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Chapter

Protein Nitrogen Determination

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13.1 INTRODUCTION

13.1.1 Background

The protein content of foods can be determined by numerous methods. The Kjeldahl method and the nitrogen combustion (Dumas) method for protein analysis are based on nitrogen determination. Both methods are official for the purposes of nutrition labeling of foods. While the Kjeldahl method has been used widely for over 100 years, the recent availability of automated instrumentation for the Dumas method in many cases is replacing use of the Kjeldahl method.

13.1.2 Reading Assignment

Chang, S.K.C., Zhang, Y. 2017. Protein analysis. Ch. 18, in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

13.1.3 Notes

Both the Kjeldahl and nitrogen combustion methods can be done without automated instrumentation, but they are commonly done with automated instruments. The descriptions below are based on the availability of such automated instrumentation. If protein content of samples analyzed by Kjeldahl and/or nitrogen combustion has been estimated in a previous experiment by near infrared analysis, values can be compared between methods.

13.2 KJELDAHL NITROGEN METHOD

13.2.1 Objective

Determine the protein content of corn flour using the Kjeldahl method.

13.2.2 Principle of Method

The Kjeldahl procedure measures the nitrogen content of a sample. The protein content then can be calculated assuming a ratio of protein to nitrogen for the specific food being analyzed. The Kjeldahl procedure can be basically divided into three parts: (1) digestion, (2) distillation, and (3) titration. In the digestion step, organic nitrogen is converted to an ammonium in the presence of a catalyst at approximately 370 °C. In the distillation step, the digested sample is made alkaline with NaOH, and the nitrogen is distilled off as NH_3. This NH_3 is "trapped" in a boric acid solution. The amount of ammonia nitrogen in this solution is quantified by titration with a standard HCl solution.

A reagent blank is carried through the analysis and the volume of HCl titrant required for this blank is subtracted from each determination.

13.2.3 Chemicals

	CAS No.	*Hazards*
Boric acid (H_3BO_3)	10043-35-3	
Bromocresol green	76-60-8	
Ethanol, 95%	64-17-5	Highly flammable
Hydrochloric acid, conc. (HCl)	7647-01-0	Corrosive
Methyl red	493-52-7	
Sodium hydroxide (NaOH)	1310-73-2	Corrosive
Sulfuric acid, conc. (H_2SO_4)	7664-93-9	Corrosive
Kjeldahl digestion tablets		Irritant
Potassium sulfate (K_2SO_4)	7778-80-5	
Cupric sulfate	7758-98-7	
Titanium dioxide (TiO_2)	13463-67-7	
Tris (hydroxymethyl) aminomethane (THAM)	77-86-1	Irritant

13.2.4 Reagents

(** It is recommended that these solutions be prepared by the laboratory assistant before class.)

- Sulfuric acid (concentrated, N-free)
- Catalyst/salt mixture (Kjeldahl digestion tablets)

  Contains potassium sulfate, cupric sulfate, and titanium dioxide

  Note: There are several types of Kjeldahl digestion tablets that contain somewhat different chemicals.
- Sodium hydroxide solution, 50%, w/v, NaOH in deionized distilled (dd) water **

  Dissolve 2000 g sodium hydroxide (NaOH) pellets in ~3.5 L dd water. Cool. Add dd water to make up to 4.0 L.
- Boric acid solution **

  In a 4-L flask, dissolve 160-g boric acid in ca. 2 L boiled, and still very hot, dd water. Mix and then add an additional 1.5 L of boiled, hot dd water. Cool to room temperature under tap water (caution: glassware may break due to sudden cooling) or leave overnight. When using the rapid procedure, the flask must be shaken occasionally to prevent recrystallization of the boric acid. Add 40 mL of bromocresol green solution (100-mg bromocresol green/100-mL ethanol) and 28 mL of methyl red solution (100-mg methyl red/100-mL ethanol). Dilute to 4 L of water and mix carefully. Transfer 25 mL of the boric acid solution to a receiver flask and distill a digested blank (a digested catalyst/salt/acid mixture). The contents of the flask should then be a neutral gray. If not, titrate with 0.1 *N* NaOH solution until this color is obtained. Calculate the amount of NaOH solution necessary to adjust the boric acid solution in the 4-L flask with the formula:

$$\text{mL } 0.1\,N \text{ NaOH} = \frac{(\text{mL titer}) \times (4000 \text{ mL})}{(25 \text{ mL})}$$

Add the calculated amount of 0.1 *N* NaOH solution to the boric acid solution. Mix well. Verify the adjustment results by distilling a new blank sample. Place adjusted solution into a bottle equipped with a 50-mL repipettor.

- Standardized HCl solution**

  Dilute 3.33-mL conc. HCl to 4 L with dd water. Empty old HCl solution from the titrator reservoir and rinse three times with a small portion of the new HCl solution. Fill the titrator with the new HCl solution to be standardized. Using a volumetric pipet, dispense 10-mL aliquots of the THAM solution prepared as described below into three Erlenmeyer flasks (50 mL). Add 3–5 drops indicator (3 parts 0.1 % bromocresol green in ethanol to 1 part of 0.2 % methyl red in ethanol) to each flask and swirl. Titrate each solution with the HCl solution to a light pink endpoint. Note the acid volume used and calculate the normality as described below.

  Calculation to standardize HCl solution:

$$\text{Normality} = \frac{\text{mL THAM} \times \text{THAM Normality}}{\text{average acid volume (AAV)}}$$
$$= \frac{20 \text{ mL} \times 0.01\,N}{\text{AAV}}$$

  Write the normality of the standardized HCl solution on the stock container.

- Tris (hydroxymethyl) aminomethane (THAM) Solution – (0.01 *N*) **

  Place 2 g of THAM in a crucible. Leave in a drying oven (95 °C) overnight. Let cool in a desiccator. In a 1-L volumetric flask, dissolve 1.2114 g of oven dried THAM in distilled water. Dilute to volume.

13.2.5 Hazards, Cautions, and Waste Disposal

Concentrated sulfuric acid is extremely corrosive; avoid breathing vapors and contact with skin and clothes. Concentrated sodium hydroxide is a corrosive. Wear corrosion-resistant gloves and safety glasses at all times. Perform the digestions in an operating hood with an aspirating fume trap attached to the digestion unit. Allow samples to cool in the hood before removing the aspirating fume trap from the digestion unit. Otherwise, adhere to normal laboratory safety procedures. The waste of combined sulfuric acid and sodium hydroxide has been largely neutralized (check pH to ensure it is pH 3–9), so it can be discarded down the drain with a water rinse. However, for disposing any chemical wastes, follow good laboratory practices outlined by environmental health and safety protocols at your institution.

For safety and accuracy reasons, dispense the concentrated sulfuric acid from a bottle fitted with a repipettor (i.e., automatic dispenser). Fit the dispenser with a thin, semirigid tube to dispense directly into the Kjeldahl tube. Set the bottle with dispenser on a tray to collect spills.

13.2.6 Supplies

(Used by students)

- Corn flour (not dried)
- 5 Digestion tubes
- 5 Erlenmeyer flasks, 250 mL
- Spatula
- Weighing paper

13.2.7 Equipment

- Analytical balance
- Automatic titrator
- Kjeldahl digestion and distillation system

13.2.8 Procedure

(Instructions are given for analysis in triplicate. Follow manufacturer's instructions for specific Kjeldahl digestion and distillation system used. Some instructions given here may be specific for one type of Kjeldahl system.)

13.2.8.1 *Digestion*

1. Turn on digestion block and heat to appropriate temperature.
2. Accurately weigh approximately 0.1 g of corn flour. Record the weight. Place corn flour in digestion tube. Repeat for two more samples.
3. Add one catalyst tablet and appropriate volume (e.g., 7 mL) of concentrated sulfuric acid to each tube with corn flour. Prepare duplicate blanks: one catalyst tablet + volume of sulfuric acid used in the sample + weigh paper (if weigh paper was added with the corn flour samples).
4. Place rack of digestion tubes on digestion block. Cover digestion block with exhaust system turned on.
5. Let samples digest until digestion is complete. The samples should be clear (but neon green), with no charred material remaining.
6. Take samples off the digestion block and allow to cool with the exhaust system still turned on.
7. Carefully dilute digest with an appropriate volume of dd water. Swirl each tube.

13.2.8.2 *Distillation*

1. Follow appropriate procedure to start up distillation system.
2. Dispense appropriate volume of boric acid solution into the receiving flask. Place receiving flask on distillation system. Make sure the tube coming from the distillation of the sample is submerged in the boric acid solution.
3. Put sample tube from Sect. 13.2.8.1 in place, making sure it is seated securely, and proceed with the distillation until completed. In this distillation process, a set volume of NaOH solution will be delivered to the tube and, a steam generator will distill the sample for a set period of time.
4. Upon completing distillation of one sample, proceed with a new sample tube and receiving flask.
5. After completing distillation of all samples, follow manufacturer's instructions to shut down the distillation unit.

13.2.8.3 *Titration*

1. Record the normality of the standardized HCl solution, as determined by the teaching assistant.
2. If using an automated pH meter titration system, follow manufacturer's instructions to calibrate the instrument. Put a magnetic stir bar in the receiver flask from Sect. 13.2.8.2 and place it on a stir plate. Keep the solution stirring briskly while titrating, but do not let the stir bar hit the electrode. Titrate each sample and blank to an endpoint pH of 4.2. Record volume of HCl titrant used.
3. If using a colorimetric endpoint, put a magnetic stir bar in the receiver flask, place it on a stir plate, and keep the solution stirring briskly while titrating. Titrate each sample and blank with the standardized HCl solution to the first faint gray color. Record volume of HCl titrant used.

13.2.9 Data and Calculations

Calculate the percent nitrogen and the percent protein for each of your duplicate or triplicate corn flour samples, then determine average values. The corn flour sample you analyzed was not a dried sample. Report percent protein results on a wet weight basis (wwb) and on a dry weight basis (dwb). Assume a moisture content of 10 % (or use the actual moisture content if previously determined on this corn flour sample). Use 6.25 for the nitrogen to protein conversion factor.

$$\%\,\mathrm{N} = \text{Normality HCl}^{*} \times \frac{\begin{array}{c}\text{corrected acid}\\ \text{vol.(mL)}^{**}\end{array}}{\text{g of sample}} \times \frac{14\,\mathrm{g\,N}}{\mathrm{mol}} \times 100$$

* Normality is in mol / 1000 mL.
** Corrected acid vol = (mL std. acid for sample) – (mL std. for blank)

$$\%\,\text{Protein} = \%\,\mathrm{N} \times \text{Protein Factor}$$

$$\frac{\%\text{ Protein, wwb}}{[\%\text{ Solids (wet)}/100\,\%]} = \%\text{ Protein, dwb}$$

	Rep	*Sample wt. (g)*	*Vol. HCl titrant (mL)*	*% Nitrogen*	*% Protein, wwb*	*% Protein, dwb*
Blank	1	–		–	–	–
	2	–		–	–	–
			$\bar{X}$ =			
Sample	1					
	2					
	3					
					$\bar{X}$ =	$\bar{X}$ =
					SD =	SD =

13.2.10 Questions

1. If the alkali pump timer on the distillation system was set to deliver 25 mL of 50 % NaOH and 7 mL of concentrated H_2SO_4 was used to digest the sample, how many milliliters of the 50 % NaOH is actually required to neutralize the amount of sulfuric acid used in the digestion? How would your results have been changed if the alkali pump timer malfunctioned and delivered only 15 mL of the 50 % NaOH? (Molarity of conc. $H_2SO_4 = 18$.)
2. Could phenolphthalein be used as an indicator in the Kjeldahl titration? Why or why not?
3. Describe the function of the following chemicals used in this determination:
   - (a) Catalyst pellet
   - (b) Borate
   - (c) H_2SO_4
   - (d) NaOH
4. Why was it not necessary to standardize the boric acid solution?
5. Explain how the factor used to calculate the percent protein for your product was obtained, and why the protein factors for some other cereal grains (e.g., wheat, oats) differ from that for corn.
6. For each of the disadvantages of the Kjeldahl method, give another protein analysis method that overcomes (at least partially) that disadvantage.

13.3 NITROGEN COMBUSTION METHOD

13.3.1 Objective

Determine the protein content of corn flour using the nitrogen combustion method.

13.3.2 Principle of Method

The nitrogen combustion method measures the nitrogen content of a sample. The protein content then is calculated assuming a ratio of protein to nitrogen for the specific food being analyzed. In the assay, the sample is combusted at a high temperature (900–1110 °C) to release nitrogen gas and other products (i.e., water, other gases). The other products are removed, and the nitrogen is quantitated by gas chromatography using a thermal conductivity detector.

13.3.3 Chemicals

	CAS No.	*Hazards*
Ethylenediaminetetraacetic acid (EDTA), disodium salt ($Na_2EDTA \cdot 2H_2O$)	60-00-4	Irritant

(The other chemicals used are specific to each manufacturer for the columns within the instrument.)

13.3.4 Hazards, Cautions, and Waste Disposal

During operation, the front panel of the instrument gets very hot. Check instructions of manufacturer for any other hazards, especially those associated with maintenance of instrument.

13.3.5 Supplies

(Used by students)

Corn flour
Sample cup

13.3.6 Equipment

Nitrogen combustion unit

13.3.7 Procedure

Follow manufacturer's instructions for start-up, analyzing samples, and shutdown.

Weigh appropriate amount of sample into a tared sample cup on an analytical balance. (Sample weight will be coordinated with sample number in autosampler, if autosampler is used.) Remove sample from balance and prepare for insertion following manufacturer's instructions. If an autosampler is used, the weighed sample must be placed into autosampler in the appropriate slot for the sample number. Repeat this procedure for EDTA standard. Sample and standard should be run in duplicate or triplicate.

13.3.8 Data and Calculations

Record the percent nitrogen content for each of your duplicate or triplicate corn flour samples. Calculate protein content from percent nitrogen data, and determine average percent protein. The corn flour sample you analyzed was not a dried sample. Report percent protein results on a wet weight basis (wwb) and on a dry weight basis (dwb). Assume a moisture content of 10 % (or use the actual moisture content if previously determined on this corn flour sample). Use 6.25 for the nitrogen to protein conversion factor.

Sample	*% Nitrogen*	*% Protein, wwb*	*% Protein, dwb*
1			
2			
3			
		$\bar{X}$ =	$\bar{X}$ =
		SD =	SD =

13.3.9 Questions

1. What are the advantages of the nitrogen combustion method compared to the Kjeldahl method?
2. Explain why ethylenediaminetetraacetic acid (EDTA) can be used as a standard to check the calibration of the nitrogen analyzer.
3. If you analyzed the corn flour sample by both the Kjeldahl and nitrogen combustion methods, compare the results. What might explain any differences?

RESOURCE MATERIALS

Chang SKC, Zhang Y (2017) Protein analysis. Ch. 18. In: Nielsen SS (ed) Food analysis, 5th edn. Springer, New York

AOAC International (2016) Official methods of analysis, 20th edn, (On-line). Method 960.52 (Micro-Kjeldahl method) and Method 992.23 (Generic combustion method). AOAC International, Rockville, MD