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Designation: D 5712 – 05

Standard Test Method for 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 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 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 test method.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this 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:* [2]

D 3577 Specification for Rubber Surgical Gloves

D 3578 Specification for Rubber Examination Gloves

D 4483 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.

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.

[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.

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[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.

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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 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 (**1-7**).[3]

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

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 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 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 of 10 to 100 µg/mL by diluting the protein stock solution with extraction buffer (for example,

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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 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 that has been concentrated in the same manner. All determinations are carried out from three individual NR 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, 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 5—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 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 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 7—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 8—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 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

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