***DRAFT:* Determination of Dietary Starch in Animal Feeds by an Enzymatic-Colorimetric Method (Modified Bach Knudsen Assay)**
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There has been increased interest in the analysis of starch in animal feeds for its positive effects on animal performance, and potential undesirable effects on glycemic response and animal health. AOAC Method **920.40** for starch in animal feeds is no longer valid because of discontinued production of the enzyme Rhozyme-S (Rohm and Haas, Philadelphia, PA) specified in the procedure. Accordingly, another approved method for starch in animal feeds is needed. Additionally, new terminology is needed to define "starch" that more accurately describes the nutritionally relevant fraction of interest and recognizes the limitations in the specificity of available methods. The goal of this collaborative study is to establish a standardized method for the measurement of dietary starch in animal feeds.

"Dietary starch" is defined as "an α-linked-glucose carbohydrate of or derived from plants, animals or microbes from which glucose is released through the hydrolytic actions of purified a-amylases and amyloglucosidases that are specifically active only on α-(1-4) and α-(1-6) linkages in samples that have been gelatinized in heated, mildly acidic buffer. Its concentration in feed is determined by enzymatically converting the a-linked-glucose carbohydrate to glucose and then measuring the liberated glucose." This definition would encompass plant starch, microbial and animal glycogen, maltodextrins, and maltose/isomaltose. As opposed to limiting the fraction to starch as a plant storage polysaccharide, dietary starch includes all alpha-glucans with linkages that could potentially be digested by salivary or small intestinal amylases or amyloglucosidases, which is what the enzymatic starch methods have always measured. Resistant starch is excluded by limiting the method of gelatinization to heating with buffer, and excluding use of dimethyl sulfoxide or alkali.

Stakeholders: Feed industry, nutritionists, veterinarians, regulators, owners of animals (pets, horses, cattle, etc.), commercial and research analytical laboratories.

The proposed method has been compared to other enzymatic starch methods and found to be simple, robust, and it avoids known defects that reduce the recover of other starch assays. It is similar in chemistry to AOAC Method **996.11**, differing in buffer used and in sample handling procedures that allow analysis of wet samples. The selectivity of the method is good, with very low values found for sucrose, which is a common source of interference. The limit of determination for the assay is 0.2% of dry matter. The standard deviation of replicates for the proposed method were 0.9 to 0.2% on foods and feeds. The method appears to meet the needs of analyzing dietary starch in animal feeds for diet formulation and regulatory purposes.

Proposed samples: alfalfa pellets, wet pet food, dry pet food, commercial low starch equine feed, corn grain, dried corn silage, soybean meal, dried distillers grains, commercial lactating dairy cattle feed, commercial swine or poultry feed. All dry samples will be ground to pass the 1 mm sieve on an abrasion mill; wet samples will be homogenized.

Apparatus

(a) *Grinding mill*. – Cyclone mill equipped with a 1 mm screen or a cutting or Wiley mill with 0.5 mm screen (give equivalent fineness of grind).

(b) *Bench centrifuge.* – Capable of centrifuging at 1000 *x g.*

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

(d) *Boiling water bath.* - Capable of boiling at 95 – 100°C.

(e) *Vortex mixer.*

(f) *pH meter.*

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

(h) *Top-loading balance*. – Capable of weighing accurately to ±0.01 g.

(i) *Analytical balance.* – Capable of weighing accurately to ±0.0001 g.

(j) *Laboratory ovens.* - With forced-convection; capable of maintaining 105 ± 1°C for determining dry weight of test sample; capable of maintaining 100 ± 1°C for carrying out incubations.

(k) *Spectrophotometer*. – Capable of operating at absorbances of 505 nm.

(l) *Pipettes.* – Capable of delivering 0.1, and 1.0 mL; with disposable tips.

(m) *Positive-displacement repeating pipette.* – Capable of accurately delivering 0.1, 1.0, and 3.0 mL.

(n) *Dispenser.* – 1000 mL or greater capacity capable of accurately delivering 20 and 30 mL.

(o) *Glass test tubes* – 16 x 100 mm.

(p) *25 x 150 mm Glass tubes with polytetrafluoroethylene (PTFE)- lined screw caps (approximate volume 55mL).*

(s) *Plastic film, or similarly nonreactive material.*

(u) *Magnetic stir plate.*

Reagents and Solutions

It is expected that good quality distilled H_2O will be used for reagent preparation and dilutions.

The reagents and method must be validated within laboratory to verify efficacy of enzymes and run conditions as well as lack of interference in the assay. Recommended validation: analyze 0.1 g samples of purified glucose, sucrose, and purified corn starch. On a dry matter basis, the glucose should give a value of 90 ± 2%, starch at 100 ± 2%, and sucrose 0.7 ± 0.3%. Enzyme preparations must not contain appreciable concentrations of glucose (>0.5%) or background absorbance readings will interfere with sample measurements.

(**a**) *Acetate buffer.— 100mM, pH 5.0.*— Weigh 6.0 g or pipette 5.71 mL of glacial acetic acid and transfer immediately to a flask; quantitatively transfer weighed acid with H_2O rinses. Bring volume to ca 850 mL. While stirring solution on a magnetic stir plate, adjust pH to 5.0 ± 0.1with 1M NaOH solution. Dilute to 1 L with H_2O. This can be done in an Erlenmeyer flask or beaker that

has been made volumetric by weighing or transferring 1 L of water into the vessel and then etching the meniscus line for the known volume.

(**b**) *Heat-stable α-amylase solution.*— Heat-stable, α-amylase, ca 20000 liquefon units/g (Examples: Termamyl 120 L, NovoNordisk; Product Multifect AA 21L, Genencor International, Rochester, NY; origin: *Bacillus licheniformis*; pH optima 5.5 – 5.8). Should not contain greater than 0.5% glucose.

(**c**) *Amyloglucosidase solution.*— 200 U/mL. Dilute concentrated amyloglucosidase with 100mM sodium acetate buffer **(a)** to give 1 mL of solution per sample with 2 to 5 mL excess (e.g., can be done with concentrated amyloglucosidase, 3260 U/mL, Product E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland; origin: *Aspergillus niger*; pH optimum 4.0; pH stability 4.0-5.5). Add one-third of needed buffer to an appropriately sized graduated cylinder. Pipette concentrated amyloglucosidase into buffer, rinsing tip by taking up and expelling the buffer. Bring to desired volume with additional buffer. Cap cylinder with plastic film and invert cylinder repeatedly to mix.

(**d**) *Glucose oxidase–peroxidase (GOPOD) reagent.*— *(1)* Mixture of glucose oxidase, 7000 U/L; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74mM. Prepare by dissolving 9.1 g of Na_2HPO_4 and 5.0 g of KH_2PO_4 in ca 300 mL H_2O in a volumetric flask. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with H_2O, swirl gently to dissolve without causing excessive foaming. Bring to 1 L volume with H_2O. Seal and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 µm retention. Store in a sealed amber bottle at ca 4°C. Determine standard curve for the reagent using a 4 point standard curve using H_2O and **(e)***(1)* according to Preparation of Reagent Blanks and Standard Curves (**b**)*(1)*. *(2)* Alternatively, use another glucose oxidase-peroxidase reagent with similar chemistry that allows measurement of glucose concentrations in the ranges described in Preparation of Reagent Blanks and Standard Curves (**b**)*(1)* and that has passed in lab validation ***(validation procedure to be described)***.

(**e**) *Glucose standard solution.*— 300, 600, and 1000 µg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥ 99.5%). Weigh approximately 75, 150, and 250 mg of glucose and record weight to 0.0001 g. Rinse from weigh paper into 250 mL volumetric flask with 0.2% benzoic acid solution and dissolve. Bring each to 250 mL volume with 0.2% benzoic acid

solution to give 3 independent glucose standard solutions. Multiply weight of glucose by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divide by 250 mL to calculate actual glucose concentrations of the solutions. Prepare solutions at least one day before use to allow equilibration of α- and β- forms of the glucose. Standard solutions may be stored at room temperature for 4 months.

Preparation of Reagent Blanks and Standard Curves

(**a**) *Reagent blank.*—For each assay, tubes containing no sample and only the reagents added for each method were carried through the entire procedure. Absorbance values for the reagent blanks are subtracted from sample absorbance values.

(**b**) *Standard curves.*— Pipette 0.1 mL of 0.2% benzoic acid solution and 300, 600, and 1000 µg/mL standard glucose solutions (**e**) in duplicate into the bottom of 16 x 100 mm glass culture tubes. Add 3.0 mL of GOPOD reagent (**d**) to each tube using a positive displacement repeating pipette aimed against wall of tube so it will mix well with the sample. No need to vortex tubes; mixing is adequate with addition of GOPOD reagent. Cover tops of tubes with plastic film. Incubate in a 50°C water bath for 20 min. Read absorbance at 505nm. All readings should be completed within 30 min of the end of incubation. Calculate the quadratic equation describing the relationship of glucose µg/mL (response variable) and absorbance at 505nm (independent variable). Use this standard curve to calculate glucose µg/mL in sample solutions. A new standard curve should be prepared with each new batch of GOPOD reagent.

(**c**) *Dietary Starch Method.*— Analyze D-glucose, corn starch, and a reagent blank with each set of test samples.

Note: Volumes of sample solutions can be determined by using accurately quantitative volumetric pipettes and dispensers to add reagents and dilute, and summing the volumes added. Alternatively, after the addition of water, samples can be filtered through Whatman 54 or equivalent into a 100 mLvolumetric flask and brought to volume to fix the sample solution volume before further dilution and analysis.

(*1*) Accurately weigh 90 to 100 mg purified and high starch samples or 190 to 200 mg of other test samples into 25 x 150 mm screw cap glass tubes.

(*2*) Dispense 30 mL of 0.1M sodium acetate buffer (pH 5.0)(**a**)*(2)* into the tube.

(*3*) Add 0.1 mL of heat-stable, α-amylase (**b**). Cap tube and vortex to mix.

Note: Vortex tube so that the solution column extends to the cap, washing the entire interior of the tube and dispersing the sample.

(*4*) Incubate tube for 1 h at 100°C in a forced-air oven or in a boiling water bath, vortexing tube at 10, 30, and 50 min of incubation.

(*5*) Cool tube on bench for 0.5 h.

(*6*) Add 1 mL of amyloglucosidase solution (**c**)*(2)*. Vortex tube.

(*7*) Incubate tube for 2 h at 50°C, vortexing at 1 h of incubation.

(*8*) Add 20 mL H_2O to tube, recap, and invert repeatedly to mix.

(9) Transfer ca 1.5 mL of sample solution to a 2 mL microcentrifuge tube, and centrifuge at 1000 *x g* for 10 min. Allow centrifuged solution to come to room temperature before preparing dilution. Alternately, filter solutions though Whatman 54 or equivalent filter paper.

(*10*) For samples containing 10 - 100% starch, an aliquot (1.0 mL) is diluted to 10 mL with H_2O and mixed thoroughly before proceeding. This is the only dilution needed to assure that samples will fall within the range of the standard curve. Reagent blanks should be analyzed undiluted and as a 1 in 10 dilution and the absorbances used to make corrections for similarly diluted samples.

Note: Preparing dilutions by weight is useful with solutions that present pipetting difficulties, such as those that adhere to the interior of pipette tips, or rise several millimeters into rinsed pipette tips when the tip is placed vertically into the sample solution. Densities of sample solutions can be determined on the remainder of a sample by centrifuging it at ca 1000 *x g* to sediment particles, allowing the centrifuged solution to come to room temperature, and determining the weight of solution held by a 10 mL volumetric flask. Sample solution densities have ranged from 0.997 to 1.00 g/mL. Water density has averaged 0.995 g/mL at 22 – 24°C. Dilutions may be prepared by volumetric methods if accuracy of sample solution pipetting is not an issue.

(*11*) Pipette 0.1 mL of H_2O and sample solutions into the bottoms of 16 x 100 mm glass test tubes in duplicate; use 2 tubes/sample solution. Add 3.0 mL of glucose oxidase-peroxidase reagent (**d**) to each tube using a positive displacement repeating pipette so it will mix well with the sample. No need to vortex tubes, mixing is adequate with addition of GOPOD reagent. Place tubes in a rack and cover with plastic film.

(*12*) Incubate in a 50°C water bath for 20 min. Read absorbance, *A*, at 505nm (or as appropriate for GOPOD reagent being used). Use 0 µg / mL standard to zero the spectrophotometer. Average *A* values for each sample and use in *Calculations*.

Note: Free glucose is determined on samples carried through steps *1* through *5* except that no α-amylase is added. Sample solutions are then subject to steps *8* through *13* and the calculations used to convert absorbance values to anhydroglucose as a percentage of dry matter. By expressing free glucose as anhydroglucose the value can be directly subtracted from the value obtained with the inclusion of amylase and amyloglucosidase, thus giving a value for dietary starch corrected for the free glucose in the sample.

Calculations

Calculate dietary starch content (percent, on a dry matter basis) in test sample as follows:

Free glucose, % = (A_F x S + I) x V_F x DF_F x 1/1000000 x W_F x DM x 162/180

Dietary Starch, % = [(A_E x S + I) x V_E x DF_E x 1/1000000 x W_E x DM x 162/180] – Free Glucose %

where subscript F represents values for samples analyzed for free glucose, and subscript E represents values for samples treated with amylase and amyloglucosidase, A = absorbance of reaction solutions minus the absorbance of the reagent blank; S = the slope and I = the intercept of the standard curve to convert absorbance values to μg glucose; V = final sample solution volume; DF = dilution factor e.g., 1.0 mL sample solution diluted into 10 mL = 10/1 = 10; 1/1000000 = conversion from μg to g; W = sample weight, as is; DM = dry matter content of the sample as a decimal; 162/180 = factor to convert from measured glucose as determined, to anhydroglucose, as occurs in starch. Correction of enzymatic starch assay values for free glucose in the sample gives results that reflect only the glucose released enzymatically from maltooligosaccharides, starch, glycogen, and other similar α-glucans.

Final sample solution volume = 30 mL acetate buffer + 0.1 mL amylase + 1 mL amyloglucosidase solution + 20 mL H_2O = 51.1 mL