Designation: D 5712 – 99

Standard Test Method for The Analysis of Aqueous Extractable Protein in Natural Rubber and Its Products Using the Modified Lowry Method[1]

This standard is issued under the fixed designation D 5712; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers an analytical test for determining the amount of total aqueous extractable protein associated with natural rubber (NR) and its products. Water soluble proteins are extracted in a buffer solution and then precipitated to concentrate them and also to separate them from water soluble substances which may interfere with the determination. The extracted protein is redissolved and quantified colorimetrically by the modified Lowry method using a protein standard.

1.2 For the purpose of this test method, the range of protein measurement will be based on the limit of detection and quantitation and recorded in micrograms per dm^2 NR specimen.

1.3 The test method is designed to be accurate and compatible with the industrial environment.

1.4 Steps are included in this test method to minimize the effects of interfering substances.

1.5 It is recognized that other methods for the analysis of leachable proteins exist and these may be used for routine quality control purposes provided they have been validated and a correlation established against the reference method specified by this Standard.

1.6 The values stated in SI units are to be regarded as the standard.

1.7 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

D 3577 Specification for Rubber Surgical Gloves[2]

D 3578 Specification for Rubber Examination Gloves[2]

D 4483 Practice for Determining Precision for Test Method Standards in the Rubber and Carbon Black Industries[3]

3. Terminology

3.1 *Definitions:*

3.1.1 *background*—the absorbance measurement of the Lowry assay in the absence of the protein analyte.

3.1.2 *calibration*—the standardization of an instrument setting.

3.1.3 *calibration solution*—the standard solution used to routinely and reproducibly calibrate a measuring instrument.

3.1.4 *dilution factor (F)*—the ratio of the volume NaOH in mL used to redissolve the test specimen extract to volume NaOH in mL used to redissolve the standard ovalbumin proteins. For example, if protein in a 1 mL test extract is acid precipitated and redissolved in 0.25 mL, and the ovalbumin protein standards are also redissolved in 0.25 mL, then the dilution factor ratio of the test extract to that of the calibration curve would equal one.

3.1.5 *concentration range*—the recommended analyte concentration range in µg/mL that produces an absorbance measurement of 0.01 to 1.5 units at 600 to 750 nm.

3.1.6 *extractant*—an aqueous buffer of pH 7.4 ± 0.2 used for the extraction process.

3.1.7 *initial setting*—the instrument setting to which the spectrophotometer is adjusted with the reference solution.

3.1.8 *interferent*—any substance which results in a false positive or negative measurement in the analytical test method.

3.1.9 *latex protein*—aqueous extractable proteins and polypeptides occurring in NR latex and its products.

3.1.10 *limit of detection (LOD)*—the lowest protein concentration that can be measured and be statistically different from the blank. The LOD is expressed as 3.3 × standard error of the y-intercept of the calibration regression line divided by the slope of the calibration line.

3.1.11 *limit of quantitation (LOQ)*—the lowest protein concentration that can be measured to produce quantitatively meaningful results with acceptable precision and accuracy. The LOQ is expressed as 10× standard error of the y-intercept of the calibration regression line divided by the slope of the calibration line.

3.1.12 *linearity*—the degree to which a graph of absorbance versus concentration approximates a straight line.

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[1] This test method is under the jurisdiction of ASTM Committee D-11 on Rubber and is the direct responsibility of Subcommittee D11.40 on Consumer Rubber Products.

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[2] *Annual Book of ASTM Standards*, Vol 09.02.

[3] *Annual Book of ASTM Standards*, Vol 09.01.

3.1.13 *Lowry*—for the purpose of this test method, the word "Lowry" is used to represent any modified form of the original Lowry assay method.

3.1.14 *repeatability*—the variability or test error between independent test results obtained within a single laboratory.

3.1.15 *reproducibility*—the variability or test error between test results obtained in different laboratories.

3.1.16 *spectrophotometric measurement*—the unit of measurement of the instrument that is proportional to absorbance.

3.1.17 *standard solution*—the standard analyte to which the test (unknown) sample being measured is compared.

3.1.18 *water* (dH_2O)—a liquid (H_2O) purified by distillation (distilled water) or deionization (deionized water).

4. Summary of Test Method

4.1 This colorimetric test method is used for the determination of protein levels in NR and its products. This test method involves the extraction of residual aqueous soluble proteins from NR followed by the precipitation of these proteins to remove interfering, aqueous soluble substances. The protein content is then determined by the Lowry method of protein analysis using a protein standard for quantitation. Spectrophotometric measurement is performed at a fixed wavelength in the range 600 to 750 Hz (nm). A wavelength of 750 nm is recommended.

5. Significance and Use

5.1 This test method, for the determination of protein levels in NR, is primarily intended to test NR materials for residual protein content. It is assumed that all who use this test method will be trained analysts capable of performing common laboratory procedures skillfully and safely. It is expected that work will be performed in a properly equipped laboratory.

6. Apparatus

6.1 Spectrophotometer and cuvettes or microplate reader and 96-well microtiter plates.

6.2 Pipettes, test tubes (for example, 1.5 mL polypropylene microcentrifuge (MC) tubes), test tube rack, vortex mixer and centrifuge for MC tubes.

7. Reagents and Materials

7.1 Whenever water is called for distilled or deionized water should be used. All other reagents should be of analytical quality.

7.2 *Extraction Buffer*—An aqueous buffer of pH 7.4 ± 0.2

NOTE 1—The following buffer solutions could be used: phosphate buffer; PBS, phosphate buffered saline; TES, N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid hemisodium salt buffer, or equivalent of sufficient buffering capacity (at least 25 mM) to maintain the extract at pH 7.4 ± 0.2.

7.3 *Modified Lowry Assay Reagents*—A more detailed description of the Lowry protein assay is discussed in Refs **(2-8)**.[4]

7.3.1 *Reagent A*—Alkaline tartrate solution prepared by dissolving 2.22 g sodium carbonate, 0.44 g sodium hydroxide, and 0.18 g sodium tartrate in water sufficient to make 100 mL.

Reagent A (alkaline tartrate):
2.22 g sodium carbonate
0.44 g sodium hydroxide
0.18 g sodium tartrate
q.s. ..... 100 mL with distilled or deionized water (dH_2O)

7.3.2 *Reagent B*—Copper sulfate solution prepared by dissolving 7.0 g cupric sulfate pentahydrate in water sufficient to make 100 mL.

Reagent B (copper sulfate):
7.0 g cupric sulfate pentahydrate
q.s. ..... 100 mL with dH_2O

7.3.3 *Reagent C*—Alkaline copper tartrate solution prepared by mixing 1 mL of Reagent B and 150 mL of Reagent A.

Reagent C (alkaline copper tartrate):
Mix reagents A & B ″Fresh″,

150 mL Reagent A
+ 1 mL Reagent B
..... 151 mL Final Volume

7.3.4 *Reagent C′ (C Prime)*—Alkaline tartrate solution prepared by mixing 1 mL of water and 150 mL of Reagent A for use in the optional correction of interferences (refer to 9.4.4).

Reagent C′ (alkaline tartrate):
150 mL Reagent A
+ 1 mL dH_2O
..... 151 mL Final Volume

7.3.5 *Reagent D*—Folin reagent diluted 50 %, prepared by diluting 1 part of Folin reagent with 1 part water.

Reagent D (dilute Folin phenol):
Mix Folin-Ciocalteu reagent with water (prepare fresh),

10 mL Folin-Ciocalteu reagent (2 N)
+ 10 mL dH_2O
..... 20 mL Final Volume

NOTE 2—Folin-Ciocalteu Phenol reagent is widely available commercially.

7.3.6 *Standard Protein Solution*—Prepare a standard protein solution (0.1 %, 1 mg/mL) by dissolving 100 mg of ovalbumin in 100 mL Extraction Buffer for 2 h at 25 °C in a polypropylene container. Filter the solution through a low protein binding 0.45 µm or smaller pore size filter and determine the absorbance at 280 nm using a UV spectrophotometer. Divide the absorbance by 0.64 to calculate the actual concentration of the ovalbumin stock solution.

0.10 g ovalbumin powder
q.s. ..... 100 mL with Extraction Buffer

NOTE 3—The ovalbumin protein can be obtained from Sigma Chemical Co.[5]

NOTE 4—The absorbance at 280 nm of 1 mg/mL of ovalbumin in a 1 cm cuvette approximates 0.64. For example, an A_{280nm} of 0.55 for a 1 mg/mL solution of ovalbumin would yield an actual concentration of 0.55/0.64 = 0.86 mg/mL.

7.3.6.1 Store the standard protein solution at 4 °C. The solution is stable for seven days under refrigeration or for

[4] The boldface numbers given in parentheses refer to a list of references at the end of the text.

[5] Catalogue No. Ovalbumin A5503, chicken egg albumin, Grade V, or equivalent.

twelve months frozen at –18° C. Thawing requires heating to 37 to 45° C for 15 min.

7.3.6.2 At least four ovalbumin standard concentrations should be prepared in the range 10 to 100 µg/mL by diluting the protein stock solution with extraction buffer (for example, 0, 10, 35, 60, 100 or 0, 2, 10, 35, 60, 100 and 200 µg/mL). Use the protein-free Extraction Buffer as the diluent and reagent blank.

NOTE 5—*Standard Solutions*—Prepare a minimum of four standard solutions to extend over the absorbance range of 0.01 to 1.5 units at 600 to 750 nm. The solutions should have concentrations spaced to produce absorbance points spanning the entire calibration range. The standard solutions are used to establish a working calibration curve of absorbance versus concentration to allow the measurement of the analyte proteins in the test extract.

7.3.7 *Sodium Deoxycholate (DOC)*—Prepare a 0.15 % (m/V) solution by dissolving 0.15 g sodium deoxycholate in water and diluting to 100 mL.

0.15 g sodium deoxycholate
q.s. ..... 100 mL with dH_2O

7.3.8 *Trichloroacetic Acid (TCA)*—Prepare a 72 % (m/V) solution by dissolving 72 g trichloroacetic acid in water and diluting to 100 mL.

72 g trichloroacetic acid
q.s. ..... 100 mL with dH_2O

7.3.9 *Phosphotungstic Acid (PTA)*—Prepare a 72 % (m/V) solution by dissolving 72 g phosphotungstic acid in water and diluting to 100 mL.

72 g phosphotungstic acid
q.s. ..... 100 mL with dH_2O

8. Hazards

8.1 Working personnel should adhere to standard good laboratory practices. Care should be exercised when working with all chemical reagents including acidic and basic solutions.

9. Extraction and Assay Procedures

9.1 The procedure involves the extraction of the NR specimen followed by concentration of the extract using acid precipitation. If the test specimen is a product, the entire product whether cut up or whole is extracted such that all functional surfaces are exposed to the extractant. The determination of the extract is performed by reference to a standard protein solution which has been concentrated in the same manner. All determinations are carried out from three individual NR specimens or products (i.e., one sample extraction each of the three specimens or products). Each of the three extracts is concentrated by acid precipitation of an aliquot from each extract. The three separate acid precipitates are redissolved in sodium hydroxide and each is assayed for protein using the Lowry test method. An average is calculated from the three protein values of a single product.

9.2 *Extraction Procedure*—Use powder free, non- NR latex gloves to handle the NR specimens used for the extraction, care being taken not to contaminate the specimen.

9.2.1 Take a single test specimen, weigh the sample, and determine the surface area (S) in dm^2.

NOTE 6—For NR medical devices such as examination and surgical gloves, the entire product whether cut up or whole is extracted such that all functional surfaces are exposed to the extractant. For other NR specimens, cut out at least one gram of material and weigh the cut piece of specimen.

9.2.2 Place the test specimen in an extraction vessel so that all surfaces of the test specimen are exposed to the extraction solution.

NOTE 7—For gloves, it is suggested to introduce at least 5 mL and no more than 10 mL (V) of extraction buffer per 1 g of glove material. A volume to weight ratio of 5 to 10 is suggested as long as all surfaces of the test specimen are evenly exposed to the extraction buffer. When the NR specimen is large, the specimen may be cut into pieces of appropriate size to accommodate the extraction vessel. The specimen should be extracted in polypropylene vessels to reduce the possible loss of proteins by adsorption to the inner surface of the container walls. The extraction vessel should be tested independently for the lack of interference with this protein assay method.

9.2.3 Extract the specimen piece(s) at 25 ± 5° C for 120 ± 5 min. Shake at least at the start, in the middle, and after 120 min.

NOTE 8—It is suggested that slow and continuous shaking of approximately 200 rpm's be used.

9.2.4 Remove the test specimen from the extraction solution. Transfer the extract into a polypropylene centrifuge tube and centrifuge for 15 min at not less than 500 × g to remove particulate matter. Alternatively, filter the extract through a low protein binding 0.45 µm or smaller pore size filter at room temperature into a polypropylene tube. Collect the supernatant liquid and store it at 2 to 8 °C. Carry out the determination within 24 h.

NOTE 9—This test method addresses the aqueous soluble proteins of the NR test specimen only, and not the aqueous insoluble protein content of the specimen.

9.3 *Acid Precipitation and Concentration of Protein Extracts and Standards*:

9.3.1 Substances that may interfere with quantifying protein during the development of the assay can be reduced by acid precipitation. For information on other options of reducing interference refer to 9.4.4.

9.3.2 Accurately transfer to separate 1.5 mL polypropylene tubes 1 mL each of the reagent blank (extraction buffer), standard protein solutions (ovalbumin standards) and the specimen extracts (NR proteins). Add 0.1 mL of DOC, mix and allow to stand for 10 min, and then add 0.2 mL of a freshly prepared solution of 50:50 TCA and PTA to acid precipitate the proteins. Mix well and allow to stand for an additional 30 min before centrifugation.

NOTE 10—The sample volume used is sufficient for analysis using 96-well microfilter plates and a micro-plate reader. To ensure sufficient volume for analysis using cuvettes, the volumes may be increased proportionately (that is, 4-fold).

9.3.3 Centrifuge the acid precipitate at 6000 × g for 15 min or equivalent. Decant off the supernatant liquid and drain by inverting each centrifuge tube on an absorbent paper towel. Remove any remaining liquid by carefully tipping the tube and wicking the solution with an absorbent paper without coming in contact with the protein precipitate. Add 0.25 mL of 0.2 M sodium hydroxide solution to each tube, including the blank so

as to redissolve the precipitated protein; use a vortex mixer or ultrasonic water bath if needed. Ensure that the protein is completely redissolved to a clear solution. Should some protein precipitates remain, add a further measured quantity of the sodium hydroxide solution up to a total of 1 mL. The redissolved protein solution may be stored prior to the determination for not more than 24 h at 3 ± 1° C.

NOTE 11—When storage of the extract for 24 h is necessary, it is preferred to store the precipitated protein pellet rather than the redissolved precipitate. The precipitate can then be redissolved after storage.

NOTE 12—Lower centrifuge speeds may leave the protein insufficiently compacted, which can lead to erroneous results. The recommended amount of sodium hydroxide solution (0.25 mL) used to redissolve the acid precipitated sample concentrates the test extract 4-fold from the original 1 mL volume. When the volume used to redissolve the test extract is different from the volume used to redissolve the ovalbumin protein standards, a dilution factor F is used in the calculation of extractable protein to adjust the ratio of the two volumes. When the spectrophotometric absorbance measurement of the redissolved test extract is outside of the limit of the calibration curve, the redissolved test extract may be diluted in 0.2 N NaOH so that the absorbance measurement of the diluted sample is within the limits of the calibration standard curve. If an additional quantity of sodium hydroxide solution is required the degree of concentration will be different and must be allowed for in subsequent calculations.

9.4 *Color Development and Reading*:

9.4.1 *Assay Procedure for 96-Well Microtiter Plate Modified Lowry Method*:

9.4.1.1 Add 125 µL of Reagent C,

NOTE 13—*Optional Correction of Interferences*—To prepare the reagent to correct for interferences, repeat all reagent additions but replace Reagent C with Reagent C′ (C prime, no copper sulfate present) and subtract the absorbance in the absence of copper sulfate from the test sample absorbance containing copper sulfate (refer to 7.3.4 and 9.4.4).

9.4.1.2 Add 60 µL of redissolved specimen extracts (NR proteins), standard protein (ovalbumin), or reagent blank (minus protein analyte), mix well and let set for 15 min at room temperature (RT),

9.4.1.3 Add 15 µL of Reagent D, thoroughly mix immediately and let set for 30 min at RT,

9.4.1.4 The absorbance of the final assay mixture in a 96-well microtiter plate using a microplate reader (spectrophotometer) is measured at a wavelength of 750 nm (600 to 750 nm optional) within 1 h of adding the Folin reagent. All determinations are carried out from extractions of three individual NR specimens or products. Each of the three extracts is concentrated by acid precipitation, and an average is calculated from the three extracts.

9.4.2 *Assay Procedure for Cuvette Modified Lowry Method*:

9.4.2.1 Add 2.5 mL of Reagent C,

NOTE 14—*Optional Correction of Interferences*—To prepare the reagent to correct for interferences, repeat all reagent additions but replace Reagent C with Reagent C′ (C prime, no copper sulfate present) and subtract the absorbance in the absence of copper sulfate from the test sample absorbance containing copper sulfate (refer to 7.3.4 and 9.4.4).

9.4.2.2 Add 1.2 mL of redissolved specimen extracts, standard protein, or reagent blank (minus protein analyte), mix well and let set for 15 min at RT,

9.4.2.3 Add 0.3 mL of Reagent D, thoroughly mix immediately and let set for 30 min at RT,

9.4.2.4 Transfer 4 mL or less of the final assay mixture to a cuvette and measure the absorbance in a spectrophotometer at a wavelength of 750 nm (600 to 750 nm optional) within 1 h of adding the Folin reagent. All determinations are carried out from sample extractions of three individual NR specimens or products. Each of the three extractions is concentrated by acid precipitation, and an average is calculated from the three extracts.

9.4.3 *Color Development:* Following the addition of dilute Folin Reagent, color development reaches a maximum in approximately 20 to 30 min at room temperature. There may be a gradual loss of signal of a few percent per hour.

NOTE 15—A standard calibration curve should be run at the same approximate time as the test samples for each Lowry assay. It is important for uniform results that in all subsequent determinations the time scales, equipment and wavelength be consistent.

9.4.4 *Optional Correction of Interferences*—Universal methods to eliminate interferences do not yet exist for this assay. Aqueous extractable chemicals that are added to NR for compounding and curing may interfere with the Lowry protein assay. Interfering chemicals (for example, accelerators, synthetic polymers, etc.) can cause a change in the color development; absorbance values are usually inflated. It is known that the Lowry Folin phosphomolybdate/tungstate reagent can form a color which absorbs in the 600 to 750 nm range when reducing chemicals are present. The variation of the Folin reagent color can be a result of contamination from chemicals external to the Lowry assay that affects the accuracy and reliability of low-level protein determinations. Since the protein-induced color formation of the Lowry Folin reagent depends less on the reducing potential of aromatic aminoacyl residues in proteins, and more on the reductant reaction of the copper-polypeptide bond complexes in proteins **(2-8)**, it is possible to correct for some interferences. A modification of the Lowry method where the difference in color formation determined by assaying protein extracts in the presence and absence of copper can be used to approximate the amount of peptide bonds in the protein extract. This correction method is included as an option in this test method to reduce the effects of aqueous-soluble interfering chemicals in the assay. This approach involves subtracting the test extract response prepared in the absence of copper sulfate from the protein measurement in the presence of copper sulfate to produce a protein signal, by difference. Equivalent care should be given to measuring the signal in the absence of copper as the test sample itself since any variability of the measurement can contribute to the final measured value.

NOTE 16—The test method may not remove all substances which interfere with the Lowry colorimetric assay. Other methods of reducing the signal of interfering chemicals other than protein in the Lowry assay may be used. These include dialysis of the protein test extract in an aqueous buffer to remove the interfering chemicals, organic phase extraction of the protein test extract to remove the interfering chemicals from the aqueous phase, and double acid precipitation of the protein test extract. These approaches are for informational purposes only and their adequacy for use in this test method must be validated separately.

10. Calculation

10.1 The absorbance measurements of the test extracts are converted to µg protein/mL using a calibration curve. The concentration of the protein analyte in the test extract is read from the calibration curve. A standard curve should be prepared at the time the test samples are evaluated.

NOTE 17—When the option of correction of interferences is used, the average absorbance readings of the standard protein solutions minus that of the correction signals are plotted against the concentration of standard protein added (refer to 9.4.4).

10.1.1 *Calibration Curve*—The spectrophotometric absorbance measurements of the redissolved standard protein solutions in the Lowry assay are plotted on the ordinate against their concentration in µg/mL on the abscissa. The calibration curve is curvilinear over the protein concentration range of 0 to 200 µg/mL of standard solutions. The calibration data should be curve-fit to a second degree polynomial function (quadratic) and forced through the origin of the calibration plot. The concentration (C) of the protein analyte in the test specimen extract is read from the calibration curve in µg/mL.

NOTE 18—Some protein is lost during the concentration process; it is assumed that the same percentage of protein is lost from the standards as from the test samples during the concentration process. Provided that all of the precipitated protein standards and test sample extracts have been concentrated to the same degree, the precipitated protein standards may be used for the calibration curve to determine the test extracts directly. It is not necessary to plot the unprecipitated protein standard concentrations.

10.2 Determine the concentrations of the extracted samples (C) in µg/mL extract by reading them directly from the calibration curve.

10.3 A nonlinear relationship between absorbance and concentration exists when the dose-response profile of the absorbance readings versus protein concentration of the Lowry assay is curvilinear. Since the Lowry calibration curve is typically curvilinear, the calibration data of protein standards should be fitted to a quadratic nonlinear equation that represents the shape of the data. Curve-fitting of data may be performed by the spectrophotometer or attached instrument with a preprogrammed microprocessor or independently through the use of an external computer. In the later case, the calibration data may be curve-fit using the following quadratic equation:

$$A_{std} = a_1 * C + a_2 * C^2 \quad (1)$$

where:

A_{std} = absorbance readings of the standard protein solutions,
a_1 = the slope coefficient at low standard protein concentrations,
a_2 = coefficient that defines the curvature of the standard curve, and
C = concentration of the standard protein solution in µg/mL.

10.3.1 When the absorbance value of the protein test extract is in the linear region of the calibration curve of protein standards, the protein concentration may be calculated either directly from the standard curve or from the following mathematical relationship:

$$C\ (\mu g/mL) = C_{low} + \{(C_{high} - C_{low}) \times (A - A_{low})/(A_{high} - A_{low})\} \quad (2)$$

where:

A = units of absorbance reading of the test extract,
A_{low} = units of absorbance reading of the low standard protein solution,
A_{high} = units of absorbance reading of the high standard protein solution,
C = concentration of the test extract in µg/mL,
C_{low} = concentration of the low standard protein solution in µg/mL, and
C_{high} = concentration of high standard protein solution in µg/mL.

NOTE 19—This equation can only be used in the region of the calibration curve where the relationship between absorbance and concentration is linear.

10.4 The aqueous extractable protein content is determined in µg/mL for each test specimen. The total protein content is determined for each test specimen by multiplying the µg/mL quantity by the total volume of extractant in mL used for the specimen. Multiply the result by the dilution factor ratio of the test extract to protein standard volume used to redissolve the protein precipitates. Then divide the result by the total surface area in square decimeters of the test specimen to give units of µg/dm^2. To determine the results in µg/g, calculate the weight of the test specimen in grams and divide the result by the weight instead of surface area to convert to units of µg/g.

NOTE 20—For NR glove specimens, the area of the glove can be determined from the dimensions for glove size given in the Specification D 3578 for examination gloves and Specification D 3577 for surgical gloves. The total area of four sides of a glove (palm inside and outside, back inside and outside) is calculated by multiplying the minimum length (L) in millimeters by the nominal width (W) in millimeters from the ASTM Standards of glove dimensions and converting this value to dm^2 using the following formula: L × W × 4/10 000.

10.4.1 The protein content of the test specimen in µg protein/dm^2 is given by:

$$\text{extractable protein } (E)\ \mu g/dm^2 = [(C \times V \times F)/S] \quad (3)$$

where:

V = volume of Extraction Buffer used in mL,
C = protein concentration of the extract in µg/mL,
F = dilution factor (ratio of the volume NaOH in mL used to redissolve the test extract to volume NaOH in mL used to redissolve the standard ovalbumin protein),
S = Surface area in dm^2 of the NR specimen.

NOTE 21—When the absorbance value of the protein test extract is in the curvilinear region of the calibration curve, the protein concentration can be calculated by non-linear regression curve-fitting of a second degree polynomial that is forced to zero (for example, $A_{std} = a_1 * C^1 + a_2 * C^2$). It is suggested that commercial computer software for curve-fitting and calculation of the unknown concentrations be used.

11. Report

11.1 The working laboratory should maintain a record of all observations, calculations, derived data, and test reports for an appropriate period. The records of each test should contain all of the information necessary to allow the test to be satisfactorily repeated. All determinations are carried out from sample extractions of three individual NR specimens or products. Each

of the three extracts is concentrated by acid precipitation, and an average value is calculated from the three extracts. See Fig. 1.

12. Statistical Information: Precision and Bias, and Limit of Quantitation

12.1 Several laboratories participated in evaluating this test method for the precision and bias of protein extraction and determination using the modified Lowry method. The data was collected by extracting whole test specimens and determining the residual protein content as described in this test method. The data is summarized statistically in Table 1 for intra-laboratory repeatability and inter-laboratory reproducibility.

12.1.1 Calibration data of several laboratories was used to determine the limit of detection (LOD) and the limit of quantitation (LOQ) as defined in the International Conference on Harmonization: Validation of Analytical Procedures: Methodology **(10)**. The LOD is defined as the lowest protein concentration that can be measured and be statistically different from the blank.

12.1.2 The LOD is expressed as 3.3 times the standard error of the y-intercept of the calibration regression line divided by the slope of the calibration line. The LOQ is the lowest protein concentration that can be measured to produce quantitatively meaningful results with acceptable precision and accuracy. The LOQ is expressed as ten times the standard error of the y-intercept of the calibration regression line divided by the slope of the calibration line. Experiments to determine the LOD and the LOQ using several standard ovalbumin (chicken egg albumin) protein concentrations in the range of 1.25 to 100 µg/mL were performed among six independent laboratories. The LOD and LOQ were determine as 4.7 and 14.1 µg/mL, respectively. A more detailed description of the statistics used in the test method is found in Test Method D 4483, and **(9)** and **(10)**.

TABLE 1 Test Method Statistical Information[A]

				Within Laboratories			Between Laboratories		
Material Specimen	Mean Protein µg/g	Labs p	% C.V.	S_r	r	(r)	S_R	R	(R)
NR Sample "A"	106.5	8	39.0	8.1	23.0	21.6	41.5	117.6	110.4
NR Sample "B"	574.0	6	23.2	50.4	142.7	24.9	133.0	376.4	65.6

[A] %C.V. = percent coefficient of variation of the mean values,
p = number of laboratories used to obtain the test results,
q = 2 (number of specimens),
S_r = within laboratory standard deviation,
r = repeatability between test results of a single laboratory,
(r) = repeatability as a percent,
S_R = standard deviation of between-laboratory variability,
R = reproducibility between laboratories, and
(R) = reproducibility as a percent.
Average specimen weight was 7.0 ± 0.2 g and the total surface area was 8.74 dm^2 per specimen.

12.2 No certified standard reference material is currently available to assess the accuracy of this method. It is assumed that any bias in the method will be less than the reproducibility of the test. The testing laboratory should verify that the accuracy of the method is adequate for the intended use.

12.3 If the method yields results that appear erroneous due to interferences, then it is the responsibility of the test laboratory to determine that the spectrophotometric measurements of the NR specimen or product extracts are due to interference and not protein alone.

1	2	3	4	5	6	7
Test Specimen Extract	Protein precip-itate (C) µg/*mL*	Weight of Specimen (W) g	Volume of Extract (V) *mL*	Dilution Factor (F) #	Surface Area of specimen (S) dm^2	Aqueous Extractable Protein (C x V x F) / S = (E) µg/dm^2
1						
2						
3						
					Average:	(µg/dm^2)

1, Extractions of a three NR specimens or products,
2, Precipitation of the extract of each test specimen,
3, Weight in grams (W) of the NR specimen extracted,
4, Volume (V) of Extraction Buffer in *mL* used to extract,
5, Dilution factor (F) is the ratio of volume of NaOH in *mL* used to redissolve the precipitated test extract to the volume of NaOH in *mL* used to redissolve the precipitated standard ovalbumin protein,
6, Surface area (S) in dm^2 of NR specimen,
7, Extractable protein (E) µg/dm^2 = (C x V x F) / S,
Calculate the µg/dm^2 of the three test extracts and record as the average of a single specimen.

FIG. 1 Test Specimen Example Data Sheet

REFERENCES

(**1**) *Form and Style for ASTM Standards*, 8th ed., 13-000001-89, ASTM 1989.

(**2**) Bensadoun, A. and Weinstein, D., "Assay of Proteins in the Presence of Interfering Materials," *Anal. Biochem. 70:* 1976, pp. 142-250.

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