

2017

U.S. Pharmacopeia
National Formulary

USP 40 NF 35

**USP 40 Published General Chapter
<507> *Protein Determination Procedures***

The official version can be found in the *USP–NF*.
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Official: August 1, 2017

(507) PROTEIN DETERMINATION PROCEDURES

INTRODUCTION

Several procedures exist to determine the total protein in pharmaceutical drug substances and products. Procedures may differ based on the physical characteristic of the protein, which serves as the basis of the measurement principle (e.g., absorbance of ultraviolet light by aromatic amino acid residues or by dye binding). Understanding the protein structure and sample matrix is important when selecting a total protein quantitation procedure. Users must verify that the chosen validated method below is suitable for their specific purposes (see [Verification of Compendial Procedures \(1226\)](#)).

PROCEDURE

Two options for Method I are presented: *Method IA*, using denaturing conditions; and *Method IB*, using native conditions. Depending on the protein structure and the nature of the protein sample (e.g., denaturing conditions may be more suitable for a strong coiled structure with most aromatic amino acids in the interior of the protein), one method may prove superior to the other, and users should select which is the most suitable method based on their protein and verify, as appropriate. At a minimum, both methods are dependent on the following conditions being met: 1) the amino acid sequence and molecular weight of the protein must be known; and 2) the protein must contain tyrosines or tryptophans, and no other chromophores (other than cystines) that absorb near 280 nm (including nucleic acids and diluent components) should be present.

● METHOD IA. ULTRAVIOLET LIGHT ABSORBANCE UNDER DENATURING CONDITIONS

Sample buffer A: Prepare 20 mM sodium phosphate, pH 6.5 by dissolving 0.24 g of sodium dihydrogen phosphate in about 80 mL of water. Adjust the pH with sodium hydroxide solution (10% w/w), and fill with water to 100 mL. Filter.

Sample buffer B: Prepare 7 M guanidine hydrochloride and 20 mM sodium phosphate, pH 6.5 by dissolving 0.24 g of sodium dihydrogen phosphate and 66.87 g of guanidine hydrochloride in about 50 mL of water. Adjust with sodium hydroxide solution (10% w/w), and fill with water to 100 mL. Filter.

Reference solution: Prepare 6 M guanidine hydrochloride in *Sample buffer A* by mixing 330 μ L of *Sample buffer A* and 2000 μ L of *Sample buffer B*.

Sample preparation A: Dilute the test sample by adding *Sample buffer A* and *Sample buffer B* to achieve a final concentration of 6 M guanidine hydrochloride and an absorbance of about 0.4–0.6. [NOTE—Gravimetric dilutions can be used and sometimes improve accuracy.] Prepare at least three replicates per test sample.

Analysis

Samples: *Sample buffer A*, *Sample buffer B*, *Reference solution*, and *Sample preparation A*

Determination of the protein concentration in denaturing buffer: Using a suitable spectrophotometer (see [Ultraviolet-Visible Spectroscopy \(857\)](#)), measure the absorbance of *Sample preparation A* in 1-cm cuvettes at an optical density of 280 nm against the *Reference solution*, and subtract the absorbance value at 330 nm to obtain $A_{corrected}$.

Calculate the protein concentration (c) in the test sample:

$$c = A_{corrected} \times DF \times MW / (\epsilon_{280\text{ nm}} \times 1\text{ cm})$$

$A_{corrected}$ = absorbance at 280 nm minus the absorbance at 330 nm

DF = dilution factor

MW = calculated molecular weight of the protein

$\epsilon_{280\text{ nm}}$ = extinction coefficient of the test sample at 280 nm

$$\epsilon_{280\text{ nm}} = (n_{\text{Trp}} \times 5690 + n_{\text{Tyr}} \times 1280 + n_{\text{Cys}} \times 120) \text{ M}^{-1} \times \text{cm}^{-1}$$

n_{Trp} = number of tryptophans

n_{Tyr} = number of tyrosines

n_{Cys} = number of cystines

System suitability

The test sample UV spectra must show a maximum between 270 and 285 nm and must show a minimum between 243 and 257 nm. The measured absorbance at 280 nm must be NLT 0.4 and NMT 0.6. The relative standard deviation (RSD) of the calculated concentration of all replicates must be NMT 3.0%.

● METHOD IB. ULTRAVIOLET LIGHT ABSORBANCE UNDER NATIVE CONDITIONS

Sample buffer A: Prepare 20 mM sodium phosphate, pH 6.5 by dissolving 0.24 g of sodium dihydrogen phosphate in about 80 mL of water. Adjust the pH with sodium hydroxide solution (10% w/w), and fill with water to 100 mL. Filter.

Sample preparation A: Dilute the test sample by adding *Sample buffer A* to achieve an absorbance of about 0.4–0.6. [NOTE—Gravimetric dilutions can be used and sometimes improve accuracy.] Prepare at least three replicates per test sample.

Analysis

Samples: *Sample buffer A* and *Sample preparation A*

Determination of the protein concentration in native buffer: Using a suitable spectrophotometer (see [\(857\)](#)), measure the absorbance of *Sample preparation A* in 1-cm cuvettes at 280 nm against *Sample buffer A* and subtract the absorbance value at 330 nm to obtain $A_{\text{corrected}}$.

Calculate the protein concentration (c) of the test sample:

$$c = A_{\text{corrected}} \times DF \times MW / (\epsilon_{280\text{ nm-native2}} \times 1\text{ cm})$$

$A_{\text{corrected}}$ = absorbance at 280 nm minus the absorbance at 330 nm

DF = dilution factor

MW = calculated molecular weight of the protein

$\epsilon_{280\text{ nm-native2}}$ = extinction coefficient of the test sample at 280 nm

where the extinction coefficient $\epsilon_{280\text{ nm-native2}}$ is calculated as:

$$\epsilon_{280\text{ nm-native2}} = (n_{\text{Trp}} \times 5500 + n_{\text{Tyr}} \times 1490 + n_{\text{Cys}} \times 125) \text{ M}^{-1} \times \text{cm}^{-1}$$

n_{Trp} = number of tryptophans

n_{Tyr} = number of tyrosines

n_{Cys} = number of cystines

System suitability

The test sample UV spectra must show a maximum between 270 and 285 nm and must show a minimum between 243 and 257 nm. The measured absorbance at 280 nm must be NLT 0.4 and NMT 0.6. The RSD of the calculated concentration of all replicates must be NMT 3.0%.

● METHOD II. BICINCHONIC ACID METHOD

BCA reagent solution:¹ Prepare a solution of 10 g/L of bicinchoninic acid (BCA), 20 g/L of sodium carbonate monohydrate, 1.6 g/L of sodium tartrate, 4 g/L of sodium hydroxide, and 9.5 g/L of sodium bicarbonate in water to a pH of 11.25.

Copper sulfate reagent solution: Prepare a solution of 40 g/L of cupric sulfate pentahydrate in water.

BCA working reagent solution: Mix 50 volumes of *BCA reagent solution* with 1 volume of *Copper sulfate reagent solution*. Mix until the green solution is clear.

Sample dilution buffer: Water or another suitable, non-interfering buffer

Standard solutions: Ideally, use the USP Reference Standard for the protein under test. If the protein of interest is unknown, is a mixture, or is a specific USP Reference Standard that is unavailable, use USP BSA for Protein Quantitation RS. Dilute portions of this solution with the *Sample dilution buffer* to obtain NLT five evenly spaced *Standard solutions* in triplicate. The selected *Standard solution* concentrations should result in a linear curve, typically in the range of 25–1000 µg/mL of protein.

Suitability standard solution: Prepare an independent dilution of the same protein used to make the *Standard solutions* above using *Sample dilution buffer* to obtain a *Suitability standard solution* with a concentration near the midpoint of the standard curve. Prepare the *Suitability standard solution* in triplicate.

Sample solution: Prepare a *Sample solution* with an expected concentration within the standard curve by diluting with the *Sample dilution buffer*. Prepare triplicate samples.

Analysis

Samples: *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution*

Accurately transfer 100 µL of *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* to individual test tubes. [NOTE—96-well, plate-based, and automated methods can also be used with suitable volume adjustments to the procedure below.] Add 2.0 mL of *BCA working reagent solution* to each tube, and mix well.

Cover and incubate the solutions at $37 \pm 2^\circ$ for 30 ± 1 min, then allow the samples to cool to room temperature for NLT 5 min and NMT 60 min. Determine the absorbances of the solutions at 562 nm with a suitable spectrophotometer (see [\(857\)](#)). Autozero the instrument with the *Sample dilution buffer* sample.

Measure the absorbance of all *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples within 10 min.

Calculations: Plot the absorbances of the *Standard solutions* versus the protein concentrations of the *Standard solutions*, and prepare a standard curve using the linear regression method. Use this standard curve and the absorbance of the *Sample solution* to determine the concentration of protein in each *Suitability standard solution* and *Sample solution*.

System suitability

Samples: *Standard solutions* and *Suitability standard solution*

Suitability requirements

Linearity: The coefficient of determination (r^2) for a linear fit of all *Standard solutions* is NLT 0.99.

Relative standard deviation: NMT 5% for triplicate results of the *Suitability standard solution*

Accuracy: The calculated concentration of the *Suitability standard solution* must be within 90%–110% of the theoretical concentration.

Acceptance criteria

Relative standard deviation: NMT 5% for triplicate *Sample solution* results

● METHOD III. BRADFORD METHOD

Sample dilution buffer: Water or another suitable, non-interfering buffer

Standard solutions: Use the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS if the protein of interest is unknown, is a mixture, or is a specific USP

Reference Standard that is unavailable. Dilute portions of this solution with the *Sample dilution buffer* to obtain NLT five evenly spaced *Standard solutions* in triplicate. Two standard curve ranges are provided for use: either 0.1–1.0 mg/mL of protein (see *Analysis* procedure A, below) or 5–25 µg/mL of protein (see *Analysis* procedure B, below).

Suitability standard solution: Dilute the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS with *Sample dilution buffer* to obtain a *Suitability standard solution* having a concentration near the midpoint of the standard curve. Prepare the *Suitability standard solution* in triplicate.

Sample solution: Prepare a *Sample solution* with an expected concentration within one of the two concentration ranges in options A or B below by diluting with the *Sample dilution buffer*. Prepare triplicate samples.

Coomassie reagent solution: Dissolve 100 mg of brilliant blue G² in 50 mL of alcohol. Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman No. 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.]

Analysis: Select one of the two concentration ranges listed below that is suitable for the expected concentrations of the *Sample solutions*.

A. Protein concentration range: 0.1–1.0 mg/mL

Samples: *Sample dilution buffer*, *Standard solutions*, and *Sample solution*

Add 1 mL of the *Coomassie reagent solution* to 20 µL of each *Sample dilution buffer*, *Standard solution*, and *Sample solution* sample. Mix by inversion, avoiding foaming. Incubate 10–30 min. By using a suitable spectrophotometer (see [\(857\)](#)) [NOTE—Do not use quartz (silica) cuvettes.] , use the *Sample dilution buffer* to set the instrument to zero at 595 nm. Autozero the instrument with the *Sample dilution buffer* sample.

Measure the absorbance of all *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples within 60 min.

B. Protein concentration range: 5–25 µg/mL

Samples: *Sample dilution buffer*, *Standard solutions*, and *Sample solution*

Add 0.5 mL of the *Coomassie reagent solution* to 0.5 mL each of *Sample dilution buffer*, *Standard solution*, and *Sample solution* sample. Mix by inversion, avoiding foaming. Incubate 10–30 min. By using a suitable spectrophotometer (see [\(857\)](#)) [NOTE—Do not use quartz (silica) cuvettes.] , use the *Sample dilution buffer* to set the instrument to zero at 595 nm. Autozero the instrument with the *Sample dilution buffer* sample. Measure the absorbance of all *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples within 60 min.

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 absorbances of the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From this standard curve and the absorbance of the *Sample solution*, determine the concentration of protein in the *Sample solution*.

System suitability

Samples: *Standard solutions* and *Suitability standard solutions*

Suitability requirements

Linearity: r^2 for a fit of all *Standard solutions* is NLT 0.99.

Relative standard deviation: NMT 15% for triplicate results of the *Suitability standard solution*

Recovery: The calculated concentration of the *Suitability standard solution* must be within 90%–110% of the theoretical concentration.

Acceptance criteria: %CV of triplicates of the *Sample solution* is NMT 15%.

● **METHOD IV. LOWRY METHOD**

SDS solution: If required, prepare a solution of 50 g/L of sodium dodecyl sulfate (SDS).

Lowry reagent A: Prepare a solution of 10 g/L of sodium hydroxide in water, and prepare a solution of 50 g/L of sodium carbonate anhydrous in water. Mix equal volumes (2 volumes and 2 volumes) of each solution, and dilute with water or *SDS solution*, if required, to 5 volumes. [NOTE—SDS may be added to *Lowry reagent A* when the test protein contains detergents or lipids or is poorly soluble after treatment for interfering substances.]

Lowry reagent B: Prepare a fresh solution of 29.8 g/L of disodium tartrate dihydrate in water. Prepare a solution of 12.5 g/L of cupric sulfate pentahydrate in water. Mix equal volumes (2 volumes and 2 volumes) of each solution, and dilute with water to 5 volumes.

Lowry reagent C: Mix 50 volumes of *Lowry reagent A* with 1 volume of *Lowry reagent B*. [NOTE—Prepare fresh and protect from light.]

Sample dilution buffer: Purified water or other non-interfering buffer

Standard solutions: Use the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS if the protein of interest is unknown, is a mixture, or is a specific USP Reference Standard that is unavailable. Dilute portions of this solution with the *Sample dilution buffer* to obtain NLT five evenly spaced *Standard solutions*. The standard concentrations should be selected to result in a linear curve, typically in the range of 10–200 µg/mL of protein.

Suitability standard solution: Dilute the USP Reference Standard for the protein under test or USP BSA for Protein Quantitation RS with *Sample dilution buffer* to obtain a *Suitability standard solution* having a concentration near the midpoint of the standard curve. Prepare and analyze the *Suitability standard solution* in triplicate.

Sample solution: Prepare a *Sample solution* with an expected concentration within the standard curve by diluting with the *Sample dilution buffer*. Prepare and analyze samples in triplicate.

Diluted Folin–Ciocalteu's phenol reagent: Dilute Folin–Ciocalteu's phenol reagent with water (1:1). Alternative dilutions may be used provided that the pH of the samples (i.e., *Standard solutions* and *Sample solutions* after addition of *Lowry reagent C* and the *Diluted Folin–Ciocalteu's phenol reagent*) is 10.0–10.6.

Analysis

Samples: *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution*

Accurately transfer 1.0 mL of *Sample dilution buffer*, *Standard solutions*, *Suitability standard solution*, and *Sample solution* samples to individual test tubes. To each test tube add 5.0 mL of *Lowry reagent C*. Allow to stand at room temperature for 10 min. Add 0.5 mL of *Diluted Folin–Ciocalteu's phenol reagent* to each test tube, mix immediately, and incubate at room temperature for 30 min. Determine the absorbance of each solution at 750 nm with a suitable spectrophotometer (see [\(857\)](#)). Autozero the instrument with the *Sample dilution buffer*.

Calculations: Plot the absorbances of the *Standard solutions* versus the protein concentrations of the *Standard solutions*, and prepare a standard curve using the linear regression method. Use this standard curve and the absorbances of the *Sample solutions* to determine the concentration of protein in each of the *Sample solution* samples and *Suitability standard solution*.

System suitability

Sample: *Suitability standard solution*

Suitability requirements

Linearity: r^2 for a linear fit of all standards is NLT 0.995.

Relative standard deviation: NMT 5% for triplicate results of the *Suitability standard solution*

Recovery: The calculated concentration of the *Suitability standard solution* must be within 90%–110% of the theoretical concentration.

Acceptance criteria

Relative standard deviation: NMT 5% for triplicate sample results

● METHOD V. AMINO ACID ANALYSIS

Separation and determination of hydrolyzed amino acids by ion-exchange chromatography, followed by postcolumn ninhydrin derivatization, is described for protein samples containing a protein of known molecular weight and amino acid composition. [NOTE—For additional information, see [Biotechnology-Derived Articles—Amino Acid Analysis \(1052\)](#), which may be a helpful but not mandatory resource.]

Hydrolysis solution: 6 N hydrochloric acid

Sample hydrolysate preparations: Prepare a protein sample such that the content of amino acids is within the established linear working range of the procedure. [NOTE—Glassware used for hydrolysis must be clean.] Place suitable volumes of the sample and *Hydrolysis solution* in a hydrolysis tube. [NOTE—Common volumes range from 100 to 500 μ L for the sample and from 1 to 2 mL for the *Hydrolysis solution* such that complete protein digestion is demonstrated within the hydrolysis incubation time.] Cool the mixture until solidification, and evacuate until the pressure is <0.2 bar. Flame-seal the hydrolysis tube in vacuum by melting the neck of the tube. [NOTE—Alternative commercially available hydrolysis vials also can be used.] Hydrolyze at 110° for 22 h. Allow the tube to cool, and remove the solvent by centrifugal vacuum concentrator.

Chromatography

Solution A: Sodium citrate buffer (0.2 N in sodium), containing 2.0% trisodium citrate dihydrate, 0.1% phenol, 0.2% thiodiglycol, and 2.0% propan-2-ol. Adjust with concentrated hydrochloric acid to a pH of 3.2.

Solution B: Sodium citrate buffer (0.2 N in sodium), containing 2.0% trisodium citrate dihydrate and 0.1% phenol. Adjust with concentrated hydrochloric acid to a pH of 4.25.

Solution C: Sodium citrate buffer (1.2 N in sodium), containing 2.0% trisodium citrate dihydrate, 0.1% phenol, and 5.8% sodium chloride. Adjust with concentrated hydrochloric acid to a pH of 6.45.

Solution D: 0.4 M sodium hydroxide containing 0.1% ethylenediaminetetraacetic acid

Mobile phase: See [Table 1](#). Equilibrate the system before each run.

Table 1

Time (min)	Solution (100%)	Column Temperature	Flow, Mobile Phase (mL/h)	Flow, Postcolumn Reagent (mL/h)
0	A	49°	35	25
8.5	A	49°	35	25
8.51	B	51°	35	25
23.0	B	51°	35	25
23.01	C	51°	35	25

Time (min)	Solution (100%)	Column Temperature	Flow, Mobile Phase (mL/h)	Flow, Postcolumn Reagent (mL/h)
24.0	C	51°	35	25
24.01	C	95°	35	25
41.5	C	95°	35	25
41.51	D	95°	35	25
45.5	D	95°	35	25
45.51	A	49°	35	25
49.0	A	49°	35	25
49.01	50% Isopropanol	49°	0	0
51.0	50% Isopropanol	49°	0	0
51.01	A	49°	44	0
62.0	A	49°	44	0
62.01	A	49°	35	25
64.0	A	49°	35	25

Loading buffer: Sodium citrate buffer (0.2 N in sodium), containing 2.0% trisodium citrate dihydrate, 0.1% phenol, and 2.0% thiodiglycol. Adjust with concentrated hydrochloric acid to a pH of 2.2.

Postcolumn reagent

Solution 1: Solution containing 7.6% acetic acid, 47.7% ethylene glycol, 28.7% water, and 16.0% potassium acetate

Solution 2: Solution containing 9% methanol, 8% ninhydrin, 46.4% diethylene glycolmethyl ether, 36% ethylene glycol, and 0.6% hydrindantin. Prepare *Postcolumn reagent* by mixing 1.75 L of *Solution 1* and 0.25 L of *Solution 2*. Use nitrogen sparging during preparation and mixing. Keep the solution under nitrogen pressure.

Standard solution: Prepare a solution having known equimolar amounts of L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine with half the equimolar amount of L-cysteine. [NOTE—Suitable concentrations are 250 and 125 nmol/mL, respectively.]

Sample solution: Reconstitute the *Sample hydrolysate preparations* in a suitable volume of *Loading buffer*.

Blank: *Hydrolysis solution* that has been taken through analytical steps including hydrolysis

Chromatographic system

(See [Chromatography \(621\), System Suitability.](#))

Mode: LC

Detector: 440 and 570 nm

Columns

Prewash: 4.6-mm × 10-cm; 20-µm sodium form, packing L58

Analytical: 4.6-mm × 20-cm; 8-μm sodium form, packing L58

Injection volume: 40 μL, at 1 min with baseline reset

Postcolumn reaction: As the amino acids are eluted from the column, *Postcolumn reagent* is added at a rate of 25 mL/h. After mixing, the column effluent and the *Postcolumn reagent* are passed through a reaction coil of approximately 10-m × 0.3-mm PTFE tubing at a temperature of approximately 135°, where a purple color is developed.

Reaction coil temperature: 135°

System suitability

Sample: *Standard solution*

Suitability requirements: All 17 amino acid peaks must be visible in the *Standard solution*.

Resolution: NLT 1.2 between the pairs of L-threonine and L-serine, L-cystine and L-valine, and L-isoleucine and L-leucine

Tailing factor: 0.8–1.5 for the peak of L-aspartic acid

Relative standard deviation: NMT 2.0% determined from the L-aspartic acid peak

Analysis

Samples: *Blank, Standard solution, and Sample solution*

Record and measure the responses for each amino acid peak in the *Standard solution* and *Sample solution*. Use the responses obtained from the *Standard solution* to calculate the content of each amino acid in the sample. Analyze the *Blank* to confirm purity of the reagent and diluent.

Calculations: Use well-recovered amino acids to quantify the protein. [NOTE—For additional information, see [Biotechnology-Derived Articles—Amino Acid Analysis \(1052\)](#), [Protein Hydrolysis](#), which may be a helpful but not mandatory resource.] Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. Discard protein content values that deviate >5% from the mean. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample.

- [USP REFERENCE STANDARDS \(11\)](#)

USP BSA for Protein Quantitation RS

¹ Suitable BCA reagents are available from Thermo Scientific catalog #23225 or equivalent.

² Suitable brilliant blue G dye is available from Sigma-Aldrich, catalog #B0770.

Auxiliary Information— Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
General Chapter	Elena Curti	(GCBA2015) General Chapters-Biological Analysis 2015
Reference Standards	RS Technical Services 1-301-816-8129 rstech@usp.org	

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Previously Appeared In:

Pharmacoepial Forum: Volume No. 41(2)