18 chapter

Protein Analysis

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S. Nielsen (ed.), *Food Analysis*, Food Science Text Series,
DOI 10.1007/978-3-319-45776-5_18,

18.1 INTRODUCTION

18.1.1 Classification and General Considerations

Proteins are an abundant component in all cells, and almost all except storage proteins are important for biological functions and cell structure. Food proteins are very complex. Many have been purified and characterized. Proteins vary in molecular mass, ranging from approximately 5,000 to more than a million Daltons. They are composed of elements including hydrogen, carbon, nitrogen, oxygen, and sulfur. Twenty α-amino acids are the building blocks of proteins; the amino acid residues in a protein are linked by peptide bonds. Nitrogen is the most distinguishing element present in proteins. However, nitrogen content in various food proteins ranges from 13.4 to 19.1% [1] due to the variation in the specific amino acid composition of proteins. Generally, proteins rich in basic amino acids contain more nitrogen.

Proteins can be classified by their composition, structure, biological function, or solubility properties. For example, simple proteins contain only amino acids upon hydrolysis, but conjugated proteins also contain non-amino acid components. Proteins have unique conformations that could be altered by denaturants such as heat, acid, alkali, 8 *M* urea, 6 *M* guanidine-HCl, organic solvents, and detergents. The solubility as well as functional properties of proteins could be altered by denaturants. The analysis of proteins is complicated by the fact that some food components possess similar physicochemical properties. Nonprotein nitrogen could come from free amino acids, small peptides, nucleic acids, phospholipids, amino sugars, porphyrin, and some vitamins, alkaloids, uric acid, urea, and ammonium ions. Therefore, the total organic nitrogen in foods would represent nitrogen primarily from proteins and to a lesser extent from all organic nitrogen-containing nonprotein substances. Depending upon methodology, other major food components, including lipids and carbohydrates, may interfere physically with analysis of food proteins.

Numerous methods have been developed to measure protein content. The basic principles of these methods include the determinations of nitrogen, peptide bonds, aromatic amino acids, dye-binding capacity, ultraviolet absorptivity of proteins, and light scattering properties. In addition to factors such as sensitivity, accuracy, precision, speed, and cost of analysis, what is actually being measured must be considered in the selection of an appropriate method for a particular application.

18.1.2 Importance of Analysis

Protein analysis is important for:

1. **Nutrition labeling**
2. **Pricing:** The cost of certain commodities is based on the protein content as measured by nitrogen content (e.g., cereal grains, milk for making certain dairy products, e.g., cheese).
3. **Functional property investigation**: Proteins in various types of food have unique food functional properties: for example, gliadin and glutenins in wheat flour for bread making, casein in milk for coagulation into cheese products, and egg albumen for foaming. (See Chap. 24, Sect. 24.3.3.)
4. **Biological activity determination:** Some proteins, including enzymes or enzyme inhibitors, are relevant to food science and nutrition: for instance, the proteolytic enzymes in the tenderization of meats, pectinases in the ripening of fruits, and trypsin inhibitors in legume seeds are proteins. To compare between samples, enzyme activity often is expressed in terms of specific activity, meaning units of enzyme activity per mg of protein.

Protein analysis is required when you want to know:

1. Total protein content
2. Content of a particular protein in a mixture
3. Protein content during isolation and purification of a protein
4. Nonprotein nitrogen
5. Amino acid composition (see Chap. 24, Sect. 24.3.1)
6. Nutritive value of a protein (see Chap. 24, Sect. 24.3.2)

18.1.3 Content in Foods

Protein content in food varies widely. Foods of animal origin and legumes are excellent sources of proteins. The protein contents of selected food items are listed in Table 18.1 [2].

18.1.4 Introduction to Methods

Principles, general procedures, and applications are described below for various protein determination methods. See Table 18.2 for a summary of methods described, including more details about their applications and AOAC numbers [3]. Advantages and disadvantages of methods are included in the summary table, rather than in the text. Please refer to the references cited within the text for detailed instructions of the procedures. Many of the methods covered in this chapter are described in somewhat more detail in recent books on food proteins [4–6]. The Kjeldahl, Dumas (N combustion), infrared spectroscopy, and anionic dye-binding methods described are from the *Official Methods of Analysis* of AOAC International [3] and are used commonly in nutrition labeling and/or quality control. The other methods described are used

18.1 table Protein content of selected foods

Food item	*Percent protein (wet weight basis)*
Cereals and pasta	
Rice, brown, long-grain, raw	7.9
Rice, white, long-grain, regular, raw, enriched	7.1
Wheat flour, whole-grain	13.7
Corn flour, whole-grain, yellow	6.9
Spaghetti, dry, enriched	13.0
Cornstarch	0.3
Dairy products	
Milk, reduced fat, fluid, 2%	3.2
Milk, nonfat, dry, regular, with added vit. A	36.2
Cheese, cheddar	24.9
Yogurt, plain, low fat	5.3
Fruits and vegetables	
Apple, raw, with skin	0.3
Asparagus, raw	2.2
Strawberries, raw	0.7
Lettuce, iceberg, raw	0.9
Potato, whole, flesh, and skin	2.0
Legumes	
Soybeans, mature seeds, raw	36.5
Beans, kidney, all types, mature seeds, raw	23.6
Tofu, raw, firm	15.8
Tofu, raw, regular	8.1
Meats, poultry, fish	
Beef, chuck, arm pot roast	21.4
Beef, cured, dried beef	31.1
Chicken, broilers or fryers, breast meat only, raw	23.1
Ham, sliced, regular	16.6
Egg, raw, whole, fresh	12.6
Finfish, cod, Pacific, raw	17.9
Finfish, tuna, white, canned in oil, drained solids	26.5

From the US Department of Agriculture, Agricultural Research Service [2]

commonly in research laboratories working on proteins.

18.2 NITROGEN-BASED METHODS

18.2.1 Kjeldahl Method

18.2.1.1 *Principle*

In the Kjeldahl procedure, proteins and other organic food components in a sample are digested with sulfuric acid in the presence of catalysts. The **total organic nitrogen** is converted to ammonium sulfate. The digest is neutralized with alkali and distilled into a boric acid solution. The borate anions formed are titrated with standardized acid, which is converted to nitrogen in the sample. The result of the analysis represents the crude protein content of the food since nitrogen also comes from nonprotein components (note that the Kjeldahl method also measures nitrogen in any ammonia and ammonium sulfate).

18.2.1.2 *Historical Background*

In 1883, Johann Kjeldahl developed the basic process of today's Kjeldahl method to analyze organic nitrogen. An excellent book to review the Kjeldahl method for total organic nitrogen was written by Bradstreet [7]. Several important modifications have improved the original Kjeldahl process, but the original method and the current procedure (as described in detail below) both include the same basic steps: (1) digestion, (2) neutralization and distillation, and (3) titration.

18.2.1.3 *General Procedures and Reactions*

18.2.1.3.1 Sample Preparation

Solid foods are ground to pass a 20-mesh screen. Samples for analysis should be homogeneous. No other special preparations are required.

18.2.1.3.2 Digestion

Place sample (accurately weighed) in a Kjeldahl flask. Add acid and catalyst; digest until clear to get complete breakdown of all organic matter. Nonvolatile ammonium sulfate is formed from the reaction of nitrogen and sulfuric acid.

$$\text{Protein} \xrightarrow[\text{Heat, catalyst}]{\text{Sulfuric acid}} (NH_4)_2\,SO_4 \qquad (18.1)$$

During digestion, protein nitrogen is liberated to form ammonium ions; sulfuric acid oxidizes organic matter and combines with ammonium formed; carbon and hydrogen elements are converted to carbon dioxide and water.

18.2.1.3.3 Neutralization and Distillation

The digest is diluted with water. Alkali-containing sodium thiosulfate is added to neutralize the sulfuric acid. The ammonia formed is distilled into a boric acid solution containing the indicators methylene blue and methyl red (AOAC Method 991.20).

$$(NH_4)_2\,SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O \qquad (18.2)$$

$$NH_3 + H_3BO_3\,(\text{boric acid}) \rightarrow NH_4 + H_2BO_3^-\,(\text{borate ion}) \qquad (18.3)$$

18.2 table Protein analysis method comparison

Method	*Chemical basis*	*Principle*	*Advantages*	*Disadvantages*	*Applications*
Kjeldahl	Nitrogen (total organic)	Determine N by method that involves digestion, neutralization, distillation, and titration. Use N content to calculate protein content	Inexpensive (if not automated system). Widely used and accepted method for over a century	Measures total organic N, and not just protein N. Time consuming. Uses corrosive reagents. Lower precision than some other methods	Applicable to all foods. Little used now, due to availability of automated Dumas systems
Dumas	Nitrogen (total organic and inorganic)	N is released upon combustion of sample at very high temperature. N gas is quantitated by gas chromatography using a thermal conductivity detector. Use N content to calculate protein content	Requires no hazardous chemicals. Rapid (few minutes). Automated instruments allow for analyzing many samples without attention	Expensive equipment. Measures total organic and inorganic N, and not just protein N	Applicable to all foods. Widely used now, compared to Kjeldahl method, for both official and quality control purposes
Infrared spectroscopy	Peptide bond	Presence of peptide bond in protein molecules causes absorption of radiation at specific wavelength in mid- or near infrared region	Rapid way to estimate protein content. Requires minimal training	Expensive equipment. Only provides an estimate of protein content. Instrument must be calibrated against results from official methods	Applicable to wide range of food products (grains, cereal, meat, dairy). Used as rapid, quality control method
Anionic dye-binding	Basic amino acid residues (of histidine, arginine, and lysine) and N-terminus of protein molecule	Residues identified react with anionic sulfonic acid dye to form an insoluble complex. Unbound soluble dye is measured by absorbance and related to protein concentration	Rapid (15 min. or less for non-automated method; much less for automated method). Relatively accurate. No corrosive reagents. Does not measure nonprotein N. More precise than Kjeldahl method. Can be used to estimate changes in available lysine content, since the dye does not bind altered, unavailable lysine	Not as sensitive as some other colorimetric methods. Requires a calibration curve for a given food commodity, since proteins differ in basic amino acid content so differ in dye-binding capacity. Not suitable for hydrolyzed proteins due to dye binding to N-terminal amino acids. Some nonprotein components bind dye or protein, to cause error	Automated version used for quality control purposes, especially as a method to compare results against a nitrogen-based method (to check for economic adulteration)

(continued)

18.2 table (Continued)

Method	*Chemical basis*	*Principle*	*Advantages*	*Disadvantages*	*Applications*
Bicinchoninic acid	Peptide bond and specific amino acids (cysteine, cystine, tryptophan, and tyrosine)	Peptide bond is complexed with cupric ions under alkaline conditions. Cuprous ions are chelated by BCA reagent to give color measured by spectroscopy	Good sensitivity, and micro-BCA method is even better (0.5–10 ug). Nonionic detergents and buffer salts do not interfere with the reaction, nor do medium concentrations of denaturing reagents	Color is not stable with time. Any compound capable of reducing Cu^{+2} to Cu^{+} will lead to color formation. Reducing sugars and high concentrations of ammonium sulfate interfere. Get color variation among proteins	Widely used method for protein isolation and purification. Has largely replaced other quantitative research colorimetric methods
Absorbance at 280 nm	Tyrosine and tryptophan	Aromatic amino acids, tryptophan and tyrosine, cause proteins to absorb at 280 nm. Absorbance can be used to estimate protein content	Rapid. Relatively sensitive (100 ug protein required). No interference from ammonium sulfate and other buffer salts. Nondestructive (so samples can be used after protein determination)	Nucleic acids can absorb at 280 nm. Aromatic amino acid contents in proteins vary between food sources, so results are qualitative. Requires relatively pure, clear, and colorless samples	Best used in purified protein systems (e.g., postcolumn detection of intact proteins)
Absorbance at 220 nm	Peptide bond	Peptide bonds cause proteins to absorb at 220 nm. Absorbance can be used to estimate protein content	Rapid. Nondestructive (so samples can be used after protein determination)	Many things other than peptide bonds absorb at 220 nm. Requires relatively pure, clear, and colorless samples	Best used with purified, hydrolyzed protein systems (i.e., postcolumn detection of hydrolyzed proteins)
Biuret	Peptide bond	Peptide bond is complexed with cupric ions under alkaline conditions to give color that is quantitated by spectroscopy	Less expensive, faster, and simpler than Kjeldahl method. Does not detect nopeptide or nonprotein sources. Few interferences		

18.2.1.3.4 Titration

Borate anion (proportional to the amount of nitrogen) is titrated with standardized HCl.

$$H_2BO_3^- + H^+ \rightarrow H_3BO_3 \quad (18.4)$$

18.2.1.3.5 Calculations

$$\text{Moles of HCl} = \text{moles } NH_3 = \text{moles N in the sample} \quad (18.5)$$

A reagent blank should be run to subtract reagent nitrogen from the sample nitrogen.

$$\%N = N\text{HCl} \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\text{g N}}{\text{mol}} \times 100 \quad (18.6)$$

where:

N HCl = normality of HCl in moles/1,000 mL
Corrected acid vol. = (mL std. acid for sample) – (mL std. acid for blank)
14 = atomic weight of nitrogen

A factor is used to convert percent N to percent crude protein. Most proteins contain 16% N, so the conversion factor is 6.25 (100/16 = 6.25).

$$\%N / 0.16 = \%\text{ protein}$$

OR

$$\%N \times 6.25 = \%\text{ protein} \quad (18.7)$$

Conversion factors for various foods are given in Table 18.3 [1, 8].

18.2.1.4 *Applications*

The Kjeldahl method is an AOAC official method for crude protein content and has been the basis for evaluation of many other protein methods. The Kjeldahl method is still used for some applications, but now has limited use in many countries due to the availability and advantages of automated nitrogen combustion (Dumas) systems (Sect. 18.2.2) (see also Table 18.2 for advantages and disadvantages of the methods).

18.2.2 Dumas (Nitrogen Combustion) Method

18.2.2.1 *Principle*

The combustion method was introduced in 1831 by Jean-Baptiste Dumas. It has been modified and automated to improve accuracy since that time. Samples are combusted at high temperatures (700–1,000 °C) with a flow of pure oxygen. All carbon in the sample is converted to carbon dioxide during the flash combustion. Nitrogen-containing components produced include N_2 and nitrogen oxides. The nitrogen oxides are reduced to nitrogen in a copper reduction column at a high temperature (600 °C). The total nitrogen (including inorganic fraction, i.e., including nitrate and nitrite) released is carried by pure helium and quantitated by **gas chromatography** using a **thermal conductivity detector** (TCD) [9]. Ultrahigh purity acetanilide and EDTA (ethylenediaminetetraacetate) may be used as the standards for the calibration of the nitrogen analyzer. The nitrogen determined is converted to protein content in the sample using a protein conversion factor.

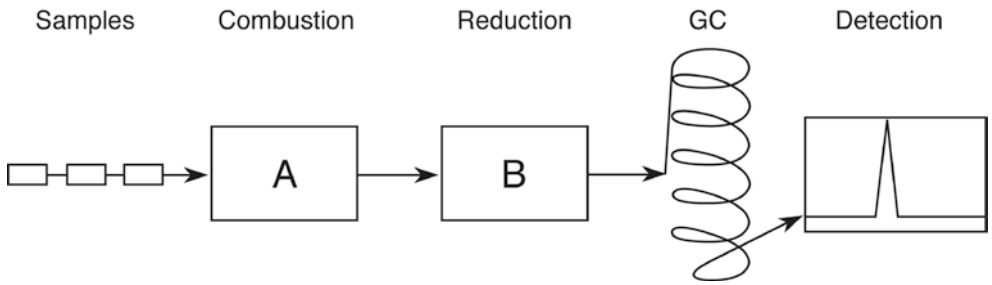
18.2.2.2 *Procedure*

Samples (approximately 100–500 mg) are weighed into a tin capsule and introduced to a combustion reactor in automated equipment. The nitrogen released is measured by a built-in gas chromatograph. Figure 18.1 shows the flow diagram of the components of a Dumas nitrogen analyzer.

18.3 table Nitrogen to protein conversion factors for various foods

Food	*Percent N in protein*	*Factor*
Egg or meat	16.0	6.25
Milk	15.7	6.38
Wheat	18.76	5.33
Corn	17.70	5.65
Oat	18.66	5.36
Soybean	18.12	5.52
Rice	19.34	5.17

Data from [1, 8]



18.1 figure General components of a Dumas nitrogen analyzer. (**A**) the incinerator. (**B**) copper reduction unit for converting nitrogen oxides to nitrogen, gas chromatography (GC) column, and detector

18.2.2.3 *Applications*

The combustion method is a faster and safer alternative to the Kjeldahl method [10] and is suitable for all types of foods. As an AOAC method, the Dumas method is widely used for official purposes, but its speed also allows for quality control applications. The industry uses different units/systems, depending on sample size and protein content. Freeze drying can be used to concentrate diluted liquid samples, e.g., waste steam samples.

18.3 INFRARED SPECTROSCOPY

18.3.1 Principle

Infrared spectroscopy measures the **absorption of radiation** (near- or mid-infrared regions) by molecules in food or other substances. Different functional groups in a food absorb different frequencies of radiation. For proteins and peptides, various **mid-infrared** bands (6.47 μm) and **near-infrared** (NIR) bands (e.g., 3,300–3,500 nm, 2,080–2,220 nm, 1,560–1,670 nm) characteristic of the **peptide bond** can be used to estimate the protein content of a food. By irradiating a sample with a wavelength of infrared light specific for the constituent to be measured, it is possible to predict the concentration of that constituent by measuring the energy that is reflected or transmitted by the sample (which is inversely proportional to the energy absorbed) [11].

18.3.2 Procedure

See Chap. 8 for a detailed description of instrumentation, sample handling, and calibration and quantitation methodology.

18.3.3 Applications

Mid-infrared spectroscopy is used in infrared milk analyzers to determine milk protein content, while near-infrared spectroscopy is applicable to a wide range of food products (e.g., grains, cereal, meat, and dairy products) [3, 12, 13], especially as a rapid method to test nonstandard milk.

18.4 COLORIMETRIC METHODS

When protein reacts with specific reagents under certain conditions, colorful compounds are generated, and the absorbance is measured by spectrophotometer. The protein content is expressed on the basis of standard protein such as bovine serum albumin (BSA), and thus this method is not an absolute method. Due to the differences in the composition of proteins, these methods have limited use. However, because of high sensitivity, these methods have the advantage of requiring a small sample size.

18.4.1 Dye-Binding Methods

18.4.1.1 *Anionic Dye-Binding Method*

18.4.1.1.1 Principle

The protein-containing sample is mixed with a known excess amount of **anionic dye** in a buffered solution. Proteins bind the dye to form an insoluble complex. The unbound soluble dye is measured after equilibration of the reaction and the removal of insoluble complex by centrifugation or filtration.

$$\text{Protein} + \text{excess dye} \rightarrow \text{Protein} - \text{dye insoluble complex} + \text{unbound soluble dye} \quad (18.8)$$

The anionic sulfonic acid dye, including acid orange 12, orange G, and Amido Black 10B, binds cationic groups of the **basic amino acid residues** (imidazole of histidine, guanidine of arginine, and ε-amino group of lysine) and the **free amino terminal group** of the protein [14]. The amount of the unbound dye is inversely related to the protein content of the sample [14].

18.4.1.1.2 Procedure

1. The sample is finely ground (60 mesh or smaller sizes) and added to an excess dye solution with known concentration.
2. The content is vigorously shaken to equilibrate the dye-binding reactions and filtered or centrifuged to remove insoluble substances.
3. Absorbance of the unbound dye solution in the filtrate or supernatant is measured and dye concentration estimated from a dye standard curve.
4. A straight calibration curve can be obtained by plotting the unbound dye concentration against total nitrogen (as determined by Kjeldahl method) of a given food covering a wide range of protein content.
5. Protein content of the unknown sample of the same food type can be estimated from the calibration curve or from a regression equation calculated by the least squares method.

18.4.1.1.3 Applications

Anionic dye-binding has been used to estimate proteins in milk [15, 16], wheat flour [17, 18], soy products [18, 19], and meats [20]. The anionic dye-binding method may be used to estimate the changes in available lysine content of cereal products during processing since the dye does not bind altered, unavailable lysine. Since lysine is the limiting amino acid in cereal products, the available lysine content represents pro-

tein nutritive value of the cereal products [21]. An automated Sprint™ Rapid Protein Analyzer has been developed by the CEM Company (Matthews, NC) based on the anionic dye-binding method. This rapid protein analyzer combines and automates all steps (sample weighing, homogenization, predetermined dye addition, filtration, absorption measurement, and protein calculation) in a compact bench top unit. This dye-binding method has been approved in AOAC Method 2011.04 for raw and processed meats. This analyzer has shown great agreement with Kjeldahl method in protein analysis of milk, cream, soybean, and soymilk [22, 23]. This method is safe (the proprietary dye iTag is nonhazardous), easy to use, and efficient. Because different types of sample matrixes and proteins exhibit different responses on the Sprint Rapid Protein Analyzer, this automated method requires calibration for each type of food protein determined using other official methods.

18.4.1.2 *Bradford Dye-Binding Method*

18.4.1.2.1 Principle

When **Coomassie Brilliant Blue G-250** binds to protein, the **dye changes color** from reddish to bluish, and the absorption maximum of the dye is shifted from 465 to 595 nm. The change in the absorbance at 595 nm is proportional to the protein concentration of the sample [24]. Like other dye-binding methods, the Bradford relies on the **amphoteric nature of proteins**. When the protein-containing solution is acidified to a pH less than the isoelectric point of the protein(s) of interest, the dye added binds electrostatically. Binding efficiency is enhanced by hydrophobic interaction of the dye molecule with the polypeptide backbone adjoining positively charged residues in the protein [4]. In the case of the Bradford method, the dye bound to protein has a change in absorbance spectrum relative to the unbound dye.

18.4.1.2.2 Procedure

1. Coomassie Brilliant Blue G-250 is dissolved in 95% ethanol and acidified with 85% phosphoric acid.
2. Samples containing proteins (1–100 μg/mL) and standard BSA solutions are mixed with the Bradford reagent.
3. Absorbance at 595 nm is read against a reagent blank.
4. Protein concentration in the sample is estimated from the BSA standard curve.

18.4.1.2.3 Applications

The Bradford method has been used successfully to determine protein content in worts and beer products [25] and in potato tubers [26]. This procedure has been improved to measure microgram quantities of proteins [27]. Due to its rapidity, sensitivity, and fewer interferences than the Lowry method, the Bradford method has been used widely for the analysis of low concentrations of proteins and enzymes in their purification and characterizations. Improved Coomassie dye-based protein assays based on the Bradford method have been developed (e.g., CB™ and CB-X™, G-Biosciences, Geno Technology, Inc.) for increased compatibility with buffers and conditions commonly used in protein isolation. Other dye-based protein assays (e.g., RED 660™ Protein Assay, Thermo Scientific Pierce) are being developed to improve the linearity, color stability, and interferences of the Bradford and improved Coomassie/Bradford assays.

18.4.2 Copper Ion-Based Methods

Since the biuret method was first developed to measure protein content based on the reaction with copper ions, several improved methods have been developed. Following the description of the basic biuret method below are the modified Lowry and the bicinchoninic acid (BCA) methods, which are both based in part on the biuret method.

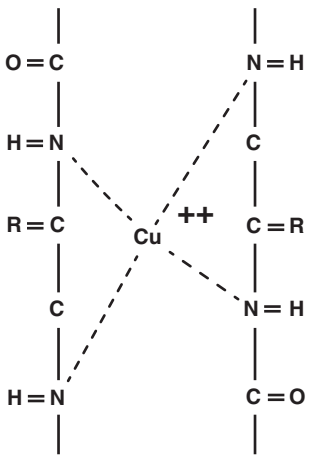
18.4.2.1 *Biuret Method*

18.4.2.1.1 Principle

A violet-purplish color is produced when **cupric ions** are complexed with **peptide bonds** (substances containing at least two peptide bonds, i.e., oligopeptides, large peptides, and all proteins) under **alkaline conditions** (Fig. 18.2). The absorbance of the color produced is read at 540 nm. The color intensity (absorbance) is proportional to the protein content of the sample [28].

18.4.2.1.2 Procedure

1. A 5-mL biuret reagent is mixed with a 1-mL portion of protein solution (1–10 mg protein/mL).



18.2 figure Reaction of peptide bonds with cupric ions

The reagent includes copper sulfate, NaOH, and potassium sodium tartrate, which is used to stabilize the cupric ion in the alkaline solution.
2. After the reaction mix is allowed to stand at room temperature for 15 or 30 min, the absorbance is read at 540 nm against a reagent blank.
3. Filtration or centrifugation before reading absorbance is required if the reaction mixture is not clear.
4. A standard curve of concentration versus absorbance is constructed using **bovine serum albumin** (BSA).

18.4.2.1.3 Applications

The biuret method has been used to determine proteins in cereal [29, 30], meat [20], soybean proteins [19], and as a qualitative test for animal feed [31]. The biuret method also can be used to measure the protein content of isolated proteins, but it has been largely replaced for this use by more sensitive methods such as the modified Lowry method and especially the BCA methods for the reasons described in Sect. 18.4.2.

18.4.2.2 *Lowry Method*

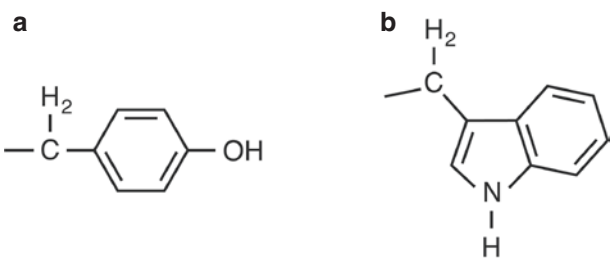
18.4.2.2.1 Principle

The Lowry method [32, 33] combines the **biuret reaction** with the reduction of the **Folin-Ciocalteu phenol reagent** (phosphomolybdic-phosphotungstic acid) by **tyrosine** and **tryptophan** residues in the proteins (Fig. 18.3). The bluish color developed is read at 750 nm (high sensitivity for low protein concentration) or 500 nm (low sensitivity for high protein concentration). The original procedure has been modified by Miller [34] and Hartree [35] to improve the linearity of the color response to protein concentration and replace the use of two unstable reagents with one stable reagent.

18.4.2.2.2 Procedure

The following procedure is based on the modified procedure of Hartree [35]:

1. Proteins to be analyzed are diluted to an appropriate range (20–100 µg).
2. K Na Tartrate-Na_2CO_3 solution is added after cooling then incubated at room temperature for 10 min.
3. $CuSO_4$-K Na Tartrate-NaOH solution is added after cooling then incubated at room temperature for 10 min.
4. Freshly prepared Folin's reagent is added and then the reaction mixture is mixed and incubated at 50 °C for 10 min.
5. Absorbance is read at 650 nm.
6. A standard curve of BSA is carefully constructed for estimating protein concentration of the unknown.



18.3 figure Side chains of amino acids tyrosine (**a**) and tryptophan (**b**)

18.4.2.2.3 Applications

Because of its simplicity and sensitivity, the Lowry method has been widely used in protein biochemistry. However, it has not been widely used to determine proteins in food systems without first extracting the proteins from the food mixture.

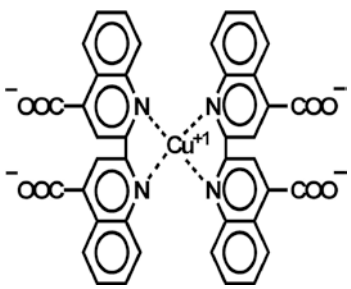
18.4.2.3 *Bicinchoninic Acid Method*

18.4.2.3.1 Principle

Proteins and peptides (as short as di-peptides) reduce **cupric ions** to **cuprous ions** under **alkaline conditions** [36], which is similar in principle to that of the biuret reaction. The cuprous ion then reacts with the apple-greenish **bicinchoninic acid reagent** (BCA) to form a purplish complex (one cuprous ion is chelated by two BCA molecules) (Fig. 18.4). The color measured at 562 nm is near linearly proportional to protein concentration over a wide range of concentration from micrograms up to 2 mg/mL. Peptide bonds and four amino acids (cysteine/cystine, tryptophan, and tyrosine) contribute to the color formation with BCA.

18.4.2.3.2 Procedure

1. Mix (one step) the protein solution with the BCA reagent, which contains BCA sodium salt,



18.4 figure Protein reaction with cupric ions under alkaline conditions to form cuprous ions, which react with bicinchoninic acid (*BCA*) to form purple color, measured at 562 nm (Figure courtesy of Pierce Biotechnology Technical Library, Thermo Fisher Scientific, Inc., Rockford, IL)

sodium carbonate, NaOH, and copper sulfate, pH 11.25.
2. Incubate at 37 °C for 30 min, or room temperature for 2 h, or 60 °C for 30 min. The selection of the temperature depends upon the sensitivity desired. A higher temperature gives a greater color response.
3. Read the solution at 562 nm against a reagent blank.
4. Construct a standard curve using BSA.

18.4.2.3.3 Applications

The BCA method is widely used in protein isolation and purification due to its advantage (over the modified Lowry method and any Coomassie dye-based assay) of being compatible with samples containing up to 5% detergents. While most dye-binding methods are faster, the BCA method is less affected by protein compositional differences, so there is better protein-to-protein uniformity.

18.5 ULTRAVIOLET ABSORPTION METHODS FOR PROTEINS AND PEPTIDES

18.5.1 Ultraviolet 280 nm Absorption for Protein

18.5.1.1 *Principle*

Proteins show strong absorption in the **ultraviolet** (UV) region at **UV 280 nm**, primarily due to **tryptophan** and **tyrosine** residues in the proteins. Because the content of tryptophan and tyrosine in proteins from each food source is fairly constant, the absorbance at 280 nm could be used to estimate the concentration of proteins, using **Beer's law**. Since each protein has a unique aromatic amino acid composition, the extinction coefficient (E_{280}) or molar absorptivity (E_m) must be determined for individual proteins for protein content estimation.

18.5.1.2 *Procedure*

1. Proteins are solubilized in buffer or alkali.
2. Absorbance of protein solution is read at 280 nm against a reagent blank.
3. Protein concentration is calculated according to the following equation:

$$A = abc \tag{18.9}$$

where:

A = absorbance
a = absorptivity
b = cell or cuvette path length
c = concentration

18.5.1.3 *Applications*

The UV 280 nm method has been used to determine the protein contents of milk [37] and meat products [38]. It has not been used widely in food systems. This technique is better applied in a purified protein system or to proteins that have been extracted in alkali or denaturing agents such as 8 *M* urea. Although peptide bonds in proteins absorb more strongly at 190–220 nm than at 280 nm, the low UV region is more difficult to measure.

18.5.2 Peptide Measurement at 190–220 nm

Peptides without or with low level of tyrosine or tryptophan residues can be quantified at 190–220 nm at which peptide bonds have maximum absorption. The extinction coefficients in the far UV range can be calculated with the consideration of contribution of tyrosine and tryptophan to the absorption [39]. Protein can also be measured in this UV range. For example, NanoDrop™ 2,000/2,000 c from Thermo Scientific can be used for protein and peptide quantification at 205 nm with extinction coefficient being 31.

18.6 NONPROTEIN NITROGEN DETERMINATION

18.6.1 Principle

Protein is precipitated by trichloroacetic acid (TCA) and separated from nonprotein nitrogen (NPN)-containing compounds.

18.6.2 Procedure

1. Add appropriate amount of TCA into a sample solution to make the final TCA concentration around 10%. Alternatively, prepare a 10% TCA solution and add dry sample powder into the solution.
2. Mix the reaction mixture thoroughly and let precipitate settle for 5 min.
3. Filter the mixture through Whatman No. 1 filter paper. Alternatively, centrifugation at 30,000 g can be used to separate precipitated protein from the supernatant that contains NPN.
4. Determine nitrogen content of the filtrate by the Kjeldahl method.
5. Convert nonprotein nitrogen to protein equivalent with conversion factor.

18.6.3 Applications

The NPN determination is used to check economic adulteration with nitrogen-rich compounds such as

urea, ammonia, and melamine. NPN for milk can be tested by AOAC methods (991.21 and 991.22). The results obtained are not absolute, but are relative to the standardized conditions used in the procedure [40].

18.7 COMPARISON OF METHODS

1. **Sample preparation:** The Kjeldahl, Dumas, and infrared spectroscopy methods require little preparation. Sample particle size of 20 mesh or smaller generally is satisfactory for these methods. Some of the newer NIR instruments can make measurements directly on whole grains and other coarsely granulated products without grinding or other sample preparation. Other methods described in this chapter require fine particles for extraction of proteins from the complex food systems.
2. **Principle:** The Dumas and Kjeldahl methods measure directly the nitrogen content of foods. However, the Kjeldahl method measures only organic nitrogen plus ammonia, while Dumas measures total nitrogen, including the inorganic fraction. (Therefore, Dumas gives a higher value for products that contain nitrates/nitrites.) Other methods of analysis measure the various properties of proteins. For instance, the biuret method measures peptide bonds, and the Lowry and BCA methods measure a combination of peptide bonds and specific amino acids. Infrared spectroscopy is an indirect method to estimate protein content, based on the energy absorbed when a sample is subjected to a wavelength of infrared radiation specific for the peptide bond.
3. **Sensitivity:** Kjeldahl, Dumas, and biuret methods are less sensitive than Bradford, Lowry, BCA, or UV methods.
4. **Speed:** After the instrument has been properly calibrated, infrared spectroscopy is likely the most rapid of the methods discussed. In most other methods involving spectrophotometric (colorimetric) measurements, one must separate proteins from the interfering insoluble materials before mixing with the color reagents or must remove the insoluble materials from the colored protein-reagent complex after mixing. However, the speed of determination in the colorimetric methods and in the Dumas method is faster than with the Kjeldahl method.
5. **Applications:** Although both Kjeldahl and Dumas methods can be used to measure N content in all types of foods, in recent years the Dumas method has largely replaced the Kjeldahl method for nutrition labeling (since Dumas method is faster, has a lower detection limit, and is safer). However, the Kjeldahl method is the preferred method for high fat samples/products since fat may cause an instrument fire during the incineration procedure in the Dumas method. The Kjeldahl method or the nitrogen combustion method is specified to correct for protein content in the most recent AOAC official method to measure the fiber content of foods (see Chap. 19, Sect. 19.5). Melamine, a toxic nitrogen adulterant, is included in the total nitrogen content if measured by the Kjeldahl or Dumas methods. NIR, CEM-Sprint dye binding, and Dumas methods are good for quality control in food processing plants.

18.8 SPECIAL CONSIDERATIONS

1. To select a particular method for a specific application, sensitivity, accuracy, and reproducibility as well as physicochemical properties of food materials must be considered. The data should be interpreted carefully to reflect what actually is being measured.
2. Food processing methods, such as heating, may reduce the extractability of proteins for analysis and cause an underestimation of the protein content measured by methods involving an extraction step.
3. Except for the Dumas and Kjeldahl methods, and the UV method for purified proteins, all methods require the use of a standard or reference protein or a calibration with the Kjeldahl method. In the methods using a standard protein, proteins in the samples are assumed to have similar composition and behavior compared to the standard protein. The selection of an appropriate standard for a specific type of food is important.
4. **Nonprotein nitrogen** is present in practically all foods. To determine **protein nitrogen**, the samples usually are extracted under alkaline conditions then precipitated with trichloroacetic acid or sulfosalicylic acid. The concentration of the acid used affects the precipitation yield. Therefore, nonprotein nitrogen content may vary with the type and concentration of the reagent used. Heating could be used to aid protein precipitation by acid, alcohol, or other organic solvents. In addition to acid precipitation methods used for nonprotein nitrogen determination, less empirical methods such as dialysis, and

ultrafiltration and column chromatography could be used to separate proteins from small nonprotein substances.

5. In the determination of the nutritive value of food proteins, including **protein digestibility** and **protein efficiency ratio** (PER), the Kjeldahl or Dumas method with a 6.25 conversion factor is used to determine crude protein content. The PER could be underestimated if a substantial amount of nonprotein nitrogen is present in foods. A food sample with a higher nonprotein nitrogen content (particularly if the nonprotein nitrogen does not have many amino acids or small peptides) may have a lower PER than a food sample containing similar protein structure/composition and yet with a lower amount of nonprotein nitrogen.

18.9 SUMMARY

Methods based on the unique characteristics of proteins and amino acids have been described to determine the protein content of foods. The Kjeldahl and Dumas methods measure nitrogen. Infrared spectroscopy is based on absorption of a wavelength of infrared radiation specific for the peptide bond. Copper-peptide bond interactions contribute to the analysis by the biuret, Lowry, and BCA methods. Amino acids are involved in the Lowry, BCA, dye-binding, and UV 280-nm methods. The BCA method also utilizes the reducing power of proteins in an alkaline solution. The various methods differ in their speed and sensitivity.

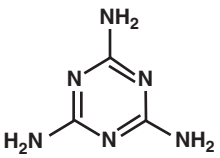
In addition to the commonly used methods discussed, there are other methods available for protein quantification. Because of the complex nature of various food systems, problems may be encountered to different degrees in protein analysis by available methods. Rapid methods may be suitable for quality control purposes, while a sensitive method is required for work with a minute amount of protein. Indirect colorimetric methods usually require the use of a carefully selected protein standard or a calibration with an official method.

18.10 STUDY QUESTIONS

1. What factors should one consider when choosing a method for protein determination?
2. The Kjeldahl method of protein analysis consists of three major steps. List these steps in the order they are done and describe in words what occurs in each step. Make it clear why milliliters of HCl can be used as an indirect measure of the protein content of a sample.
3. Why is the conversion factor from Kjeldahl nitrogen to protein different for various foods, and how is the factor of 6.25 obtained?
4. Differentiate the principles of protein determination by dye binding with an anionic dye such as Amido Black vs. the Bradford method, which uses the dye Coomassie Blue G-250.
5. With the anionic dye-binding method, would a sample with a higher protein content have a higher or a lower absorbance reading than a sample with a low protein content? Explain your answer.
6. For each of the situations described below, identify a protein assay method most appropriate for use and indicate the chemical basis of the method (i.e., what does it really measure?)

   (a) Nutrition labeling
   (b) Intact protein eluting from a chromatography column; qualitative or semiquantitative method
   (c) Intact protein eluting from a chromatography column; colorimetric, quantitative method
   (d) Rapid, quality control method for protein content of cereal grains

7. The FDA found melamine (see structure below) in pet food linked to deaths of pets in the United States. The FDA also found evidence of melamine in wheat gluten imported from China used as one of the ingredients in the production of the pet food. Melamine is a nitrogen-rich chemical used to make plastic and sometimes used as a fertilizer.



   (a) Knowing that each ingredient is tested and analyzed when imported, explain how melamine in wheat gluten could have escaped detection.
   (b) How can the adulteration of wheat gluten be detected (not necessarily detecting melamine specifically) using a combination of protein analysis methods? Explain your answer.

18.11 PRACTICE PROBLEMS

1. A dehydrated precooked pinto bean was analyzed for crude protein content in duplicate using the Kjeldahl method. The following data were recorded:

Moisture content = 8.00 %
Wt of Sample 1 = 1.015 g
Wt of Sample 2 = 1.025 g
Normality of HCl used for titration = 0.1142 *N*
HCl used for Sample 1 = 22.0 mL
HCl used for Sample 2 = 22.5 mL
HCl used for reagent blank = 0.2 mL

Calculate crude protein content on both wet and dry weight basis of the pinto bean, assuming pinto bean protein contains 17.5 % nitrogen.

2. A 20-mL protein fraction recovered from a column chromatography was analyzed for protein using the BCA method. The following data were the means of a duplicate analysis using BSA as a standard:

BSA mg/mL	*Mean absorbance at 562 nm*
0.2	0.25
0.4	0.53
0.6	0.74
0.8	0.95
1.0	1.15

The average absorbance of a 1-mL sample was 0.44. Calculate protein concentration (mg/mL) and total protein quantity of this column fraction.

3. A turkey frankfurter was analyzed for crude protein content using the Kjeldahl method. The following data were recorded:

Wt of sample = 0.5172 g
Normality of HCl = 0.1027 *N*
Vol of HCl used for sample = 8.8 mL
Vol of HCl used for reagent blank = 0.2 mL

Calculate % crude protein content on wet weight basis.

For calculating the conversion factor, assume that amino acids in meat protein contain 16 % nitrogen (or look up the conversion factor).

$$\%\ \text{Nitrogen} = \frac{(\text{mL HCl sample} - \text{mL HCl blank}) \times N\ \text{HCl} \times 1.4007}{\text{Sample weight (g)}}$$

$$\%\ \text{N} \times \text{Conversion Factor} = \%\ \text{Protein}$$

$$100 / \%\ \text{N} = \text{Conversion Factor}$$

4. Why is it difficult to accurately determine protein content of a composite food such as a sausage pizza when using the Kjeldahl and combustion methods for protein?
5. A standard procedure for the Dumas combustion method for protein states that a sample should contain between 10 and 50 mg N. Using the USDA Nutrient Database, calculate the weight of sample to use for analysis. Sample: turkey thigh meat.
6. Estimate the weight of sample (Wheat Thins) you should use for Kjeldahl protein determination if you want to titrate the sample with at least 7 mL 0.1 N acid. Show calculation. Use the nutritional label as a guide (serving size: 29 g; protein: 2 g).
7. A stock solution of bovine serum albumin (BSA) was prepared previously by a coworker. Before using the BSA to prepare a standard curve for biuret determination, you need to confirm that the stock solution contains 20 mg protein/mL using UV spectroscopy. The $E^{1\%}$ for BSA is 6.3 at 280 nm. If the stock solution was correctly prepared, calculate the absorbance you would expect to find when the stock solution is diluted tenfold (0.3 mL stock with 2.7 mL water) and absorbance of this solution determined at 280 nm in a 3-mL cuvette with a 0.5-cm path length.
8. Colorimetric protein assay. Calculate the concentration of protein in an unknown solution X using the biuret test for protein. The stock solution contains 20-mg protein/mL. The standard curve was prepared using the stock solution as follows (used 1-cm path length cuvette):

Standard curve	*1*	*2*	*3*	*4*	*5*	*6*
Vol. of H_2O (mL)	1	0.8	0.6	0.4	0.2	0.0
Vol. of protein standard (mL)	0.0	0.2	0.4	0.6	0.8	1.0
Vol. of biuret reagent (mL)	4.0	4.0	4.0	4.0	4.0	4.0
Absorbance at 540 nm	0.0	0.174	0.343	0.519	0.691	0.823

Solution X was prepared for analysis as follows:

Sample	*Tube 1*	*Tube 2*
Vol. of H_2O (mL)	0.8	0.2
Vol. of solution X (mL)	0.2	0.8
Vol. of biuret reagent (mL)	4.0	4.0
Absorbance at 540 nm	0.451	0.857

(a) Explain why the absorbance of the Biuret assay is measured at 540 nm.
(b) Calculate the concentration of protein (mg/tube) in each tube used to prepare the standard curve.
(c) Prepare a standard curve (in mg/tube). Label the graph properly.

(d) What sample dilution (tube 1 or tube 2) should you use to determine the concentration of the unknown sample? Why?
(e) Calculate the concentration of protein in solution X. List the concentration as mg protein/cuvette and then express results as mg protein/mL of solution X.

Answers

1. Protein content = 19.75% on a wet weight basis; 21.47% on a dry weight basis.

Calculations:

$$\%\text{N} = N\,\text{HCl} \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\,\text{g N}}{\text{mol}} \times 100 \qquad (18.6)$$

where:

NHCl = normality of HCl in mol/1,000 mL
Corrected acid vol. = (mL std. acid for sample) – (mL std. acid for blank)
14 = atomic weight of nitrogen

Corrected acid volume for Sample 1 = 22.0 mL – 0.2 mL = 21.8 mL

Corrected acid volume for Sample 2 = 22.5 mL – 0.2 mL = 22.3 mL

% N for Sample 1

$$= \frac{0.1142\ \text{mole}}{1000\ \text{ml}} \times \frac{21.8\ \text{ml}}{1.015\ \text{g}} \times \frac{14\text{g N}}{\text{mol}} \times 100\ \% = 3.433\ \%$$

% N for Sample 2

$$= \frac{0.1142\ \text{mole}}{1000\ \text{ml}} \times \frac{22.3\ \text{ml}}{1.025\ \text{g}} \times \frac{14\text{g N}}{\text{mol}} \times 100\ \% = 3.478\ \%$$

Protein conversion factor
= 100%/17.5% N = 5.71

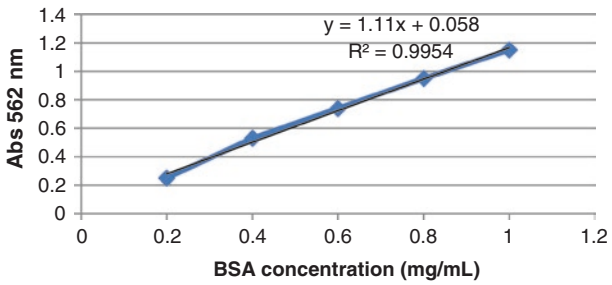
Crude protein content for Sample 1
= 3.433 % × 5.71 = 19.6%

Crude protein content for Sample 2
= 3.478% × 5.71 = 19.9%

The average for the duplicate data
= (19.6% + 19.9%)/2 = 19.75%
= ~19.8% wet weight basis

To calculate protein content on a dry weight basis: Sample contains 8% moisture, and therefore, the sample contains 92% dry solids, or 0.92 g out of 1 g sample. Therefore, protein on a dry weight basis can be calculated as follows: 19.75%/0.92 g dry solids = 21.47% = ~21.5% dry weight basis.

2. Protein content = 0.68 mg/mL. Total protein quantity = 6.96 mg
Calculations:
Plot absorbance (y-axis, absorbance at 562 nm) versus BSA protein concentration (x-axis, mg/mL) using the data above. Determine the equation of the line ($y = 1.11\ x + 0.058$) and then use this equation and the given absorbance ($y = 0.44$) to calculate the concentration ($x = 0.344$ mg/mL). Since 1 mL of sample gives a concentration of 0.344 mg/mL and we have a total of 20 mL collected from column chromatography, we will have a total of (0.344 mg/mL × 20 mL) = 6.88 mg protein in this collected column fraction.



3. $\frac{100}{16\%} = 6.25$

$$\%\ \text{N} = \frac{(8.8 - 0.2\ \text{mL})(0.1027\ \text{N})(1.4007)}{0.5172}$$
$$= 2.39\%\ \text{N} \times 6.25 = 14.95\ \%\ \text{Protein}$$

4. The composite food contains many different proteins with different proportions of amino acids of varying nitrogen contents. The following is the conversion factors of some ingredients:

Meat: 6.25
Dairy: 6.38
Wheat flour: 5.7

5. Database: 19.27 g protein/100 g
Estimate:

$$\%\ \text{N} = \frac{19.27\%\ \text{protein}}{6.25} = 3.05\%\ \text{N}$$
$$\frac{3.08\ \text{g}}{100\ \text{g}} = \frac{0.010\,\text{g}}{x}$$
$$x = 0.3247\,\text{g}$$
$$5x = 5 \times 0.3247\ \text{g} = 1.6234\ \text{g}$$
$$\text{Weight of sample} = 0.32 - 1.62\,\text{g}$$

6. $\%\ \text{Protein} = \frac{2\,\text{g}}{29\,\text{g}} \times 100 = 6.9\%$

Conversion factor for wheat flour is 5.7,

$$\text{so N\%} = \frac{6.9\%}{5.7} = 1.2\%$$

$$1.2\% = \frac{(7\text{mL})(0.1N)(1.4007)}{\text{sample weigh}}$$

$$\text{Sample weight} = 0.82\,\text{g}$$

7. Concentration of BSA after tenfold dilution = 2 mg/mL = 0.002 g/g = 0.2 %

$$A = a \times b \times c = 6.3 \times 0.5 \times 0.2 = 0.63$$

8. (a) The complex has maximum absorbance at this wavelength.
   (b) Tube 1: 0 mg/tube
   Tube 2: 4 mg/tube
   Tube 3: 8 mg/tube
   Tube 4: 12 mg/tube
   Tube 5: 16 mg/tube
   Tube 6: 20 mg/tube
   (c) Standard curve prepared as in Problem 2.
   (d) Tube 1 is used because its absorbance is within the range of the standard curve.
   (e) Based on the standard curve and absorbance of X solution:
   Protein concentration calculated is about 10 mg/tube
   10 mg/0.2 mL = 50 mg/mL

Acknowledgments The authors thank Dr. Denise Smith of the Washington State University for her contribution of several valuable practice problems and answers to this chapter. We also thank Dr. S. Suzanne Nielsen for her valuable suggestions for improvement of this chapter.

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