1.0 Purpose

The purpose of this procedure is to describe a method for the quantitative analysis of protein content in raw materials and finished products using the Protein Lowry method. The method is based on the USP monograph <1057> Biotechnology-Derived Articles- Total Protein Assay, method 2.

2.0 Scope

The principle of the Protein Lowry method involves the reaction of protein, cupric sulfate and tartrate in alkaline solution, resulting in formation of tetradentate copper-protein complexes. When Folin-Ciocalteu Reagent is added, it is effectively reduced in proportion to these chelated copper complexes, producing a water-soluble product whose blue color can be measured at 750nm. Some excipients and dietary ingredients used in finished products can interfere with the analysis of protein thus each finished product containing protein with a label claim needs to be verified against interference and/or suppression before using the method. The methods are structured based on the monograph <1057> Biotechnology-Derived Articles- Total Protein Assay, method 2. This method was validated for use with dietary supplements under protocol MV-LAB-14-013.

3.0 Responsibility

3.1 It is the responsibility of QC and Analytical Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **STD** – Standard

4.2 **SAM** – Sample

4.3 **CofA –** Certificate of Analysis

4.4 **NaOH** – Sodium Hydroxide

4.5 **NaCl** – Sodium Chloride

4.6 **SDS** – Sodium Dodecyl Sulfate

4.7 **QC** – Quality Control

5.0 References

5.1 USP monograph <1057> Biotechnology-Derived Articles- Total Protein Assay, method-2

5.2 MV-LAB-14-013, Protocol, Total Protein Determination using the Protein Lowry and Bradford methods coupled with Visible Light Spectroscopy

6.0 Reagents, Supplies and Equipment

6.1 Reagents: general- reagent grade or better

6.1.1 Millipore water

6.1.2 Sodium Chloride (NaCl)

6.1.3 Sodium Hydroxide (NaOH)

6.1.4 Sodium Dodecyl Sulfate (SDS)

6.1.5 Lowry Reagents- Formulated as a kit by Thermo Scientific, Cat# 23240 or equivalent

6.1.6 Protein Standard(s)

6.2 Supplies and Glassware

6.2.1 13mm X 100mm glass test tubes with caps

6.2.2 Scintillation vials

6.2.3 25ml, 50ml, and 100ml volumetric flask

6.2.4 200µL, 1ml and 10ml pipette tips

6.2.5 1.5ml and 2.0ml Eppendorf tubes

6.2.6 Plastic luer lock syringes

6.2.7 Weigh boats

6.2.8 Nylon syringe filters, 0.45µm

6.2.9 Whatman #1 filter paper

6.2.10 Disposable Cuvettes- regular or semi-micro

6.3 Equipment

6.3.1 Lambda 45 Spectrophotometer, Perkin Elmer, with Win-Lab Software or equivalent

6.3.2 Analytical Balance

6.3.3 Vortex

6.3.4 Wrist-Action Shaker

6.3.5 Eppendorf Centrifuge

6.3.6 200µl, 1ml and 10ml pipettes

6.4 Buffers

6.4.1 Lowry Dissolution Buffer (1)- 0.9% NaCl, 0.05M NaOH, 0.1% SDS in Millipore water, prepared by adding 9.0g NaCl, 1.0g SDS and 2.0g NaOH to a 1L volumetric flask and dissolving to 1L with Millipore water. Other final amounts can be made by adjusting all ratios to meet that final volume.

6.4.2 Lowry Dissolution Buffer (2) <for egg protein> 0.9% NaCl and 0.1%SDS in Millipore water, prepared by adding 9.0g NaCl and 1.0g SDS to a 1L volumetric flask and dissolving to 1L with Millipore water. Other final amounts can be made by adjusting all ratios to meet that final volume.

7.0 Sample and Standard preparation

7.1 Standard Certification

7.1.1 For raw materials that do not have a protein standard that is commercially available, a raw material consisting of a similar protein composition can be certified for use as a standard by determining the protein content using nitrogen pyrolysis or other appropriate source on a dry basis providing no other appreciable nitrogen sources are contained within the material.

7.1.2 Finished product protein standards will be generated by mixing in proportion to the formula all protein types. The finished product protein blend will be certified for use as a standard by determining the protein content using nitrogen pyrolysis on a dry basis providing no other appreciable nitrogen sources are contained within the blend.

7.1.3 If, during the validation of the standard a slope is generated in the linear range

that is equivalent to another standard, the standards with matching slopes can be used interchangeably.

7.1.4 Protein standards are certified yearly.

7.2 Standard Preparation

7.2.1 Use the tested purity value from nitrogen pyrolysis on a dry basis or the CofA for the specific protein standard in your calculations. The Stock Standard Preparation reflects 100% content.

Example: Egg White, 81.3% protein standard

Prepare 50 ml of a 1mg/ml solution

1mg/ml X 50ml = 50mg

50mg/0.813 = 61.5mg

Dissolve 61.5mg up to 50ml = 1.0mg/ml

7.2.2 Dry approximately one gram of standard material under vacuum at 105ºC for 2 hours using a scintillation vial. Allow material to cool before weighing.

7.2.3 All Standards are prepared by weighing no less than the limit of the scale used, during that time period and transferring to a volumetric flask of the appropriate volume. Bring up to 2/3's final volume using the appropriate Dissolution Buffer. Vortex for 1 minute to facilitate dissolution. Attach the flask to a wrist action shaker and mix for an additional 15 minutes. Bring standard to final volume before using.

7.2.4 Begin with a 1 mg/ml standard stock. Dilute the standard to a concentration reflective of the estimated protein concentration of the sample using the appropriate Dissolution Buffer.

7.3 Sample Preparation

7.3.1 If samples are being reported on a dry basis, dry approximately one gram of sample material under vacuum at 105ºC for 2 hours in a scintillation vial. Allow material to cool before weighing.

7.3.2 No less than the limit of the scale used, during that time period of material may be weighed.

7.3.3 Weigh an exact amount of material (not corrected for purity) to make a 1 mg/ml sample.

- Other concentrations can be prepared to address solubility or other related issues.

7.3.4 Samples can be dissolved in Dissolution Buffer at any volume starting from 25mL.

7.3.5 All samples are shaken on a wrist action shaker for 15 minutes at RT in two thirds their initial volumes then brought up to final volume.

7.3.6 Typical high protein content samples should be diluted to 0.75mg/ml using Dissolution Buffer for testing. Other concentrations can be used.

7.3.7 The final diluted sample must be filtered or centrifuged before analyzing.

7.3.8 For finished products or raw materials being analyzed for the first time using either method an in process verification is required to demonstrate the absence of interference and/or suppression before quantitation can be implemented.

8.0 Lowry Method for Identification

8.1 Prepare 1X (1N) Folin-Ciocalteu Reagent by diluting supplied 2X (2N) reagent 1:1 with Millipore water by adding 500ul 2X Reagent and 500ul Millipore water to a 2ml Eppendorf tube and vortex.

8.2 Add solution to test tubes as follows:

8.2.1 Add 300ul of each sample or standard into an appropriately labeled test tube.

8.2.2 Add 1.5ml of Modified Lowry Reagent to each test tube. Mix well and incubate each tube at room temperature for exactly 10 minutes.

8.2.3 At the end of the 10-minute incubation period, add 150µl of prepared 1X Folin-Ciocalteu Reagent and vortex to mix the contents.

8.2.4 If a color change is not immediately observed, cover sample mixture and incubate up to 30 minutes.

8.2.5 A color change to blue or purple is acceptable as passing unless otherwise specified.

9.0 Lowry Method for Quantitation

9.1 Parameters

9.1.1 Wavelength= 750nm

9.1.2 Slit width- 2mm

9.1.3 Range- 1µg to 1.5mg

9.2 See Attachment I for dilution schedule

9.3 Prepare 1X (1N) Folin-Ciocalteu Reagent by diluting supplied 2X (2N) reagent 1:1 with Millipore water by adding 500ul 2X Reagent and 500ul Millipore water to a 2ml Eppendorf tube and vortex.

9.4 Add solution to test tubes as follows:

9.4.1 Add 300ul of each sample or standard into an appropriately labeled test tube.

9.4.2 At 15-second intervals, add 1.5ml of Modified Lowry Reagent to each test tube. Mix well and incubate each tube at room temperature for exactly 10 minutes.

9.4.3 Exactly at the end of the 10-minute incubation period, add 150µl of prepared 1X Folin-Ciocalteu Reagent, immediately vortex to mix the contents.

9.4.4 Cover (completely eliminate light exposure) and incubate all tubes at RT for 30 minutes.

9.4.5 Transfer samples into cuvettes.

9.4.6 Measure absorbance at 750nm.

10.0 Data Collection

9.1 Turn on the Lambda 45 UV/VIS Spectrophotometer and allow the bulbs to warm up for at least 15 minutes.

9.2 Open <WinLab> Software.

9.3 Click on <Methods>.

9.4 Open task folder.

9.5 Click on <D-744> Test Method.

9.6 Click on <Run>.

9.7 Choose the number of samples.

9.8 Complete sample spreadsheet to include:

9.8.1 Sample ID

9.8.2 Description

- Type – sample, standard, blank, control

- Concentration

9.8.3 Press <Start> to initiate testing.

9.8.4 Press <OK> to Zero the spectrophotometer.

9.8.5 Place the cuvette(s) in the designated slot(s) on the carousel.

9.8.6 Press <OK> to start data Collection

9.8.7 The results can be previewed by clicking on the <Results> icon.

9.8.8 Save as <New Task>.

- Label file using Date-Sample ID-Concentration format.

9.8.9 Click on the <Output> icon.

9.8.10 Select <Default-Wavelength Program (Rev1)> from the drop down menu.

9.8.11 Print.

9.9 Calculations for determining quantity

9.9.1 The protein concentration in a single dose can be calculated by the following equation when a single point standard is used:

- $\%wt = ((A(ABS_{750sam} / ABS_{750std}))/B)$

A = Standard concentration in mg/mL

B = Sample concentration in mg/mL

ABS_{750sam} = Average absorbance of sample after blank subtraction

ABS_{750std} = Average absorbance of standard after blank subtraction

%wt = The percent protein in the sample

9.9.2 The protein concentration in the sample can be calculated by the following equation when a multipoint standard curve is generated:

- Equation 1: Y = mX, linear regression forced through 0,0

  Equation 2: %wt = mX/B

  Y = Protein concentration of sample in mg/ml

  B = Sample concentration in mg/ml

  %wt = The percent protein in the sample

  m = slope of standard curve

  X = Absorbance of sample average after blank subtraction

9.9.3 Calculation for determining protein content per serving

- **C** = %wt*wt_{dose}

  C = weight of protein in a dose

  %wt = The percent protein in the dose

  wt_{dose} = The weight of a single dose

9.9.4 The % of label in a single dose of protein can be calculated by the following equation:

- % of label = C \ LC

  C = weight of protein in a dose

  LC = Label content for a single dose

11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	05/17/14	New	14-0417	B. Johns
1	09/21/16	Biennial review: Updated SOP format. Updated stability and release requirements.	16-0850	N. Zhang
2	04/14/20	Aligned SOP with current practices, Removed release requirements as it is in test tickets.	CC-20-0274	J. Maignan
3	08/08/24	Split out section 8.0 to differentiate between identification and quantitation of samples.	CC-24-0366	A. Shannon

12.0 Attachments

12.1 Attachment 1 – Standard Dilution Schedule for Sample Tubes

Attachment 1 – Standard Dilution Schedule for Sample Tubes

Sample	[mg/ml]	Std4Dilution	Std µl	Buffer µl
1	2.0			
2	1.0	2.0mg/ml	300	300
3	0.50	1.0 mg/ml	300	300
4	0.75	1.0 mg/ml	450	150
5	0.25	0.5 mg/ml	300	300
6	0.05	0.25 mg/ml	120	480

Sample	[STD] mg/ml	Absolute (ug)	Std µl	Buffer µl	Using
1	1.4	140	140	60	2.0mg/ml stock
2	1.0	100	100	100	2.0mg/ml stock
3	0.75	75	75	125	2.0mg/ml stock
4	0.50	50	50	150	2.0mg/ml stock
5	.025	25	50	150	1.0 mg/ml std
6	0.150	15	30	170	1.0 mg/ml std
7	0.125	12.5	50	150	0.5 mg/ml std
8	0.100	10	40	160	0.5 mg/ml std
9	0.050	5	40	160	0.25 mg/ml std
10	0.010	1	40	160	0.05 mg/ml std