AOAC Official Method 2001.11
Protein (Crude) in Animal Feed, Forage (Plant Tissue), Grain, and Oilseeds
Block Digestion Method Using Copper Catalyst and Steam Distillation into Boric Acid
First Action 2001

[Applicable to the determination of 0.5–50% Kjeldahl N (3–300% equivalent crude protein) in forage, animal feed and pet food, grain, and oilseeds, and applicable to the same matrixes as **976.05** (4.2.05), **976.06** (4.2.06), **984.13** (4.2.09), **988.05** (4.2.03), and **990.02** (4.2.07); the method does not measure oxidized forms of N or heterocyclic N compounds.]

See Tables **2001.11A** and **B** for the results of the interlaboratory study, expressed on a protein basis (N × 6.25), supporting acceptance of the method.

A. Principle

The material is digested in H_2SO_4 to convert the protein N to $(NH_4)_2SO_4$ at a boiling point elevated by the addition of K_2SO_4 with a Cu catalyst to enhance the reaction rate. Ammonia is liberated by alkaline steam distillation and quantified titrimetrically with standardized acid. Aluminum block heaters increase the efficiency of the digestion.

The digest must contain residual H_2SO_4 to retain the NH_3. Water is added manually or automatically to the digest to avoid mixing concentrated alkali with concentrated acid and to prevent the digest from solidifying. Concentrated NaOH is added to neutralize the acid and make the digest basic, and the liberated NH_3 is distilled into a boric acid solution and titrated with a stronger standardized acid, HCl, to a colorimetric endpoint. The same endpoint detection system (e.g., indicator, wavelength) must be used for the standardization of the HCl and for the analyte.

The analyte is referred to as "crude" protein because the method determines N, a component of all proteins. In addition, N from sources other than true protein is also determined. (Additional digestion procedures must be used in order to include N from nitrate.) The amount of protein in most materials is calculated by multiplying % N by 6.25, because most proteins contain 16% N.

The H_2SO_4 and NaOH used are in concentrated form and are highly corrosive. Wear gloves and eye protection while handling the chemicals. Do not mix concentrated acid and NaOH directly. If chemicals are splashed on the skin or in the eyes, flush with copious amounts of water. Seek medical attention. Do not breathe the sulfur oxide fumes produced during digestion.

B. Apparatus

(**a**) *Digestion block.*—Aluminum alloy block with adjustable temperature device for measuring and controlling block temperature (Tecator Digestion System 20, 1015 Digestor, Foss North America, 7682 Executive Dr, Eden Prairie, MN 55344, USA; +1-952-974-9892, Fax: +1-952-974-9823, info@fossnorthamerica.com; or equivalent).

(**b**) *Digestion tubes.*—250 mL.

(**c**) *Distillation units.*—(*1*) *For steam distillation.*—Foss Tecator 2200, or equivalent, to accept 250 mL digestion tubes and 500 mL titration flasks. (*2*) *For steam distillation and autotitration.*—Foss Tecator 2300, or equivalent.

(**d**) *Titration flask.*—500 mL graduated Erlenmeyer flask (for collection and titration of distillate).

(**e**) *Fume exhaust manifold.*—With Teflon ring seals, connected to a water aspirator in a hooded sink.

(**f**) *Weighing paper.*—Low N, Alfie Packers No. 201 (Alfie Packers, Inc., 8901 J St, Ste 10, Omaha, NE 68127, USA), or Fisher 09-898-12A, 3 ×3 in. (76 × 76 mm), or equivalent.

(**g**) *Pipetting dispenser.*—25 mL, adjustable volume, attached to a 5 pint (2.4 L) acid bottle.

C. Reagents

(**a**) *Sulfuric acid.*—Concentrated, 95–98% H_2SO_4, reagent grade.

Table 2001.11A Interlaboratory study results for the determination of crude protein by block digestion with a copper catalyst and distillation into 4% boric acid

ID	No. of labs[a]	Mean, %	RSD_r, %	RSD_R, %	HORRAT
Protein block	10(1)	40.19	0.45	0.76	0.333
Swine pellets	10(1)	37.04	0.47	0.60	0.256
Corn silage	11	7.10	1.64	2.16	0.726
Grass hay	11	7.11	1.94	1.94	0.650
Fish meal	11	64.67	0.73	0.98	0.460
Dog food	11	24.50	0.87	0.91	0.369
Chinchilla food	11	18.01	0.89	0.99	0.383
Albumin	10(1)	79.14	0.40	0.44	0.212
Birdseed	11	13.48	0.88	1.29	0.475
Meat and bone meal	11	50.06	1.90	1.90	0.857
Milk replacer	11	20.78	1.39	1.39	0.550
Soybeans	9(2)	38.76	0.49	0.54	0.236
Sunflower seeds	11	17.43	2.38	2.38	0.916
Legume hay	11	18.81	1.45	1.45	0.565

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

Table 2001.11B Interlaboratory study results for the recovery of nitrogen from standard compounds by block digestion with a copper catalyst and distillation into boric acid

Compound	No. of labs[a]	Theoretical yield, % N	Avg. found, % N	Avg. rec., %	RSD_R, %	HORRAT
Acetanilid	10(0)	10.36	10.37	100.1	1.50	0.53
Lysine·HCl	10(0)	15.34	13.32	86.8	4.16	1.53
Tryptophan	10(0)	13.72	13.55	98.8	1.04	0.39

[a] Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

(**b**) *Catalyst.*—7.0 g K_2SO_4 + 0.8 g $CuSO_4$. (Commercially available in tablet form as 3.5 g K_2SO_4 and 0.4 g $CuSO_4$ per tablet.)

(**c**) *Sodium hydroxide solution.*—40% (w/w) NaOH, low N (≤5 μg N/g).

(**d**) *Methyl red indicator solution.*—Dissolve 100 mg methyl red in 100 mL methanol.

(**e**) *Bromocresol green indicator solution.*—Dissolve 100 mg bromocresol green in 100 mL methanol.

(**f**) *Boric acid solution.*—4% (w/v). Dissolve 400 g H_3BO_3 in 5–6 L hot deionized water. Mix and add more hot deionized water to a volume of about 9 L. Cool to room temperature, add 100 mL bromocresol green solution and 70 mL methyl red solution, and dilute to a final volume of 10 L. Adjust to obtain a positive blank of 0.05–0.15 mL with 30 mL H_3BO_3 solution, using 0.1M NaOH (to increase blank) or 0.1M HCl (to decrease blank). Commercially available.

(**g**) *Boric acid solution.*—1% (w/v). (Optional trapping solution for titrators that automatically begin titration when distillation begins.) Dissolve 100 g H_3BO_3 in 5–6 L hot deionized water, mix, and add more hot deionized water to a volume of about 9 L. Cool to room temperature, add 100 mL bromocresol green solution and 70 mL methyl red solution, and dilute to a final volume of 10 L. Commercially available.

(**h**) *Hydrochloric acid standard solution.*—0.1000M. Prepare as in **936.15** (*see* A.1.06) or use premade solution of certified specification range 0.0995–0.1005M, and use 0.1000M for calculation. Commercially available.

(**i**) *Reference standards*.—Ammonium sulfate, tryptophan, lysine·HCl, or glycine *p*-toluenesulfonic acid, for use as standard; 99.9%.

(**j**) *Sucrose.*—N-free.

D. Preparation of Analytical Sample

Grind dry laboratory sample to fineness of grind (ca 0.7–1 mm), which gives a relative standard deviation (RSD) of ≤2.0% for 10 successive determinations of N in ground mixture of corn grain and soybeans (2 + 1). Fineness required to achieve this precision must be used for all dry mixed feeds and other nonuniform materials. Mix liquids to uniformity.

E. Determination

(**a**) *Digestion.*—Turn on block digestor and heat to 420°C. Weigh materials, as indicated below, recording each test portion weight (W) to the nearest mg for weights of ≥1 g, and to the nearest 0.1 mg for weights of <1.0 g. Do not exceed 1.2 g. For materials with 3–25% protein, weigh approximately 1.0 g test portion; with 25–50% protein, approximately 0.5 g test portion; and >50% protein, approximately 0.3 g test portion.

(*1*) *Dry feed, forage, cereal, grain, oilseeds*.—Weigh 1 g test portion of ground, well-mixed test portion onto a tared, low N weighing paper. Fold paper around material and drop into a numbered Kjeldahl tube.

(*2*) *Liquid feed.*—Weigh slightly >1 g test portion of well-mixed analytical sample into a small tared beaker. Quantitatively transfer to a numbered Kjeldahl tube with <20 mL deionized water. Alternatively, weigh slightly >1 g well-mixed test portion into a small tared beaker. Transfer to a numbered Kjeldahl tube and reweigh beaker. The differential weight loss corresponds to the amount of test portion actually transferred to the tube.

(**b**) *Standards.*—Perform quality control analysis and analyses of standards with each batch. The standards available from Hach Co. (PO Box 389, Loveland, CO 80539, USA; +1-800-227-4224 or +1-970-669-3050), Sigma (St. Louis, MO), J.T. Baker (Phillipsburg, NJ), the National Institute of Standards and Technology (NIST; Gaithersburg, MD) are listed in Table **2001.11C**.

The various ammonium salts and glycine *p*-toluenesulfonate serve primarily as a check on distillation efficiency and accuracy in titration steps because they are digested very readily. Lysine and nicotinic acid *p*-toluenesulfonate serve as a check on digestion efficiency because they are difficult to digest.

Include a reagent blank tube containing a folded low N weighing paper with each batch.

(**c**) *Digestion.*—Add 2 catalyst tablets to each tube. Add 12 mL H_2SO_4 to each tube, using pipetting dispenser; add 15 mL for high fat materials (>10% fat). Mixtures may be held overnight at this point. If mixture foams, slowly add 3 mL 30–35% H_2O_2. Let reaction subside in perchloric acid fume hood or in exhaust system.

Attach heat side shields to tube rack. Place fume manifold tightly on tubes, and turn water aspirator on completely. Place rack of tubes in preheated block. After 10 min, turn water aspirator down until acid fumes are just contained within exhaust hood. A condensation zone should be maintained within the tubes. After bulk of sulfur oxide fumes are produced during initial stages of digestion, reduce vacuum source to prevent loss of H_2SO_4. Digest additional 50 min. Total digestion time is approximately 60 min.

Turn digestor off. Remove rack of tubes with exhaust still in place, and put in the stand to cool for 10–20 min. Cooling can be increased by using commercial air blower or by placing in hood with hood sash pulled down to increase airflow across tubes. When fuming has stopped, remove manifold, and shut off aspirator. Remove side shields. Let tubes cool. Wearing gloves and eye protection, predilute digests manually before distilling. Carefully add a few milliliters of deionized water to each tube. If spattering occurs, the tubes are too hot. Let cool for a few more minutes. Add water to each tube to a total volume of approximately 80 mL (liquid level should be about half way between the 2 shelves of the tube rack). This is a convenient stopping point.

If digest solidifies, place tube containing diluted digest in block digester, and carefully warm with occasional swirling until salts dis-

Table 2001.11C Standards

Standard	Approximate weight, g	Theoretical yield, % N
Ammonium *p*-toluenesulfonate (Hach 22779-24)	0.5	7.402
Glycine *p*-toluenesulfonate (Hach 22780-24)	0.6	5.665
Nicotinic acid *p*-toluenesulfonate (Hach 22781-24)	0.2	4.743
Lysine monohydrochloride (Sigma L-5626)	0.1	15.34
Acetanilide (Baker A068-05)	0.3	10.36
Tryptophan (Sigma T 8659)	0.2	13.72
Ammonium salts		
Diammonium hydrogen phosphate (100% assay)	0.2	21.21
Ammonium chloride (100% assay)	0.2	26.18
Ammonium sulfate (100% assay)	0.2	21.2
Ammonium dihydrogen phosphate (NIST 200)	0.3	12.18
Citrus leaves (NIST 1572)	1.0	2.86
Urea (NIST 2141)	0.1	46.63

solve. If distilling unit equipped with steam addition for equilibration is used, the manual dilution steps can be omitted. About 70 mL deionized water is then automatically added during the distillation cycle.

(**e**) *Distillation.*—Place 40% NaOH in alkali tank of distillation unit. Adjust volume dispensed to 50 mL. Attach digestion tube containing diluted digest to distillation unit, or use automatic dilution feature, if available. Place graduated 500 mL Erlenmeyer titration flask containing 30 mL H_3BO_3 solution with indicator on receiving platform, and immerse tube from condenser below surface of H_3BO_3 solution. (When an automatic titration system is used that begins titration immediately after distillation starts, 1% H_3BO_3 may be substituted.) Steam distill until ≥150 mL distillate is collected (≥180 mL total volume). Remove receiving flask. Titrate H_3BO_3 receiving solution with standard 0.1000M HCl to violet endpoint (just before the solution goes back to pink). Lighted stir plate may aid visualization of endpoint. Record milliliters of HCl to at least the nearest 0.05 mL.

This is done automatically by using a steam distiller with automatic titration. Follow the manufacturer's instructions for operation of the specific distiller or distiller/titrator.

F. Verification of Nitrogen Recovery

Run N recoveries to check accuracy of procedure and equipment.

(**a**) *Nitrogen loss.*—Use 0.12 g $(NH_4)_2SO_4$ and 0.67 g sucrose per flask. Add all other reagents as in **E**, and distill under same conditions as in **E**. Recoveries must be ≥99%.

(**b**) *Distillation and titration efficiency.*—Distill 0.12 g $(NH_4)_2SO_4$, omitting digestion. Recoveries must be ≥99.5%.

(**c**) *Digestion efficiency.*—Use 0.3 g acetanilide or 0.18 g tryptophan, with 0.67 g sucrose per flask. Add all other reagents as stated in **E**. Digest and distill under same conditions as used for a determination. Recoveries must be ≥98%.

G. Calculations

$$\text{Kjeldahl nitrogen, \%} = \frac{(V_S \quad V_B) \times M \times 14.01}{W \times 10}$$

$$\text{Crude protein, \%} = \text{\% Kjeldahl N} \times F$$

where V_S = volume (mL) of standardized acid used to titrate a test; V_B = volume (mL) of standardized acid used to titrate reagent blank; M = molarity of standard HCl; 14.01 = atomic weight of N; W = weight (g) of test portion or standard; 10 = factor to convert mg/g to percent; and F = factor to convert N to protein.

F factors are 5.70 for wheat, 6.38 for dairy products, and 6.25 for other feed materials.

Reference: *J. AOAC Int.* (future issue).