Kjeldahl Standard Operating Procedure

Specific references to Labconco equipment are made in this SOP. As the SOP was not provided by Labconco, those references remain unchanged. The method may be adapted to other similar equipment without difficulty.

Background information for the Kjeldahl procedure.

Required Equipment

- • Micro digestion apparatus. Labconco 25 place block digestor; 25 digestion tubes of 250 mL capacity.
- • Appropriate ventilation system to rid noxious fumes of digestion. The system of choice is a high capacity water vacuum aspirator.
- • Analytical balance; accurate to 0.0001 g.
- • Distillation system. Labconco Rapid Still II; capable of addition of 50% NaOH to neutralize digestate and production of 100 mL distillate in approximately 8 minutes.
- • Appropriate safety equipment including: cotton lab coat; safety glasses or goggles; appropriate device for handling hot (> 100 degrees C) digestion tubes (large flask tongs or a "hot hands" holder is highly recommended.)
- • 250 mL Erlenmeyer flasks. One is required for each sample or standard to be analyzed. Minimum of 25 recommended.
- • An electrical stirrer and stir bars are recommended, but not required.
- • 25 mL manual burette, or appropriate auto titration system.

Required Chemicals and Supplies

- • Low nitrogen weighing paper. Approximately 2 x 2 inch or larger.
- • Glass, porcelain or aluminum oxide boiling stones.
- • Catalyst (CuSO4) / acid / potassium sulfate solution. Ricca 2551 or equivalent.
- • Concentrated sulfuric acid.
- • 50% aqueous NaOH.
- • Receiver solution; 4% aqueous boric acid with bromocresol green/methyl red mixed indicator. Ricca 1070 or equivalent.
- • 0.1N HCl or 0.1N H2SO4. Ricca 3600, 8250 or equivalent.
- • Distilled or de-ionized water.
- • Assorted measuring pipettes; 20 mL, 30mL, 50 mL, 100 mL. Appropriate bottle-top dispenser or other accurate measuring device may be substituted without prejudice.
- • Micro spatula(s).

Method

The method will be separated into three categories; Digestion (conversion of nitrogen in the sample to ammonia), Distillation (separation of the ammonia from the digestate and collection for analysis), and Titration (quantification of the ammonia and calculation of the initial protein concentration).

Digestion

1. 1. Prepare sufficient 250 mL digestion tubes for the number of samples, standards and blanks to be analyzed. The tubes should be clean and rinsed with distilled or deionized water in order to eliminate contamination from previously run samples or foreign material.
2. 2. Place a single sheet of weighing paper on the analytical balance and tare the balance. Using a micro spatula, transfer 1.0000 g of sample to be analyzed to the weighing paper. The acceptable range of sample weight is 0.9995 to 1.0000g. Sample should be homogenous. Samples that may not be homogenous in their native state should be thoroughly ground and mixed prior to being analyzed. For liquid or semi-solid samples, the 250 mL digestion tube may be tared on the balance and the sample directly weighed into the tube. **Note 1 at conclusion of Digestion.
3. 3. Carefully fold the weighing paper, avoiding spilling the weighed sample. Transfer the paper to a clean 250 mL digestion tube. Label the tube with the sample identification number using an indelible marker.
4. 4. Add four to five boiling stones or glass or ceramic chips to the digestion tube. Continue until all samples to be analyzed are completed.
5. 5. Prepare one blank for each 10 samples to be analyzed. The blank preparation is accomplished by folding and placing a clean weighing paper into a 250 mL digestion tube and labeling the tube appropriately.
6. 6. Add 30 mL of catalyst solution to each tube; sample, standard or blank. Carefully "aim" the tube away from the analyst or other present in the laboratory as the reaction with some analytes can be violent and result in the sample / catalyst mixture erupting from the tube. Add the solution slowly to minimize the likelihood of violent reaction.
7. 7. Add 20 mL of concentrated sulfuric acid to each tube. The same precautions should be taken with the addition of acid. Significant heat will be formed in the addition of concentrated sulfuric acid to an aqueous solution and care should be taken to add the acid slowly to minimize boiling or violent reaction.
8. 8. Continue steps 6 and 7 until all samples, standards and blanks have been prepared.
9. 9. Transfer all samples simultaneously, using the carrier provided in the Labconco Digestion system, to the distillation unit.
10. 10. When the samples are in place, turn the vacuum aspirator on its highest flow. Place the hood device over the top of the tubes to be digested. Do not place empty tubes in places of the block where no samples are to be analyzed.
11. 11. Turn the temperature dial on the digestion to approximately 300 degrees C.
12. 12. Significant fuming will occur early in the digestion process. A second ventilator such as a fume hood is recommended to eliminate the possibility of exposing the analysts to noxious fumes. Allow the reaction to occur for 15 to 20 minutes. At that time, increase the temperature setting to 350 degrees C.
13. 13. Allow the reaction to continue for at least 15 additional minutes. If the fuming is still vigorous, continue an additional 15 for a total of 30 minutes at the higher temperature setting and full vacuum.
14. 14. The total elapsed time will be a maximum of 45 minutes at this point. Lower the vacuum to approximately ½ the original water flow. Remove the sides from the Labconco tube support to allow air flow across the tubes. The goal at this point is to achieve a reflux of acid vapor in the tube. A "cloud" of acid vapor should be visible approximately 2/3 of the total height of the tube.

15. 15. Allow the reflux to continue until the digestates are clear. Some adjustment of the temperature and vacuum flow may be needed to assure complete reaction without evaporating the sample(s) to dryness. Total digestion time should not exceed 1 ¾ hours.
16. 16. When the digestion is complete, remove the samples from the digestor and set aside to cool. Samples may begin to form crystals as they cool. A solid cake should not be allowed to form. If crystals begin to form, proceed quickly to the next step. If crystals do not form, samples may be safely stored overnight in this form.
17. 17. Carefully add 50 mL of distilled or deionized water to each sample. Again, the addition of water to a concentrated sulfuric acid solution will cause significant heat, and the sample(s) may boil. Carefully aim the opening of the digestion tube away from the analyst and anyone in the laboratory in order to assure their safety. Continue until all samples are diluted.

** Note 1. Some materials being digested will react very vigorously in the beginning or will form large amounts of black particles on the sides of the digestion tube, which will not disappear during the digestion. The cause may be the sample matrix, or the high concentration of protein in the sample. The result will be poor reproducibility in the calculated results. In either case, the problem will likely be eliminated by reducing the sample weight by one-half to 0.4997 to 0.5003 grams.

Distillation

1. 1. Turn on the power to the Rapid Still II. Confirm that it is connected to a tap water source, and that the water is flowing vigorously.
2. 2. Visually inspect the 1 L water supply to the steam source and verify that it is filled to the indicator mark with tap water. If it is not, press the fill button on the front of the Rapid Still II until the fill level is reached. When the reservoir is full, turn on the power to the steam source.
3. 3. Verify that the 50% NaOH supply is attached to the Rapid Still II, and that there is sufficient solution in the supply to run the samples to be analyzed. It will require approximately 50 mL of NaOH per digested sample.
4. 4. Add 100 mL of indicating boric acid solution to a sufficient number of 250 mL Erlenmeyer flasks to distill each sample, standard or blank that has been digested. The graduated markings on the side of the flasks are sufficiently accurate for this purpose.
5. 5. Place a filled 250 mL Erlenmeyer flask on the receiving station of the Rapid Still II. Verify that the ball of the receiving tube is below the surface of the receiving solution.
6. 6. Carefully place the first sample to be distilled on the Rapid Still II. Turn the digestion tube ¼ to ½ turn while pressing upward toward the rubber seat to assure a vapor tight fit of the digestion tube to the still.
7. 7. Verify that the power is on, the steam source is boiling, and the distillation timer is set to 0 minutes.
8. 8. Using the volume indicator on the back of the Rapid Still II, estimate the volume of liquid in the digestion tube. Within 10 mL is sufficiently accurate for the estimate.
9. 9. “Tap” the NaOH addition switch on the front of the still to add NaOH in small increments. Violent reaction will occur, which is minimized by slow addition. After a few mLs are added, the solution may begin to turn dark and cloudy. This is normal and will not affect the analysis. Continue addition until 50 mL of NaOH have been added, or until the addition of continued amounts of the solution does not result in a vigorous reaction within the vessel, whichever comes

later. The absence of vigorous reaction is confirmation that the acid solution has been neutralized. During the NaOH addition process, significant bubbling may occur in the receiver vessel, and the solution may change colors from red/pink to gray, blue or green. This is due the emission of NH3 by the sample and is normal.

10. 10. When sufficient NaOH has been added, set the timer to 8 minutes and begin distillation. The goal is to distill 100 mL of liquid to the receiving flask, which will reach a total volume of 200 mL. Determine experimentally the correct time for 100 mL distillate and use this time on future analyses. (6 to 7 minutes is normal for the Rapid Still II.)
11. 11. When the distillation is complete, turn the power off to the steam generator. Slowly add sufficient water to the generator to bring it back to the fill mark.
12. 12. Remove the 250 mL Erlenmeyer (containing 200 mL of solution). Rinse the glass bulb with a small amount of distilled water, collecting it in the Erlenmeyer to clean the tube and avoid contamination of the following sample(s). Set the sample aside for titration.
13. 13. Using an appropriate device for handling very hot glassware, remove the digestion tube from the holder. The dark solution may be disposed of by pouring it down a drain with copious amounts of water. **Note 2 at the end of the Distillation section.
14. 14. Wipe the white tube that goes into the center of the digestion tube during distillation with a paper towel or similar material to remove any possible contamination for the next sample.
15. 15. Return to step 5 and repeat steps 5 through 14 for all samples to be analyzed.
16. 16. When all samples to be analyzed are distilled, place a sample that contains only 50 mL of distilled or deionized water in a 250 mL digestion tube on the sample stand.
17. 17. Add approximately 20 mL of NaOH and distill for 6 minutes into an Erlenmeyer flask. It is not necessary to have receiver solution in the flask. 100 mL of tap or distilled water is sufficient.
18. 18. When the blank sample is distilled, remove the digestion tube. Clean the rubber seal with a clean damp cloth. Apply a very thin film of stopcock grease or similar lubricant to the rubber seal. Place a clean, dry digestion tube in position. Remove the Erlenmeyer and rinse the round receiver tube with a small amount of distilled water. Turn off the power to the steam source. Refill the steam source with water, then turn off the power to the system. Turn off the water supply.

** Note 2. The drain disposal assumes use of a Cu, Ti or similar catalyst. Use of a mercury (Hg) catalyst requires proper disposal per local environmental regulations. Mercury catalyst should NOT be poured down a drain that empties into a public waste system.

Titration

(This method assumes manual titration using a 25 mL burette.)
The receiver solution was originally a bright pink to red color. The titration will be to a moderate pink. Do not try to duplicate the original color, as this would significantly over titrate the sample(s). The normal progression of color is from green (for a sample containing nitrogen. The blanks will in some cases not change color) to a deep gray with pink overtones, to a light pink. The first “pure” pink color with no hint of gray should be taken as the endpoint.

1. 1. If a magnetic stirrer is to be used, add a stir bar to the sample. Otherwise, hold the sample and swirl it in a circular motion throughout the titration to assure rapid mixing of the acid with the sample.

2. 2. Fill the burette to the 0 mark or record the initial reading on the burette.
3. 3. Add titrant slowly to the sample. An effective technique is to adjust the burette so that a steady, slow drip of titrant occurs. Stir or swirl continuously. As titrant is added, a pink color will be seen where the titrant enters the solution. As the endpoint nears, this colored area will increase in size, and persist for longer periods before being dispersed into the solution. That is an indicator to slow the addition of acid.
4. 4. Continue to add titrant slowly, dropwise. A gray solution with a pink tint throughout will develop. The addition of a few more drops of titrant should result in a pink solution without the gray color. Record the mL of titrant used.
5. 5. Repeat steps 2 through 5 until all blanks, standards and samples are titrated.
6. 6. For automatic titration, refer to the operator's manual of the auto titrator for setup instructions.

Calculation of Results

The general equation for the protein content is:

[(Vb – Vs)(N)(1.4007) / (W)] * F = percent protein

Vb = mL titrant for the blank(s)
Vs = mL titrant for the individual samples
N = Normality of the acid titrant (nominally 0.1)
1.4007 = a single factor that takes into account the molecular weight of nitrogen, the conversion of the milli-equivalent result of V*N, and the conversion to %.
W = the weight of sample in grams. The error is sufficiently small that, for samples weighed to 1.0000g +/- 0.0005 G, this can be assumed to be 1.

F = the factor for converting the percent nitrogen in a sample to percent protein. This factor varies based on sample type and can vary greatly. (From approximately 5.2 for some nuts, to 6.7 for some meat products.) The regulating body for the analysis performed will usually provide the factor. If not, refer to the appropriate method, and use the factor located in that method.

The method can be validated by digesting organic materials of known nitrogen content, for example, aniline. By performing the above calculation and assuming a factor of 1, the correct percent nitrogen should be determined. The correct nitrogen percentage for aniline is 15.05%. A smaller sample of aniline should be analyzed due to the high nitrogen content. 100 mg (0.1000g) is recommended.

Technical information provided by Kevin Lackey.

Background for the Kjeldahl determination of Organic Nitrogen

The Kjeldahl method is an analytical method for the determination of nitrogen in the trinegative state in certain organic compounds. The method was developed in 1883 by Johan Kjeldahl, a Danish chemist, and is used extensively in the determination of protein in foods, since protein is a macromolecule made up of nitrogen containing amino acids linked together. When used for protein determination, the percent nitrogen measured is converted to the equivalent protein content by use of an appropriate numerical factor. For meat samples, this factor is 6.25 since meat protein is approximately 16%nitrogen. The Kjeldahl method does not account for N-N and N-O linkages (e.g., azides, nitrates, nitrites, nitro groups, etc.). Such samples must be pre-treated or subjected to reducing conditions before Kjeldahl analysis. The amino nitrogen in the sample is converted to ammonium bisulfate as the organic material in the sample is destroyed by digestion with boiling, concentrated sulfuric acid. Potassium sulfate is added to raise the boiling point of the mixture, thus speeding the decomposition. The amounts of sulfuric acid and potassium sulfate used must be controlled, depending on the amount of organic material present in the sample, to ensure that a proper digestion temperature range of 370 - 400oC. is maintained. Too low a temperature may lead to long digestion times and/or incomplete digestion, while too high a ratio of potassium sulfate to sulfuric acid may raise the temperature above 400oC., thus resulting in pyrolytic loss of nitrogen and low results. A metal catalyst is also added to accelerate the digestion. Mercury has been the most common catalyst used, but copper and selenium are being used more now, for safety and environmental reasons.

After digestion of the sample is complete, excess sodium hydroxide is added to the digestion mixture to neutralize the remaining sulfuric acid and release the ammonia formed as the nitrogen containing molecule was oxidized. The ammonia is then distilled over into a measured excess of a standard acid, and the excess acid is backtitrated with a standard base. As an alternative, the ammonia can be distilled over into a boric acid solution, and the ammonia is then determined by direct titration with a standard acid. The ammonia can also be determined colorimetrically (e.g., via reaction with phenate ion) or by using an ammonia selective electrode. In these cases, a standard mineral acid should be used as the ammonia absorbent. If mercury or a mercuric salt is used as the catalyst, thiosulfate or sulfide is added with the sodium hydroxide to decompose any mercuric-ammonium complex, thus releasing the ammonia and precipitating the mercuric compound which may interfere with the distillation of the ammonia. Zinc granules, pumice or other suitable boiling stones are added to the distillation flask to prevent "bumping" which may lead to erroneous results due to carry-over of some of the caustic in addition to the ammonia. The reactions involved are summarized as follows:

Sample Digestion
Organic N + H_2SO_4 + Heat + Catalyst => CO_2 + H_2O + NH_4HSO_4

Neutralization of Digestion Mixture and Release of Ammonia
NH_4HSO_4 + 2NaOH => NH_3 + Na_2SO_4 + H_2O

Direct Titration of Ammonia
NH_3 + HCl [or H_2SO_4] => NH_4Cl [or $(NH_4)_2SO_4$]

Back Titration of Standardized Acid
$NaOH + HCl [or H_2SO_4] \Rightarrow NaCl [or Na_2SO_4] + H_2O$

If the ammonia, after absorption in boric acid solution, is titrated directly with a standardized acid, methyl red, methyl purple, or bromocresol green - methyl red mixed indicator can be used as the indicator. These indicators can also be used in a back-titration using standard sodium hydroxide, if an excess standard acid is used as the absorbent for the ammonia. Alternatively, the ammonia may quantitated by ion selective electrode (ISE).

For a discussion of the use of the Ammonia ISE, see the appropriate Technical Service note in this webpage.