Designation: D5712 – 15

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

This standard is issued under the fixed designation D5712; 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 NR, latex, and elastomeric 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 that 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 test 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 test method.

1.6 This test method has not been validated for use with lubricated products such as condoms. Condoms with different lubricants as typically marketed, have not been tested in an ASTM ILS to determine if, and if so to what degree, the lubricant interferes with the assay.

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:*[2]

D3577 Specification for Rubber Surgical Gloves

D3578 Specification for Rubber Examination Gloves

D4483 Practice for Evaluating Precision for Test Method Standards in the Rubber and Carbon Black Manufacturing Industries

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 *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.

3.1.5 *dilution factor (F)*—the ratio of the volume NaOH in millilitres used to redissolve the test specimen extract to volume NaOH in millilitres 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.

3.1.6 *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 that results in a false positive or negative measurement in the analytical test method.

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

Current edition approved July 1, 2015. Published September 2015. Originally approved in 1995. Last previous edition approved in 2010 as D5712 – 10. DOI: 10.1520/D5712-15.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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

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, latex, and elastomeric products. This test method involves the extraction of residual aqueous soluble proteins from NR, latex, and elastomeric products 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 quantification. 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 latex, is primarily intended to test NR, latex, and elastomeric 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 **(1-7)**.[3]

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 ± 5 min at 25°C in a polypropylene container. Filter the solution through a low protein binding 0.45 µm or smaller pore size filter and

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

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 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 1 to 8°C. The solution is stable for seven days under refrigeration or for twelve months frozen at ≤–10°C. Thawing requires heating to between 37 and 45°C for 15 min.

7.3.6.2 At least four ovalbumin standard concentrations should be prepared in the range of 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 4—*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 test 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 that has been concentrated in the same manner. All determinations are carried out from three individual test specimens or products (that is, 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, synthetic polymer gloves to handle the test 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 5—For 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 test 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 6—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 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 7—It is suggested that slow and continuous shaking by mechanical means (rotator, rocker plate, shaker, etc.) be used. The speed chosen will depend on the device used and the speed chosen should ensure that adequate mixing of the test sample and extract buffer occurs.

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 1 to 8°C. Carry out the determination within 24 h.

NOTE 8—This test method addresses the aqueous soluble proteins of the 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 test specimen extracts. 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 9—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 1 to 8°C.

NOTE 10—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 11—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 12—*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 test specimen extracts, 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 test 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 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).

9.4.2.2 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 test 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 14—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, latex, and elastomeric products for compounding and curing may interfere with the Lowry protein assay. Interfering chemicals (for example, accelerators, synthetic polymers, and so forth) 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 that 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 (**1-7**), 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 15—The test method may not remove all substances that 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 16—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 17—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 18—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 millilitres 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 decimetres 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 19—For glove specimens, the area of the glove can be determined from the dimensions for glove size given in the Specification D3578 for examination gloves and Specification D3577 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 millimetres by the nominal width (W) in millimetres 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 test specimen.

NOTE 20—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 test 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 (**8**). 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 determined 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 D4483, and Refs (**9**) and (**8**).

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

TABLE 1 Test Method Statistical Information[A]

Material Specimen	Mean Protein µg/g	Labs *p*	% *C.V.*	Within Laboratories S_r	Within Laboratories *r*	Within Laboratories (*r*)	Between Laboratories S_R	Between Laboratories *R*	Between Laboratories (*R*)
Sample "A"	106.5	8	39.0	8.1	23.0	21.6	41.5	117.6	110.4
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.

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

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 test specimen or product extracts are due to interference and not protein alone.

13. Keywords

13.1 amino acid analysis; Lowry; natural rubber latex; protein

APPENDIX

(Nonmandatory Information)

X1. AMINO ACID ANALYSIS OF PROTEINS FROM NR, LATEX, AND ELASTOMERIC PRODUCTS

X1.1 Significance and Use

X1.1.1 This test method, for the determination of protein levels in latex gloves, is intended to test materials for residual protein content. It may be used as a procedure for validating other protein quantitation methods, or supplementing the modified Lowry method, or both. The method itself can be validated against a standard protein solution of bovine serum albumin (BSA), which is available from the National Institute of Standards and Technology (NIST)–SRM 927c and against a NR protein standard available from ARDL–Hevea Natural Rubber (HNR) IRM 913.

NOTE X1.1—Pearson correlation between HPLC amino acid analysis and Test Method D5712 (modified Lowry method) is 0.567 ($p<0.05$) (Guthrie Labs}.

X1.2 Materials/Apparatus

NOTE X1.2—Most reagents can be ordered from Sigma.

X1.2.1 6N Hydrochloric acid containing 2 % Phenol.

X1.2.2 *o*-Phthalaldehyde.

X1.2.3 3-Mercaptopropionic acid.

X1.2.4 Amino acid standard containing 16 amino acids (standard calibration mixture).

X1.2.5 Vacuum concentrator.

X1.2.6 Autosampler.

X1.2.7 Gradient system.

X1.2.8 Fluorescent detector.

X1.2.9 Glass screw cap test tubes with Teflon liners.

X1.2.10 Bovine serum albumin standard.

X1.2.11 Norvaline/norleucine (internal standard).

X1.2.12 Protein hydrolysate amino acid standards mixture: Sigma Catalog #AA-S-18 or an equivalent alternative from a laboratory reagents supplier.

X1.2.13 NR protein standard (IRM 913).

X1.3 Product Extraction

X1.3.1 Natural rubber latex products can be extracted by a technique similar to that used by Test Method D5712. Use five millilitres of water/gram of product and extract with agitation. Amine-based buffers must not be used in any step of the analysis. Two identical aliquots of this extract are removed before proceeding. See Note X1.3.

NOTE X1.3—Some glove extracts contain free amino acids. Therefore, identical aliquots of the extract should be analyzed *with* and *without* HCl hydrolysis (that is, perform two analyses on each extract, one with, and without HCl hydrolysis). The free amino acid values obtained from the "minus hydrolysis" control are subtracted from the hydrolysate values before calculating the total protein content of the sample.

X1.4 Hydrolysis

X1.4.1 Proteins must be hydrolyzed to free the amino acids prior to separation and analysis. See an example in Table X1.1. A50-µL aliquot of extract (which should contain 1 to 10 µg of protein), is placed in a hydrolysis tube, spiked with 5 nanomoles of norvaline and evaporated to dryness. The hydrolysis tube is a 2-mL glass screw cap test tube with a Teflon liner. The dried sample is hydrolyzed in 200 µL of 6N HCl at 110°C for 24, 48, or 72 hours. The hydrochloric acid should be of highest available purity. Oxygen is removed prior to hydrolysis by displacement with a stream of nitrogen. After hydrolysis the sample is evaporated to dryness (for example, in a SpeedVac vacuum concentrator). Hydrolysis is done in a heating block calibrated to 110°C (**10**).

NOTE X1.4—Acid hydrolysis must be carried out in a liquid state, as vapor phase hydrolysis tends to turn NRL extracts into an unusable tar.

TABLE X1.1 Example of Amino Acid Analysis Data from 24-h Hydrolysis of BSA Standard

Amino Acid (AA)	pmol Analyzed	Residue Weight	ng of each AA
ASP	6883	115.09	792.14
THR	4074	101.11	411.87
SER	3220	87.08	280.36
GLU	10124	129.12	1307.26
PRO	3906	97.12	379.34
GLY	2116	57.05	120.69
ALA	5916	71.08	420.49
VAL	4424	99.13	438.56
MET	522	131.2	68.44
ILE	1690	113.16	191.25
LEU	7678	113.16	868.83
TYR	2498	163.18	407.69
PHE	3412	147.18	502.13
HIS	2326	137.14	318.98
LYS	7445	128.18	954.38
ARG	3135	156.19	489.60

X1.5 Precolumn Derivatization with *o*-Phthaldialdehyde (OPA) and 3-Mercaptopropionic Acid (MPA)

X1.5.1 The amino acids that are released by hydrolysis of the proteins are converted into detectable derivatives. Any commercially available instrumentation that uses the same derivatization chemistry can be used.

X1.5.1.1 The derivatization is carried out automatically using the autosampler. Dissolve the dried hydrolysate in 100 µL of 0.1M HCl. Five microlitres of the redissolved hydrolysates are incubated for 2.5 min at room temperature with 12.5 µL of 83 mM OPA in 400 mM sodium borate buffer (pH 10.4). The reaction is terminated by addition of 25 µL 1M $KHSO_4$ (pH 7.0).

X1.5.1.2 Twenty microlitres of the solution are injected onto the HPLC column. The fluorescent isoindole derivatives that arise from derivatization are separated and detected with a fluorescence detector.

X1.5.2 The sample process steps are as follows:

X1.5.2.1 Fifty-microlitre glove extract.

X1.5.2.2 Dry.

X1.5.2.3 Hydrolyze with 200 µL 6N HCl.

X1.5.2.4 Dry.

X1.5.2.5 Dissolve hydrolysate in 100 µL 0.1N HCl.

X1.5.2.6 Take 5 µL hydrolysate, add 12.5 µL reagent (5 µL/100 µL).

X1.5.2.7 Terminate with 25 µL buffer (total volume 42.5 µL).

X1.5.2.8 Load 20 µL on to analyzer (20 µL/42.5 µL).

X1.6 HPLC Separation Conditions

X1.6.1 *Precolumn*—3 cm Hypersil ODS 3 microns.

X1.6.2 *Column*—Grom Amino OPA (15 cm Hypersil ODS: 3 micron).

X1.6.3 *Flow Rate*—1 mL/min.

X1.6.4 *Eluent A*—25 mM sodium phosphate, pH = 6.8, 1.5 % tetrahydrofuran (HPLC grade) (v:v).

X1.6.5 *Eluent B*—65 % 25 mM sodium phosphate, pH = 6.8, 25 % acetonitrile (HPLC grade), 10 % tetrahydrofuran (HPLC grade) (v:v:v).

X1.6.6 *Wavelength*—Excitation 330 nm, emission 450 nm.

X1.6.7 *Gradient*—Computer-controlled gradient system with a binary high pressure gradient system is recommended (stepwise and linear gradients).

0 % Eluent B for 0 to 3 min (time after sample injection)
10 % Eluent B for 3 to 8 min
10 to 40 % Eluent B, for 8 to 13 min
40 % Eluent B, for 13 to 24 min
40 to 80 % Eluent B, for 24 to 26 min
80 % Eluent B, for 26 to 30 min
80 % Eluent B to 0 % Eluent B, for 30 to 32 min

X1.6.8 Each laboratory needs to calibrate the analysis system using the standard amino acid calibration mixture prior to analyzing unknown samples.

X1.7 Calculations

X1.7.1 The addition of 5 nanomoles of norvaline/norleucine to the initial aliquot of glove extract as an internal standard means that any subsequent handling losses can be corrected in the final calculation of protein concentration. The concentration of the individual amino acids in the sample loaded on to the analyzer must be quantitated, converted to weight, summed, and corrected for losses during handling, before the final protein content can be determined.

X1.7.2 Quantification through an internal standard (norvaline/norleucine), the subsequent summation of the individual amino acids, and normalization via a reference protein standard reveal the total protein content. This can be achieved by the following steps:

X1.7.2.1 The concentration for the individual amino acids are calculated by their area ratio when compared with those of the standard calibrant mixture.

X1.7.2.2 The amount of norvaline/norleucine is also quantitated at this step. Initially, 5.0 nanomoles of internal standard were added to the glove extract, 50 µL was hydrolyzed, 5 % was derivatized (5 µL/100 µL), and 20 µL/42.5 µL analyzed. Thus the expected value for the internal standard should be 5000 × 5/100 × 20/42.5 = 117.6 picomoles. If the observed value is lower than this value, then all of the amino acid values are corrected by the appropriate factor. This process is repeated for the control glove extract that had not been hydrolyzed.

X1.7.2.3 Subtract the free amino acid values (non-hydrolysed control) from the protein-derived amino acids (see Note X1.3).

X1.7.2.4 The corrected amino acid values are an accurate representation of the protein content of the glove. They are quantitated as picomoles of amino acid per 20 µL of derivatization mix. The next step is to convert the concentration of each amino acid to weight by multiplying the "pmoles analyzed" column by the appropriate residue weight as indicated in Table X1.1 (see also Note X1.5). This gives the number of nanograms of each amino acid present in the 20-µL aliquot. These weights are summed to obtain the weight (in ng) of protein recovered from the aliquot of extract that was hydrolyzed.

X1.7.2.5 The next step is to convert this value to the actual amount of protein present in the aliquot by multiplying by the normalization factor (See Note X1.5 and Table X1.2).

X1.7.2.6 To obtain the amount of protein present in the original 50-µL glove extract, the total protein weight has to be corrected for derivatization (42.5 µL/20 µL) × hydrolysate sampling (100 µL/5 µL) volumes = 42.5; that is, µg protein in 50 µL extract = 42.5 × µg protein in 20 µL aliquot.

X1.7.2.7 The weight of protein per gram of glove is calculated as follows: wt of protein (µg)/volume of aliquot (50 µL) × volume of extract (mL)/wt of glove (g).

NOTE X1.5—*Example*—Using ASP in Table X1.1, pmole analyzed

TABLE X1.3 Normalization Factors for Absolute Protein Content

Timepoint (h)	BSA Yield (%)	NR Protein Yield (%)	Factor
24	0.85	0.91	1.28
48	0.84	0.91	1.29
72	0.83	0.91	1.30

NOTE 1—For example, the 24-h normalization factor is calculated by: 100/85 × 100/91 = 1.28.

(6883) × residue wt (115) = 792 ng present. Mathematically, the protein concentration is determined by taking the measured quantity of each amino acid found multiplied by its molecular weight minus 18 (water eliminated for the peptide bond) and summing them (the residue weight for each amino acid is the molecular weight minus water). An average amino acid molecular weight value of 112 mass units (m.u.) is typically used in industry when an approximation is required. Although the 18 m.u. is present on the N and C termini of every protein, their contribution is insignificant relative to the weight of a typical protein (BSA) and can be ignored (that is, 18 m.u./40000 m.u. × 100 % = 0.045 % error).

NOTE X1.6—*Data Normalization*—During hydrolysis some amino acids are destroyed totally (cysteine/cystine, tryptophan) or partially (serine, threonine), and consequently will not be included in the total protein estimation calculated above. This situation is most easily corrected by using a standard protein solution to develop a "normalization factor" for converting the observed protein content to absolute protein content. Two standards are available for this normalization: a BSA standard from NIST and an NR protein standard from ARDL. Amino acid analysis calibration factors have been determined for each of these solutions using multiple replicates and 24, 48, and 72-h hydrolysis timepoints. These values are presented in Table X1.2. Individual laboratories should determine their own normalization factor for their particular analysis system using the same standard solutions.

REFERENCES

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

(2) Bollag, D. M. and Edelstein, S. J., *Protein Methods*, John Wiley & Sons, Inc., 1991.

(3) Fryer, H. J. L., Davis, G. E., Manthorpe, M., and Varon, S.,"Lowry Protein Assay Using an Automatic Microtiter Plate Spectrophotometer," *Analytical Biochemistry* 153: 1986, pp. 262-266.

(4) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., "Protein Measurement with the Folin Phenol Reagent," *Journal of Biological Chemistry* 193: 1951, pp. 265-275.

(5) Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E., "A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples," *Analytical Biochemistry 87:* 1978, pp. 206-210.

(6) Raghupathi, R. N. and Diwan, A. M., "A Protocol for Protein Estimation That Gives a Nearly Constant Color Yield with Simple Proteins and Nullifies the Effects of Four Known Interfering Agents: Microestimation of Peptide Groups," *Analytical Biochemistry* 219: 1994, pp. 356-359.

(7) Peterson, G. L.,"Determination of Total Protein," *Meth. Enzymol*. 91: 1983, pp. 95-121.

(8) International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: *Methodology*, 6 November 1996.

(9) Krull, I. and Swartz, M., *Determination Limits of Detection and Quantitation, LC-GC 16*, #10, 1998, pp. 922-924.

(10) Schegg, K. M., et al, "Quantitation and Identification of Proteins by Amino Acid Analysis," *Techniques in Protein Chemistry*, D. R. Marshak, ed., Academic Press, 1997, pp. 207-216.