatant as tests but is not treated with β-glucosidase. Alternatively, filter supernates through filter paper, **B**(**r**).]

(*7*) Carefully and accurately transfer 0.1 mL each supernate (or filtrate) to bottoms of separate test tubes using 3 tubes/supernate (or filtrate). [Treat only 2 tubes (reaction solution) with β-glucosidase in step (*8*). Tube that is not treated with β-glucosidase will yield blank value (on treatment with glucose oxidase–peroxidase–buffer mixture).]

(*8*) Add 0.1 mL 50mM acetate buffer, **C**(**f**)(*2*), to blank supernate (or filtrate). Add 0.1 mL aliquots of β-glucosidase solution, **C**(**b**), to reaction solutions from test and control flours. Incubate tubes 10 min at 50°C.

(*9*) Add 3.0 mL glucose oxidase–peroxidase–buffer mixture, **C**(**c**), to each tube (reaction solution from test and control flours, reagent blank, D-glucose standard working solutions, and blanks), and incubate 20 min at 50°C.

(*10*) Measure and record *A* of each test at 510 nm against reagent blank. Test solutions containing >10% β-D-glucan will have *A* values greater than those of 100 µg D-glucose standard working solution. In such case, reanalyze test sample by diluting aliquot of sample supernate (or filtrate) from (*6*) 1 + 2 with 50mM acetate buffer, and proceed with step (*7*). Include dilution factor when calculating β-D-glucan content. Average *A* values for each test sample and use in *Calculations*, **F**.

F. Calculations

Calculate content of β-D-glucan (percent, on as is basis) as follows:

$$\beta\text{-D-glucan, \%} = \Delta A \times F \times 94 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta A \times \frac{F}{W} \times 8.46$$

where Δ*A* = absorbance of reaction solution (i.e., after β-glucosidase treatment minus blank absorbance for the same test sample); *F* = factor to convert absorbance values to µg glucose = 100 µg glucose/absorbance values for 100 µg glucose; 94 = volume correction factor (0.1 of solution from 9.4 mL was analyzed); 1/1000 = conversion from µg to mg; 100/*W* = conversion to 100 mg test portion; *W* = test portion weight, mg; 162/180 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in β-D-glucan.

Calculate β-D-glucan contents (percent, on dry weight basis) as follows:

$$\beta\text{-D-glucan, \%} = \frac{\beta\text{ - glucan (as is basis) } \times 100}{[100 - \text{ moisture content (\%)}]}$$

Reference: (1) *J. AOAC Int*. **80,** 580(1997).

(2) Proceedings of the 4th International Oat Conference (1992) (A.R. Barn, Ed.) Adelaide, SA, Australia, pp 104–107.

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