Click to view page images in PDF format.

Click to view page images in PDF format (Collaborative Study).

4.1.11 - Animal Feed / Animal Feed--General

AOAC Official Method 994.12
Amino Acids in Feeds

Performic Acid Oxidation
with Acid Hydrolysis-Sodium Metabisulfite Method
First Action 1994
Final Action 1997

(Applicable to determination of amino acids [including methionine and cystine] in feeds. Not applicable to determination of tyrosine and tryptophan.)

See Tables **994.12A-E** for the results of the interlaboratory study supporting acceptance of method.

A. Principle

Performic acid oxidation is performed prior to hydrolysis to oxidize cystine and methionine to cysteic acid and methionine sulfone, respectively. Sodium metabisulfite is added to decompose performic acid. Amino acids are liberated from protein by hydrolysis with 6M HCl. Hydrolysates are diluted with sodium citrate buffer or neutralized, pH is adjusted to 2.20, and individual amino acid components are separated on ion-exchange chromatograph. Tyrosine is destroyed by oxidation. Tryptophan is destroyed by hydrolysis, so those amino acids cannot be determined.

B. Apparatus

(**a**) *Amino acid analyzer.*—Ion-exchange resin with ninhydrin post-column derivatization.

(**b**) *Analytical balance.*—Accurate to ±0.1 mg.

(**c**) *Balance*.—Top loading.

(**d**) *Bottle*.—50 mL; polyethylene.

(**e**) *Digestion tubes*.—Boiling flasks are suitable.

(**f**) *Digestion block*.—Heating mantle is suitable.

(**g**) *Filter units*.—0.22 µm (Millex GS, Millipore are suitable).

(**h**) *Magnetic stirring plate*.

(**i**) *pH meter*.—Calibrated with buffers of pH 2.0, 4.0, and 7.0.

(**j**) *Reflux condensers*.

(**k**) *Rotary evaporator*.

(**l**) *Vacuum flask*.—250 mL.

(**m**) *Glassware*.—Glass beakers, 250 and 1000 mL; Erlenmeyer flask, 150 mL; round-bottom evaporating flask, 1000 mL; graduated cylinders, 100, 500, and 1000 mL; volumetric flask, 1000 mL; volumetric pipets, 10 and 20 mL.

(**n**) *Sintered glass filter*.—Porosity 10-15 µm.

(**o**) *Ice bath*.

(**p**) *Syringes*.

C. Reagents

(**a**) *Formic acid*.—88%.

(**b**) *Hydrogen peroxide*.—30%.

(**c**) *Sodium metabisulfite*.

(**d**) *DL-Norleucine*.—Crystals.

(**e**) *HCl*.—Concentrated.

(**f**) *NaOH*.—30% solution (30 g/100 mL).

(**g**) *Phenol.*—Crystals.

(**h**) *Thiodiglycol.*—98% solution.

(**i**) *Tri-sodium citrate dihydrate.*

(**j**) *pH buffer.*—pH 2.0, 4.0, and 7.0.

(**k**) *Amino acid standard kit.*—To calibrate amino acid analyzer; available from Aldrich Chemical Co., Inc., 1001 West Saint Paul Ave, Milwaukee, WI 53233, USA.

D. Preparation of Solutions

(**a**) *Sodium citrate buffer, pH 2.20.*—Weigh 19.60 g tri-sodium citrate dihydrate in 1000 mL beaker and dissolve in ca 800 mL H_2O. While stirring, add 10 mL 98% thiodiglycol solution and 15 mL concentrated HCl. Transfer solution quantitatively into 1000 mL volumetric flask and dilute to mark with H_2O. Filter buffer solution through sintered glass filter, **B**(**n**). Adjust pH to 2.20 with concentrated HCl or 2M NaOH.

(**b**) *6M HCl-phenol solution.*—Weigh 1 g phenol crystals into tared 1000 mL beaker. Dissolve crystals in 500 mL H_2O. While stirring, slowly add 500 mL concentrated HCl.

(**c**) *HCl solutions.*—(*1*) *1M HCl.*—Pour ca 800 mL H_2O into 1000 mL volumetric flask, and then add 83.3 mL concentrated HCl, using pipet. Dilute to the mark with H_2O and mix thoroughly. (*2*) *0.1M HCl.*—Pour ca 800 mL H_2O into 1000 mL volumetric flask, and then add 100 mL 1M HCl, using pipet. Dilute to the mark with H_2O and mix thoroughly.

(**d**) *NaOH solutions.*—(*1*) *7.5M NaOH.*—Weigh 300.0 g NaOH in tared 1000 mL beaker. (*2*) *2M NaOH.*—Weigh 80.0 g NaOH in tared 1000 mL beaker. Slowly dissolve pellets in each beaker in ca 600 mL H_2O. Cool solutions and transfer quantitatively to separate 1000 mL volumetric flasks. Dilute to mark with H_2O and mix thoroughly.

(**e**) *Norleucine standard solution.*—Accurately weigh 195-200 mg DL-norleucine crystals into tared 150 mL Erlenmeyer flask. Dissolve crystals with 100 mL 1M HCl. Transfer solution quantitatively into 1000 mL volumetric flask and dilute to mark with H_2O.

(**f**) *Performic acid reagent.*—Prepare in hood. Weigh 25 mg phenol crystals in 25 mL test tube; then add 0.5 mL 30% H_2O_2, using micropipet, and 4.5 mL 88% formic acid solution. Cover test tube with stopper, and let mixture stand 30 min at room temperature. After 30 min, place test tubes in ice bath and cool performic acid mixture for 15 min. Prepare reagent just before use.

E. Performic Acid Oxidation

Finely grind test sample to pass 0.25 mm sieve. Accurately weigh ca 100-1000 mg test portions to the nearest 0.1 mg (equivalent to ca 10 mg nitrogen content) into labeled digestion tubes.

Calculate approximate amount of test portion to use as follows:

$$W_s =$$

where N_s = nitrogen content of test portion, %; W_s = weight of test portion equivalent to 10 mg nitrogen content, mg.

Put magnetic stirrer into each tube and place digestion tubes in ice bath (0°C).

After both the performic acid and test portion have cooled at least 15 min, add 5 mL performic acid into digestion tube, cover all tubes with glass stoppers and stir 15 min on magnetic stirring plate.

Return digestion tubes to ice bath and let oxidize 16 h.

Remove glass stoppers and add ca 0.84 g sodium metabisulfite to decompose performic acid. Stir for 15 min to liberate SO_2.

F. Hydrolysis

Add 50 mL 6M HCl-phenol solution, **D**(**b**), to test solution and briefly stir. Remove stirring bar using magnetic stirring rod, and rinse with small volume of 0.1M HCl into tube. Add 2-3 pieces of boiling chips to test solution.

Hydrolyze under reflux for 24 h at 110°-120°C using digestion block, **B**(**f**). (*Caution*: Perform this step inside fume hood with adequate ventilation.)

Remove digestion tubes from heat and cool to room temperature. Add 20 mL norleucine standard solution, **D**(**e**), to each hydrolysate using volumetric pipet. Mix solutions by swirling flasks. Proceed with (**a**) or (**b**) below. {*Note:* If low sodium

concentration is required for chromatography, evaporate HCl carefully [perform step (**a**)]. If low sodium concentration is not required perform neutralization step, (**b**).}

(**a**) Filter hydrolysates through sintered glass filter into labeled 1000 mL evaporating flasks. Connect flasks to rotary evaporators, and evaporate under vacuum at 40°C to ca 5.0 mL. (*Note:* Do not let solution evaporate to dryness.) Remove flasks from evaporator. Add 50 mL sodium citrate buffer, **D**(**a**), to evaporated test solution, mix well, and transfer into labeled 50 mL polyethylene bottle, **B**(**d**). Proceed to **G**, or freeze until measurement.

(**b**) Filter hydrolysates into 250 mL vacuum flask, **B**(**l**), through sintered glass filter, then transfer filtrate to 250 mL beaker. Place beaker in ice bath. Partly neutralize hydrolysates with ca 40 mL 7.5M NaOH, **D**(**d**)(*1*), while stirring. (*Note:* Temperature can not exceed 40°C.) Adjust pH to 2.20 using 2M NaOH, **D**(**d**)(*2*). Proceed to **G**.

G. Determination

Dilute aliquot of evaporated hydrolysate **F**(**a**) with sodium citrate buffer, **D**(**a**), and adjust pH to 2.20 with 2M NaOH. When neutralized hydrolysates **F**(**b**) are used, dilute aliquot with H_2O. Filter through filter unit, **B**(**g**), into autosampler tube and inject into analyzer. (*Note:* Volume of aliquot and dilution depends on response of analyzer.)

Calibrate amino acid analyzer with amino acid standard kit solution, **C**(**k**), containing norleucine. Operate amino acid analyzer according to manufacturer's specifications. Adjust analyzer conditions to ensure baseline separation of peaks. Minimum resolution between 2 peaks should be 90%.

H. Calculations

Calculate response factor (RF_{aa}) for each amino acid as follows:

$$RF_{aa} =$$

where P_{aa} = peak area of amino acid; P_n = peak area of norleucine; W_{aa} = weight of amino acid, mg; W_n = weight of norleucine, mg.

Calculate internal standard (*IS*) factor as follows:

$$IS = W_n \quad 2 \quad 10^{-2}$$

where mg norleucine = norleucine content in 20 mL norleucine standard, **D**(**e**).

Calculate amino acid (AA) content of the test sample as follows:

$$AA, \% = $$

where P_{aa} = peak area of amino acid; P_n = peak area of norleucine; W_s = weight of test portion, mg; RF_{aa} = amino acid response factor; IS = internal standard factor.

Performic Acid Oxidation with Acid Hydrolysis-Hydrobromic Acid Method

(Applicable to determination of amino acids [including methionine and cystine] in feeds. Not applicable to determination of phenylalanine, tyrosine, histidine, and tryptophan.)

See Tables **994.12F-J** for the results of the interlaboratory study supporting acceptance of method.

A. Principle

Performic acid oxidation is performed prior to hydrolysis to oxidize cystine and methionine to cysteic acid and methionine sulfone, respectively. Hydrobromic acid is added to decompose performic acid. Amino acids are liberated from protein by hydrolysis with 6M HCl. Hydrolysates are diluted with sodium citrate buffer and individual amino acid components are separated by ion-exchange chromatography. Tryptophan is destroyed by hydrolysis. Tyrosine, phenylalanine, and histidine are destroyed during oxidation process and by reaction with bromine, and cannot be accurately analyzed.

B. Apparatus

(**a**) *Amino acid analyzer.*—Ion-exchange resin with ninhydrin post-column derivatization.

(**b**) *Analytical balance.*—Accurate to ±0.1 mg.

(**c**) *Balance.*—Top loading.

(**d**) *Bottle.*—50 mL; polyethylene.

(**e**) *Digestion tubes.*—Boiling flasks are suitable.

(**f**) *Digestion block.*—Heating mantle is suitable.

(**g**) *Filter units.*—0.22 µm (Millex GS, Millipore are suitable).

(**h**) *Magnetic stirring plate.*

(**i**) *pH meter.*—Calibrated with buffers of pH 2.0, 4.0, and 7.0.

(**j**) *Reflux condensers.*

(**k**) *Rotary evaporator.*

(**l**) *Glassware.*—Glass beakers, 250 and 1000 mL; Erlenmeyer flask, 150 mL; round-bottom evaporating flask, 1000 mL; graduated cylinders, 100, 500, and 1000 mL; volumetric flask, 1000 mL; volumetric pipets, 10 and 20 mL.

(**m**) *Sintered glass filter.*—Porosity 10-15 µm.

(**n**) *Ice bath.*

(**o**) *Syringes.*

C. Reagents

(**a**) *Formic acid.*—88%.

(**b**) *Hydrobromic acid.*—48%.

(**c**) *Hydrogen peroxide.*—30%.

(**d**) *DL-Norleucine.*—Crystals.

(**e**) *HCl.*—Concentrated.

(**f**) *NaOH.*—30% solution (30 g/100 mL).

(**g**) *Phenol.*—Crystals.

(**h**) *Thiodiglycol.*—98% solution.

(**i**) *Tri-sodium citrate dihydrate.*

(**j**) *pH buffer.*—pH 2.0, 4.0, and 7.0.

(**k**) *Amino acid standard kit.*—To calibrate amino acid analyzer; available from Aldrich Chemical Co., Inc., 1001 West Saint Paul Ave, Milwaukee, WI 53233, USA.

D. Preparation of Solutions

(**a**) *Sodium citrate buffer, pH 2.20.*—Weigh 19.60 g tri-sodium citrate dihydrate in 1000 mL beaker and dissolve in ca 800 mL H_2O. While stirring, add 10 mL 98% thiodiglycol solution and 15 mL concentrated HCl. Transfer solution quantitatively into 1000 mL volumetric flask and dilute to mark with H_2O. Filter buffer solution through sintered glass filter, **B**(**m**). Adjust pH to 2.20 with concentrated HCl or 2M NaOH.

(**b**) *6M HCl-phenol solution.*—Weigh 1 g phenol crystals into tared 1000 mL beaker. Dissolve crystals in 500 mL H_2O. While stirring, slowly add 500 mL concentrated HCl.

(**c**) *HCl solutions.*—(*1*) *1M HCl.*—Pour ca 800 mL H_2O into 1000 mL volumetric flask, and then add 83.3 mL concentrated HCl, using pipet. Dilute to the mark with H_2O and mix thoroughly. (*2*) *0.1M HCl.*—Pour ca 800 mL H_2O into 1000 mL volumetric flask, and then add 100 mL 1M HCl, using pipet. Dilute to the mark with H_2O and mix thoroughly.

(**d**) *Norleucine standard solution.*—Accurately weigh 195-200 mg DL-norleucine crystals into tared 150 mL Erlenmeyer flask. Dissolve crystals with 100 mL 1M HCl. Transfer solution quantitatively into 1000 mL volumetric flask and dilute to mark with H_2O.

(**e**) *Performic acid reagent.*—Prepare in hood. Weigh 25 mg phenol crystals in 25 mL test tube; then add 0.5 mL 30% H_2O_2, using micropipet, and 4.5 mL 88% formic acid solution. Cover test tube with stopper, and let mixture stand 30 min at room temperature. After 30 min, place test tubes in ice bath and cool performic acid mixture for 15 min. Prepare reagent just before use.

E. Performic Acid Oxidation

Finely grind test sample to pass 0.25 mm sieve. Accurately weigh ca 100-1000 mg test portions to the nearest 0.1 mg (equivalent to ca 10 mg nitrogen content) into labeled digestion tubes.

Calculate approximate amount of test portion to use as follows:

$$W_s =$$

where N_s = nitrogen content of test portion, %; W_s = weight of test portion equivalent to 10 mg nitrogen content, mg.

Put magnetic stirrer into each tube and place digestion tubes in ice bath.

After both the performic acid and test portion have cooled at least 15 min, add 5 mL performic acid into digestion tube, cover all tubes with glass stoppers and stir 15 min on magnetic stirring plate.

Return digestion tubes to ice bath and let samples oxidize 16 h.

After oxidation, remove glass stoppers and decompose performic acid by adding 0.70 mL 48% hydrobromic acid, **C**(**b**). Stir (held in ice bath) for 30 min to liberate bromine.

Transfer digestion tube to rotary evaporator, and swirl solution under vacuum at room temperature until color turns from bright orange to yellowish tint. Remove tube from evaporator and place on tube rack.

F. Hydrolysis

Add 50 mL 6M HCl-phenol solution, **D**(**b**), to test solution and briefly stir. Remove stirring bar using magnetic stirring rod, and rinse with small volume of 0.1M HCl into tube. Add 2-3 pieces of boiling chips to test solution.

Hydrolyze under reflux for 24 h at 110-120°C using digestion block, **B**(**f**).
(*Caution:* Perform this step inside fume hood with adequate ventilation.)

Remove digestion tubes from heat and cool to room temperature.

Add 20 mL norleucine standard solution, **D**(**d**), to each test solution using volumetric pipet. Mix solution by swirling flask. Filter hydrolysates through sintered glass filter into labeled 1000 mL round-bottom evaporating flasks.

Evaporate hydrolysate at 60°C to dryness using rotary evaporator. Wash by adding ca 20 mL H_2O to hydrolysate and evaporate again. Repeat washing and evaporating steps 2 .

Remove flask from evaporator, add 50 ml sodium citrate buffer to hydrolysate and mix well. Transfer buffered hydrolysate into labeled 50 mL polyethylene bottles, **B**(**d**). Proceed to **G**, or freeze until measurement.

G. Determination

Dilute aliquot of hydrolysate with sodium citrate buffer, **D**(**a**), filter through 0.22 µm filter unit into autosampler tube, and inject into analyzer. (*Note:* Volume of aliquot and dilution depends on response of analyzer.)

Calibrate amino acid analyzer with amino acid standard kit solution, **C**(**k**), containing norleucine. Operate analyzer according to manufacturer's specifications. Adjust analyzer conditions to ensure baseline separation of peaks. Minimum resolution between 2 peaks should be 90%.

H. Calculations

Proceed as in Performic Acid Oxidation with Acid Hydrolysis-Sodium Metabisulfite Method **H**.

Acid Hydrolysis Method

(Applicable to determination of amino acids in feeds except methionine, cystine, and tryptophan.)

Results of Interlaboratory Study:
See Tables **994.12K-O** for the results of the interlaboratory study supporting acceptance of method.

A. Principle

Amino acids are liberated from protein by hydrolysis with 6N HCl. Internal standard is added and HCl is evaporated. Hydrolysates are diluted with sodium citrate buffer and individual amino acid components are separated by ion-exchange chromatograph. Cystine and methionine are partially oxidized, and tryptophan is completely destroyed; therefore, they cannot by accurately quantified.

B. Apparatus

(**a**) *Amino acid analyzer.*—Ion-exchange resin with ninhydrin post-column derivatization.

(**b**) *Analytical balance.*—Accurate to ±0.1 mg.

(**c**) *Balance.*—Top loading.

(**d**) *Bottle.*—50 mL; polyethylene.

(**e**) *Digestion tubes.*—Boiling flasks are suitable.

(**f**) *Digestion block.*—Heating mantle is suitable.

(**g**) *Filter units.*—0.22 µm (Millex GS, Millipore are suitable).

(**h**) *pH meter.*—Calibrated with buffers of pH 2.0, 4.0, and 7.0.

(**i**) *Reflux condensers.*

(**j**) *Rotary evaporator.*

(**k**) *Glassware.*—Glass beakers, 250 and 1000 mL; Erlenmeyer flask, 150 mL; round-bottom evaporating flask, 1000 mL; graduated cylinders, 100, 500, and 1000 mL; volumetric flask, 1000 mL; volumetric pipets, 10 and 20 mL.

(**l**) *Sintered glass filter.*—Porosity 10-15 µm.

(**m**) *Syringes.*

C. Reagents

(**a**) *DL-Norleucine.*—Crystals.

(**b**) *HCl.*—Concentrated.

(**c**) *NaOH.*—30% solution (30 g/100 mL).

(**d**) *Phenol.*—Crystals.

(**e**) *Thiodiglycol.*—98% solution.

(**f**) *Tri-sodium citrate dihydrate.*

(**g**) *pH buffer.*—pH 2.0, 4.0, and 7.0.

(**h**) *Amino acid standard kit.*—To calibrate amino acid analyzer; available from Aldrich Chemical Co., Inc., 1001 West Saint Paul Ave, Milwakee, WI 53233.

D. Preparation of Solutions

(**a**) *Sodium citrate buffer, pH 2.20.*—Weigh 19.60 g tri-sodium citrate, dihydrate in 1000 mL beaker and dissolve in ca 800 mL H_2O. While stirring, add 10 mL 98% thiodiglycol solution, and 15 mL concentrated HCl. Transfer solution quantitatively into 1000 mL volumetric flask and dilute to mark with H_2O. Filter buffer solution

through sintered glass filter, **B**(**l**). Adjust pH to 2.20 with concentrated HCl or 2M NaOH.

(**b**) *6M HCl-phenol solution.*—Weigh 1 g phenol crystals into tared 1000 mL beaker. Dissolve crystals in 500 mL H_2O. While stirring, slowly add 500 mL concentrated HCl.

(**c**) *HCl solutions.*—(*1*) *1M HCl.*—Pour ca 800 mL H_2O into 1000 mL volumetric flask, and then add 83.3 mL concentrated HCl, using pipet. Dilute to the mark with H_2O and mix thoroughly. (*2*) *0.1M HCl.*—Pour ca 800 mL H_2O into 1000 mL volumetric flask, and then add 100 mL 1M HCl, using pipet. Dilute to the mark with H_2O and mix thoroughly.

(**d**) *NaOH solution.*—To make 2M NaOH: Weigh 80.0 g NaOH in tared 1000 mL beaker. Slowly dissolve pellets in beaker in ca 600 mL H_2O. Cool solution and transfer quantitatively to 1000 mL volumetric flask. Dilute to mark with H_2O and mix thoroughly.

(**e**) *Norleucine standard solution.*—Accurately weigh 195-200 mg DL-norleucine crystals into tared 150 mL Erlenmeyer flask. Dissolve crystals with 100 mL 1M HCl. Transfer solution quantitatively into 1000 mL volumetric flask and dilute to mark with H_2O.

E. Hydrolysis

Finely grind test sample to pass 0.25 mm sieve. Accurately weigh ca 100-1000 mg test portions to the nearest 0.1 mg (equivalent to ca 10 mg nitrogen content) into labeled digestion tubes.

Calculate approximate amount of test portion to use as follows:

$$W_s =$$

where N_s = nitrogen content of test portion, %; W_s = weight of test portion equivalent to 10 mg nitrogen content, mg.

Add 50 mL 6M HCl-phenol solution to test portion and briefly stir. Add 2-3 pieces of boiling chips to sample solution.

Hydrolyze under reflux for 24 h at 110°-120°C using digestion block, **B**(**f**). (*Caution:* Perform this step inside fume hood with adequate ventilation.)

Remove digestion tubes from heat and cool to room temperature. Add 20 mL norleucine standard solution, **D**(**e**), to each test solution using volumetric pipet. Mix solutions by swirling flasks.

Filter hydrolysates through sintered glass filter into labeled 1000 mL round-bottom evaporating flasks. Connect flasks to rotary evaporators, and evaporate at 60°C to dryness. Wash by adding ca 20 mL H_2O and evaporate again. Repeat washing and evaporating steps 2 .

Remove flasks from evaporator. Add 50 mL sodium citrate buffer, **D**(**a**), to evaporated hydrolysate, mix well, and transfer into labeled 50 mL polyethylene bottle, **B**(**d**). Proceed to **F**, or freeze until ready for measurement.

F. Determination

Dilute aliquot of evaporated hydrolysate with sodium citrate buffer, **D**(**a**), and adjust pH to 2.20 with 2M NaOH. When neutralized hydrolysates are used, dilute aliquot with H_2O. Filter through filter unit, **B**(**g**), into autosampler tube and inject into analyzer. (*Note:* Volume of aliquot and dilution depends on response of analyzer.)

Calibrate amino acid analyzer with amino acid standard kit solution, **C**(**h**), containing norleucine. Operate analyzer according to manufacturer's specifications. Adjust analyzer conditions to ensure baseline separation of peaks. Minimum resolution between two peaks should be 90%.

G. Calculations

Proceed as in Performic Acid Oxidation with Acid Hydrolysis-Sodium Metabisulfite Method **H**.

Reference:
J. AOAC Int. **77**, 1362(1994).

Revised: March 1998

Table 994.12A: Results of interlaboratory study for determination of amino acids in broiler finisher feed by sodium metabisulfite method

Table 994.12B: Results of interlaboratory study for determination of amino acids in broiler starter feed by sodium metabisulfite method

Table 994.12C: Results of interlaboratory study for determination of amino acids in corn by sodium metabisulfite method