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Medical gloves made from natural rubber latex — Determination of water-extractable protein using the modified Lowry method

Gants médicaux à base de latex de caoutchouc naturel — Détermination des protéines extractibles par l'eau par la méthode modifiée de Lowry

Reference number
ISO 12243:2003(E)

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 12243 was prepared by Technical Committee ISO/TC 45, *Rubber and rubber products*, Subcommittee SC 3, *Raw materials (including latex) for use in the rubber industry*.

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Introduction

There have been problems of allergic reactions experienced by some users of medical gloves manufactured from natural rubber latex. ISO 12243 specifies a method for the determination of the water-extractable protein in such gloves.

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Medical gloves made from natural rubber latex — Determination of water-extractable protein using the modified Lowry method

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This International Standard specifies a method for the determination of the amount of water-extractable protein in natural rubber (NR) gloves for medical use. The method is potentially suitable for the determination of extractable protein in other articles made from NR latex; however the extraction procedures and times have not been validated and will vary with the type of article to be tested. Other methods for the determination of specific proteins in medical gloves exist (see Annex C) but they are not of general applicability.

This International Standard is concerned solely with the method of assay. It is not concerned with sampling nor does it purport to address the safety implications of the values obtained or requirements for labelling.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10282:2002, *Single-use sterile rubber surgical gloves — Specification*

ISO 11193-1:2002, *Single-use medical examination gloves — Part 1: Specification for gloves made from rubber latex or rubber solution*

3 Principle

Water-soluble proteins are extracted into a buffer solution and then precipitated to concentrate them and separate them from other water-soluble substances which may interfere with the determination (see Annexes A and D). The precipitated protein is redissolved and quantified colorimetrically by the modified Lowry method using a protein standard (for a general review of the method, see reference [1] in the Bibliography).

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4 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

4.1
concentration factor
F
extent to which a protein extract is concentrated by precipitation followed by redissolution in a smaller volume of sodium hydroxide solution

NOTE Thus if the protein in 4 cm^3 of solution is precipitated and redissolved in 0,8 cm^3, then the concentration factor F would be 4/0,8 (= 5).

4.2
protein
proteins and protein-like substances (e.g. polypeptides) occurring in articles made from NR latex and which are extractable with water

4.3
modified Lowry method
modification of the original Lowry assay method, which involves the precipitation and isolation of the proteins to reduce the level of other water-extractable substances that may interfere in the determination

5 Apparatus

Unless otherwise stated, all laboratory equipment (i.e. flasks, tubes, etc.) shall be made of polypropylene or polyethylene.

NOTE Polypropylene or polyethylene equipment is specified rather than glass to minimize surface adsorption. A method for the determination of protein-binding capacity is described in Annex B.

5.1 Protein-free gloves, made from synthetic rubber latex or plastic and that are free of powder and other materials capable of being transferred to the test samples or extractant solutions.

5.2 Centrifuge, capable of reaching not less than 60 000 m/s^2 (6 000 $\times g$).

NOTE A refrigerated centrifuge is preferred as it is possible for the temperature to rise considerably when centrifugation is carried out for prolonged periods.

5.3 Centrifuge tubes, capacity 200 cm^3, 50 cm^3, 10 cm^3, 2 cm^3 and 1,5 cm^3, made of polypropylene or polyethylene (if available) with a low protein-binding capacity.

5.4 Conical flasks, capacity 250 cm^3.

5.5 Micropipettes.

5.6 Test tube shaker, operating at between 3 Hz and 6 Hz.

5.7 Vortex mixer or **ultrasonic bath**.

5.8 Disposable filter, with a low protein-binding capacity and a pore size of 0,45 µm or less.

5.9 Clamps, for sealing gloves watertight during extraction. Pairs of aluminium bars lined with foam rubber which can be screwed together, or 170-mm-long plastic clips as used for haemodialysis, are suggested.

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5.10 Spectrophotometric equipment.

5.10.1 Spectrophotometer, with disposable polystyrene cuvettes (quartz cuvettes may be used but require careful cleaning).

Or

5.10.2 Microplate reader, with flat-bottom polystyrene microtitre plates having 96 wells of 0,25 cm^3 to 0,5 cm^3 capacity.

NOTE Wells with a capacity of 0,5 cm^3 are preferred. Wells with a smaller capacity may be used but will reduce the sensitivity of the assay.

5.11 Balance, accurate to 0,000 1 g.

6 Reagents

During the assay, use only reagents of recognized analytical grade and distilled or deionized water.

6.1 Dye solution: Bromophenol blue, sodium salt, prepared by dissolving 0,1 g of bromophenol blue in 1 l of water. Discard the solution after four weeks.

6.2 Extractant solution: A buffer solution capable of maintaining pH 7,4 ± 0,4 throughout the extraction.

NOTE 1 Suitable buffers include phosphate buffer saline (PBS) solution (0,01 mol/l) and *N*-tris-(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid hemisodium salt (TES) solution (0,1 mol/l). The PBS buffer is prepared by dissolving a PBS tablet in distilled water in accordance with the manufacturer's instructions. In the event that, at the conclusion of the extraction, pH 7,4 ± 0,4 is not achieved, it would be necessary to use a more concentrated buffer solution. The TES solution is prepared by dissolving 24 g of TES in 500 cm^3 of water and making the volume up to 1 l.

NOTE 2 PBS tablets and TES are widely available.

6.3 Modified Lowry protein assay reagents

6.3.1 Reagent A: Alkaline copper citrate, prepared fresh daily by mixing 10 parts of reagent C with 0,2 parts of reagent D.

Alkaline copper tartrate is also considered to be suitable. It shall also be prepared fresh daily. The material available in kits can contain undeclared preservatives which may affect the determination.

6.3.2 Reagent B: Dilute Folin reagent prepared by diluting 72 cm^3 of 2 N Folin reagent with 28 cm^3 of water.

NOTE 2 N Folin reagent is available commercially. It can, for example, be obtained from Sigma Chemical Co. (Catalogue No. F 9252), Box 14508, St Louis, MO 63178, USA. The concentration of some commercial Folin reagents may not be 2 N.

6.3.3 Reagent C: A solution of 6 g of sodium carbonate in 100 cm^3 of water.

6.3.4 Reagent D: A solution containing 1,5 g of copper sulfate and 3 g of sodium citrate in 100 cm^3 of water.

6.3.5 Sodium hydroxide solution, c(NaOH) = 0,2 mol/l.

6.3.6 Sodium deoxycholate (DOC) solution, prepared by dissolving 0,15 g of sodium deoxycholate in water and diluting with water to 100 cm^3. Store the solution in a refrigerator, discarding it after 4 weeks.

6.3.7 Trichloroacetic acid (TCA) solution, prepared by diluting 72 g of trichloroacetic acid to 100 cm^3 with water and mixing thoroughly. Store the solution in a refrigerator. The solution is stable over a long period.

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6.3.8 Phosphotungstic acid (PTA) solution, prepared by diluting 72 g of phosphotungstic acid to 100 cm^3 with water and mixing thoroughly. Store the solution in a refrigerator, discarding it after 4 weeks.

It may be convenient to premix the TCA and PTA solutions in equal volumes and to add them simultaneously in 7.4.2. Such a mixture shall be prepared daily in the absence of data on its storage life.

6.4 Ovalbumin protein stock solution.

Use ovalbumin prepared by ammonium sulfate fractionation and repeated crystallization at pH 4,5 such as Sigma A 5503 from Sigma Chemical Co., Box 14508, St Louis, MO 63178, USA.

Prepare a solution of 100 mg of ovalbumin in 100 cm^3 of the preferred extractant (6.2) to give a concentration of 1 mg/cm^3. Filter the solution through a low-protein-binding filter of 0,45 µm or smaller pore size and determine the absorbance at 280 nm using a UV spectrophotometer with a 1 cm path length cuvette and employing extractant solution (6.2) as a blank. Divide the absorbance by 0,64 [1)] to obtain the precise concentration of the ovalbumin stock solution. The solution is stable for 2 days when stored at a temperature of not more than 7 °C or for 2 months frozen at −10 °C. Thawing requires heating to 45 °C for 15 min.

NOTE The length of time under refrigeration is cumulative. In order to avoid repeated thawing and freezing, it is recommended that the stock solution be stored as aliquot portions each sufficient for the preparation of a single calibration curve or for use in the verification procedure (see Annex A).

7 Procedure

7.1 Principle

The procedure involves the extraction of a whole glove followed by purification and concentration of the extract. The concentration of protein in the extract is determined by reference to a standard calibration curve prepared using dilutions of the protein stock solution (6.4 and 7.3) which has been concentrated in the same manner. The analytical technique of the analyst must previously have been verified as described in Annex A.

The extraction is run in triplicate using three gloves or pairs of gloves from a given lot; the purification and concentration of each extract and the subsequent determination are run singly.

7.2 Extraction procedure

7.2.1 General

The entire surface of the glove shall be exposed to the extractant at 25 °C ± 5 °C for a period of 120 min ± 5 min. Two extraction procedures are permitted, the so-called “cut-glove” procedure and also the “glove-in-glove” procedure. The procedure used shall be noted in the test report and all samples in a given series shall be extracted by the same procedure. The extraction shall be carried out in triplicate and single determinations run on each extract.

Use protein-free gloves (5.1) to handle the glove samples used for the extraction.

NOTE The frequency of sampling and left- or right-handedness of gloves are outside the scope of this document.

7.2.2 Procedure A — Cut-glove procedure

7.2.2.1 Record the mass of the glove (m) to an accuracy of not less than 0,001 g.

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1) The precise value of the extinction coefficient of ovalbumin is subject to confirmation.

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7.2.2.2 Cut the glove along the periphery. To facilitate the extraction, it is permissible to cut the glove into smaller pieces (but see 7.2.2.3).

7.2.2.3 If the result is to be reported in micrograms per unit area of the glove (e.g. µg/dm^2), determine the surface area of the glove as follows:

Cut a rectangular piece from the back of the glove of about 0,5 dm by 0,5 dm and measure its dimensions accurately. Calculate the area A_1.

Determine the mass (m_p) of the rectangular piece to the nearest 0,001 g.

The total surface area A of both sides of the glove is given by $A = 2A_1 \times m/m_p$.

7.2.2.4 Transfer all the pieces of the glove to a suitable conical flask (5.4).

7.2.2.5 Add accurately a volume V of extractant (6.2). The total volume V of extractant used shall be between 10 cm^3 and 15 cm^3 per gram of glove and sufficient to cover the pieces.

7.2.2.6 Extract the test sample at 25 °C ± 5 °C for 120 min ± 5 min, shaking for 15 s initially and thereafter at intervals not greater than 30 min. If practical, continuous slow shaking is desirable.

7.2.2.7 Decant off the extract and remove any particulate matter by centrifuging at not less than 20 000 m/s^2 (2 000 × g) for 15 min. The extract is preferably used immediately but may be stored for up to 48 h at a temperature of not more than 7 °C or frozen for up to 15 days at below −10 °C.

7.2.3 Procedure B — Glove-in-glove procedure

7.2.3.1 Take two gloves and determine the mass of each one to an accuracy of not less than 0,001 g (m_1 and m_2). Mark each glove at a point on the cuff 20 cm from the tip of the middle finger. Take one glove and insert it inside the other so that they fit together (this can be done conveniently using rods to insert the thumb into the thumb, etc.; however, the method of doing this is not critical as long as the gloves are exposed to minimum handling). Repeat the process with two further pairs of gloves of the same size.

7.2.3.2 Pour sufficient dye solution (6.1) into the inner glove to fill all of the fingers. Introduce 25 cm^3 of extractant (6.2) between the inner and outer glove. Manipulate gently to remove any air bubbles and seal the gloves with a clamp (5.9) at the 20 cm mark.

7.2.3.3 Fix the gloves to a shaker and shake for 120 min ± 5 min at 25 °C ± 5 °C. If small droplets of liquid are noted on the outer surface, suggesting the presence of pinholes in the outer glove, discard the samples and repeat the extraction with a fresh pair of gloves.

7.2.3.4 Remove the clamp and separate the gloves carefully, taking care not to contaminate the extract with the dye solution in the inner glove.

7.2.3.5 Decant the extract from the outer glove into a centrifuge tube (5.3). If it is coloured blue, it is indicative of a pin-hole or cross-contamination. In such cases, discard the solution and repeat the extraction with a fresh pair of gloves. Clarify the extract by centrifugation at not less than 20 000 m/s^2 (2 000 × g) for 15 min. Store the extract at a temperature of not more than 7 °C and carry out the determination within 48 h. Alternatively, frozen aliquots of the extract may be stored at −10 °C or lower for up to 15 days.

7.2.3.6 Cut both gloves at the 20 cm mark to remove the cuffs. Remove surplus liquid from the cuffs by blotting and allow to dry at room temperature. Determine the mass of the cuffs (m_c) to an accuracy of not less than 0,001 g. Calculate the average mass (m_s) of the extracted part of the gloves: $m_s = (m_1 + m_2 - m_c)/2$ where m_1 and m_2 are the masses of the original gloves and m_c is the combined mass of the un-extracted cuffs.

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7.3 Preparation of standard protein solutions

Prepare standard solutions of protein by dilution of the protein stock solution (6.4) with extractant solution (6.2), to make solutions with concentrations of e.g. 40 µg/cm^3, 20 µg/cm^3, 10 µg/cm^3, 5 µg/cm^3, and 2,5 µg/cm^3. Use the extractant (6.2) as a blank. The solutions are stable for 2 days refrigerated (see the Note).

NOTE The lower concentrations can readily be prepared by two-fold serial dilution of the appropriate more concentrated solution. The standard solutions should cover a wide range of concentrations the precise values of which are known since the exact concentration of the stock solution has been determined (see 6.4). These solutions are also required for the verification procedure described in Annex A.

7.4 Precipitation and concentration of protein

7.4.1 General

Carry out single determinations at 25 °C ± 5 °C.

7.4.2 Accurately transfer 4 cm^3 each of extractant (6.2) (as a blank), the standard protein solutions (see 7.3) and the three glove extracts to 10 cm^3 centrifuge tubes (5.3). Add 0,4 cm^3 of DOC (6.3.6), mix and allow to stand for 10 min, then add 0,4 cm^3 of TCA (6.3.7) and mix. Add 0,4 cm^3 of PTA (6.3.8), mix (see the Note) and allow to stand for a further 30 min.

NOTE The amount used is to ensure a sufficient quantity for analysis using a cuvette. If a micro-plate reader is used, the quantities may be reduced proportionately. If a large number of samples is involved, it is particularly important to ensure that the centrifuge tubes are clearly identified.

7.4.3 Centrifuge at not less than 60 000 m/s^2 (6 000 × g) for 30 min. It is important that the protein is properly compacted. If necessary, extend the time of centrifugation. Decant the supernatant liquid and drain by inverting each centrifuge tube on an absorbent paper towel. Add 0,8 cm^3 of 0,2 mol/l sodium hydroxide solution (6.3.5) to each tube, including the blank, to redissolve the precipitated protein. Use a vortex mixer or ultrasonic water bath (5.7) if needed.

Ensure that the protein has completely redissolved to give a clear solution. Should some protein precipitate remain, add a further measured quantity of sodium hydroxide solution up to 3,2 cm^3 (i.e. a total of 4,0 cm^3). The same amount of sodium hydroxide shall be used for each of the solutions. The recommended amount of sodium hydroxide solution (0,8 cm^3) gives a concentration factor F of 5. If the same amount of sodium hydroxide is not used for each sample, then F will vary from one sample to another:

$$F = \frac{\text{Volume of extract before precipitation}}{\text{Volume of NaOH used to redissolve the protein}}$$

The redissolved-protein solution should preferably be used the same day. If the determination cannot be carried out at once, the pellet may be stored for not more than 24 h at a temperature not exceeding 7 °C.

In cases where complete dissolution in not achieved after addition of 4,0 cm^3 of NaOH, centrifuge at 60 000 m/s^2 (6 000 × g) for 15 min to give a clear protein solution.

7.5 Colour development

7.5.1 Switch on the spectrophotometer and zero it in accordance with the manufacturer’s instructions.

7.5.2 To 0,8 cm^3 of the redissolved-protein solutions, including the blank from 7.4.2, add 0,3 cm^3 of reagent A (6.3.1) and mix well. Add 0,1 cm^3 of reagent B (6.3.2), mix, and allow to stand for at least 15 min but no longer than 1 h before measuring the absorbance.

NOTE Only 0,8 cm^3 of the redissolved-protein solution is used for the colour reaction, regardless of the final volume of the redissolved-protein solution.

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If precipitation occurs on standing due to the presence of certain interferants, centrifuge to give a clear solution prior to colour measurement.

7.5.3 Spectrophotometric measurement

Transfer the solutions prepared in 7.5.2 to cuvettes and measure the absorbance versus the blank at 750 nm (preferred) or a specific wavelength in the range 600 nm to 750 nm within 1 h of adding reagent B. For uniform results, the time scales, equipment and chosen wavelength must remain consistent. Determine the protein content, in micrograms per gram of glove, as described in 8.3.

Or

7.5.4 Measurement using a micro-plate reader

Transfer 0,49 cm^3 of the solutions prepared in 7.5.2 to a flat-bottom microtitre plate (see 5.10.2) and measure the absorbance versus the blank at a specific wavelength in the range 600 nm to 750 nm within 1 h of adding reagent B. Determine the protein content, in micrograms per gram of glove, as described in 8.3.

8 Calculation of results

8.1 Calibration curve

Prepare a calibration curve by plotting the concentration of the original protein solutions (see 7.3) against their absorbance after undergoing precipitation and being redissolved (see 7.5.3 or 7.5.4).

NOTE Some protein is lost during the concentration process. The method assumes that the same percentage of protein is lost from the standards as from the test samples during concentration.

8.2 Calculation of concentrations

Determine the concentration c of each of the three extracted samples, in micrograms per cubic centimetre of extract, by using their absorbance to read them directly from the curve. Report the median value.

NOTE In the event that the calibration curve is non-linear, the value can be calculated by polynomial regression. It is suggested that commercial computer software for curve fitting and calculation of unknown concentrations is more practical.

8.3 Calculation of extractable-protein content

8.3.1 Procedure A — Cut-glove procedure

Calculate the extractable-protein content E, in micrograms per gram of glove, from the equation:

$$E = \frac{V \times c \times 5}{F \times m}$$

where

- V is the volume of extractant used, in cubic centimetres;
- c is the protein concentration in the redissolved-protein solution, in micrograms per cubic centimetre;
- F is the concentration factor;
- m is the mass, in grams, of the whole glove.

NOTE The value of $5/F$ will be 1 unless it has been necessary to use other than the recommended amount of sodium hydroxide — see comment in 7.4.3.

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The extractable protein per glove, in micrograms, is obtained by multiplying the result obtained above by m:

Extractable protein per glove $= E \times m$

8.3.2 Procedure B — Glove-in-glove procedure

Calculate the extractable-protein content E, in micrograms per gram of glove, from the equation:

$$E = \frac{V \times c \times 5}{F \times m_s}$$

where

V is the volume of extractant used, in cubic centimetres;

c is the protein concentration in the redissolved-protein solution, in micrograms per cubic centimetre;

F is the concentration factor;

m_s is the mass, in grams, of the glove sample extracted (see 7.2.3.6).

NOTE The value of $5/F$ will be 1 unless it has been necessary to use other than the recommended amount of sodium hydroxide — see comment in 7.4.3.

The extractable protein per glove, in micrograms, is obtained by multiplying the result obtained above by m:

Extractable protein per glove $= E \times m$

where

m is the mass, in grams, of a whole glove $\left[= (m_1 + m_2)/2\right]$;

m_1 and m_2 are the respective masses of the original pair of gloves.

8.3.3 Conversion to mass per unit surface area

Regulatory authorities may require the results to be expressed in terms of surface area, e.g. micrograms per unit area. Conversion to these values is as follows:

Extractable protein in µg/dm^2 $= \dfrac{V \times c \times 5}{F \times A}$

where A is the total surface area of the glove (see 7.2.2.3), in square decimetres.

9 Precision

9.1 Background

An interlaboratory test programme (ITP) to evaluate the precision of the method was conducted in 2002 using the precision procedures and guidelines described in ISO 9272 (in preparation). The existing ISO/TR 9272 may be consulted for other details and terminology.

Both extraction procedures were evaluated: the cut-glove procedure and the glove-in-glove procedure. The ITP was conducted with four materials with increasing measurement levels. Seven laboratories participated in the ITP, and a Type 1 precision was evaluated. A test result is the mean of three replicates on each of two separate test days, and precision is given in terms of test results, i.e. a mean value for each of two test days.

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The precision results as determined by this ITP shall not be applied to acceptance or rejection testing for any group of materials or products without documentation that the results of this precision evaluation actually apply to the products or materials tested.

9.2 Precision results

For each of the four materials, the precision results for both procedures are given in Table 1. These results were obtained using the outlier replacement procedures and outlier deletion procedures as described in ISO 9272. General statements for the use of the precision results are cited below. These are given in terms of both the absolute precision, r and R, and also for the relative precision, (r) and (R). See additional comments below.

The repeatability and reproducibility statements are as follows:

Repeatability: The repeatability, or local-domain precision, for each of these procedures has been established by the values found in Table 1, for each measurement level (for the materials) as listed in the table. Two single mean test results (obtained by the proper use of this International Standard) that differ by more than the tabulated values for r, in measurement units, and (r), in percent, shall be considered as suspect, i.e. to have come from different populations. Such a decision suggests that some appropriate investigative action be taken.

Reproducibility: The reproducibility, or global-domain precision, for each of these procedures has been established by the values found in Table 1, for each measurement level (for the materials) as listed in the table. Two single mean test results obtained in different laboratories (by the proper use of this International Standard) that differ by more than the tabulated values for R, in measurement units, and (R), in percent, shall be considered as suspect, i.e. to have come from different populations. Such a decision suggests that some appropriate investigative action be taken.

9.3 Additional comments

For the cut-glove procedure, the analysis showed that two laboratories had excessive outliers. Although an outlier replacement operation was conducted using ISO 9272 procedures, both repeatability and reproducibility were still quite poor. The results shown in Table 1 for the cut-glove procedure are for the analysis with both outlying laboratories deleted from the database, i.e. for five participating laboratories. For the glove-in-glove procedure, one of the same laboratories also had excessive outliers, which again resulted in poor precision. The results given in Table 1 for the glove-in-glove procedure are for the analysis with this one laboratory deleted from the database, i.e. for six participating laboratories.

9.4 Bias

Bias is the difference between a measured average test result and a reference or true value for the measurement in question. Reference values do not exist for these procedures and therefore bias cannot be evaluated.

10 Test report

The test report shall contain at least the following information:

a) a reference to this International Standard;

b) sufficient information to adequately identify the sample tested;

c) the date and results of the test;

d) the source of standard protein used and its identification;

e) the nature of the buffer used;

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f) the extraction procedure followed (procedure A or procedure B);

g) the name and address of the test laboratory, if different from the glove manufacturer;

h) any unusual behaviour noted and any deviation from the specified procedure.

Table 1 — Precision data

Cut-glove procedure (procedure A)								
Material	**Mean value** µg/g	**Within lab**			**Between labs**			**No. of labs**
		s_r	r	(r)	s_R	R	(R)	
1	14,3	3,48	9,7	68,3	7,57	21,2	148,5	5
2	68,3	6,46	18,1	26,5	12,6	35,2	51,5	5
3	162,2	6,79	19,0	11,7	25,1	70,3	43,3	5
4	200,6	13,6	37,9	18,9	28,2	78,9	39,3	5
Glove-in-glove procedure (procedure B)								
Material	**Mean value** µg/g	**Within lab**			**Between labs**			**No. of labs**
		s_r	r	(r)	s_R	R	(R)	
1	13,8	1,66	4,64	33,6	4,70	13,2	95,2	6
2	53,1	4,97	13,93	26,3	16,3	45,6	86,0	6
3	140,0	5,25	14,70	10,5	21,7	60,9	43,5	6
4	164,2	11,21	31,40	19,1	32,6	91,4	55,6	6

Notation used:

s_r is the within-laboratory standard deviation (in measurement units);

r is the repeatability, i.e. within-lab precision (in measurement units);

(r) is the repeatability (in percent of mean level);

s_R is the between-laboratory standard deviation (for the total between-laboratory variation in measurement units);

R is the reproducibility, i.e. between-lab precision (in measurement units);

(R) is the reproducibility (in percent of mean level);

No. of labs is the number after deletion of excessive-outlier laboratories.

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Annex A
(normative)

Verification

A.1 General

Chemicals such as surfactants, accelerators and antioxidants added to the NR latex during the manufacture of the gloves can interfere with the colour development during the determination; some chemicals may reduce colour development while others can increase it.

The process of concentrating the protein by precipitation and redissolving is intended to purify the protein by ridding it of these interferants. It is inevitable that during this process a certain amount of protein is lost and it is assumed for the purposes of the test that the same percentage will be lost from the protein standard solutions as from the test sample extracts.

In order to ensure that the operation is carried through with the minimum of losses, it is mandatory that the technique of new laboratories and/or new operators be verified by determining the actual level of recovery achieved when precipitating and redissolving the protein standards, as described below.

A.2 Principle

Standard protein solutions are concentrated in duplicate and the resultant solutions then tested in duplicate to assess the consistency of the operator's work.

A.3 Procedure

A.3.1 Preparation of unprecipitated-protein standard solutions

Using the protein stock solution (6.4), prepare dilutions with 0,2 mol/l sodium hydroxide solution (6.3.5) to give protein standard solutions having concentrations of 80 μg/cm^3, 40 μg/cm^3, 20 μg/cm^3, 10 μg/cm^3 and 5 μg/cm^3.

A.3.2 Preparation of standard protein solutions for precipitation

Similarly, using the protein stock solution (6.4), prepare dilutions using the extractant (6.2) to give standard protein solutions having concentrations of 40 μg/cm^3, 20 μg/cm^3, 10 μg/cm^3, 5 μg/cm^3 and 2,5 μg/cm^3.

A.3.3 Precipitation and concentration of protein

Carry out the procedure in duplicate.

Using the procedure described in 7.4, precipitate the standard protein solutions diluted with extractant (see A.3.2). Redissolve the precipitated protein in 0,2 mol/l sodium hydroxide (6.3.5) to give two solutions for each concentration having a concentration factor F = 5.

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A.3.4 Colour development and determination

Carry out the procedure in duplicate.

Using the procedure described in 7.5, carry out the determination on the unprecipitated protein solutions (see A.3.1) and each of the solutions obtained from the protein which was precipitated and redissolved (see A.3.3).

A.3.5 Calculation

Prepare a calibration curve by plotting the average absorbances of the unprecipitated standard protein solutions against their concentrations (see A.3.1), and use the curve thus produced to determine the concentration c of the protein solutions after precipitation and concentration (see A.3.3). Average c for each group of four determinations (each protein standard was precipitated in duplicate and the resultant concentrate also tested in duplicate to give four results for each protein standard).

A.3.6 Percent recovery

The percent recovery is c/F, expressed as a percentage of the concentration of the original standard protein solution (see A.3.2) before precipitation. Plot the percent recovery against the original concentrations.

EXAMPLE Protein from an original dilution of 50 μg/cm^3 was precipitated and redissolved to give a 5× concentration (F = 5) and c found to be 200 μg/cm^3. Thus c/F = 40 μg/cm^3 and the difference from the original 50 μg/cm^3 reflects the material lost during the process. Expressing the new value as a percentage of the true value, (40/50) × 100 = 80 %, gives the percent recovery.

A.3.7 Requirement

The percent recovery shall not be less than 80 % at concentration levels lower than 100 μg/cm^3. If this has not been achieved, repeat the process, paying particular attention to technique. The operator's technique shall be verified before undertaking determinations on glove samples.

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Annex B
(normative)

Protein adsorption on polypropylene and polyethylene tubes

B.1 General

Polypropylene or polyethylene tubes are used throughout since they are known to have a low protein-binding capacity. To check the actual adsorption, the following method is appropriate.

The test shall be started and completed in not more than one day.

B.2 Procedure

B.2.1 Prepare 50 cm^3 of a reference solution containing 10 µg/cm^3 of ovalbumin by dilution of the standard solution (6.4) with the extractant solution (6.2).

B.2.2 Transfer 10 cm^3 test portions of the ovalbumin solution prepared in B.2.1 to each of two fresh polypropylene or polyethylene tubes (5.3) and shake the tubes on a test tube shaker (5.6), ensuring that the whole surface of the tube is wetted by the solution. After 30 min, transfer the solutions to a further two tubes and shake them. Repeat the procedure until each 10 cm^3 portion has been exposed to five tubes. Store the remaining test solutions.

B.2.3 Determine the concentration of the protein in the reference solution prepared in B.2.1 and the two test solutions prepared in B.2.2 in triplicate using the method given in 7.5.

B.3 Calculation

Calculate the average mass of ovalbumin adsorbed per tube, in micrograms, from the equation:

$$\text{Ovalbumin adsorbed per tube} = \frac{10 \times (R - T)}{5}$$

$$= 2 \times (R - T)$$

where

R is the mean of the three determinations of the ovalbumin content of the reference solution;

T is the mean ovalbumin content of the test solution after passage through the tubes (i.e. the mean of six values).

B.4 Requirement

The value obtained for the adsorbed albumin shall be less than 10 µg/tube. If the value exceeds this, the tubes are unsuitable for the determination.

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Annex C
(informative)

Alternative methods of analysis

C.1 General

It is recognized that there are inherent difficulties in the modified Lowry method for the determination of water-extractable proteins. Surfactants and certain accelerators may interfere in the determination, leading to false high results or, rarely, false low results. This International Standard, involving a specified precipitation and redissolution of the extracted proteins, is intended to alleviate this problem, although it is not always satisfactory, especially with certain accelerators [4]. There is also a potential for losses during the precipitation and recovery of the protein fraction, although the analytical procedure coupled with the verification technique is intended to compensate for this. One potential method for reducing the problem of interference is “background subtraction” (see Annex D).

Other methods exist for the isolation and/or determination of proteins, and it may be desirable on occasions to use one of these as a cross-check in the event that the results obtained using this International Standard appear to be spurious. Two of these methods are briefly described below. Either of them may provide the basis of a superior analytical method in the future, but at present they also have deficiencies.

C.2 ELISA (enzyme-linked immuno-sorbent assay)

This method depends on the reaction of specific proteins with the specific antibodies associated with an allergic reaction.

The problems with the method are that it is comparatively slow and that it is, if anything, too specific.

The ideal way of doing the test would be to react a specific molecular-mass band of protein with a single (i.e. monoclonal) antibody. However, although several protein fractions that may cause an allergic reaction can be isolated from natural rubber latex concentrate, not all susceptible people react to the same fractions. It follows that, although a reaction might appear to be minimal, for other people it could be serious.

The opposite approach, i.e. using a heterogeneous mixture of antibodies from pooled serum, is also unsatisfactory since the ratios of the differing molecular-mass bands of proteins to each other are not necessarily constant. This ratio may vary both as a result of differences in the ease of extraction and also differences between different clones of rubber trees. It is also implicit that the results of the reactions of different antibodies with different molecular-mass band proteins would need to be integrated.

C.3 HPLC (high-performance liquid chromatography)

This can be done using gel separation of the extracted proteins, or they can be hydrolysed and determined as amino acids. In both cases, the technique is slow and expensive. It would separate all of the interferants together with the various molecular-mass band proteins. However, it would be necessary to identify each of the separated bands so that the correct fractions could be integrated. If the proteins are hydrolysed and determined as amino acids, there is no indication of what proteins are involved.

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Annex D
(informative)

Background subtraction

D.1 General

A method has been developed which appears to provide a high degree of correlation with the presence of interferants in the glove extract. The test relies on the observation originally made by Lowry *et al* [5] that the colour development associated with proteins in the presence of Folin reagent is largely dependent on the presence of copper, whereas the interfering materials develop colour with Folin reagent in the absence of copper. The method is included here for the benefit of those who wish to determine the quantity of the interferants in gloves.

D.2 Principle

The redissolved precipitated protein extract from the gloves is divided into two separate portions. On one of these portions, the colour development is carried out as described in 7.5. On the second portion, the colour development is carried out omitting the copper sulfate from reagent D (6.3.4). The value of the absorbance thus obtained is termed the "background" and is subtracted from that of the complete determination including copper in the formulation.

D.3 Reagents

D.3.1 Reagent DA: Alkaline sodium citrate, prepared fresh daily by mixing 10 parts of reagent C with 0,2 parts of reagent DD.

D.3.2 Reagent B, as specified in 6.3.2.

D.3.3 Reagent C, as specified in 6.3.3.

D.3.4 Reagent DD: A solution containing 3 g of sodium citrate in 100 cm^3 of water.

D.4 Procedure

D.4.1 The analysis is run in duplicate simultaneously with the standard determination described in 7.5. The amount of redissolved-protein solution must be sufficient for four determinations (two duplicates). Dependent on the equipment being used, it may be necessary to increase the amount of protein extract which is precipitated, with a consequent scale-up in the amount of sodium hydroxide solution used to redissolve the protein.

D.4.2 Carry out the procedure on the standard protein solutions prepared in 7.3 at the same time, using the method described in 7.5.

D.4.3 To determine the background, follow the procedure in 7.5 but substitute reagent DA (D.3.1) for reagent A (6.3.1). Measure the absorbance at the same specific wavelength in the range 600 nm to 750 nm within 1 h. Average the values for each duplicate determination.

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D.5 Expression of results

D.5.1 Calibration curve

Prepare the calibration curve by plotting the concentrations of the standard protein solutions prepared in 7.3 against their absorbance minus the absorbance of their background.

D.5.2 Calculation

Subtract the value of the background absorbance from that determined on the protein extract in the presence of copper and read the adjusted value, in micrograms per cubic centimetre, directly from the calibration curve.

By using the value of the absorbance of the solution that does not contain copper (D.3.4) with the calibration curve, it is possible to obtain a value for the protein equivalence of the interferants.

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