
# Protein Analysis

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## 9.1 INTRODUCTION

### 9.1.1 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 5000 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.

### 9.1.2 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 breadmaking, casein in milk for coagulation into cheese products, and egg albumen for foaming (see Chap. 15).
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, enzymes 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. 15)
6. Nutritive value of a protein (see Chap. 15)

### 9.1.3 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 9-1.

## 9.2 METHODS

Principles, general procedures, and applications are described below for various protein determination methods. Refer to the referenced methods for detailed instructions of the procedures. The Kjeldahl, Dumas (N combustion), and infrared spectroscopy methods cited are from the *Official Methods of Analysis* of AOAC International (3) and are used commonly in nutrition labeling and quality control. The other methods

**9-1 table** **Protein Content of Selected Foods (2)**

| *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 vitamin 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 US Department of Agriculture, Agricultural Research Service (2009). USDA National Nutrient Database for Standard Reference. Release 22. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/ba/bhnrc/ndl

described are used commonly in research laboratories working on proteins. Many of the methods covered in this chapter are described in somewhat more detail in recent books on food proteins (4–6).

## 9.2.1 Kjeldahl Method

### *9.2.1.1 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).

### *9.2.1.2 Historical Background*

*9.2.1.2.1 Original Method* In 1883, Johann Kjeldahl developed the basic process of today's Kjeldahl method to analyze organic nitrogen. General steps in the original method include the following:

1. **Digestion** with sulfuric acid, with the addition of powdered potassium permanganate to complete oxidation and conversion of nitrogen to ammonium sulfate.
2. **Neutralization** of the diluted digest, followed by **distillation** into a known volume of standard acid, which contains potassium iodide and iodate.
3. **Titration** of the liberated iodine with standard sodium thiosulfate.

*9.2.1.2.2 Improvements* Several important modifications have improved the original Kjeldahl process:

1. Metallic catalysts such as mercury, copper, and selenium are added to sulfuric acid for complete digestion. Mercury has been found to be the most satisfactory. Selenium dioxide and copper sulfate in the ratio of 3:1 have been reported to be effective for digestion. Copper and titanium dioxide also have been used as a mixed catalyst for digestion (AOAC Method 988.05) (3). The use of titanium dioxide and copper poses less **safety concern** than mercury in the postanalysis disposal of the waste.
2. Potassium sulfate is used to increase the boiling point of the sulfuric acid to accelerate digestion.
3. Sulfide or sodium thiosulfate is added to the diluted digest to help release nitrogen from mercury, which tends to bind ammonium.
4. The ammonia is distilled directly into a boric acid solution, followed by titration with standard acid.
5. Colorimetry Nesslerization, or ion chromatography to measure ammonia, is used to determine nitrogen content after digestion.

An excellent book to review the Kjeldahl method for total organic nitrogen was written by Bradstreet (7). The basic AOAC Kjeldahl procedure is Method 955.04.

Semiautomation, automation, and modification for microgram nitrogen determination (micro Kjeldahl method) have been established by AOAC in Methods 976.06, 976.05, and 960.52, respectively.

### 9.2.1.3 General Procedures and Reactions

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

*9.2.1.3.2 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.

$$\text{Protein} \xrightarrow[\text{Heat, catalyst}]{\text{Sulfuric acid}} (NH_4)_2SO_2 \qquad [1]$$

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.

*9.2.1.3.3 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).

$$(NH_4)_2SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O \qquad [2]$$

$$NH_3 + H_3BO_3\,(\text{boric acid}) \rightarrow NH_4 + H_2BO_3^{-}\,(\text{borate ion}) \qquad [3]$$

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

$$H_2BO_3^{-} + H^{+} \rightarrow H_3BO_3 \qquad [4]$$

*9.2.1.3.5 Calculations*

$$\begin{aligned}\text{Moles of HCl} &= \text{moles of } NH_3 \\ &= \text{moles of N in the sample}\end{aligned} \qquad [5]$$

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

$$\%\,\text{N} = N\,\text{HCl} \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\,\text{g N}}{\text{mol}} \times \frac{100}{1000} \qquad [6]$$

**9-2 table** **Nitrogen to Protein Conversion Factors for Various Foods**

| | *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).

where:

$$\begin{aligned}N\text{HCl} &= \text{normality of HCl, in mol/1000 ml} \\ \text{Corrected acid vol.} &= (\text{ml std. acid for sample}) - (\text{ml std. acid for blank}) \\ 14 &= \text{atomic weight of nitrogen}\end{aligned}$$

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).

$$\%\,\text{N}/0.16 = \%\,\text{protein} \qquad [7]$$

or

$$\%\,\text{N} \times 6.25 = \%\,\text{protein}$$

**Conversion factors** for various foods are given in Table 9-2.

*9.2.1.3.6 Alternate Procedures* In place of distillation and titration with acid, ammonia or nitrogen can be quantitated by:

1. Nesslerization

$$4NH_4OH + 2HgI_2 + 4KI + 3KOH \rightarrow \underset{\text{ammonium dimercuric iodide, red-orange, 440 nm}}{NH_2Hg_2IO} + 7KI + 2H_2O \qquad [8]$$

   This method is rapid and sensitive, but the ammonium dimercuric iodide is colloidal and color is not stable.
2. $NH_3$ + phenol + hypochloride $\xrightarrow[OH^-]{}$ indophenol (blue, 630 nm) [9]
3. pH measurement after distillation into known volume of boric acid
4. Direct measurement of ammonia, using ion chromatographic method

#### 9.2.1.4 Applications

Advantages:

1. Applicable to all types of foods
2. Inexpensive (if not using an automated system)
3. Accurate; an official method for crude protein content
4. Has been modified (micro Kjeldahl method) to measure microgram quantities of proteins

Disadvantages:

1. Measures total organic nitrogen, not just protein nitrogen
2. Time consuming (at least 2 h to complete)
3. Poorer precision than the biuret method
4. Corrosive reagent

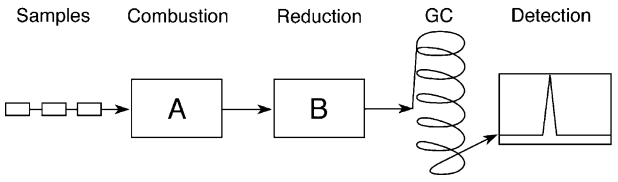
### 9.2.2 Dumas (Nitrogen Combustion) Method

#### 9.2.2.1 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 (700–1000°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). Ultra-high purity acetanilide and EDTA (ethylenediamine tetraacetate) 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.

#### 9.2.2.2 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 9-1 shows the flow diagram of the components of a Dumas nitrogen analyzer.




**9-1 figure** General components of a Dumas nitrogen analyzer. *A*, the incinerator; *B*, copper reduction unit for converting nitrogen oxides to nitrogen; and *GC*, gas chromatography column.

#### 9.2.2.3 Applications

The combustion method is an alternative to the Kjeldahl method (10) and is suitable for all types of foods. AOAC Method 992.15 and Method 992.23 are for meat and cereal grains, respectively.

Advantages:

1. Requires no hazardous chemicals.
2. Can be accomplished in 3 min.
3. Recent automated instruments can analyze up to 150 samples without attention.

Disadvantages:

1. Expensive equipment is required.
2. Measures total organic nitrogen, not just protein nitrogen.

### 9.2.3 Infrared Spectroscopy

#### 9.2.3.1 Principle

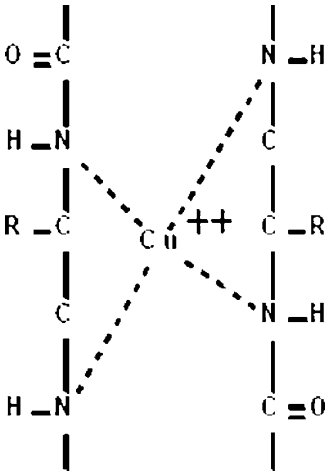
Infrared spectroscopy measures the **absorption of radiation** (near- or mid-infrared regions) by molecules in food or other substances. Different functional groups in a food absorb different frequencies of radiation. For proteins and peptides, various **mid-infrared** bands (6.47 μm) and **near-infrared** (NIR) bands (e.g., 3300–3500 nm; 2080–2220 nm; 1560–1670 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).

#### 9.2.3.2 Procedure

See Chap. 23 for a detailed description of instrumentation, sample handling, and calibration and quantitation methodology.

#### 9.2.3.3 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) (AOAC Method 997.06). Instruments are expensive and they must be calibrated properly. However, samples can be analyzed rapidly (30 s to 2 min) by analysts with minimal training.



**9-2 figure** Reaction of peptide bonds with cupric ions.

## 9.2.4 Biuret Method

### *9.2.4.1 Principle*

A violet-purplish color is produced when **cupric ions** are complexed with **peptide bonds** (substances containing at least two peptide bonds, i.e., biuret, large peptides, and all proteins) under **alkaline conditions** (Fig. 9-2). The absorbance of the color produced is read at 540 nm. The color intensity (absorbance) is proportional to the protein content of the sample (14).

### *9.2.4.2 Procedure*

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

### *9.2.4.3 Applications*

The biuret method has been used to determine proteins in cereal (15, 16), meat (17), soybean proteins (18), and as a qualitative test for animal feed [AOAC Method 935.11 (refers to Methods 22.012–22.013, AOAC, 10th edn, 1965)] (19). The biuret method also can be used to measure the protein content of isolated proteins.

Advantages:

1. Less expensive than the Kjeldahl method; rapid (can be completed in less than 30 min); simplest method for analysis of proteins.
2. Color deviations are encountered less frequently than with Lowry, ultraviolet (UV) absorption, or turbidimetric methods (described below).
3. Very few substances other than proteins in foods interfere with the biuret reaction.
4. Does not detect nitrogen from nonpeptide or nonprotein sources.

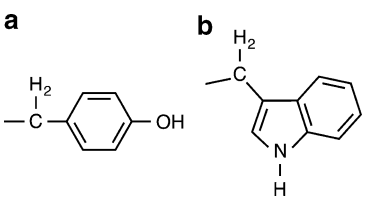
Disadvantages:

1. Not very sensitive as compared to the Lowry method; requires at least 2–4 mg protein for assay.
2. Absorbance could be contributed from bile pigments if present.
3. High concentration of ammonium salts interfere with the reaction.
4. Color varies with different proteins; gelatin gives a pinkish-purple color.
5. Opalescence could occur in the final solution if high levels of lipid or carbohydrate are present.
6. Not an absolute method: color must be standardized against known protein (e.g., BSA) or against the Kjeldahl nitrogen method.

## 9.2.5 Lowry Method

### *9.2.5.1 Principle*

The Lowry method (20, 21) combines the **biuret reaction** with the reduction of the **Folin–Ciocalteau phenol reagent** (phosphomolybdic-phosphotungstic acid) by **tyrosine** and **tryptophan** residues in the proteins (Fig. 9-3). 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 (22) and Hartree (23) to improve the linearity of the color response to protein concentration.




**9-3 figure** Side chains of amino acids tyrosine between *C* and $H_2$ (**a**) and tryptophan between *C* and $H_2$, and between *N* and *H* (**b**).

#### 9.2.5.2 Procedure

The following procedure is based on the modified procedure of Hartree (23):

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 and incubated at room temperature for 10 min.
3. $CuSO_4$-K Na Tartrate-NaOH solution is added after cooling and incubated at room temperature for 10 min.
4. Freshly prepared Folin 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.

#### 9.2.5.3 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.

Advantages:

1. Very sensitive
   (a) 50–100 times more sensitive than biuret method
   (b) 10–20 times more sensitive than 280-nm UV absorption method (described below)
   (c) Similar sensitivity as Nesslerization; however, more convenient than Nesslerization
2. Less affected by turbidity of the sample.
3. More specific than most other methods.
4. Relatively simple; can be done in 1–1.5 h.

Disadvantages:

For the following reasons, the Lowry procedure requires careful standardization for particular applications:

1. Color varies with different proteins to a greater extent than the biuret method.
2. Color is not strictly proportional to protein concentration.
3. The reaction is interfered with to varying degrees by sucrose, lipids, phosphate buffers, monosaccharides, and hexoamines.
4. High concentrations of reducing sugars, ammonium sulfate, and sulfhydryl compounds interfere with the reaction.

### 9.2.6 Dye-Binding Methods

#### 9.2.6.1 Anionic Dye-Binding Method

*9.2.6.1.1 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.

$$\text{Protein} + \text{excess dye} \rightarrow \text{Protein} - \text{dye insoluble complex} + \text{unbound soluble dye} \quad [10]$$

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 $\epsilon$-amino group of lysine) and the **free amino terminal group** of the protein (24). The amount of the unbound dye is inversely related to the protein content of the sample (24).

*9.2.6.1.2 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 is 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 Kjeldhal 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.

*9.2.6.1.3 Applications* Anionic dye binding has been used to estimate proteins in milk (25, 26), wheat flour (27), soy products (18), and meats (17). The AOAC approved methods include two dye-binding methods [Method 967.12 using Acid Orange 12 and Method 975.17 using Amido Black (10B) for analyzing proteins in milk]. AACC Method 46–14.02 uses Acid Orange 12 binding for measuring proteins in wheat flour and soy samples (28). An automated Sprint Rapid Protein

Analyzer has been developed by the CEM Company (Matthews, NC) based on the anionic dye-binding method. This automated method requires calibration for each type of food protein determined using other official methods.

Advantages:

1. Rapid (15 min or less), inexpensive, and relatively accurate for analyzing protein content in food commodities.
2. 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 protein nutritive value of the cereal products (29).
3. No corrosive reagents.
4. Does not measure nonprotein nitrogen.
5. More precise than the Kjeldahl method.

Disadvantages:

1. Not sensitive; milligram quantities of protein are required.
2. Proteins differ in basic amino acid content and so differ in dye-binding capacity. Therefore, a calibration curve for a given food commodity is required.
3. Not suitable for hydrolyzed proteins due to binding to N-terminal amino acids.
4. Some nonprotein components bind dye (i.e., starch) or protein (calcium or phosphate) and cause errors in final results. The problem with calcium and heavy metal ions can be eliminated using properly buffered reagent that contains oxalic acid.

### 9.2.6.2 Bradford Dye-Binding Method

*9.2.6.2.1 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 (30). 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.

*9.2.6.2.2 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.

*9.2.6.2.3 Applications* The Bradford method has been used successfully to determine protein content in worts and beer products (31) and in potato tubers (32). This procedure has been improved to measure microgram quantities of proteins (33). 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.

Advantages:

1. Rapid; reaction can be completed in 2 min
2. Reproducible
3. Sensitive; several fold more sensitive than the Lowry method
4. No interference from ammonium sulfate, polyphenols, carbohydrates such as sucrose, or cations such as $K^+$, $Na^+$, and $Mg^{+2}$
5. Measures protein or peptides with molecular mass approximately equal to or greater than 4000 Da

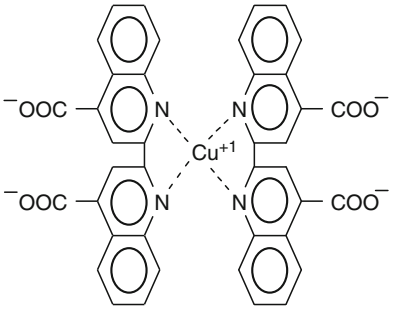
Disadvantages:

1. Interfered with by both nonionic and ionic detergents, such as Triton X-100 and sodium dodecyl sulfate. However, errors due to small amounts (0.1%) of these detergents can be corrected using proper controls.
2. The protein–dye complex can bind to quartz cuvettes. The analyst must use glass or plastic cuvettes.
3. Color varies with different types of proteins. The standard protein must be selected carefully.

## 9.2.7 Bicinchoninic Acid Method

### 9.2.7.1 Principle

Proteins and peptides (as short as dipeptides) reduce **cupric ions** to **cuprous ions** under **alkaline conditions** (34), which is similar in principle to that of the biuret reaction. The cuprous ion then reacts with the apple-greenish **bicinchoninic acid** (BCA) **reagent** to




**9-4 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.)

form a purplish complex (one cuprous ion is chelated by two BCA molecules) (Fig. 9-4). 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.

#### 9.2.7.2 Procedure

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

#### 9.2.7.3 Applications

The BCA method has been used in protein isolation and purification. The suitability of this procedure for measuring protein in complex food systems has not been reported.

Advantages:

1. Sensitivity is comparable to that of the Lowry method; sensitivity of the micro-BCA method (0.5–10 µg) is better than that of the Lowry method.
2. One-step mixing is easier than in the Lowry method.
3. The reagent is more stable than for the Lowry reagent.
4. Nonionic detergent and buffer salts do not interfere with the reaction.
5. Medium concentrations of denaturing reagents (4 *M* guanidine-HCl or 3 *M* urea) do not interfere.

Disadvantages:

1. Color is not stable with time. The analyst needs to carefully control the time for reading absorbance.
2. Any compound capable of reducing $Cu^{+2}$ to $Cu^{+}$ will lead to color formation.
3. Reducing sugars interfere to a greater extent than in the Lowry method. High concentrations of ammonium sulfate also interfere.
4. Color variations among proteins are similar to those in the Lowry method.

### 9.2.8 Ultraviolet 280 nm Absorption Method

#### 9.2.8.1 Principle

Proteins show strong absorption in the region at **ultraviolet** (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.

#### 9.2.8.2 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 equation

$$A = abc \qquad [11]$$

where:

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

#### 9.2.8.3 Applications

The UV 280-nm method has been used to determine the protein contents of milk (35) and meat products (36). 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.

Advantages:

1. Rapid and relatively sensitive; At 280 nm, 100 μg or more protein is required; several times more sensitive than the biuret method.
2. No interference from ammonium sulfate and other buffer salts.
3. Nondestructive; samples can be used for other analyses after protein determination; used very widely in postcolumn detection of proteins.

Disadvantages:

1. Nucleic acids also absorb at 280 nm. The absorption 280 nm/260 nm ratios for pure protein and nucleic acids are 1.75 and 0.5, respectively. One can correct the absorption of nucleic acids at 280 nm if the ratio of the absorption of 280 nm/260 nm is known. Nucleic acids also can be corrected using a method based on the absorption difference between 235 and 280 nm (37).
2. Aromatic amino acid contents in the proteins from various food sources differ considerably.
3. The solution must be clear and colorless. Turbidity due to particulates in the solution will increase absorbance falsely.
4. A relatively pure system is required to use this method.

## 9.3 COMPARISON OF METHODS

- **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.
- **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 method measures a combination of peptide bonds and the amino acids tryptophan and tyrosine. 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.
- **Sensitivity:** Kjeldahl, Dumas, and biuret methods are less sensitive than Lowry, Bradford, BCA, or UV methods.
- **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.
- **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. Also, the Kjeldahl method is specified to correct for protein content in an official method to measure the fiber content of foods (see Chap. 10, Sect. 10.5). Melamine, a toxic nitrogen adulterant, is included in the total nitrogen content if measured by the Kjeldahl or Dumas methods.

## 9.4 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 (9).
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 with 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 method with a 6.25 conversion factor usually 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.

## 9.5 SUMMARY

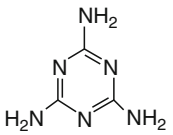
Methods based on the unique characteristics of proteins and amino acids have been described to determine the protein content of foods. The Kjeldahl and Dumas methods measure nitrogen. Infrared spectroscopy is based on absorption of a wavelength of infrared radiation specific for the peptide bond. Copper–peptide bond interactions contribute to the analysis by the biuret and Lowry methods. Amino acids are involved in the Lowry, dye-binding, and UV 280 nm methods. The BCA method 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 (e.g., Kjeldahl).

## 9.6 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. How can Nesslerization or the procedure that uses phenol and hypochlorite be used as part of the Kjeldahl procedure, and why might they be best for the analysis?
5. Differentiate and explain the chemical basis of the following techniques that can be used to quantitate proteins in quality control/research:
   (a) Kjeldahl method
   (b) Dumas method (N combustion)
   (c) Infrared spectroscopy
   (d) Biuret method
   (e) Lowry method
   (f) Bradford method
   (g) Bicinchoninic acid method
   (h) Absorbance at 280 nm
   (i) Absorbance at 220 nm
6. Differentiate the principles of protein determination by dye binding with an anionic dye such as Amido Black vs. with the Bradford method, which uses the dye Coomassie Blue G-250.
7. 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.
8. 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
9. 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.

## 9.7 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.

### Answers

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

Calculations:

$$\%\,\mathrm{N} = N\,\mathrm{HCl} \times \frac{\text{Corrected acid volume}}{\text{g of sample}} \times \frac{14\,\text{g N}}{\text{mol}} \times 100 \quad [6]$$

where:

$N$HCl = normality of HCl, in mol/1000 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

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

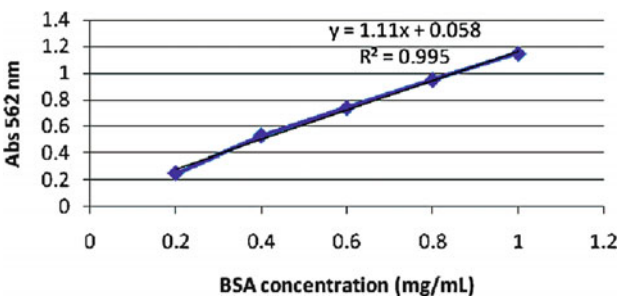
%N for Sample 2

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

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 contain 8% moisture, 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) vs. BSA protein concentration (*x*-axis, mg/ml)) using the data above. Determine the equation of the line ($y = 1.11x + 0.058$), 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.



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