Scope
Applicable to nitrate containing samples and animal feeds

Apparatus
Kjeldahl flasks for digestion, total capacity about 500-800 ml, made of hard thick well annealed glass.

Digestion heaters, 600 W. Heater unit should bring 250 ml of water at 25 C to boil in 5 minutes.

Distillation flask. 500-800 ml capacity fitted with rubber stopper through which passes lower end of efficient bulb or trap to prevent NaOH being carried over mechanically during distillation. Connect upper end of bulb tube to condenser tube by means of rubber tubing.

Reagents
1. H2SO4 93-98%, nitrogen free
2. Mercuric oxide or metallic mercury, nitrogen free
3. Potassium Sulfate N free
4. Salicylic acid N free
5. Sulfide of thiosulfate solution
6. NaOH pellets or solution
7. Zinc granules
8. Zinc dust, an impalpable powder
9. Methyl red indicator
10. Standard HCl or H2SO4
11. Standard alkali solution
Test reagents before using by blank determination with 2 g sugar which ensures partial reduction of any nitrates.

**Procedure**

1. Place weighed sample (.7-2.2 g) in digestion flask. Add 40 ml H2SO4 containing 2 g salicyclic acid. Shake until thoroughly mixed and let stand with occasional shaking for 30 minutes, then add 2 g Zinc dust. Shake and let stand 5 minutes. Then heat over low flame until frothing ceases. Turn off heat.
2. Add .7 g of mercury oxide or .65 g of metallic Hg, 15 g powdered potassium sulfate and 25 ml H2SO4. If more than 2.2 g of sample were used increase H2SO4. Place flask in inclined position and heat until gently frothing ceases. Boil briskly until solution clears and then for at least 30 min longer.
3. Cool, add approximately 200 ml water, cool below 25 degrees, add 25 ml sulfide or thio sulfate solution, and mix to precipitate mercury. Add a few zinc granules to prevent bumping, tilt flask and add layer of NaOH without agitation.
4. Immediately connect flask to distilling bulb on condenser and with tip of condenser immersed in 25 ml standard acid receiver, rotate flask to mix contents thoroughly. Then heat until all ammonia has distilled.
5. Titrate excess standard acid in distillate with standard alkali solution, using methyl red indicator.
6. Correct for blank determinations on reagents.

**CALCULATIONS**

\[
%N = \frac{[(B-S)\times N \times 1.4007]}{\text{sample weight}}
\]

B = ml alkali back titration of blank

S = ml alkali back titration of sample

N = Normality of alkali

AOAC Official Method 942.05

Ash of Animal Feed
Weigh 2 g sample into porcelain crucible and place in temperature controlled furnace preheated to 600. Hold at this temperature 2 h. Transfer crucible directly to desiccator, cool, and weigh immediately, reporting % ash to first decimal place.

References: JAOAC 25, 857(1942); 26, 220(1943).

AOAC Official Method 976.06
Protein (Crude) in Animal Feed and Pet Food
Semiautomated Method
First Action 1976
Final Action 1977

A. Principle

Samples are digested in 250 mL calibrated tubes, using block digestor. A of NH3-salicylate complex is read in flowcell at 660nm, or NH3 is distilled into standard acid and back-titrated with standard alkali.

B. Apparatus

(a) Block digestor- Model BD-20 (Technical Instruments Corp., instrument now available from Bran and Luebbe,Inc. 1025 Bush Parkway, Buffalo Grove, IL 60089) or Model DS-20 (Tecator Inc., instruments now available from Perstorp Analytical In., Silver Spring, MD 20904). Capable of maintaining 410 and digesting 20 samples at a time in 250 mL calibrated volumetric tubes constricted at top. Block must be equipped with removable shields to enclose exposed are of tubes completely at or above height of constriction.

(b) Automatic analyzer- AutoAnalyzer with following modules (Technical Instruments Corp., instrument now available from Bran and Luebbe,Inc. 1025 Bush Parkway, Buffalo Grove, IL 60089) or equivalent: Sampler II or IV with 40/h (2:1) cam (higher ratio result in carry-over and poorer peak separation); proportioning pump III; NH3 analysis cartridge No. 116-D531-01 (or construct equivalent manifold from flow diagram); AAII single channel colorimeter with 15 X
1.5-2.0 mm id tubular flowcell, matched 660 nm interference filters, and voltage stabilizer; and recorder of appropriate span.

C. Reagents

(a) Phosphate-tartrate buffer solution - pH 14.0. Dissolve 50 g sodium potassium tartrate and 26.8 g Na2HPO47H2O in 600 mL H2O. Add 54 g NaOH and dissolve. Add 1 mL Brij-35 (Technicon Instruments Corp.), dilute to 1 L with H2O, and mix.

(b) Sodium chloride-sulfuric acid solution - Dissolve 200 g NaCl in H2O in 2 L volumetric flask. Add 15 mL H2SO4 and 2 mL Brij-35. Dilute to volume with H2O and mix.

(c) Sodium hypochlorite solution - Dilute 6 mL commercial bleach solution containing 5.25% available Cl to 100 mL with H2O and mix. Prepare fresh daily.

(d) Sodium nitroprusside-sodium salicylate solution - Dissolve 150 g NaC7H5O5 and 0.3 g Na2Fe(CN)5NO2H2O in 600 mL H2O. Add 1 mL Brij-35, dilute to 1 L with H2O, and mix.

(e) Nitrogen standard solution - Prepare 6 standards by accurately weighing (± 10 mg) 59, 118, 236, 295, and 354 mg (NH4)2SO4 primary standard (Fisher Scientific Co. No A-938, or equivalentL dry 2 h at 105 before use and assume theoretical value of 21.20% N after drying) into individual 250 mL digestion tubes. Proceed as in 976.06G, beginning "Add 9 g K2SO4, 0.42 g H2O and 15 mL H2SO4..." Standards may be stored and reused until exhausted.

(f) Sodium hydroxide-potassium sulfide solution - Dissolve 400 g NaOH in H2O. While still warm, dissolve 30 g K2S in solution and dilute to 1L.

D. Analytical System

If manifold is to be constructed, use clear standard pump tubes for all air and solution flows. All fittings, coils, and glass transmission lines are AAII type and size. Use glass transmission tubing for all connections after pump to colorimeter. Construct modified AO fitting on sample dilution loop using AO fitting, N13 stainless steel nipple connector, and ½" length of 0.035" id Tygon tubing. Insert N13 nipple approximately halfway into 0.035" Tygon tubing. Insert tubing into side arm of AO fitting far enough so resample line will not pump any air. Space pump tubes equally across pump rollers. Cut 0.16 mL/min resample pumptube 1" at entrance before connecting to side arm of AO fitting. In operation, add buffer and hypochloric solutions through metal side arms of A10 type fittings: add salicylate solution through metal insert to 20T coil. Air, reagents, and sample are combined immediately after pump through injection fittings.

E. Start-up

Start automatic system and place all lines except salicylate line in respective solutions. After 5 min, place salicylate line in respective solution and let system equilibriate. If precipitate forms
after addition of salicylate, pH is too low. Immediately stop proportioning pump and flush coils with H2O using syringe. Before restarting system, check concentrations of NaCl-H2SO4 solution and phosphate-tartrate buffer solution.

Pump lowest concentration N standard solution continuously through system 5 min

and adjust baseline control on colorimeter to read 10% full scale. Pump highest concentration N standard solution continuously through system until no drift exists (usually 10 min) and adjust "standard calibration" control to read 85% full scale. Recorder tracings must be stable and show <0.3 division noise. If noisy conditions exist, replace dialyzer membrane. When recorder tracing indicates stable condition, immediately start sampling.

F. Shut-Down

Place reagent lines in H2O, removing salicylate line first. Let system was out 20 min.

G. Colorimetric Determination

Weigh samples (see Table 976.06) into dry digestion tubes. Add 9 g K2SO4, 0.42 g HgO, and 15 mL H2SO4 to each tube. (Calibrated metal scoops may be used for solids) Insert tubes into digester block preheated to 410, place shields around tubes, and digest for 45 min.

After digestion, remove rack of tubes from block, place in hood, and let cool 8-10 min. (Time depends upon air flow around tubes.) Direct rapid spray of H2O (kitchen sink dish rinsing sprayer works well) to bottom of each tube to dissolve acid digest completely. If precipitate forms, place tube in ultrasonic bath to aid in redissolving salt. Let cool, dilute to volume, and mix thoroughly. Transfer portion of each sample solution to AutoAnalyzer beaker.

Place standards in tray in increasing order of concentration, followed by group of samples. Analyze lowest concentration standard in duplicate, discarding first peak. Precede and follow each group of samples with standard reference curve to correct for possible drift. Analyze standards and samples at rate of 40/h, 2/1 sample-to-wash ratio. Prepare standard curve by averaging peak heights of first and second set of standards. Plot average peak height standards against N concentration contained in each 250 mL tube.

\[
\text{%Protein} = \frac{[(mg \text{ N/250 mL from graph}) \times 6.25 \times 100]}{mg \text{ sample}}
\]

H. Titrimetric Determination

Digest as in 976.06G. Cool 5 min. and add only enough H2O to dissolve salts (70-75 mL). Cool and attach digestion tube to distillation head according to manufacturer's directions. Place receiver flask containing 25 mL standard acid, 936.15A or 890.01A, add 5-7 drops methyl red indicator on platform. Condenser tip must be below surface of standard acid solution. Add 50
mL NaOH-K2S solution to tube and steam distill vigorously until 125 mL distillate collects. Titrate excess acid with standard 0.1N NaOH solution, 936.16. Correct for reagent blank.

\[ \%N = \left( \frac{mL \text{ standard acid} \times \text{normality acid}}{1.4007/g \text{ sample}} \right) - \left( \frac{mL \text{ standard NaOH} \times \text{normality NaOH}}{1.4007/g \text{ sample}} \right) \]

\% crude protein = \%N * 6.25

References: JAOAC 59, 134(1976); 62, 290 (1979)

**AOAC official Method 971.09**

**Pepsin Digestibility**

**of Animal Protein Feeds**

Filtration Method

First Action 1971, Final Action 1973

A. Principle

Defatted sample is digested 16 h with warm solution of pepsin under constant agitation. Insoluble residue is isolated by filtering, washed, dried, and weighed to determine % residue. Residue is examined microscopically and analyzed for protein. Filtration method is applicable to all animal proteins. Methods are not applicable to vegetable proteins or mixed feeds, because of presence of complex carbohydrates and other compounds not digested by pepsin.

B. Apparatus

(a) Agitator.-- Continuous, slow speed (15 rpm), end-over-end type, to operate inside incubator at 45°+/- 2° and carry 8 oz screw-cap prescription bottles, or equivalent Agitator and bottles
available from D.E. Sims, 2107 Cherry Lane Estates, Quincy, IL 62301. Stirring or reciprocating (shaking) type agitator cannot be used because solid particles collect on sides of bottle and do not contact pepsin solution. If heat from agitator motor raises incubator temperature to >45°, mount motor outside incubator by drilling hole through side of incubator and connecting motor to agitator with extension shaft and coupling (available from agitator supplier).

(b) Settling rack.-- Wood or metal to hold digestion bottles at 45° angle. May be made from 2 boards nailed horizontally into "V" cut into vertical end pieces. Also available from agitator supplier, (a).

(c) Filtering device.-- Modified California Buchner, 962.09 C(d) (see 4.4.01), available from Labconco Corp., 8811 Prospect Ave, Kansas City, MO 64132. No. 55100. (If edge of screen is rough, smooth with small-tip soldering iron.) Use with retainer sleeve, 2X2.75" od stainless steel tube, available from agitator supplier (a).

(d) Glass fiber filter.-- 7 cm, Whartman, Inc., 934-AH, or equivalent.

(e) Moisture dishes.-- Al, 78 mm od x 20 mm, with outside cover and vertical sides (Curtin Matheson Scientific, Inc., No. 19370-30, or equivalent).

C. Reagent

Pepsin solution.-- 0.25 pepsin (activity 1:10000) in 0.075N HCL; do not use pepsin NF or pepsin of activity other than 1:10,000. Prepare just before use by diluting 6.1 mL HCL to 1 L and heating to 42-45°. Add pepsin and stir gently until dissolved. Do not heat pepsin solution on hot plate or overheat.

D. Preparation of Sample

Sieve sample, 956.16, through No. 20 sieve. Grind portion retained on sieve to pass No. 20 sieve. Combine both portions and blend by stirring and shaking in pint (500 mL) jar. Thorough blending is essential. Because of high fat content of many animal products, grinding without sieving may cause sticking in mill, loss of moisture or fat, or poorly blended sample.

E. Extraction

(Caution: See Appendix B, safety notes on distillation, flammable solvents, and diethyl ether.)

Prepare extraction thimble from 11 cm Whatman No. 2 paper, or equivalent, as follows: Fold paper in half; straighten paper and refold at right angles to first fold; turn paper over and repeat process with folds at 45° to original fold; while holding creased paper in one hand, place short
test tube (6-8 mm smaller in diameter than extractor sample holder or cup in which thimble is to be used) at its center; fold along natural crease lines to form 4-pointed star around tube; and wrap points in same direction around tube to complete thimble.

Weigh 1000 g ground sample (0.500 g of poultry byproducts or hydrolyzed feathers because of gummy nature and amount of residue into thimble and extract 1 h with ether at condensation rate of 3-4 drops/s. (If Soxhlet is used, top of thimble should extend above siphon tube to avoid loss of solid particles. If paper containing sample is totally submerged in siphon cup, sample must be completely wrapped in paper.) Observe ether extract to determine that no solid particles were carried into solvent. For approximate fat content determination, evaporate ether, and dry and weigh residue. Remove paper from sample container or cup and let dry at room temperature. Unfold, and quantitatively brush defatted sample into digestion bottle, avoiding contamination by brush bristles or filter paper fibers. Use of powder funnel is helpful in avoiding loss.

F. Pepsin Digestion

To defatted sample in agitator bottle add 150 mL freshly prepared pepsin solution prewarmed to 42-45°. Be sure sample is completely wetted by pepsin solution. Stopper bottle, clamp in agitator, and incubate with constant agitation 16 h at 45°.

G. Treatment of Residue

Dry individual sheets of glass fiber filter, (d), 30 min at 110° in moisture dishes with cover open. Cool in desiccator 30 min with cover closed, and weigh (W₁).

Remove bottles from agitator. Place in 45° settling rack and loosen caps. Let residue settle >/= 15 min. Place weighed filter in California Buchner, (c), apply suction, and moisten with H₂O. Carry bottle from rack to filter at same angle as settled and slowly pour contents through filter as continuous small stream, avoiding all unnecessary agitation. Liquid passes through paper as rapidly as poured, with residue spreading over surface of filter but not covering it completely until all or practically all of liquid has passed through. If filtration rate become slow, it may be accelerated by adding acetone washes described below, but only if no significant amount of digestion mixture remains on funnel when acetone is added. (Filtration [passage of aqueous mixture through filter] should be complete within 1 min with most proteins.) After supernate has passed through filter, quantitatively transfer residue onto filter as follows:

Add 15 mL acetone to bottle. Hold thumb over bottle neck and shake vigorously. Release pressure, replace thumb over bottle neck, and shake bottle in inverted position over filter. Remove thumb, letting acetone and residue discharge onto filter. Repeat rinse with second 15 mL portion acetone, shaking and releasing pressure as above. Inspect bottle, and rinse further with acetone, using policeman, is necessary. If > 3mm liquid remains on paper when acetone washes are started, it may be necessary to use three 15 mL acetone washer instead of 2 to increase filtration rate.
After all liquid passes through funnel, wash residue and inside surface of retainer sleeve with 2 small portions acetone from wash bottle or hypodermic syringe, and suck dry. Remove retainer sleeve from funnel. Transfer filter to original moisture dish. Scrape or brush any residue particles of filter clinging to retainer sleeve or funnel onto filter in moisture dish. Dry in oven, cool, and weigh as before \((W_2)\). Calculate % indigestible residue = \((W_2-W_1)\) x100/g sample.

Determine indigestible protein by transferring filter containing residue directly to Kjeldahl flask. Proceed as in 954.01. (Caution: Violent reaction may take place when NaOH is mixed with diluted digestion mixture, caused by large excess \(\text{H}_2\text{SO}_4\) due to small amount organic material from residue and none from glass filter. Avoid by thoroughly mixing and cooling digestion mixture before addition of NaOH or by using 20 mL \(\text{H}_2\text{SO}_4\) in Kjeldahl digestion instead of 25mL.) Make blank determination on 1 sheet of glass filter and subtract from each sample determination, if necessary. Calculate % protein based on original sample weight. Result represents % indigestible protein in sample. Convert to % crude protein content of sample not digested, \("\text{protein indigestible}"\) = % indigestible protein in sample x 100% total crude protein in sample.

AOAC Official Method 941.04
Urea and Ammoniacal Nitrogen in Animal Feed
Urease Method
Final Action

A. Reagents

(a) Defoaming solution.-- Dow Corning Corp. Antifoam B Emulsion.

(b) Urease solution.-- Prepare fresh solution by dissolving standardized urease in \(\text{H}_2\text{O}\) so that each 10 mL neutralized solution will convert N of greater than or equal to 0.1 g pure urea.

Standardization.-- To determine alkalinity of commercial urease preparation dissolve 0.1 g in 50 mL \(\text{H}_2\text{O}\) and titrate with 0.1 \(\text{N HCl}\), using methyl red, 984.13B(c) (see 4.2.09). Add same volume 0.1\(\text{N HCl}\) to each 0.1 g urease in preparing urease solution. To determine enzyme activity, prepare ca 50 mL neutralized 1% solution. Add different amounts of solution to 0.1 g samples pure urea and follow with enzyme digestion and distillation as in determination. Calculate activity of urease preparation from amount of this urease solution that completely converted urea, as determined by complete recovery of N by distillation.

(c) Calcium chloride solution.-- Dissolve 25 g CaCl2 in 100 mL \(\text{H}_2\text{O}\).

B. Determination
Place 2 g sample in Kjeldahl flask with ca 250 mL H2O. Add 10 mL urease solution, stopper tightly, and let stand 1 h at room temperature or 20 min at 40 degrees. Cool to room temperature if necessary. Use additional urease solution if feed contains >5% urea (ca 12% protein equivalent). Rinse stopper and neck with few mL H2O. Add at least 2g MgO (heavy type), 5 mL CaCl2 solution, and 3 mL defoaming solution, and connect flask with condenser by Kjeldahl connecting bulb. Distill 100 mL into measured volume standard acid, 936.15 (see A.1.06) or 890.01 (see A.1.14), and titrate with standard alkali, 936.16 (see A.1.12), using methyl red, 984.13B(c) (see 4.2.09).

References: JAOAC 24, 867(1941); 25, 874(1942); 27, 494(1944).

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AOAC Official Method 967.07
Urea in Animal Feed
Colorimetric Method
First Action 1967
Final Action 1970

(Applicable to animal feeds and their ingredients.)

A. Apparatus

Spectrophotometer.-- Instrument with maximum band width 2.4 nm at 420 nm, with 1 cm cells.

B. Reagents

(a) \textit{p-Dimethylaminobenzaldehyde (DMAB) solution}. Dissolve 16.00 g (Eastman Kodak Co. No. 95 only) in 1 L alcohol and add 100 mL HCl. Stable 1 month. Prepare new standard curve with each new batch of reagent.

(b) \textit{Zinc acetate solution}. Dissolve 22.0 g Zn(CH3COO)2*2H2O in H2O, add 3 mL CH3COOH, and dilute to 100 mL.
(c) Potassium ferrocyanide solution. Dissolve 10.6 g K₄Fe(CN)₆·3H₂O in H₂O and dilute to 100 mL.

(d) Vegetable charcoal. Darco G-60

(e) Phosphate buffer solution. pH 7.0. Dissolve 3.403 g anhydrous KH₂PO₄ and 4.355 g anhydrous K₂HPO₄ separately in ca 100 mL portions freshly distilled H₂O. Combine solutions and dilute to 1 L with H₂O.

(f) Urea standard solutions. (1) Stock solution. 5 mg/mL. Dissolve 5.000 ± 0.001 g reagent grade urea in H₂O and dilute to 1 L with H₂O. (2) Working solutions: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mg urea/5 mL. Pipet 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 mL stock solution into 250 mL volumetric flasks and dilute to volume with phosphate buffer. (3) Reference solution. Use standard solution containing 1.0 mg urea/5 mL as reference standard. Store at < 24 degrees. Stable 1 week.

C. Preparation of Standard Curve

Pipet 5 mL aliquots of working standard solutions into 20 x 150 mm (25 mL) test tubes and add 5 mL DMAB solution to each. Prepare reagent blank of 5 mL buffer solution and 5 mL DMAB solution. Shake tubes thoroughly and let stand 10 min in H₂O bath at 25 degrees. Read A in 1 cm cell at 420 nm with reagent blank at zero A. Plot A against concentration urea. Plot should be straight line; if not, repeat, using new lot of DMAB.

D. Determination

Weigh 1.00 g ground sample into 500 mL volumetric flask. Add 1 g charcoal, ca 250 mL H₂O, 5 mL Zn (CH₃COO)₂ solution, and 5 mL K₄Fe(CN)₆ solution. Shake mechanically 30 min and dilute to volume with H₂O. Let stand until precipitate settles. Decant through Whatman No. 40 paper and collect clear filtrate. Pipet 5 mL filtrate into test tube, add 5 mL DMAB solution, and shake thoroughly. Include reference standard [5 mL solution (f)(3) and 5 mL DMAB solution] and reagent blank with each group of samples. Let stand 10 min in H₂O bath at 25 degrees. Read A at 420 nm against reagent blank.

% Urea = (1.0 x A (sample) x 100)/(A(standard) x mg sample in aliquot)

Reference: JAOAC 50, 56(1967).