FOOD COMPOSITION AND ADDITIVES

Determination of β-Glucan in Barley and Oats by Streamlined Enzymatic Method: Summary of Collaborative Study

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A collaborative study was conducted involving 8 laboratories (including the authors' laboratories) to validate the streamlined enzymatic method for determination of β-D-glucan in barley and oats. In the method, the flour sample is cooked to hydrate and gelatinize β-glucan, which is subsequently hydrolyzed to soluble fragments with the lichenase enzyme. After volume and pH adjustments and filtration, the solution is treated with β-glucosidase, which hydrolyzes β-gluco-oligosaccharides to D-glucose. D-Glucose is measured with glucose oxidase–peroxidase reagent. Other portions of lichenase hydrolysate are treated directly with glucose oxidase–peroxidase reagent to measure free glucose in test sample. If levels of free glucose are high, the sample is extracted first with 80% ethanol. For all samples analyzed, the repeatability relative standard deviation (RSD_r) values ranged from 3.1 to 12.3% and the reproducibility relative standard deviation (RSD_R) values ranged from 6.6 to 12.3%. The streamlined enzymatic method for determination of β-D-glucan in barley and oats has been adopted first action by AOAC INTERNATIONAL.

The original enzymatic method for measurement of β-D-glucan was developed and validated in an interlaboratory study in 1985 (1). The method, as applied to barley, was evaluated by an analytical subcommittee of the Cereal Division of the Royal Australian Chemical Institute and became the recommended Australian method. The analytical subcommittee of the European Brewing Convention evaluated the method on barley, malt, wort, and beer samples and subsequently adopted it as a recommended procedure.

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The recommendation was approved by the Methods Committee on Commodity Foods and Commodity Products and was adopted by the Official Methods Board of the Association. See "Official Methods Board Actions" (1995) *J. AOAC Int.* **78**, 179A, and "Official Methods Board Actions" (1995) *The Referee*, October issue.

The original method was modified in 1991 (2) to simplify the procedure and make it more robust, without any loss in precision, accuracy, or reliability. In this modification, 30–40 samples can be analyzed in less than 2 h (more than a hundred samples per day) and most of the analytical steps are performed in a single test tube. The modified streamlined enzymatic method was validated by 8 laboratories from Australia, United States, Canada, and Europe (3). The study was performed according to the guidelines of the American Association of Cereal Chemists (AACC) and AOAC and included split-level design. Five pairs of samples were analyzed in replicate by the streamlined enzymatic method and by AACC Method 32–22. Test samples consisted of Australian oat products that included whole groat, flour, and bran and various breakfast cereals. Two control samples were provided to each collaborator to gain experience with the methods. No significant difference was found between the β-D-glucan results of the 2 methods ($P > 0.05$.) This report describes the streamlined enzymatic method adopted first action by AOAC INTERNATIONAL.

995.16 β-D-Glucan in Barley and Oats, Streamlined Enzymatic Method, AACC–AOAC Method

First Action 1995

(Applicable for determination of β-D-glucan in flours from whole oat grains, milling fractions, and unsweetened cereal products and in 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.)

Caution: *See* Appendix B, safety notes on ethanol. Glucose oxidase–peroxidase–buffer mixture contains sodium azide. Avoid contact with skin and eyes. In case of contact, immediately flush contact surfaces with plenty of water. Disposal of this reagent into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential explosive hazards. Dispose of waste solvents according to applicable environmental rules and regulations.

Method Performance:

Table 995.16. Method performance for determination 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] $R = 2.8 \times s_R$.

See Table **995.16** for method performance data.

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 by 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 with glucose oxidase–peroxidase–buffer mixture.

B. Apparatus

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

(b) *Bench centrifuge.*—Capable of holding 16 × 120 mm glass test tubes, with rating to ca 1000 × *g*.

(c) *Water bath.*—Capable of maintaining 50° ± 1°C.

(d) *Boiling water bath.*—Capable of boiling (e.g., appropriate deep-fat fryer filled with water) at 95°–100°C.

(e) *Vortex mixer.*

(f) *pH Meter.*

(g) *Stop-clock timer (digital).*

(h) *Top-loading balance.*

(i) *Analytical balance.*

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

(k) *Spectrophotometer.*—Equipped with flow-through cell; capable of operating at 510 nm.

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

(m) *Positive displacement pipetter.*—Equipped with 5.0 mL tips capable of accurately delivering 100 or 200 mL, and 50 mL tips capable of 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, suitable for centrifugation at ca 1000 × *g*.

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

(q) *Thermometer.*—Capable of reading 103° ± 1°C.

(r) *Filter paper.*—Fast, ashless.

C. Reagents

(a) *Lichenase solution.*—50 U/mL. Dilute 1 mL lichenase–ammonium sulfate solution to 20.0 mL with 20 mM sodium phosphate buffer, **(e)**. Divide enzyme solution into 5 mL aliquots, and store frozen in polypropylene containers to prevent microbial contamination. When not diluted in buffer, lichenase suspension in ammonium sulfate 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 µmol 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–ammonium sulfate solution to 20 mL with 50 mM sodium acetate buffer, **(f)**(2). Divide enzyme solution into 5 mL aliquots, and store frozen in polypropylene containers to prevent microbial contamination. When not diluted in buffer, β-glucosidase suspension in ammonium sulfate 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* with corn starch as test sample. Obtained content of β-D-glucan must be 0% (i.e., sample 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 (i.e., up to several hours instead of recommended 10 min).

(c) *Glucose oxidase–peroxidase–buffer mixture.*—Mixture of glucose oxidase, >12 000 U/L; peroxidase, >650 U/L; and 4-aminoantipyrine, 0.4 mM.

Prepare buffer concentrate by dissolving 13.6 g potassium dihydrogen orthophosphate, 4.2 g sodium hydroxide, and 3.0 g 4-hydroxybenzoic acid in 96 mL distilled H_2O. Adjust pH to 7.4 with either 2M HCl or 2M NaOH. Dilute solution to 100 mL, add 0.4 g sodium azide, and mix until dissolved. Buffer concentrate is stable up to 3 years when stored at 4°C.

To prepare glucose oxidase–peroxidase–buffer mixture, dilute 25 mL buffer concentrate to 1.0 L and then add entire content of vial containing freeze-dried glucose oxidase–peroxidase mixture to obtain required concentration. Reagent is stable

2–3 months when stored at 4°C and 2–3 years when stored 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 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 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.*—20 mM, pH 6.5. Dissolve 3.12 g sodium dihydrogen orthophosphate dihydrate in 900 mL H_2O and adjust pH to 6.5 with 100 mM sodium hydroxide solution. Buffer is stable 1 month when stored 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) 50 mM, pH 4.0.*— Dilute aliquot 200 mM acetate buffer 4-fold 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 sample in grinding mill to pass 0.5 mm sieve. Transfer all material into wide-mouthed plastic jar and mix well by shaking and inversion.

Determine H_2O content in sample by drying 2–5 g ground sample 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 50 mM sodium acetate buffer, **C(f)***(2)*. Prepare solutions immediately before use.

(c) *Reagent blank.*—Transfer 0.2 mL 50 mM 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 test samples. Use reagent blank to zero spectrophotometer.

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

(1) Accurately weigh 80–100 mg ground test sample directly into glass test tube. Tap tube gently on laboratory bench to ensure that all particles of sample drop to bottom of tube. (*Note*: When analyzing cereal products containing high levels of glucose, pre-extract 80–100 mg ground test sample 2× with 10 mL aqueous ethanol, *C*(**d**), at ca 80°C over 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 sample is wet. Add 4.0 mL sodium phosphate buffer, *C*(**e**), and mix contents of tube vigorously on Vortex mixer to ensure complete dispersion of sample.

(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 contents vigorously on Vortex mixer. *Note*: Part of sample will adhere to side of test tube, but this will not affect analysis because tube content will be treated with enzyme in next step.

(4) Place tubes in H_2O bath set at 50°C and allow to equilibrate 5 min. Add 0.2 mL lichenase solution, *C*(**a**), into tube and mix on Vortex mixer. To ensure hydrolysis of β-D-glucan in whole sample, 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 tube contents 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 hotplate magnetic stirrer set at 50°C; or use stirring incubation bath.)

Note: It is not necessary to stir tube 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 200 mM 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, but this does not affect accuracy of assay because sample blank contains the same supernatant as test samples 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 sample blank value (on treatment with glucose oxidase–peroxidase–buffer mixture).]

(8) Add 0.1 mL 50 mM of acetate buffer, *C*(**f**)(*2*), to sample blank supernate (or filtrate). Add 0.1 mL aliquots of β-glucosidase solution, *C*(**b**), to reaction solutions from test samples 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 sample and control flours, reagent blank, D-glucose standard working solutions, and sample blanks), and incubate 20 min at 50°C.

(10) Measure and record *A* of each sample at 510 nm against reagent blank. Test samples containing >10% β-D-glucan will have A values greater than those of 100 μg D-glucose standard working solution. In such case, reanalyze sample by diluting aliquot of sample supernate (or filtrate) from *(6)* 3-fold with 50 mM acetate buffer, and then proceed with step *(7)*. Include dilution factor when calculating β-D-glucan content. Average A values for each sample and use in calculations, *F*.

F. Calculations

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

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

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

where ΔA = absorbance of reaction solution (i.e., after β-D-glucosidase treatment) minus blank absorbance for the same 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 sample; W = sample 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 (%, on dry weight basis) as follows:

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

Ref.: *J. AOAC Int.* **80**, 580—583(1997)

References

(1) McCleary, B.V., & Glennie-Holmes, M. (1985) *J. Inst. Brewing* **91**, 285–295

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(3) McCleary, B.V., & Mugford, D.C. (1991) *Proceedings of the 4th International Oat Conference*, Vol. 1, October 19, 1992, Adelaide, Australia, 104–107