⟨1057⟩ BIOTECHNOLOGY-DERIVED ARTICLES—TOTAL PROTEIN ASSAY

Delete the following:

▲This chapter provides guidance and procedures used for characterization of biotechnology-derived articles. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are provided in *Biotechnology-Derived Articles—Amino Acid Analysis* ⟨1052⟩, *Capillary Electrophoresis* ⟨1053⟩, *Biotechnology-Derived Articles—Isoelectric Focusing* ⟨1054⟩, *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩.▲ (USP 1-Aug-2019)

Change to read:

INTRODUCTION

▲A number of factors needs to be considered when choosing a procedure for the measurement of total protein content in a pharmacopeial preparation. Those factors include the complexity of the sample, constraints that impact the procedure, the required accuracy/variability of the procedure, and the desired analyst contact or automation associated with the procedure (e.g., sample availability, analysis time). The choice of a total protein measurement procedure balances these and other factors to meet the application need.

An analytical separation technique (e.g., HPLC, capillary electrophoresis) may be the best option as a total protein measurement procedure when analyzing a complex sample. Separating the sample into various components and integrating relevant peaks on a chromatogram or electropherogram can be used to manage 1) sample components that interfere with spectrophotometric protein measurements; 2) a mixture of proteins or peptides in the sample, including excipients (e.g., human serum albumin), where the individual protein components need to be quantitated; 3) other sample attributes that are being assessed (e.g., identity, impurity quantitation) during the analysis; 4) a limited sample quantity available for analysis; or 5) the balance between analysis time, instrument complexity, and other factors that favor a separation procedure over a spectrophotometric procedure.

Amino acid quantitation may also be suitable for quantitation of total protein and is often used as a primary method of calibration for other total protein measurement procedures (e.g., determination of an extinction coefficient used with protein absorbance at 280 nm).

Spectrophotometric procedures for total protein measurement are often employed for the analysis of pharmacopeial preparations due to their simplicity, high sample throughput for a given analysis time, and low cost.▲ (USP 1-Aug-2019)

Many of the total protein assay methods described below can be performed successfully using kits from commercial sources. ▲▲ (USP 1-Aug-2019)

Add the following:

▲SAMPLE EXTRACTION METHODS FOR TOTAL PROTEIN ASSAYS

Accurate measurement of total protein cannot always be accomplished if there are interfering substances in the sample. This interference can be observed with spectrophotometric methods of total protein measurement. In order to accurately measure the total protein content in samples with these interfering substances, a method to remove these substances needs to be part of the procedure and the total procedure (extraction in combination with a measurement method) should be validated as suitable for its intended purpose.

It is possible that an interfering substance exhibits a known reproducible effect on the assay that can be eliminated by using an appropriate blank containing the interfering substance to calibrate the spectrophotometer to zero. As an alternative, a spike control added to the *Test solution* or sample matrix can be used to adjust the test result based on the spike recovery.

Sample extraction techniques often involve precipitating protein from the sample, leaving the interfering substances in the liquid phase that is subsequently removed, and then solubilizing the precipitated protein in the assay buffer for analysis. Precipitating protein from the sample can also have the advantage of concentrating proteins from a dilute solution, thereby allowing a sample that was too dilute to use with a particular spectrophotometric procedure to now be within the useful range of the method. Gel filtration procedures to remove an interfering substance can result in sample dilution which should also be considered when developing the method of analysis. With all techniques, volumes of solutions used must be measured quantitatively to allow accurate calculation of concentration and dilution factors that are used in the final calculation of protein concentration in the starting sample.

The use of an extractive procedure may introduce a sample preparation error and affect the precision of the protein concentration result. When an extraction procedure is performed on *Test solutions*, it should also be performed for *Standard solutions*, system suitability solutions, and the *Blank*. Samples should be homogeneous and free of particles. Examples of several possible sample extraction procedures, depending upon the nature of the interfering substance, are described below but others may be demonstrated as suitable for use too.

Procedure 1—Protein Precipitation with Acetone

1. Add acetone to the sample to bring the concentration of acetone to 85%–90%.
2. Let the sample stand for 1 h. If necessary for appropriate recovery, hold the sample at 4° overnight.
3. Centrifuge the sample at 14,000 × g or greater for 10 min. Discard the supernatant.

4. Dissolve the pellet in the appropriate buffer for analysis.

Procedure 2—Protein Precipitation with Trichloroacetic Acid

SODIUM DEOXYCHOLATE REAGENT

Prepare a solution of 1.5 g/L sodium deoxycholate in water.

TCA REAGENT

Prepare a solution of 720 g/L trichloroacetic acid (TCA) in water.

PROCEDURE

1. Add 0.1 mL of *Sodium deoxycholate reagent* to 1.0 mL of *Test solution*.
2. Mix using a vortex mixer, and then incubate for 10 min.
3. Add 0.1 mL of *TCA reagent*, and mix on a vortex mixer.
4. Centrifuge at a minimum of 6700 × g for 30 min.
5. Discard the supernatant.
6. Dissolve the protein pellet in 1.0 mL of the appropriate buffer for analysis.

Procedure 3—Solid Phase Device Extraction

BUFFER PREPARATION

Prepare the appropriate buffer or solvent mixture depending upon the solid phase extraction (SPE) device being used for fractionation.

PROCEDURE

1. Load the sample onto the SPE device.
2. Wash with a suitable buffer or solvent mixture.
3. Elute the protein with an appropriate buffer or solvent mixture.

Procedure 4—Column Gel Filtration

BUFFER PREPARATION

Prepare the appropriate buffer for the test method.

PROCEDURE

The protein is purified using either gravity or a centrifugal procedure per the manufacturer's instructions.[1] ▲ (USP 1-Aug-2019)

Change to read:

▲TOTAL PROTEIN MEASUREMENT PROCEDURES

Ideally, the protein in the *Standard solution* that is used to create the calibration curve should be the same protein as in the *Test solution*. This may not be practical and the use of a generic protein in the *Standard solution* may be substituted. Bovine serum albumin is often used because it is readily available and can be purchased at high purity. However, using a generic protein to create the standard curve may not be the ideal choice for all assays depending on the principle of the assay and the protein structure responsible for the response. Due to the potential variation in responses between different proteins that may be used to create the standard curve, the protein used for the standard curve should always be reported. In addition (unless otherwise specified in the individual monograph) prepare the Reference Standard or reference material for the protein under test in the same buffer used to prepare the *Test solution*.▲ (USP 1-Aug-2019)

Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of *Method 1*. ▲▲ (USP 1-Aug-2019)If the buffer used to ▲solubilize▲ (USP 1-Aug-2019) the protein has a high absorbance relative to ▲▲ (USP 1-Aug-2019) water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. The results may be compromised if the

[1] Suitable commercial columns are available. For example, PD-10 columns (available from GE Healthcare) for samples ranging from 1.0–2.5 mL, NAP-10 columns for samples up to 1.0 mL, NAP-5 columns for samples up to 0.5 mL, or another suitable alternative.

interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation.

▲To determine whether the protein sample is contaminated with nucleic acid, a 260/280 ratio is recommended if no other methods are used to quantitate the nucleic acids. *Table 1* can be used as a guide for protein purity.

Table 1. 260/280 Ratio for Estimating Nucleic Acid Contamination and Protein Purity of the Sample

Protein (%)	Nucleic Acid (%)	260/280 Ratio
100	0	0.57
95	5	1.06
90	10	1.32
70	30	1.73▲ (USP 1-Aug-2019)

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2–2 mg/mL.

STANDARD SOLUTION

Unless otherwise specified in the individual monograph, prepare a solution of Reference Standard or reference material for the protein under test in the same buffer and at the same concentration as the *Test solution*.

PROCEDURE

Concomitantly determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Standard solution* and *Test solution* in quartz cells at a wavelength of 280 nm with a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩), using the buffer as the *Blank*. ▲ [NOTE—Keep the *Test solution*, *Standard solution*, and buffer at the same temperature during testing.]▲ (USP 1-Aug-2019) To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

LIGHT SCATTERING

The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the ▲*Test solution*.▲ (USP 1-Aug-2019)If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250–300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light scattering, determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Test solution* at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light scattering at 280 nm. Subtract the absorbance due to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-µm porosity or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

CALCULATIONS

Calculate the concentration, C_U, of protein in the test specimen by the formula:

$$\text{Result} = C_S(A_U/A_S)$$

in which C_S is the concentration of the *Standard solution*; and A_U and A_S are the corrected ▲absorbance values▲ (USP 1-Aug-2019) of the *Test solution* and the *Standard solution*, respectively (see ⟨857⟩).

Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdic–tungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. ▲Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.▲ (USP 1-Aug-2019)

The effect of interfering substances can be minimized by dilution, provided the concentration of the protein under test remains sufficient for accurate measurement. ▲Alternatively, procedures described in *Sample Extraction Methods for Total Protein Assays* could be used to remove interfering substances from the test sample.

The Lowry assay can be used for protein concentrations of 5–100 µg/mL. Wider ranges may be acceptable if the range of standard concentrations selected results in a linear curve.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations between 5 and 100 µg of protein per mL, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*. An appropriate buffer will produce a pH in the range of 10.0–10.5.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

REAGENTS AND SOLUTIONS

Copper sulfate reagent: Dissolve 100 mg of cupric sulfate and 200 mg of sodium tartrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.
SDS solution: Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.
Sodium hydroxide solution: Dissolve 3.2 g of sodium hydroxide in water, dilute with water to 100 mL, and mix.
Alkaline copper reagent: Prepare a mixture of *Copper sulfate reagent, SDS solution,* and *Sodium hydroxide solution* (1:2:1). This reagent may be stored at room temperature for up to 2 weeks.
Diluted Folin-Ciocalteu's phenol reagent: Mix 10 mL of Folin-Ciocalteu's phenol TS with 50 mL of water. Store in an amber bottle at room temperature.

PROCEDURE

To 1 mL of each *Standard solution*, the *Test solution*, and the *Blank*, add 1 mL of *Alkaline copper reagent*, and mix. Allow to stand at room temperature for 10 min. Add 0.5 mL of the *Diluted Folin-Ciocalteu's phenol reagent* to each solution, mix each tube immediately, ▲and hold at room temperature for about 30 min.

[NOTE—Color development reaches a maximum in 20–30 min during incubation at room temperature, after which there is a gradual loss of color.]

Determine the absorbance values of the *Standard solutions* and *Test solution* at 750 nm with a suitable spectrophotometer, using the solution from the *Blank* to set the instrument to zero (see ⟨857⟩).▲ (USP 1-Aug-2019)

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the ▲absorbance values▲ (USP 1-Aug-2019) of the solutions from the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test solution*, determine the concentration of protein in the *Test solution*.

▲▲ (USP 1-Aug-2019)

Method 3

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the Brilliant Blue G dye binds to protein. The Brilliant Blue G dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins. ▲There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 100 µg–1 mg/mL of protein, the concentrations being evenly spaced.

TEST SOLUTION

▲Solubilize▲ (USP 1-Aug-2019) a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

COOMASSIE REAGENT

Dissolve 100 mg of Brilliant Blue G[2] in 50 mL of alcohol. [NOTE—Not all dyes have the same Brilliant Blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman #1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [NOTE—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

PROCEDURE

Add 5 mL of the *Coomassie reagent* to 100 µL of each *Standard solution*, the *Test solution*, and the *Blank,* and mix by inversion. Avoid foaming, which will lead to poor reproducibility. ▲Incubate at room temperature for a suitable period of time, and then determine the absorbance values▲ (USP 1-Aug-2019) of the solutions from the *Standard solutions* and *Test solution* at 595 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument to zero. [NOTE—Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same. ▲For consistent and accurate results, the absorbance values for all *Standard solutions*, *Blank*, and *Test solutions* should be determined within a suitable period of time to assure reproducible results of adding the *Coomassie reagent*.▲ (USP 1-Aug-2019)]

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] ▲Plot the absorbance values of the *Standard solutions* versus the protein concentrations, and use linear regression to establish the standard curve. Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

Method 4

This method, commonly referred to as the bicinchoninic acid (BCA) assay, is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{1+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement. ▲If substances that will cause interference in the test are present, proceed as directed in *Sample Extraction Methods for Total Protein Assays*. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲solubilize▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 10–1200 µg/mL of protein, the concentrations being evenly spaced.

TEST SOLUTION

Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

REAGENTS

BCA reagent: Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium bicarbonate in water. Adjust, if necessary, with sodium hydroxide or sodium bicarbonate to a pH of 11.25. Dilute with water to 1 L, and mix.
Copper sulfate reagent: Dissolve about 2 g of cupric sulfate in water to a final volume of 50 mL.
Copper–BCA reagent: Mix 1 mL of *Copper sulfate reagent* and 50 mL of *BCA reagent*.

[2] Dye purity is important in the reagent preparation. Serva Blue G (Crescent Chemical Company, ▲Islandia,▲ (USP 1-Aug-2019) NY) is an acceptable grade.

PROCEDURE

Mix 0.1 mL of each *Standard solution*, the *Test solution*, and the *Blank* with 2 mL of the *Copper–BCA reagent*. Incubate the solutions at 37° for 30 min, note the time, and allow ▲the mixtures▲ (USP 1-Aug-2019) to ▲cool▲ (USP 1-Aug-2019) to room temperature. Within 60 min following the incubation time, determine the ▲absorbance values for▲ (USP 1-Aug-2019) the *Standard solutions* and the *Test solution* ▲▲ (USP 1-Aug-2019) at 562 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to set the instrument ▲calibration▲ (USP 1-Aug-2019) to zero. The color intensity continues to increase gradually after the solutions are cooled to room temperature. ▲▲ (USP 1-Aug-2019)

CALCULATIONS

[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] ▲Plot the absorbance values of the solutions from the *Standard solutions* versus the protein concentrations and determine the standard curve best fitting the plotted points. Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

Method 5

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu^{2+}) ion with protein in an alkaline solution and the ▲▲ (USP 1-Aug-2019) development of absorbance at 545 nm. ▲This test shows minimal difference between IgG and albumin samples at the same concentration. Addition of the sodium hydroxide and the *Biuret reagent* as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the *Biuret reagent* will give IgG samples a higher response than albumin samples. The trichloroacetic acid method (described in *Procedure 2* of *Sample Extraction Methods for Total Protein Assays*) used to minimize the effects of interfering substances can also be used to determine the protein content in test specimens at concentrations below 500 µg/mL.▲ (USP 1-Aug-2019)

STANDARD SOLUTIONS

▲Prepare the▲ (USP 1-Aug-2019) Reference Standard or reference material for the protein under test in ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution. ▲▲ (USP 1-Aug-2019) Dilute portions of this solution with ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution ▲▲ (USP 1-Aug-2019) to obtain NLT 3 *Standard solutions* having concentrations of 0.5–10 mg/mL, the concentrations being evenly spaced. ▲▲ (USP 1-Aug-2019)

TEST SOLUTION

Prepare a solution of the test protein in ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution ▲▲ (USP 1-Aug-2019) having a concentration within the range of the concentrations of the *Standard solutions.*

BLANK

Use ▲0.9%▲ (USP 1-Aug-2019) sodium chloride solution. ▲▲ (USP 1-Aug-2019)

BIURET REAGENT

Dissolve about 3.46 g of cupric sulfate in 10 mL of hot water, and allow to cool (Solution 1). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of sodium carbonate in 80 mL of hot water, and allow to cool (Solution 2). Mix Solution 1 and Solution 2, and dilute with water to 200 mL. The *Biuret reagent* is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

PROCEDURE

To 1 volume of ▲▲ (USP 1-Aug-2019) the *Test solution* add an equal volume of ▲6%▲ (USP 1-Aug-2019) sodium hydroxide solution ▲▲ (USP 1-Aug-2019) and mix. Immediately add a volume of *Biuret reagent* equivalent to 0.4 of the volume of the *Test solution*, and mix. Allow to stand at a temperature between 15°–25° for NLT 15 min. Within 90 min after the addition of the *Biuret reagent*, determine the ▲absorbance values▲ (USP 1-Aug-2019) of the *Standard solutions* and the solution from the *Test solution* at 545 nm with a suitable spectrophotometer (see ⟨857⟩), using the *Blank* to ▲calibrate▲ (USP 1-Aug-2019) the instrument to zero. [NOTE—Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

CALCULATIONS

▲Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear. Plot the absorbance values of the *Standard solutions* versus the protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the line.▲ (USP 1-Aug-2019) A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. ▲Determine the concentration of protein in the *Test solution* from the standard curve and the absorbance of the *Test solution*.▲ (USP 1-Aug-2019)

Method *6*

This fluorometric method is based on the derivatization of the protein with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH_2-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the OPA reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with OPA and must be avoided or removed. Ammonia at high concentrations will react with OPA as well. The fluorescence obtained when amine reacts with OPA can be unstable. The use of automated procedures to standardize ▲the analysis▲ (USP 1-Aug-2019) may improve the accuracy and precision of the test.

STANDARD SOLUTIONS

Unless otherwise specified in the individual monograph, ▲prepare▲ (USP 1-Aug-2019) the Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test solution*. Dilute portions of this solution with the same buffer to obtain NLT 5 *Standard solutions* having concentrations of 10–200 µg/mL of protein, the concentrations being evenly spaced.

TEST SOLUTION

▲Solubilize▲ (USP 1-Aug-2019) a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard solutions*.

BLANK

Use the buffer used to prepare the *Test solution* and the *Standard solutions*.

REAGENTS

Borate buffer: Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1 L, and mix.
Stock OPA reagent: Dissolve about 120 mg of OPA in 1.5 mL of methanol, add 100 mL of *Borate buffer*, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.
OPA reagent: To 5 mL of *Stock OPA reagent* add 15 µL of 2-mercaptoethanol. Prepare at least 30 min prior to use. This reagent is stable for 1 day.

PROCEDURE

Adjust each of the *Standard solutions* and the *Test solution* to a pH between 8▲.0▲ (USP 1-Aug-2019) and 10.5. Mix 10 µL of the *Test solution* and each of the *Standard solutions* with 100 µL of *OPA reagent*, and allow to stand at room temperature for 15 min. Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see *Fluorescence Spectroscopy* ⟨853⟩), determine the fluorescent intensities of solutions from the *Standard solutions* and the *Test solution* at an excitation wavelength of 340 nm and an emission wavelength between 440–455 nm. [NOTE—The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

CALCULATIONS

The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. ▲Determine the concentration of protein in the *Test solution* from the standard curve and the fluorescence of the *Test solution*.▲ (USP 1-Aug-2019)

Method *7*

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test specimen can affect the determination of protein by this method ▲since non-proteinaceous nitrogen will also be detected.▲ (USP 1-Aug-2019) Nitrogen analysis techniques destroy the protein under test and are not ▲stand-alone methods since they quantitate all nitrogen present. If either *Procedure 1* or *Procedure 2* below is used to determine protein content, then a suitable orthogonal method must also be used to verify that no other nitrogen-containing sources are present and contributing to the apparent total protein content.▲ (USP 1-Aug-2019)

PROCEDURE 1

Determine the nitrogen content of the protein under test as directed under *Nitrogen Determination* ⟨461⟩. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

PROCEDURE 2

Commercial instrumentation is available for nitrogen ▲(elemental)▲ (USP 1-Aug-2019) analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°), which produces nitric oxide (NO) and similar oxides of nitrogen (NO_x) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material or reference standard that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

CALCULATIONS

The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitrogen content of the Reference Standard or reference material.