

Regulatory aspects of latex allergy (CEN; extractable protein and allergen assays for latex gloves)

H.U. KOCH

SUMMARY: Due to the dramatic increase of type I allergies to natural rubber latex gloves the working group 3 of CEN TC 205 decided to include the biological evaluation into the standard EN 455, medical gloves for single use. A study supported by the European Community and a harmonisation between ASTM, ISO and CEN finally led to a standard procedure for the determination of extractable proteins in latex gloves. This method was shown to be the most useful method that revealed (except of some outliers) allergological relevant protein values. The most important steps of the method are: 1. Double glove extraction (or equivalent), 2. TES buffer pH 7.4 (or equivalent), 3. 2 h, 25 C°, moderate agitation, 4. Modified Lowry, 5. Values shall be given in µg/g glove. Although not useful as a standard procedure, the amino acid analysis (by HPLC) for total protein determination is recommended as reference method, because it revealed the best correlation to the allergological response. The immunological methods are not recommended at present, because standardized antibodies and standard allergens are not available.

During the last ten years a dramatic increase in adverse reactions to medical gloves for single use was observed especially in employees of medical health services.

Medical gloves may cause irritant reactions (glove powder, preservatives, endotoxin), type I allergies (natural rubber latex proteins) or type IV allergies (accelerators, preservatives, dyes). Within the last 8 years 48% of 1335 patients with adverse reactions to medical gloves revealed an allergy to glove material. Most of these allergies (78.9%) were identified as type I reactions to latex proteins. Whereas the number of type IV allergies Erlangen decreased since 1990, type I allergies are still increasing [6].

Several authors reported different numbers of allergic patients in medical professions using latex

gloves. Among 2500 prick tested health care workers in hospitals in Finland, USA, France, Canada, Sweden, Belgium, Venezuela and Germany 8.04 % were allergic against natural rubber latex (range 2.5 in Venezuela - 17.0 % in USA) [1, 5, 7, 10, 11, 12, 13].

Due to this increasing problem with natural rubber latex gloves about five years ago the working group 3 of CEN TC 205 decided to include the biocompatibility of gloves into the standard EN 455, medical gloves for single use. Finally, in October 1997 the prEN 455-3 (Medical gloves for single use - Part 3: Requirements and testing for biological evaluation) was published.

An important part of the scientific progress and the harmonisation of the existing methods of protein determination in latex gloves was the study:

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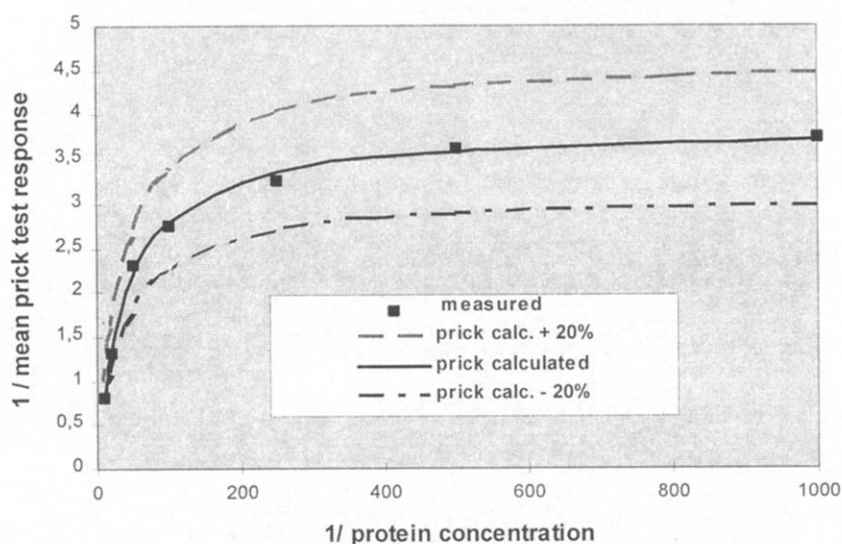


Fig. 1. – Mean prick test response in 16 volunteers to a series of diluted freeze dried latex sera.

Determination of allergological relevant compounds in disposable gloves - Correlation of chemical, allergological and immunological data (Contract-nr. MATI-CT 940060). This study was supported by the European Community and performed by the Departments of Dermatology of the Universities of Erlangen and Copenhagen and by the Austrian Institut für Biomedizinische Werkstoffprüfung.

The aim of this study was to find standard methods for the determination of proteins and accelerators in latex gloves that correlate with their allergological potency.

In this presentation I will report the determination of soluble proteins in natural rubber latex gloves. We tested 6 different methods for protein determination, 4 chemical methods for the determination of total protein content (amino acid analysis, Bradford, modified Lowry, ESL) and 2 immunological methods for the determination of allergenic or antigenic protein content (RAST-inhibition, LEAP). The allergological relevance of these results was scrutinized with the prick test response of at least 15 volunteers to the extracts and the corresponding glove material.

In order to evaluate the correlation between the protein concentration and the allergological response we determined the mean prick test response to a dilution series of a lyophilized low ammoniated latex serum in 16 volunteers. The concentrations were adjusted to the range of protein content in glove extracts (10 µg/ml to 1 000 µg/ml). The mean prick test results plotted versus the protein concentration resulted in a curve of saturation (fig. 1), because the mean prick test results cannot exceed the highest response 4.0.

Similar curves are known in enzyme kinetics and receptor analysis described by the Michaelis - Menten - equation [9]. Therefore we tried in analogy a double reciprocal plot (fig. 2) to get a linear correlation. We found a surprisingly high correlation of the prick test response and the protein concentration (0.9994 !). In such a double reciprocal plot high values (= low values in the plot) often are underestimated. To show the influence of variations we included, beside the measured values (dots), the calculated linear regression and the $\pm 20\%$ - curves of the linear regression. That means that, a 20% variation in the measured values would lead to a dot on these two lines. The maximum response calculated by the linear regression via Michaelis-Menten equation was 3.86, which is nearly the theoretical value of 4.0.

AMINO ACID ANALYSIS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

The determination of proteins is usually based on colour reactions with particular structural elements, which are not regularly distributed in different proteins [3, 8]. Therefore the response factor differs considerably from protein to protein. Amino acid analysis by HPLC avoids these problems. Following a hydrolysis of proteins by hydrochloric acid the resulting amino acids are quantified with HPLC [4], which is independent of any structural feature of the original polymeric molecule. The summation of the individual amino acids subsequently reveals the total protein content.

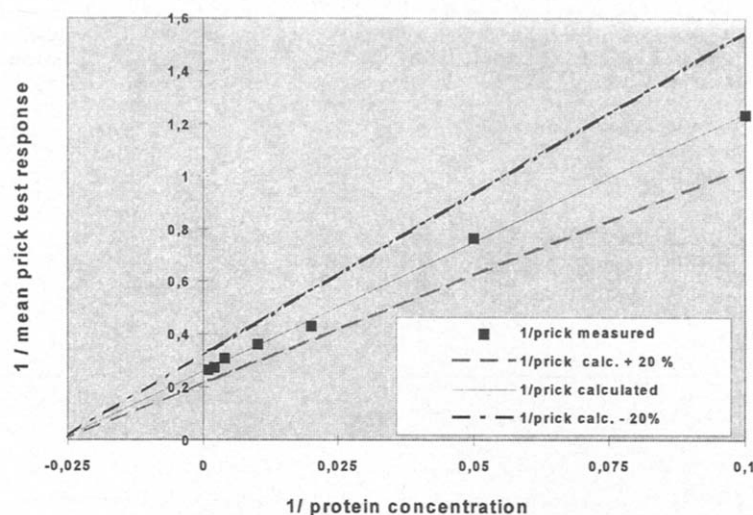


Fig. 2. – Double reciprocal plot of the mean prick test response versus the protein concentration of a dilution series of a freeze dried latex serum.

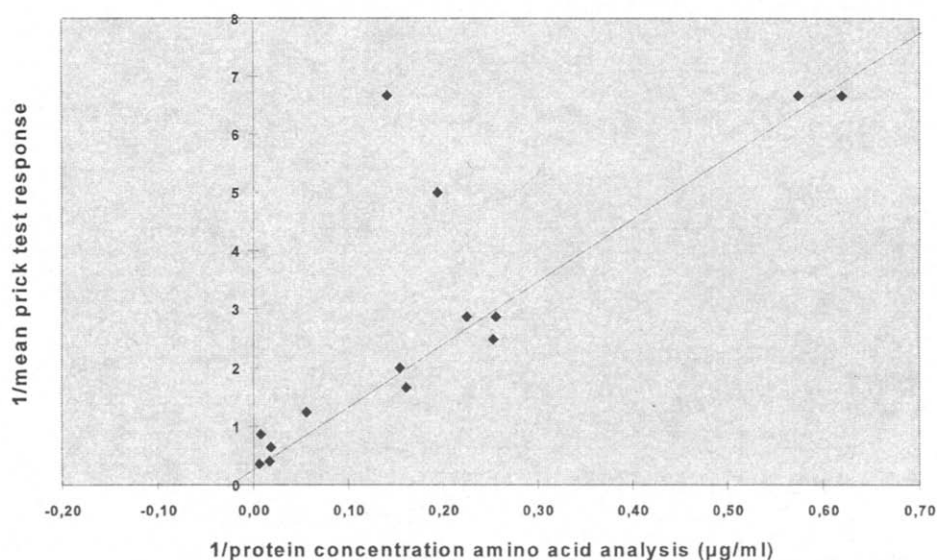


Fig. 3. – Double reciprocal plot of the mean prick test response in 18 volunteers versus the protein concentration of 15 different gloves. Protein was measured as amino acids after hydrolysis.

Accuracy and precision of the amino acid analysis were detected by the determination of two concentrations of ovalbumin in tris buffer pH 8.2 and by one glove extract (tris buffer pH 8.2). The real protein concentration of ovalbumin was measured by the absorption at 280 nm (lit). The recovery with the amino analysis was 101 and 97.6 % and the coefficient of variation 6.5 to 8.4 % (table I).

Additionally the amino acid analysis revealed the best correlation (0.97) using the double reciprocal plot (fig. 3). Among 30 different glove brands tested in two different series of volunteers

only two gloves revealed a higher protein content than we would expect by the prick test response. To one of these gloves, casein was added during the manufacturing process. Due to the theoretical approach and the excellent correlation to the prick test, the amino acid analysis by HPLC was used as the reference method within this study. However, this method is not useful as a standard procedure. It is very time consuming (at least 5 days per analysis), it is expensive (60 000 DM for the equipment with a maximum of 15 analyses per day) and it needs a lot of experience to separate and identify 20 amino acids.

Table I. – Precision and accuracy of the amino acid analysis and modified Lowry. The measurements were performed on ten different days with ten different HCl hydrolyses of every sample. Cv = coefficient of variation (%) of 10 analyses. The recovery was calculated as fractions of the ovalbumin detected by UV absorption.

Protein	UV 280 nm	Lowry			Amino acid analysis		
	µg/ml	mean µg/ml	recovery (%)	cv (%)	mean µg/ml	recovery (%)	cv (%)
Ovalbumin	5.0	5.1	102.0	10.1			
Ovalbumin	10.0	11.0	110.0	8.4	10.1	101.0	7.6
Ovalbumin	25.0	26.1	104.4	8.4	24.4	97.6	6.5
Ovalbumin	50.0	51.1	103.0	7.9			
Ovalbumin	100.0	100.4	100.4	6.9			
Glove 22	–	30.2	–	7.8	23.4	–	8.4

Table II. – Interferences of some accelerators with the modified Lowry assay simulating high protein concentrations.

Accelerator	Concentration in double distilled water	Colour	Apparent protein concentration (µg/ml)
Blank	0		0
Tetramethyl thiurammonosulfide	saturated	regular blue	8.4
Tetramethyl thiuramdisulfide	saturated	yellow (Cu+)	398
Zinc diethyl dithiocarbamate	saturated	regular blue	50.4
Sodium diethyl dithiocarbamate	1 mg/ml	yellow (Cu+)	30.8
N,N'-Diphenylthiourea	saturated	regular blue	53.2
Zinc mercaptobenzothiazole	saturated	regular blue	44.8
Zinc dibutyl dithiocarbamate	saturated	regular blue	8.4
Tetramethylthiourea	1 mg/ml	regular blue	316

BRADFORD METHOD FOR TOTAL PROTEIN CONTENT

The Bradford method (based on the colour change of Coomassie blue dye in response to various concentrations of protein) is a very sensitive and simple method for the determination of proteins [2]. Many substances strongly influence the Bradford method. In the case of glove extracts mainly surface active agents were of interest. Surface active substances are widely used in the final steps of glove processing (e.g., powdering and surface coating).

Comparing the results of the Bradford method with the amino acid analysis in 24 different gloves, we found lower values in 7 cases and higher values in 17 cases. The ratio HPLC / Bradford varies from 0.028 to 7.05 with a mean of 2.39 ± 2.00 . The results of the Bradford method therefore seemed to be too high in the most cases as expected in the presence of surface active substances.

ESL ASSAY

This new protein assay is a spectrophotometric determination of protein concentrations. The method utilizes a Biuret-like reaction and quantifies the excess of Cu^{2+} . The determination of the proteins by this assay is independent of the amino acid composition and depends only on the number of peptide bonds chelating Cu^{2+} . Additionally any interferences with reducing agents (e.g., accelerators) are not to be expected.

However, this assay is not sensitive enough for the determination of latex proteins in most of the glove extracts (detection limit 25 µg/g).

MODIFIED LOWRY METHOD

Lowry's method for the determination of proteins is a well documented and established colorimetric method. The method uses the reaction of proteins with alkaline copper tartrate solution and

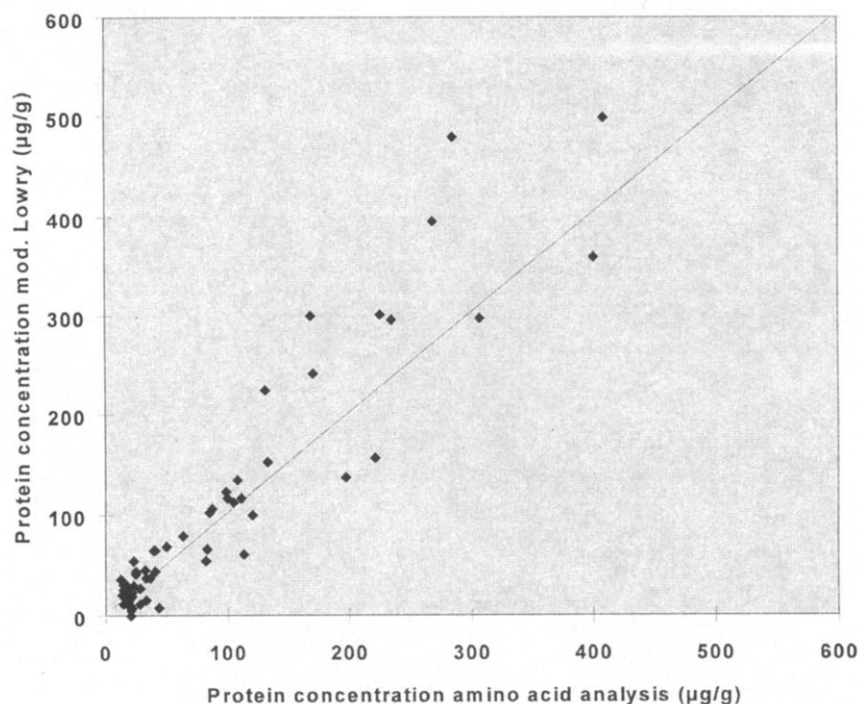


Fig. 4. – Correlation between amino acid analysis and modified Lowry in 62 different glove brands measured in spring and summer 1997

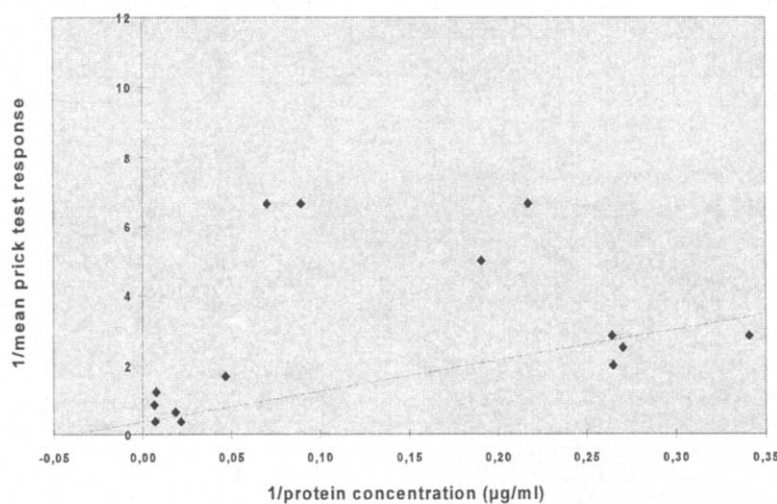


Fig. 5. – Double reciprocal plot of the mean prick test results versus protein concentration (modified Lowry) of 15 different glove brands.

the subsequent reduction of Folin reagent to give a characteristic blue colour [8].

The assay used in our investigations has been modified twice:

1. We used the Bio-Rad DC (Detergent-Compatible) Protein Assay with a special surfac-

tant solution, which enables the measurement of protein in the presence of detergents.

2. According to the ASTM, we precipitated the protein using sodium deoxycholate, trichloroacetic acid, and phosphotungstic acid prior to the analysis [14]. This reduced the influence of inter-

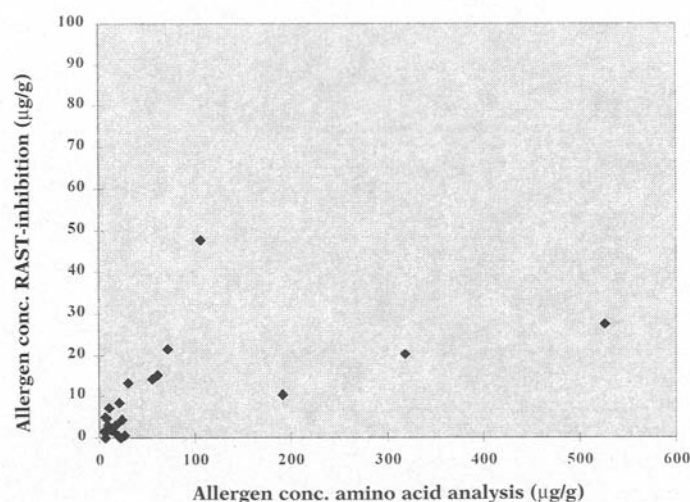


Fig. 6. – Correlation between amino acid analysis and RAST-inhibition in 23 different glove extracts

fering agents and led to a 5-fold concentration of the samples resulting in an increase of sensitivity.

The method had very good precision and accuracy (table II), but it is influenced by reducing agents, often used in gloves (table III). Figures 5 and 6 showed however an acceptable correlation (0.953) to the amino acid analysis and a good correlation to the mean prick test response. But there are some gloves that did not fit to this double reciprocal plot. Most of these gloves were from one manufacturer who changed his formulation in the mean time leading to more correct protein, values by the modified Lowry.

RAST INHIBITION

We used the well-known principle of RAST inhibition for the quantitative immunological determination of latex allergens. We used the commercial CAP-RAST-FEIA distributed by Pharmacia (Upsala, Sweden) for the determination of specific IgE antibodies in latex-allergic patients. In this assay the allergens are covalently bound to a cellulose sponge. These immobilized allergens catch the corresponding specific IgE antibodies from the patients' sera which secondary are detected by fluorescence-conjugated anti-IgE antibodies.

A pool of serum from 41 latex allergic patients was tested with the extracts of 28 different gloves. The values determined by RAST inhibition did not correlate very well to the HPLC or Lowry results (fig. 7 and 8).

Table III. – Comparison of the results of the determination of proteins from natural rubber latex gloves in the fractions < 3 kD and < 10 kD by amino acid analysis and LEAP.

The allergological relevance of the low molecular weight proteins was shown by the mean prick test response of 15 latex allergic patients.

Glove	Protein size (kD)	HPLC (µg/ml)	LEAP (µg/ml)	Mean prick test response
1	< 3	1,60	0	0,38
	< 10	2,03	0	0,63
	total	4,83	0,69	1,19
2	< 3	1,33	0	0,18
	< 10	3,50	0	0,75
	total	4,86	0,06	1,19
3	< 3	7,4	0,19	1,35
	< 10	13,2	0,24	1,90
	total	34,9	1,60	3,00
4	< 3	38,1	0,52	2,95
	< 10	76,2	4,34	3,26
	total	150	16,6	3,68

LATEX ELISA FOR ANTIGENIC PROTEINS (LEAP)

The LEAP is an immunologic method [2] often used for the determination of latex proteins. In the first step the latex proteins are absorbed onto the inner layer of polystyrene microtiter plates. Subsequently the immobilized latex proteins are detected by the reaction with a polyclonal rabbit

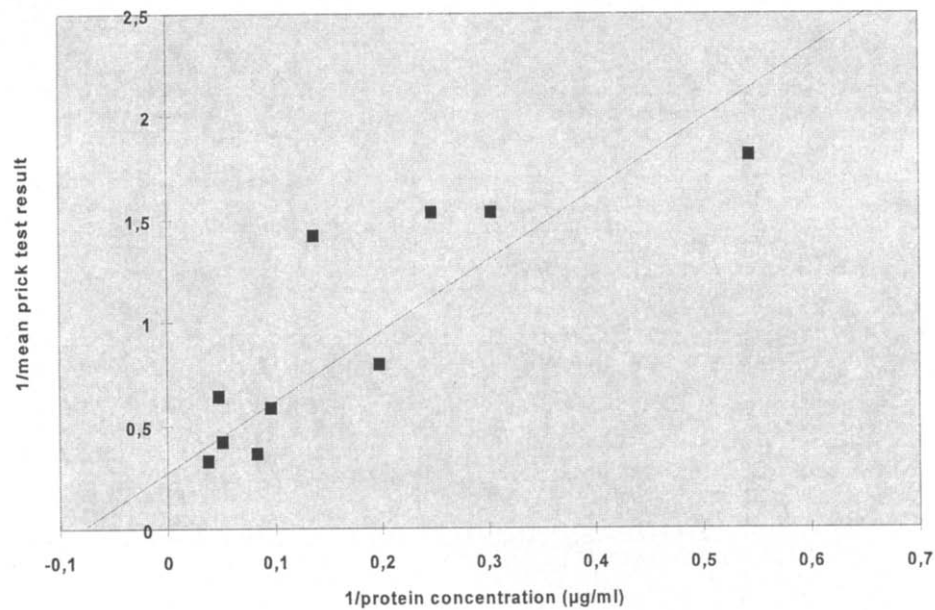


Fig. 7. – Double reciprocal plot of the mean prick test results versus allergens concentration measured by RAST-inhibition in 10 different glove brands.

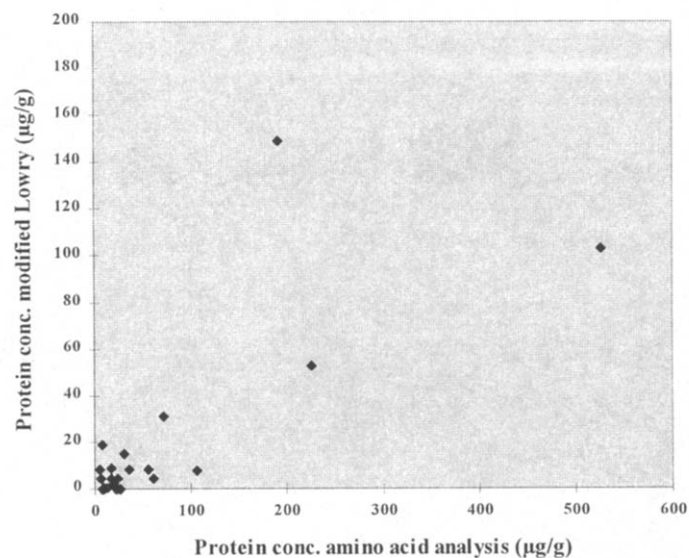


Fig. 8. – Correlation of amino acid analysis and LEAP

anti latex protein antibody followed by an enzyme conjugated goat anti-rabbit IgG antibody. The LEAP results did not correlate with amino acid analysis or prick test response (fig. 9 and 10). The reason for these unexpected results may be that most of the proteins measured in extra-

cts from natural rubber latex gloves are smaller than 10 kD and a large fraction is smaller than 3 kD (table III). Those small proteins or peptides, causing however allergic reactions in patients, do not react with the LEAP because they do not bind to the surface of polystyrene (table III).

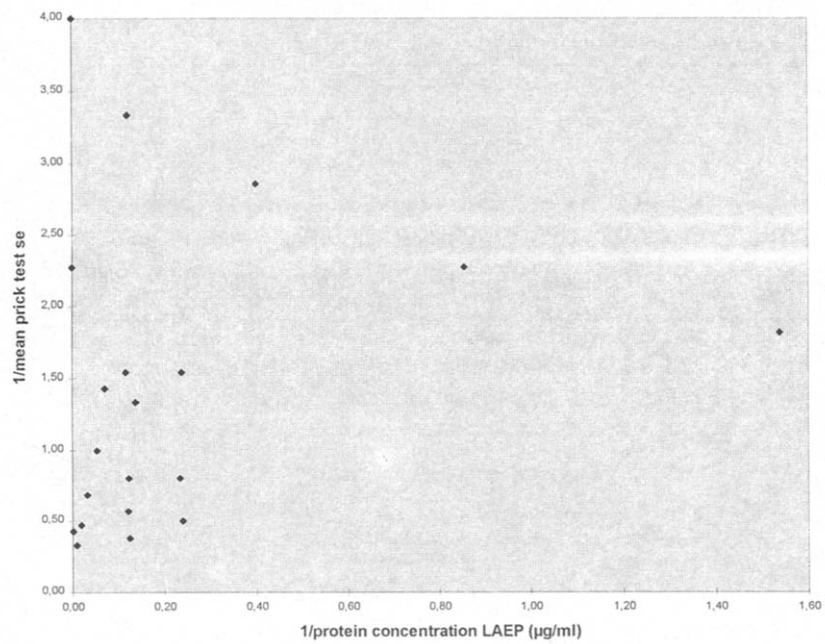


Fig. 9. – Double reciprocal plot of the mean prick test response versus protein concentration (LEAP) in 20 different gloves

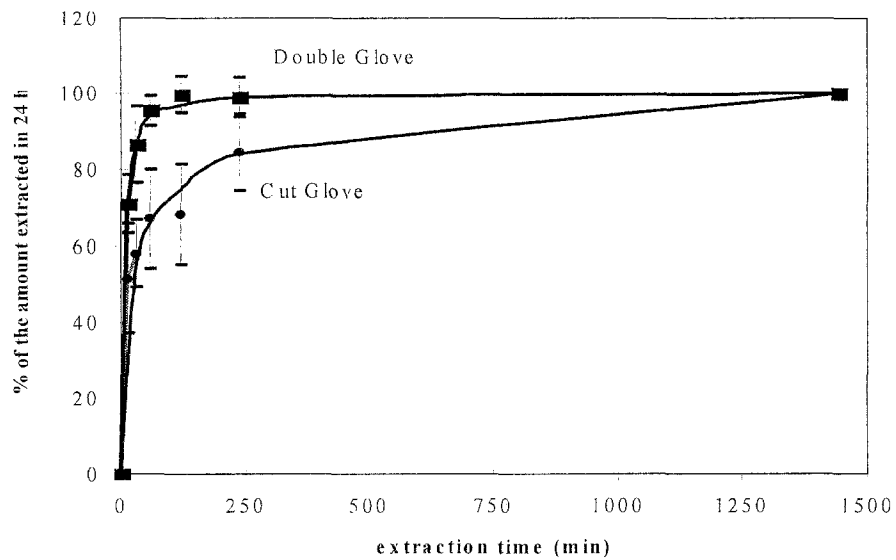


Fig. 10. – Time dependency of the double glove and the cut glove extraction of latex gloves measured in 6 different glove brands. In order to make the results of different gloves comparable, the protein values are given in fractions $\pm$ sd of the corresponding 24 h extract. The protein values were measured by the modified Lowry method.

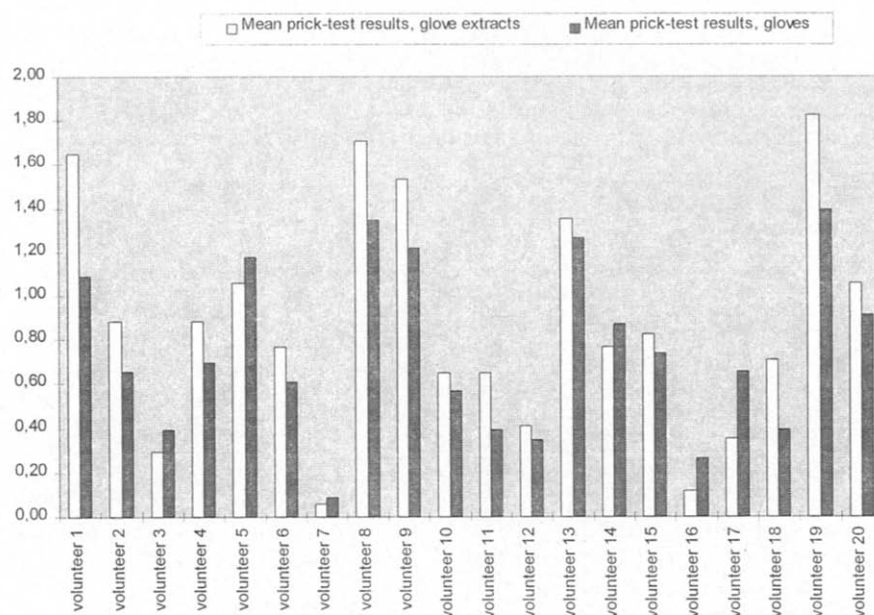


Fig. 11. – Comparison of the mean prick test responses to pieces of 21 different glove brands and their corresponding glove extracts (double glove method, pH 8.2) in 20 different volunteers. The coefficient of correlation was 0.93.

CONCLUSION

The only useful method for a standard is the modified Lowry method although wrong results are possible. Most of these wrong protein values are too high, which is from the allergological point of view no problem. During the last 12 months we observed fewer wrong results by the modified Lowry. Due to the European standard manufacturers are forced to lower the results of modified Lowry determinations. In the case of doubtful results obtained by modified Lowry we recommend the amino acid analysis as the reference method.

The described immunological methods are not useful because standard antibodies covering all allergic proteins are not available at present.

EXTRACTION OF PROTEINS FROM NATURAL RUBBER LATEX GLOVES

The extraction procedure for leachable proteins is as important as the protein assay for the standardisation of the method. The following variables may influence the solubility of proteins: extraction volume, pH, salts, agitation, time, temperature, and the material of the tubes.

The extraction volume should be minimized to get a suitable sensitivity of protein determination. This was achieved by introducing the double glove method, in which the extraction medium was placed between two gloves. The inner glove was filled with a dye solution to optimize the contact to the surface of the two gloves and to detect pinholes. An alternative and often used method was to cut the gloves in more or less defined pieces and extract them in a beaker. A comparison of these both methods is shown in fig 11, where the double glove method reached the maximum protein concentration earlier and gave more reproducible than the cut glove method. From these results we decided to extract the gloves for two hours with the double glove method.

The temperature was of minor influence, especially using the double glove method. Therefore we decided to use ambient temperature ($25^{\circ}\text{C} \pm 5^{\circ}\text{C}$).

pH and salts influenced the extractable amount of proteins considerably. The usage of a physiological pH is not possible because the skin pH after wearing gloves ranged from 4.3 to 6.2 in unpowdered gloves and 5.6 to 9.3 in powdered gloves.

In our laboratory and in two round robin tests the best reproducibility was found with a tris buffer pH 8.2. Nevertheless, during the harmonisation with ASTM and ISO we decided to use a buf-

fer of pH 7.4. In a round robin test in May/June 1997 the results of a TES buffer pH 7.4 were comparable with those of tris buffer pH 8.2 (fig.12). It is important to use a buffer concentration that is able to buffer the magnesium oxide in glove powder. The buffer should be at least 0.1 M. The often used phosphate buffered saline (PBS) is not suitable because the buffer concentration is only 0.01 M. Higher concentrations of phosphate cause problems with precipitating magnesium phosphate. Therefore we recommend TES buffer although it is expensive.

Last but not least the materials of beakers and tubes are very important, because considerable amounts of protein may be absorbed at the surface of plastics and glass. Therefore the use of polypropylene or polyethylene is recommended, and in addition the protein binding capacity of the batch of tubes used shall be tested and shall not exceed 10 µg/tube. The double glove method, this has an additional important advantage of this method, avoiding an additional container for the

extraction procedure needing only needs one tube for storing the extract.

To verify the allergological value of the extraction procedure we compared the prick test results of 20 different volunteers in 21 different gloves and their corresponding extracts. Only minor differences were found (fig 13). The extraction procedure revealed protein concentrations that correspond to the allergological potency of the gloves measured by prick testing.

Based on these results obtained from nearly 100 different gloves and evaluated in more than 60 latex allergic volunteers the standard procedure in the prEN 455-3 includes the following requirements:

1. Double glove extraction (or equivalent)
2. TES buffer pH 7.4 (or equivalent)
3. 2 h, 25 °C, moderate agitation
4. Modified Lowry
5. Values shall be given in µg/g glove

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