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PROGRAMM MEASUREMENTS AND TESTING**

Determination of allergological relevant compounds in disposable gloves

Correlation of chemical, allergological and immunological data

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ANNEX 1 pr-EN 455-3

ANNEX 2 Determination of accelerator residues in disposable latex gloves

ANNEX 3 Koch, H.U., Rev. fr. Allergol., 1997, 37 (8), 1201-1210

ANNEX 4 Knudsen et. al., draft to be published

1. INTRODUCTION

The decrease in concentration of allergens in latex gloves is a decisive prophylactic measure due to the internationally worrying increase in type I-allergies to latex proteins (about 10 % of employees in medical professions are affected) and type IV-allergies to accelerators. About 20 % of latex-allergic patients suffer from a systemic disease (generalized symptoms), which may lead to anaphylactic shock reactions. Hence, employees in medical professions are at a special risk to become unable to work. In many cases a retraining is necessary, which causes high amounts of costs. Therefore a limitation of extractable latex proteins has urgently to be set up to reduce the allergological potential of latex gloves. For this reason CEN TC 205/WG 3 has been entrusted to establish a respective norm (EN 455-3) which should lower the risk of sensitization by disposable gloves in medical professions and should consider the general rules of the guideline 93/42/EEC of the council about medical products. Many different methods were used for the determination of allergological relevant compounds in latex products. None of these methods was evaluated with respect to its allergological significance. The aim of this study was to fill this gap by statistical ensured data.

The study was performed in four parts:

2. *The determination of extractable proteins in latex gloves.*
The most common methods designed to measure protein as the main component in biological fluids were to be evaluated with regard to their suitability to determine traces of protein in latex gloves. The extraction procedure as main part of the method was to be developed, especially in view of its reflection of physiological conditions of glove use.
3. *Evaluation of the selected chemical and immunological methods for the determination of proteins in latex gloves with regard to their allergological relevance.*
The concentration of extractable protein in latex gloves measured by the selected method was to be compared with the results of prick tests and glove use tests in latex allergic volunteers in order to check the allergological relevance. In addition the immunoblotting of glove extracts with sera from latex allergic patients was to be investigated for its suitability to replace the aforementioned in-vivo tests in the classification of the allergological potential of latex gloves.
4. *The determination of extractable accelerators and related compounds in disposable gloves.*
In line with the procedure for latex proteins known and new methods for the determination of accelerators (GC, HPLC, HPTLC) were initially evaluated for their suitability to measure extractable accelerators from latex gloves. In a second step an optimal extraction method was to be developed with the objective to extract amounts of accelerators which correlate to the allergological response under physiological conditions during glove use.
5. *Evaluation of the selected methods for the determination of accelerators and related compounds with regard to their allergological relevance.*
The kind and concentrations of the extractable accelerators and related compounds measured by the selected methods were to be checked for their allergological relevance. Hence, patch tests with the same gloves and their extracts were to be performed in individual volunteers with known type IV allergies to these accelerators. The results were to be compared with those of the chemical methods.

The evaluation and final establishment of these methods for the determination of latex proteins and accelerators in latex gloves is an imperative precondition for the future manufacture and control of favourable latex gloves with a low allergological risk.

2. DESCRIPTION OF WORK

2.1. Quantification of extractable proteins and allergological relevance

Many methods for the determination of proteins are available (lit). They are usually based on colour reactions with particular structural elements, which are not regularly distributed in different proteins (2, 15, 16). Therefore the response factor considerably differs from protein to protein (2).

All common methods for the determination of proteins were adapted to their usage in biological fluids (i.e. serum, urine, spinal fluid, cell culture supernatant) or cells where the protein is one of the main components. In contrast in latex gloves traces of protein (ppm) must be detected aside from several chemical additives which interfere with most of the common methods.

The aim of our study was to find the best method(s) for the detection of traces of protein in latex gloves.

This method should fulfil the following requests:

1. Usefulness for the European Standard
The final method should be as simple as possible to be used in process control.
2. Avoidance of interferences with chemical additives in gloves
The final method should be as specific as possible and should avoid interferences by common glove ingredients.
3. Correlation to allergological results
The content of extractable protein measured by this method should correlate to the allergological potency of the glove, which had to be established in latex allergic volunteers.

During our study four different chemical methods and two immunological methods were tested for their reliability to determine proteins in latex gloves. The results were compared with the allergological response of the gloves in latex allergic volunteers. In summary only two methods were acceptable to measure proteins in latex gloves, i.e. the amino acid analysis by HPLC and the modified Lowry (table 1). A detailed description of further results and problems with the individual test methods is shown in the following chapters.

Table 1. The determination of proteins in latex gloves: Suitability of four chemical and two immunological methods.

Method	Useful as standard procedure	Interferences	Correlation to allergological response	Comment
Amino acid analysis	not acceptable	no	best correlation	reference method
Bradford	acceptable	not acceptable	not acceptable	not useful
Modified Lowry	acceptable	acceptable	acceptable	standard method
ESL-Assay	not acceptable	acceptable	not acceptable	not useful
RAST-Inhibition	not acceptable	acceptable	not acceptable	not useful
LEAP ¹	not acceptable	not acceptable	not acceptable	not useful

¹ Latex Elisa for Antigenic Protein

2.1.1 Allergological response to gloves and glove extracts in correlation to the protein concentration

The aim of this study was to determine the correlation between the chemical or immunological data of the protein concentration of natural rubber latex gloves and their allergological response in latex allergic volunteers. The most common method for the evaluation of the allergological potency of gloves or glove extracts is the prick test in volunteers known to be allergic to natural rubber latex. The prick test is a semiquantitative method. Since individuals with the same degree of sensitization may reveal differences in the prick test response with the same test substance the evaluation of at least 15 volunteers is necessary for a final mean prick test response with quantitative meaning.

Prick test

Prick tests were performed as recommended by the DKG (Deutsche Kontaktallergie Gruppe). A drop of the glove extract or a piece of the glove was put on the inner side of the forearm of the volunteer. The skin was subsequently pricked through the extract or glove using a thin needle. Readings of the test reactions were performed 20 min and 40 min later and scored according to Ring (table 2)

Table 2 Scoring of the prick test results according to Ring (20)

Score	Wheal (mm)	Flare (mm)
0	0	0
+	2-3	3-5
++	3	6-10
+++	4-6	11-20
++++	>6	>20

Volunteers

A total of 70 volunteers with a known latex allergy were investigated within our study. Most of them suffered from a localized urticaria (stage I of the contact urticaria syndrome, according to G. van Krogh and H.I. Maibach, CUS), 5 volunteers had an additional inhalant latex allergy and 4 had a dermal induced latex allergic contact urticaria syndrome stage III (symptoms of the skin and mucous membranes). Prick tests were performed with altogether about 100 different extracts and substances in a total of 1800 tests.

The investigations on these 70 latex allergic volunteers were done in 4 series:

- Series 1. Sixteen volunteers were tested with a series of diluted latex proteins to check the reliability of the method and to confirm the suitability of the mathematical model used for the quantitative correlation of the chemical results and the allergological response in latex allergic volunteers.
- Series 2. Twenty volunteers were tested with 21 different glove extracts and the corresponding glove material in order to check up the suitability of the extraction method for the reflection of the allergological potency of a natural rubber latex glove. The results of the glove extracts were additionally used for the correlation analysis with the results of the chemical determinations of latex proteins.
- Series 3. Eighteen volunteers were tested with 17 different glove extracts in order to check up the correlation between the prick test response and the results of the chemical and

immunological determination of the protein concentration.

Series 4. Sixteen volunteers were tested with 24 different extracts in order to investigate the allergological potency of latex proteins with a very low molecular weight (< 10 kD and < 3 kD), which have been found in all of the tested gloves.

The results of the different series of prick tests are presented in detail later, together with the results of the different methods for the determination of proteins and with the results of the different extraction procedures.

Correlation between the concentration of latex protein and the prick test response (series 1)

The correlation between the concentration of latex proteins and the allergological response in latex allergic volunteers was investigated in 16 different volunteers. A dilution series of a lyophilized low ammoniated latex serum was used in this study. The concentrations were adjusted to the range of protein content in glove extracts (10 : g/ml to 1000 : g/ml).

The mean prick test results of these volunteers were plotted against the protein concentration and resulted in a curve of saturation (fig. 1). However this was not surprising since the mean prick test results cannot exceed the highest response of 4.0 (++++).

Similar curves are known in enzyme kinetics and receptor analysis described by the Michaelis - Menten - equation (9). Analogously a double reciprocal plot (fig. 2) was used for a linear correlation. The results revealed an unexpected high correlation between 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. In order to visualize the influence of variations we included the $\pm 20\%$ - curves of the linear regression, beside the measured values (dots) and the calculated linear regression (i.e. a 20% variation in the measured values would lead to dots on these two lines). The maximum response calculated by the linear regression via the Michaelis-Menten equation was 3.86 which is close to the theoretical value of 4.0.

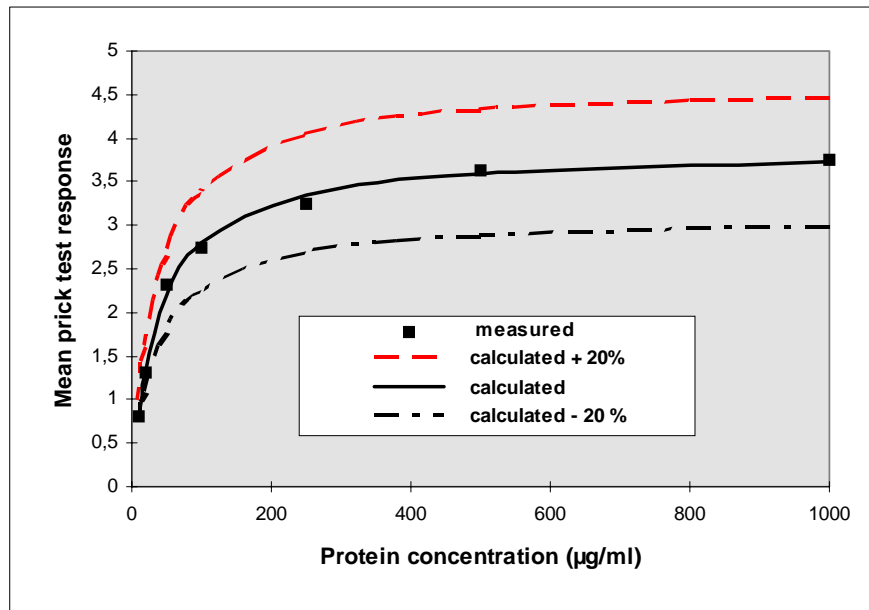


Figure 1 Plot of the mean prick test results of 16 latex allergic volunteers versus the protein concentrations of a dilution series of lyophilized natural rubber latex serum. Apart from the measured results (dots) the curve calculated by linear regression of the double reciprocal plot (figure 2) and the curves of $\pm 20\%$ deviation are shown.

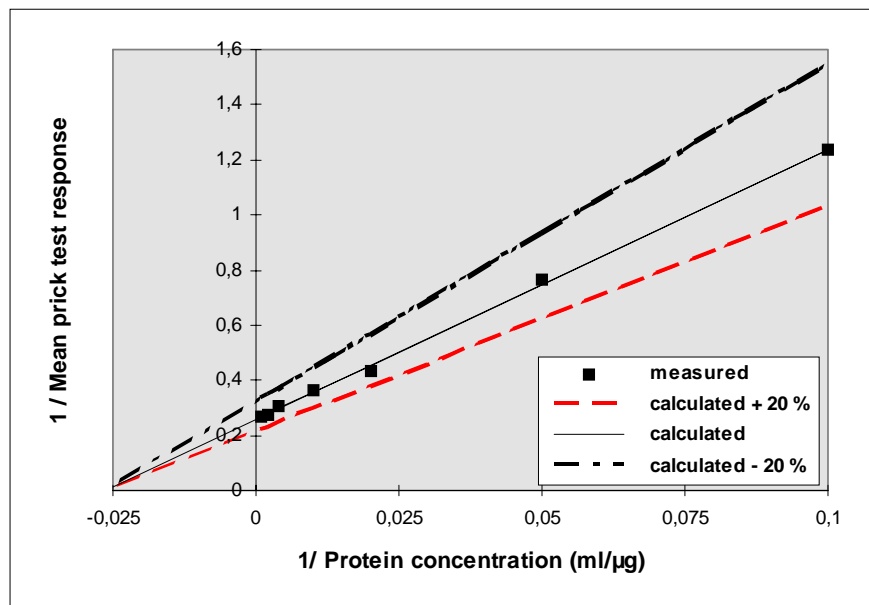


Figure 2 Double reciprocal plot of the mean prick test results in 16 latex allergic volunteers versus the protein concentrations of a dilution series of lyophilized natural rubber latex serum. Apart from the measured results (dots) the curve calculated by linear regression and the curves of $\pm 20\%$ deviation are shown.

The prick test is a tool for the quantitative determination of the allergen content

The mean prick test response of at least 15 latex allergic volunteers was shown to be very strongly dependent on the protein concentration of the tested material. The double reciprocal plot enables us to correlate the mean prick test response to latex gloves with their protein concentrations in a linear dependency. This excellent correlation shown in figure 2 enables us to evaluate the allergological relevance of the common chemical and immunological methods for the determination of proteins in gloves.

2.1.2 Assessment of a suitable method for the determination of proteins in glove extracts

2.1.2.1 Amino Acid Analysis by High Pressure Liquid Chromatography (HPLC)

Method

The amino acid analysis was used as the reference method in this study. Apart from its independency on any structural property of the proteins it is very sensitive and specific.

Following a hydrolysis of proteins by hydrochloric acid the resulting amino acids were separated and quantified by HPLC (9). The summation of the individual amino acids subsequently revealed the total protein content. The method is described in ANNEX 1 which was introduced into the European standard prEN 455-3 as an informal Annex.

The individual conditions which are dependent on the equipment and especially the HPLC-column are described in the following boxes.

Materials and apparatus:

Hydrochloric acid Suprapur® 30 %, Merck, Darmstadt
 Amino acid standard containing 20 amino acids, Sigma Chemie, Deisenhofen
 o-phthaldialdehyde (OPA), Sigma Chemie, Deisenhofen
 3-mercaptopropionic acid (MPA), Sigma Chemie, Deisenhofen
 Vacuum concentrator, Hetovac® VR1, Nunc GmbH, Wiesbaden
 Autosampler Gilson Model 231, Abimed, Düsseldorf
 Gradient System 322, Kontron Instruments, Neufahrn
 Fluorescent detector, Kontron Instruments, Neufahrn

Precolumn derivatisation:

For a sensitive amino acid detection a derivatisation is necessary. Dependent on our equipment we used the very common precolumn derivatisation with OPA and MPA (9, 10, 14, 19). The resulting fluorescent isoindol derivatives were separated on the HPLC column and detected with a fluorescent detector.

The derivatisation was carried out automatically using the autosampler. Five : 1 of the redissolved samples were incubated for 2.5 min at room temperature with 12.5 : 1 of 83 mM OPA in 400 mM sodium borate buffer (pH 10.4). The reaction was terminated by the addition of 25 : 1 1M KHSO₄ (pH 7.0) and 20 : 1 of the solution were injected immediately onto the HPLC column.

HPLC separation

Column: Grom-Amino-OPA (15 cm Hypersil ODS 3:)

Precolumn: 3 cm Hypersil ODS 3:

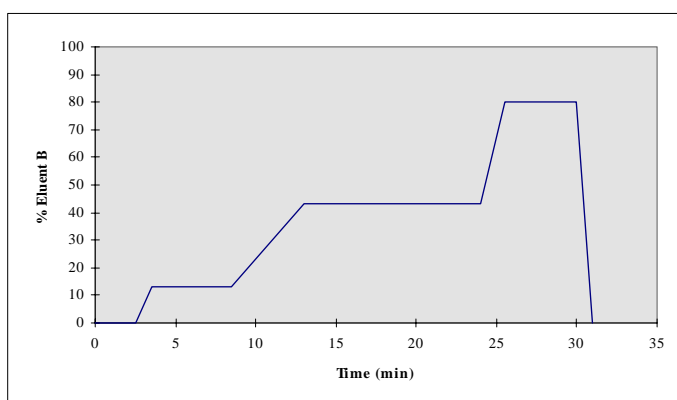
Flowrate: 1 ml/min

Eluent A: 25 mM sodium phosphate pH = 6.8, 1.5 % tetrahydrofurane (HPLC grade unstabilized) (v:v)

Eluent B: 65 % 25 mM sodium phosphate pH = 6.8 , 25 % Acetonitrile (HPLC grade), 10 % tetrahydrofurane (HPLC grade unstabilized) (v:v:v)

Wavelength: excitation 330 nm, emission 450 nm

Gradient:



Identification of the amino acids, calculation of the protein concentration and statistical data of the amino acid analysis

Typical chromatograms and a list of the detectable amino acids are shown in figure 3 and table 4. All amino acids usually found in proteins were separated well. Additionally two components derived from TES (RT 14.23 and 24.08) were resolved completely from all amino acids.

In all our experiments throughout the last 4 years we could not find any glove ingredient which interfered with the above described method. Some glove extracts contained considerable amounts of free amino acids. Therefore all extracts were measured with and without HCl hydrolysis and the total protein content was corrected by the amount of free amino acids.

The quantification limit of the amino acid analysis is about 1 : g protein / ml extract using the volumes described above. The quantification limit is influenced by the salt concentration in the extraction buffer (which may be lower in powder free gloves, see chapter 2.2) and by the amount of extractable chemical additives. In clean powder free gloves the quantification limit may be lowered to 0.2 : g/ml.

Accuracy and repeatability of the amino acid analysis was 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). For the determination of the accuracy of this method the protein concentration of ovalbumin was additionally measured by the absorption at 280 nm (absorption maximum of proteins) and by the modified Lowry method. The recovery of the amino acid analysis was 101 and 97.6 % and the coefficient of variation 6.5 to 8.4 % respectively (table 3).

Table 3

Repeatability and accuracy of the amino acid analysis. The measurements were performed at ten different days with ten different HCl hydrolyses of every sample. Cv = coefficient of variation (%) of 10 analyses. The recovery was calculated by using the values of the UV absorption (280 nm) as 100 %.

Protein	UV 280 nm : g/ml	Modified Lowry			Amino acid analysis		
		mean : g/ml	recovery (%)	cv (%)	mean : g/ml	recovery (%)	cv (%)
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
Glove extract	-	30.2	-	7.8	23.4	-	8.4

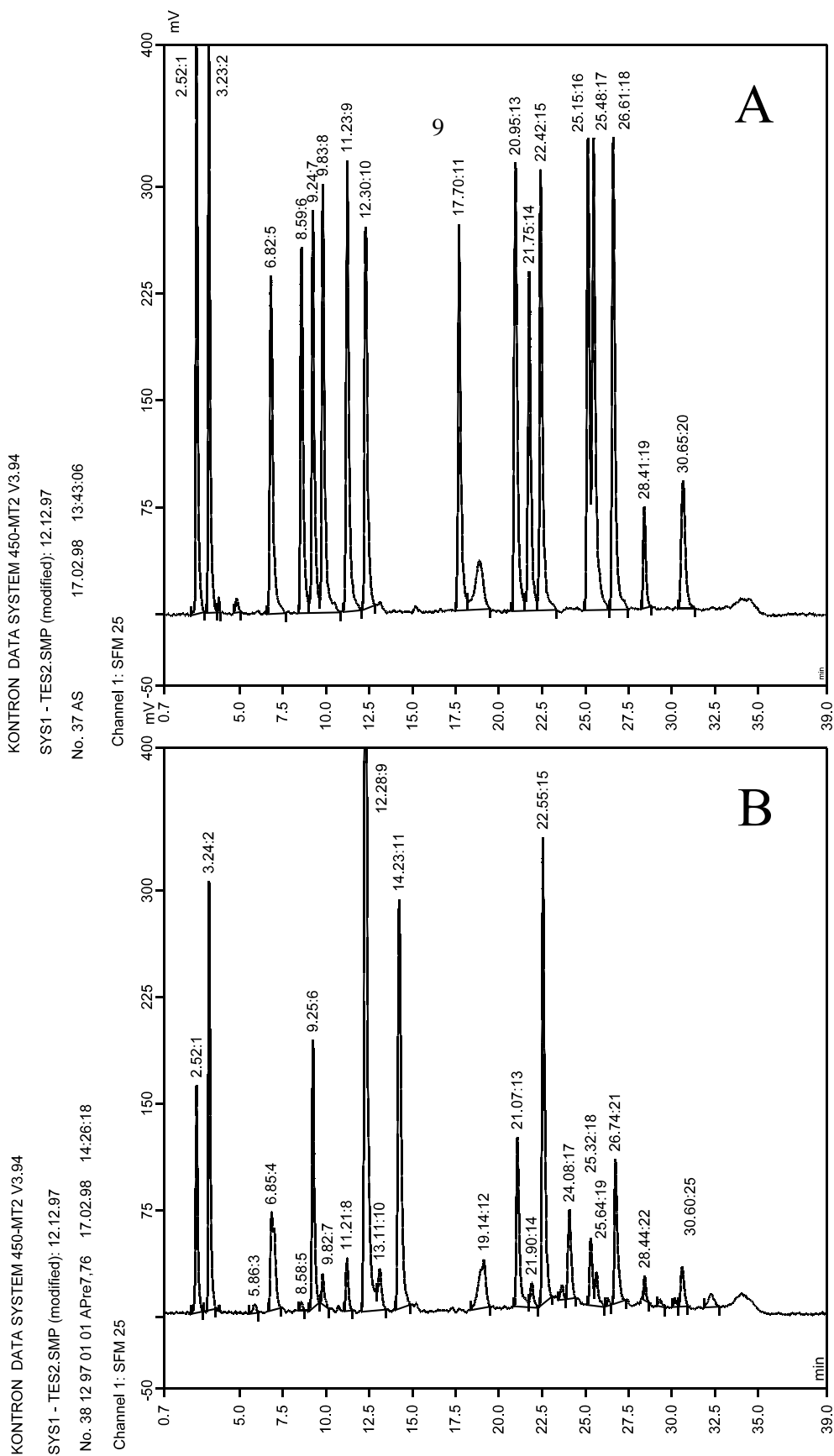


Figure 3 Typical chromatograms of an amino acid standard (A) and an analysis of a glove (35 : g protein)

Table 4 List of amino acids found in the HPLC analysis of a standard solution (fig 3 A) and in the hydrolysis of the glove extract (fig 3 B).

Amino acid	Retention time (min)		Comment
	Standard	Analysis	
Aspartic acid (ASP)	2.52	2.52	
Asparagine (ASN)			converted to ASP
Glutamic acid (GLU)	3.23	3.24	
Glutamine (GLN)			converted to GLU
Serine (SER)	6.83	6.85	
Histidine (HIS)	8.60	8.59	
Glycine (GLY)	9.25	9.25	
Threonine (THR)	9.84	9.82	
Arginine (ARG)	11.24	11.21	
Alanine (ALA)	12.30	12.29	
		14.23	TES (extraction buffer)
Tyrosine (TYR)	17.7		
Valine (VAL)	20.95	21.07	
Methionine (MET)	21.75	21.90	
Norvaline (NORVAL)	22.42	22.55	internal standard
		24.08	TES (extraction buffer)
iso-Leucine (ILE)	25.15	25.32	
Phenylalanine (PHE)	25.48	25.64	
Leucine (LEU)	26.61	26.74	
Lysine (LYS)	28.41 30.65	28.44 30.60	
Tryptophan (TRY)			destroyed by hydrolysis
Cystine, Cysteine (CYS)			destroyed by hydrolysis
Proline (PRO)			not detectable

Correlation of the protein concentrations measured by amino acid analysis to the allergological data obtained with prick tests in latex allergic volunteers

The correlation of the protein concentrations obtained by the amino acid analysis with the mean prick test response to the glove extracts was investigated in series 2 and series 3 (figures 4 and 5). In series 2 in July 1996 sixteen of 21 gloves (see chapter 2.1.1) tested in twenty volunteers known to be allergic to natural rubber latex were further evaluated (five extracts with a protein concentration $< 1 \text{ : g/ml}$ were excluded). In series 3 in November 1996 another eighteen volunteers were tested with watery extracts of seventeen additional gloves (two extracts with a protein content $< 1 \text{ mg/ml}$ were excluded). The results were nearly the same, except two outliers in series 2 (fig 4). The coefficients of correlation were 0.993 and 0.991, respectively. The maximum prick test responses calculated by linear regression of the double reciprocal plot (figures 4 and 5) were 3,85 and 3.97, respectively, which is very close to the theoretical value of 4.0 (maximum prick test response).

There was no apparent reason for these two outliers in figure 4. In both cases high protein values measured by the amino acid analysis contrasted to very low prick test responses (high values in the double reciprocal plots). The casein content of one of these gloves may in part be responsible for this discrepancy between high protein value and low allergological response.

The Amino acid analysis is useful as reference method for the determination of proteins in latex gloves

The amino acid analysis is a very selective and sensitive method for the determination of proteins. It enables to measure the real protein value independent on any structural feature of the polymeric molecule.

This method revealed an excellent correlation to the clinical data, i.e. the mean prick test response in latex allergic volunteers and hence is an appropriate method for the allergological evaluation of natural rubber latex gloves.

Our theoretically based approach to use the amino acid analysis as the reference method for protein determination was finally confirmed by these unexpected high correlations between the protein concentration and the mean prick test response.

With regard to the arrangement of a standard method, for instance for the process control of latex gloves, the amino acid analysis is too uncommon, too complex and too expensive.

However it should be designated as reference method to which any future standard method should be adapted. Regular controls shall be performed at least whenever a new batch of latex is required for the manufacturing of gloves.

Due to our results the amino acid analysis was introduced into the prEN 455-3 as an informal annex (see ANNEX 1).

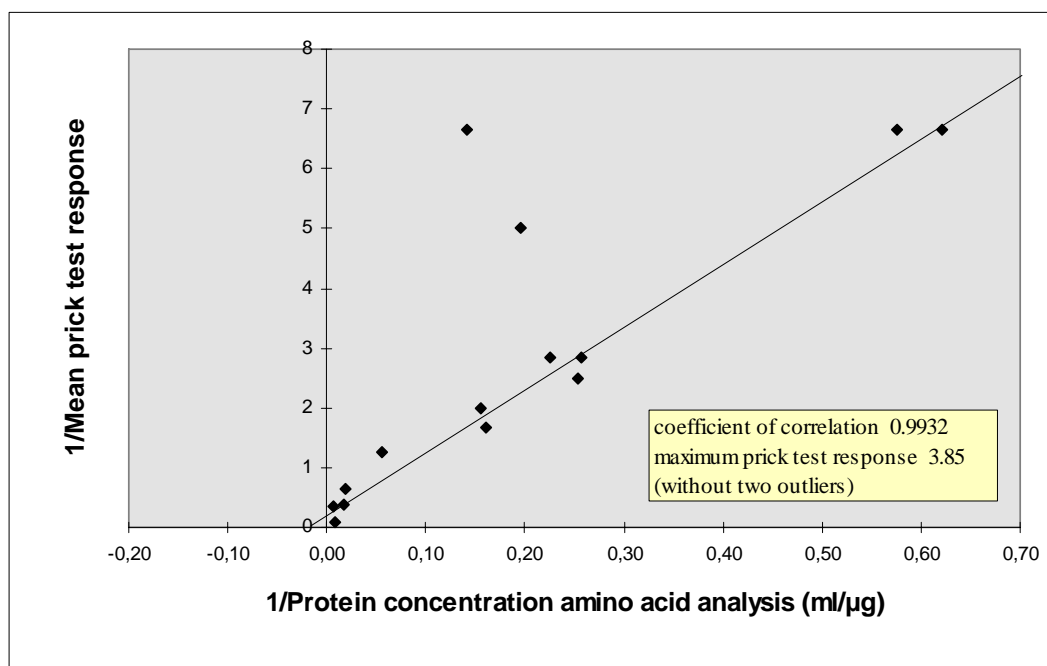


Figure 4 Double reciprocal plot of the protein concentration versus the mean prick test response to glove extracts investigated in July 1996. Sixteen different glove extracts were tested in twenty volunteers known to be allergic to natural rubber latex.

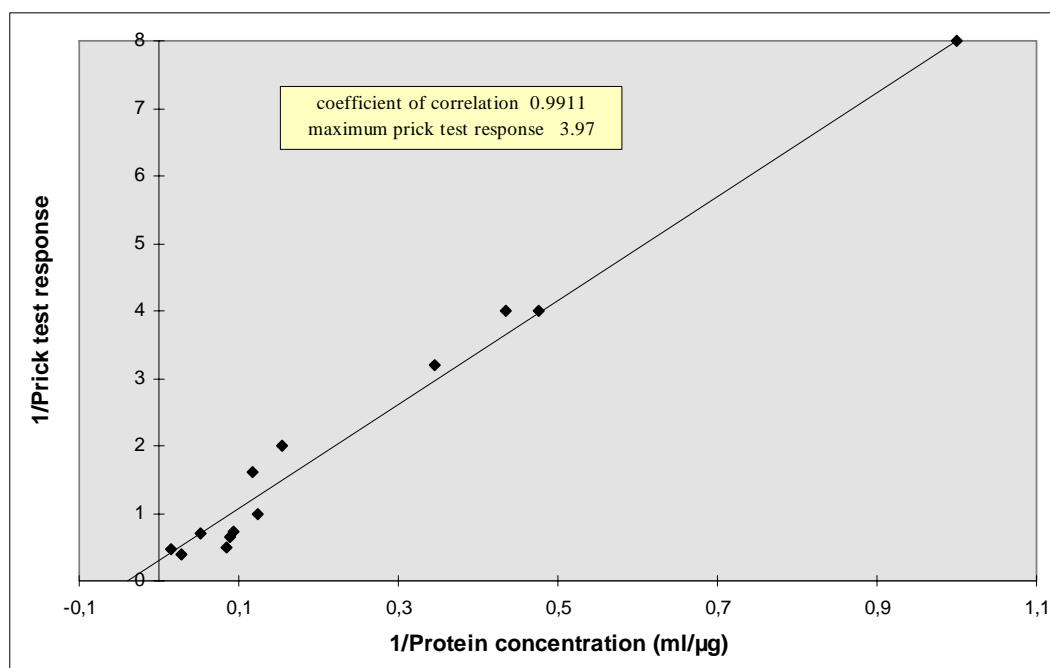


Figure 5 Double reciprocal plot of the protein concentration versus the mean prick test response to glove extracts investigated in November 1996. Fifteen different glove extracts were tested in eighteen volunteers known to be allergic to natural rubber latex.

2.1.2.2 Bradford method

Method

The Bradford method (based on the colour change of Coomassie blue dye in response to various protein concentrations) is a very sensitive and simple method for the determination of proteins. It was carried out as described by BioRad Laboratories (2, 5, 15, 16) with some minor modifications.

Materials and apparatus:

Flat bottom microtiter plates (500 : 1 per cup), Pharmacia, Freiburg

Titertek plus micro plate reader, Flow Laboratories

Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories GmbH, München

Albumin from Chicken Egg (Ovalbumin), Sigma Chemie, München

Ovalbumin standard

25 ml of a 1 mg/ml stock solution of ovalbumin were prepared in the extraction buffer. To determine the real ovalbumin concentration (commercial ovalbumin contained various amounts of water) the extinction at 280 nm (quartz cuvette, 1 cm) was measured and divided by 0.741².

Standard solutions (100 : g/ml, 50 : g/ml, 20 : g/ml, 10 : g/ml, 5 : g/ml and 2 : g/ml) were prepared by appropriate dilutions of the protein stock solution with extraction buffer. Protein free extraction buffer was used as blank.

Assay Procedure:

To every sample (1ml) of the standards and the glove extracts 0.25 ml of the Dye Reagent Concentrate was added in polypropylene micro tubes, mixed well and allowed to stand for 5 min at room temperature.

0.48 ml of the reaction mixture were transferred to a flat bottom microtiter plate and the absorbance was measured within 5 min in a plate reader at 595 nm.

Calibration curves were not exactly linear over the whole range of the standard curve. Therefore the concentration was calculated by spline approximation with a computer program (Synelisa, Elias, Freiburg).

Results

The Bradford method is known to be strongly influenced by many substances (2, 7, 8, 12, 13). Using glove extracts the extent of interferences due to surface active substances was of utmost interest, since these are widely used in the final steps of glove processing (e.g. powdering and surface coating).

In our experiments we tested anionic (sodium dodecyl sulfate), ampholytic (CHAPS) and neutral (Triton X 100, Nonidet, Tween 20) detergents for their dose dependent influence on the colour development of the Coomassie blue complex with ovalbumin (OVA) from chicken egg. All these 5 different detergents used in our experiments revealed strong interferences with the Bradford method even if low concentrations were used (table 5). All values (except SDS, 50 : g/ml) pretended higher concentrations than expected but the blanks did not reflect these differences indicating that the additional effect is dependent on the protein concentration.

Comparing the results of the Bradford method with those of the amino acid analysis we found lower

²

Assuming a molecular weight of 43 000 D and a molar extinction of 31 900 at 280 nm and pH 8.2 the extinction of 1 mg/ml (1 g/L) ovalbumin in tris buffer pH 8.2 is 0.741 using a light path of 1cm (1).

protein concentrations in 7 cases and higher ones in 18 of the 25 selected gloves (slope of the linear regression 0.65). There was no correlation between the Bradford method and the amino acid analysis (coefficient of correlation 0.325). Hence the Bradford method obviously revealed false high protein concentrations in many cases, which was mainly due to interferences of surface active substances as present in gloves.

Table 5. Influence of 5 different detergents on the determination of 10 : g/ml ovalbumin using the Bradford method.

Concentration of surfactant (: g/ml)	1000		100		50	
Concentration of Ovalbumin, weight (: g/ml)	0	10	0	10	0	10
Concentration of Ovalbumin, Bradford (: g/ml)	0	9.9	0	9.9	0	9.9
CHAPS	1.8	20.6	3	15.6	1.5	11.3
Nonidet p 40	>> 100	>> 100	2.9	22.2	2.2	16.8
Sodium dodecyl sulfate	30.7	31.7	19.5	21.6	1.7	3.7
Triton X 100	>> 100	>> 100	5.3	26.9	2.5	17.8
Tween 20	>> 100	>> 100	7.9	28.4	4.3	19.5

The Bradford method is not suitable for the determination of proteins in gloves

The Bradford method is a very sensitive and simple method, which is most rapidly performed. It is strongly influenced by several additives in gloves (especially by surfactants) and therefore often leads to false high protein values. Hence the Bradford method does not correlate with the amino acid analysis and is unsuitable for the determination of proteins in latex gloves.

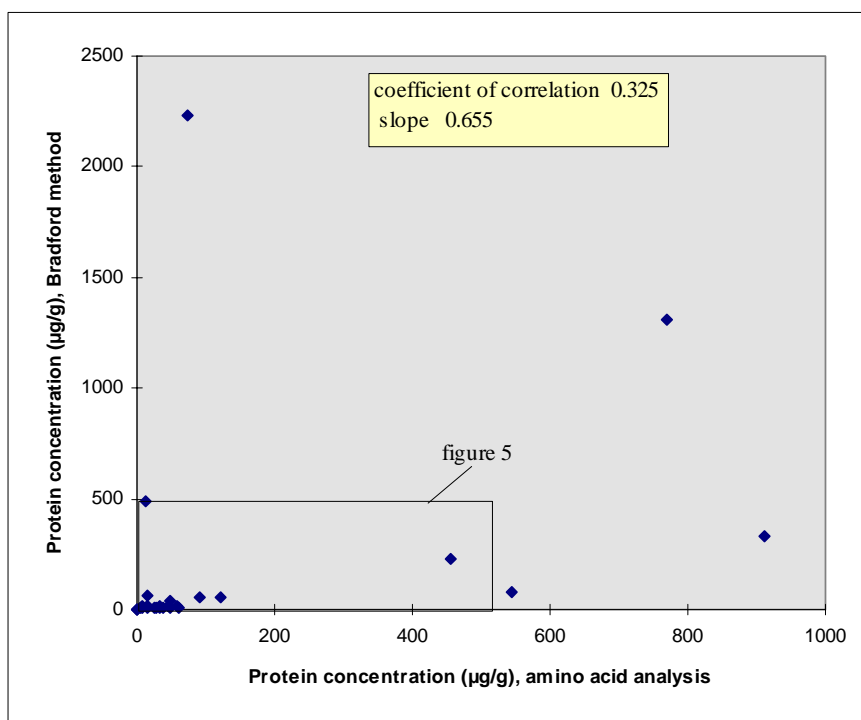


Figure 6 Correlation of the protein concentrations measured by the Bradford method and the amino acid analysis. Twenty-five extracts of different latex gloves were measured with both methods within one week. The marked detail is enlarged in figure 7.

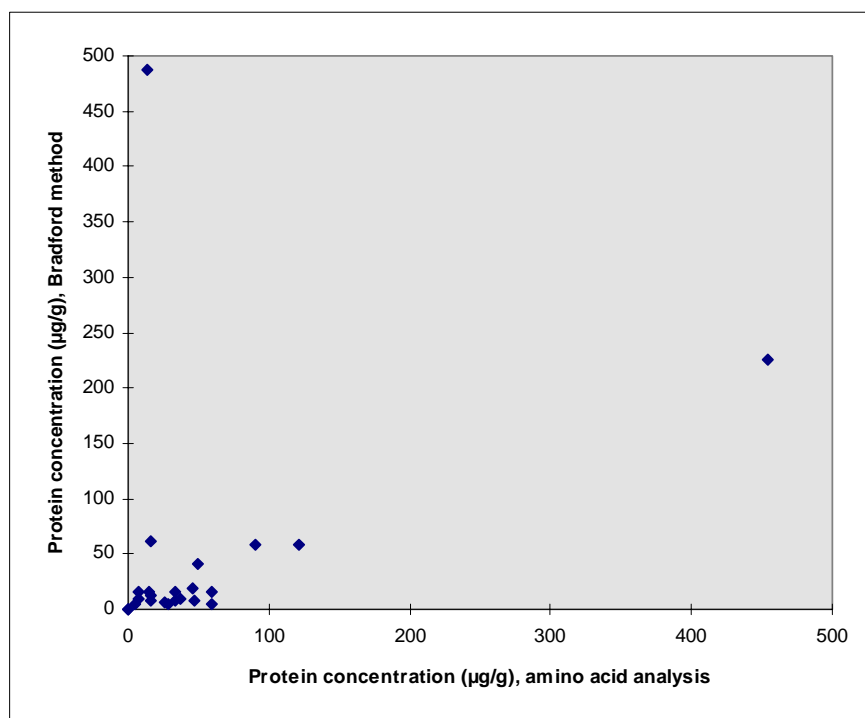


Figure7 Correlation of the protein concentrations measured by the Bradford method and the amino acid analysis, enlarged detail of figure 6.

2.1.2.3 Lowry method

Method (see ANNEX 1)

Lowry's method for the determination of proteins is a well documented and established colorimetric method. This method utilizes the reaction of proteins with alkaline copper tartrate solution and the subsequent reduction of Folin reagent to give a characteristic blue colour (3, 15, 16, 18).

The assay used in our investigations has been modified twice:

1. We used the 'Bio-Rad DC (Detergent-Compatible) Protein Assay' with a special surfactant solution, which enables the measurement of protein in the presence of detergents (3).
2. According to the ASTM (see ANNEX 1) we precipitated the protein using sodium deoxycholate, trichloroacetic acid, and phosphotungstic acid prior to the analysis (24). This led to a 5 fold concentration of the samples resulting in an increase of the sensitivity of the method and reduced the influence of interfering agents.

This method is described in detail in ANNEX 1 and is part of the European standard prEN 455-3.

Results

This modified Lowry method (ANNEX 1) is a very sensitive and precise method for the determination of proteins in natural rubber latex gloves. The coefficient of variation of the inter-day repeatability was 6.9% (100 : g/ml) to 10 % (5 : g/ml), respectively and the recovery was about 100 % (table 6).

Every microtiter plate of samples contained a set of standard solutions. The standard curves used for the calculation of the samples' protein concentrations revealed a very good repeatability as shown in figure 8 and table 7. Based on 27 standard curves (measured between July 1995 and May 1996) we found coefficients of variation of the extinction at 620 nm ranging from 13,4 % (0 : g/ml) to 7.0 % (100 and 200 : g/ml).

Table 6

Repeatability and accuracy of the modified Lowry method. The measurements were performed at ten different days using one batch of ovalbumin solutions and one glove extract. Cv = coefficient of variation (%) of 10 analyses. The recovery was calculated by using the values of UV absorption (280 nm) as 100 %.

Protein	UV 280 nm : g/ml	Lowry		
		mean : g/ml	recovery (%)	cv (%)
Ovalbumin	5.0	5.1	102.0	10.1
Ovalbumin	10.0	10.7	107.0	8.3
Ovalbumin	50.0	51.1	103.0	7.9
Ovalbumin	100.0	100.4	100.4	6.9
Glove 22	-	30.2	-	7.8

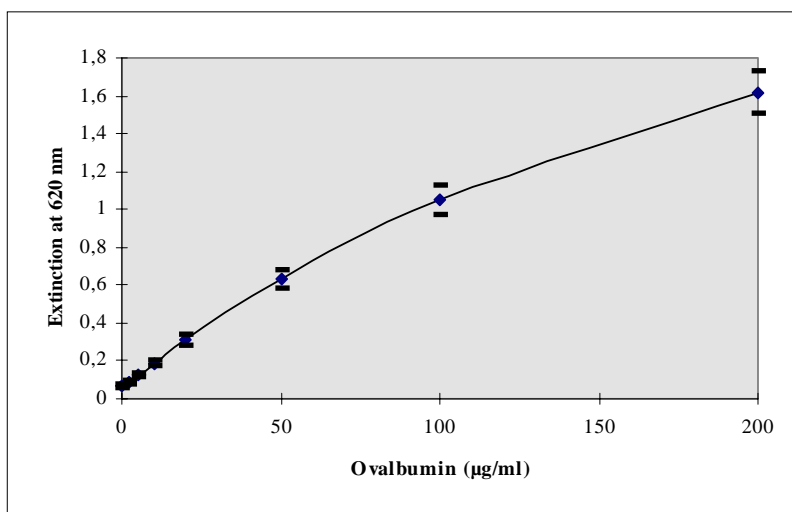


Figure 8 Mean standard curve \pm SD of 27 modified Lowry tests performed between July 1995 and May 1996

Table 7 Mean, standard deviations and coefficients of variation of the extinctions (620 nm) measured in 27 standard curves between July 1995 and May 1996

	Ovalbumin concentration (: g/ml)							
	0	2	5	10	20	50	100	200
Mean	0.068	0.087	0.127	0.187	0.312	0.631	1.051	1.619
SD	0.009	0.010	0.010	0.014	0.027	0.046	0.074	0.113
CV (%)	13.4	11.8	8.4	7.6	8.6	7.2	7.0	7.0

Although the Bio-Rad DC assay tolerates high amounts of surfactants (eg. 10 mg/ml Tween 20, Bio-Rad manual) our investigations with 5 different detergents revealed contradictory results using concentrations > 100 : g/ml which clearly interfered with the DC-assay (table 8). Probably the precipitation step was responsible for these results, since the addition of phosphotungstic acid in the presence of surfactants resulted in a voluminous precipitate, which often was incompletely soluble in NaOH, and thus induced an opacity of the measuring solution.

Aside from detergents reducing agents such as thiol groups (S-H) are the most important interfering substances for the modified Lowry method (Bio-Rad manual). Thiol groups are the functional parts of common accelerators which are used in the manufacturing process of rubber goods and thus are present in glove extracts. As shown in table 9 saturated watery solutions of some accelerators (which are poorly water soluble) pretended high protein concentrations in spite of the precipitation step used in the modified Lowry method.

Table 8 Influence of 5 different detergents on the determination of 10 : g/ml ovalbumin using the modified Lowry method.

Ovalbumin in double distilled water						
Concentration of surfactant (: g/ml)	1000		100		50	
Concentration of Ovalbumin, weight (: g/ml)	0	10	0	10	0	10
Concentration of Ovalbumin, Lowry (: g/ml)	0	10.1	0	10.1	0	10.1
CHAPS	0.6	1.8	0.7	10.2	0.9	10.5
Nonidet p 40	20	26.8	1.2	10.6	2.1	12.2
Sodium dodecyl sulfate	0.4	10.2	0.3	10.6	0.5	9.8
Triton X 100	18.7	38.7	2.3	13.7	3.3	11.8
Tween 20	11.5	22.9	1.9	12.4	1.5	10.8
Ovalbumin in TRIS buffer pH 8.2						
Concentration of surfactant (: g/ml)	1000		100		50	
Concentration of Ovalbumin, weight (: g/ml)	0	10	0	10	0	10
Concentration of Ovalbumin, Lowry (: g/ml)	0	10.2	0	10.2	0	10.2
CHAPS	0.2	12.4	0.5	11.9	0.2	11.9
Nonidet p 40	0	7	0.7	12.1	0.9	11.6
Sodium dodecyl sulfate	0	7.7	0	11.2	0.4	12.1
Triton X 100	0	5.7	0	13.8	0.5	12.5
Tween 20	2.7	19.9	0.1	12.1	0.1	13.6

Table 9. Interferences of some accelerators with the modified Lowry assay pretending high protein concentrations.

Accelerator	Concentration	Colour	pretended protein
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
<i>N,N'</i> -Diphenylthiourea	saturated	regular blue	53.2
Zinc mercaptobenzothiazole	saturated	regular blue	44.8
Zinc dibutyldithiocarbamate	saturated	regular blue	8.4
Tetramethylthiourea	1 mg/ml	regular blue	316

The determination of protein in 39 selected latex gloves revealed a poor correlation of 0.77 (figure 9) comparing the results of the modified Lowry method and the amino acid analysis. The exclusion of six gloves of one special manufacturer improved the coefficient of correlation up to 0.96.

Since 1992 the European and the American standard organisations discussed the modified Lowry method as standard procedure for the determination of proteins. Hence manufacturers tried to avoid substances which interfere with the modified Lowry method, pretending high protein concentrations. This intention was clearly reflected in our second series of 62 different gloves in summer 1997 where we found a coefficient of correlation of 0.95 comparing the results of the modified Lowry method and the amino acid analysis (figure 10). This second series comprised several gloves of the special manufacturer with former poor results in our first series.

The correlation between the protein concentrations measured by the modified Lowry method and the allergological response in latex allergic volunteers was investigated within series 2 and series 3 (July 1996 and November 1996, glove extracts with a protein content of less than the detection limit of 2 : g/g were excluded). However, both series revealed a poor correlation if all extracts were taken into account (figures 11 and 12). The exclusion of four outliers in series 2 and one in series 3 improved the coefficients of correlation from 0.337 and 0.456 up to 0.912 and 0.881, respectively, which was still lower in comparison to the amino acid analysis. The maximal prick test responses, calculated by the linear regression of the double reciprocal plot, were 2.43 and 3.28 respectively and did not fit very well to the theoretical value of 4.0.

The Modified Lowry method is useful as standard method

The modified Lowry assay is a well known very sensitive and reproducible method (see inter-laboratory test below) and therefore suitable as standard procedure in the manufacturing process control. The reagents used for the colour development are commercially available in a very good quality. However, variations of the method may impair the results. Therefore the consistent application of the BioRad assay (patent) using microtiter plates (as performed in our studies) is recommended to guarantee best results. Further commercial kits were not yet available during our studies, but may be as useful as the BioRad method.

The modified Lowry method is influenced by a number of reducing and surface active substances. Outliers therefore may occur but however are becoming less frequent within the last two years. In our study the modified Lowry revealed an acceptable correlation with the prick test response in latex allergic volunteers.

This method was introduced into the European standard (prEN 455-3) as the required standard procedure for the measurement of proteins in natural rubber latex gloves. In the mean time it has been harmonized with the ASTM D5712 and ISO 12243-2

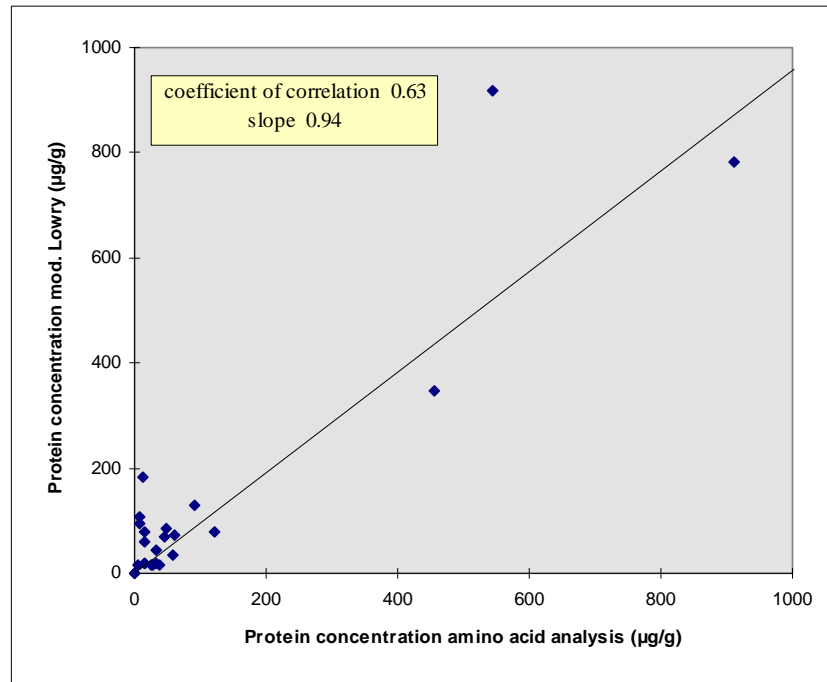


Figure 9 Correlation of the protein concentrations measured by the amino acid analysis and the modified Lowry method in 37 different glove extracts (TRIS buffer pH 8.2) in 1995

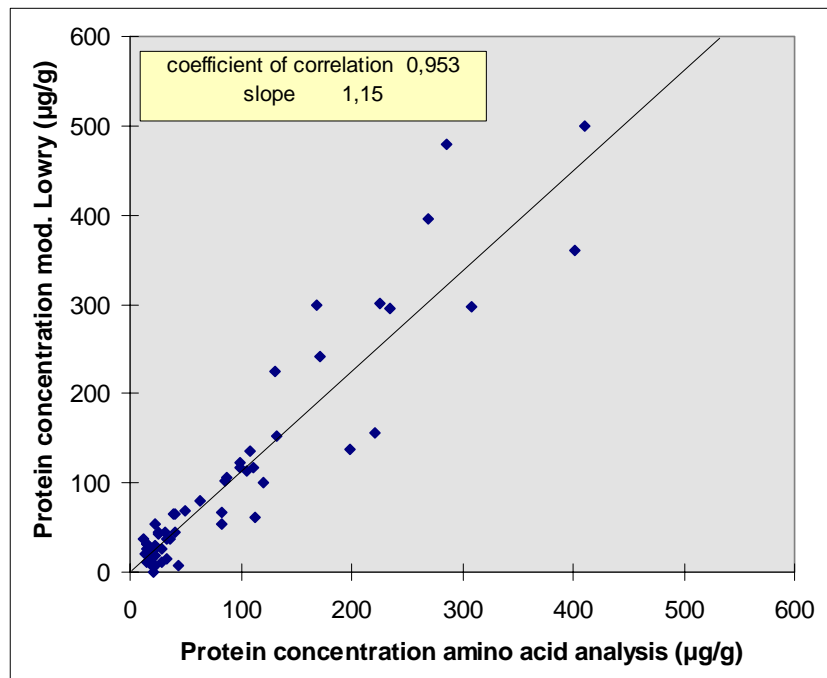


Figure 10 Correlation of the protein concentration measured by the amino acid analysis and the modified Lowry method in 62 different glove extracts (TRIS buffer pH 8.2) in summer 1997.

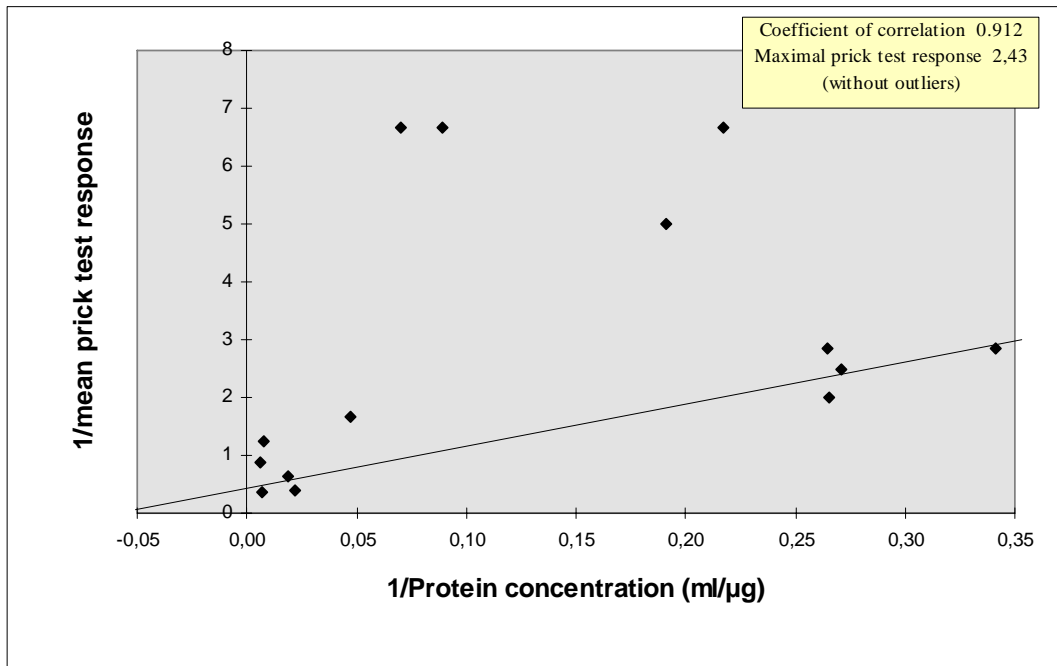


Figure 11 Double reciprocal plot of the mean prick test response versus the protein concentrations measured by the modified Lowry method in Juli 1996. 17 different glove extracts were tested in 20 different volunteers, known to be allergic against natural rubber latex.

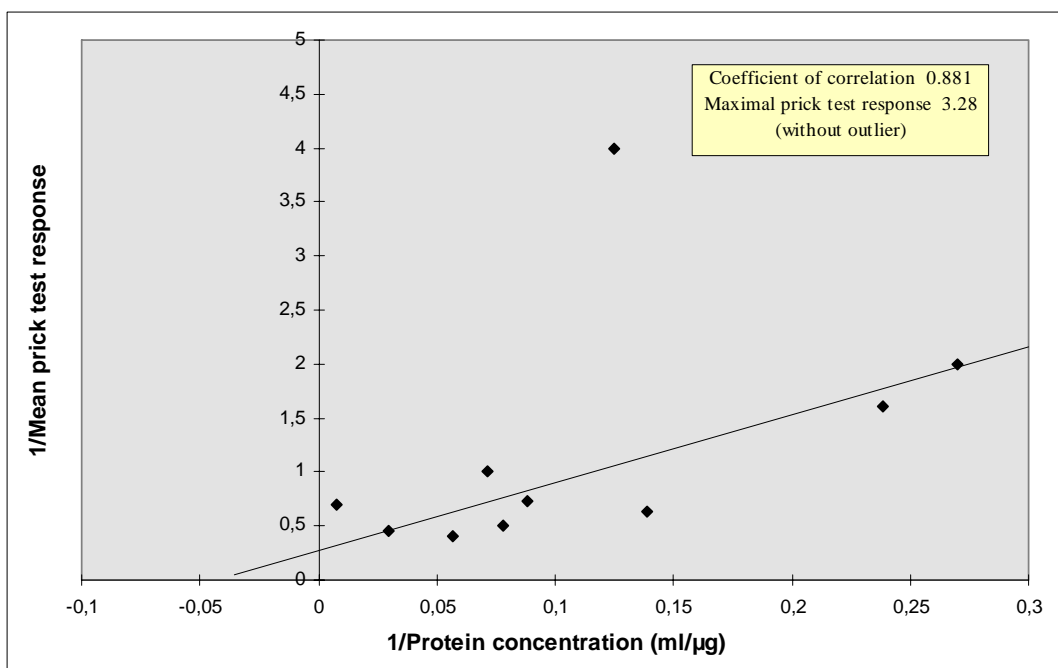


Figure 12 Double reciprocal plot of the mean prick test response versus the protein concentrations measured by the modified Lowry method in November 1996. 12 different glove extracts were tested in 17 different volunteers, known to be allergic against natural rubber latex.

2.1.2.4 ESL³-Assay for the determination of proteins

During our study we came upon a new protein assay distributed by Boehringer Mannheim which was announced to be more specific than the Lowry method especially in the presence of surface active substances.

This new protein assay is a spectrophotometric determination of the protein concentrations. The method utilizes a biuret-like reaction. The peptide bonds of the protein initially form Cu^{2+} -complexes in an alkaline solution. The protein concentration results from the measurement of the absorbance of a Cu^{2+} - bathocuproine complex which is formed with an excess of Cu^{2+} , not chelated by protein and subsequently reduced by ascorbic acid. Using this assay the determination of protein is independent of the amino acid composition and only depends on the number of peptide bonds chelating Cu^{2+} . In addition no interferences with reducing agents are to be expected.

Chelating contaminants (e.g. chelating buffer salts) should be avoided or compensated by appropriate calibration curves.

As seen in table 10 the results of this method correlated well with the amino acid analysis. Unfortunately the protein content of most of the gloves (25 of 32) was lower than the method's detection limit of 25 : g/g.

The presence of large amounts of non protein material in the glove extracts foiled any attempts to concentrate the extracts and lower the detection limit of the ESL-assay.

The ESL assay is not sensitive enough for the determination of proteins in latex gloves

Although the ESL assay is not influenced by chemical additives its low sensitivity does not allow the method to be used for the determination of proteins in latex gloves.

³ Exact Sensitive Low interference

Table 10 Comparison of the results measured by the ESL Assay and the amino acid analysis

Glove	HPLC (: g/g)	ESL (: g/g)
1 A	5	< 25
2 A	10.3	< 25
3 A	< 5,0	< 25
4 A	8	< 25
5 A	6.5	< 25
6 A	7.5	< 25
7 B	20.6	< 25
8 B	55.8	< 25
9 B	106	51.7
10 B	21.8	< 25
11 B	6.8	< 25
12 C	192	180
13 D	9.4	< 25
14 E	72	57.3
15 E	319	275
16 E	7.6	< 25
17 E	12.8	< 25
18 F	< 5	< 25
19 G	26.6	< 25
20 G	23.2	< 25
21 G	61.1	< 25
22 H	23.4	< 25
23 H	17.6	< 25
24 H	9.5	< 25
25 I	30.6	< 25
26 K	527	510
27 K	226	95.4
28 K	36.4	< 25
29 K	16.7	< 25
30 K	312	246
31 K	16.9	< 25
32 L	24.5	< 25

2.1.2.5 RAST-Inhibition

Method

We used the well known principle of RAST-Inhibition for the quantitative immunological determination of latex proteins. The commercial Pharmacia CAP System distributed by Pharmacia (Uppsala, Sweden) was used for the determination of specific IgE antibodies in latex-allergic volunteers. In this assay the allergens are covalently bound to a cellulose sponge. These immobilized allergens catch the corresponding specific IgE antibodies from the patient's serum which secondary are detected by fluorescence conjugated anti-IgE antibodies. For the determination of RAST-Inhibition the patient's serum is pre-incubated with a solution of latex allergens (e.g. glove extracts). Depending on the allergen content more or less IgE antibodies are on disposal for the secondary RAST assay, since they have already been fixed to glove allergens during the initial step of the RAST-inhibition assay.

A pool serum of 41 latex allergic volunteers was tested with the extracts of 28 different latex gloves. Equal volumes of the pool serum and the extract were pre-incubated for 1 h. Subsequently the CAP-FEIA assay was performed as recommended by Pharmacia. The content of allergenic protein was calculated by a standard curve which was established by measuring a dilution series of a lyophilized latex serum in concentrations from 1 to 100 : g/ml.

Results

The values determined by RAST inhibition did not correlate to the results of the amino acid analysis (figure 13), but however revealed a low correlation to the prick test response in latex allergic volunteers if two outliers were excluded (figure 14).

The RAST inhibition comprises three not standardized variables:

1. *The latex proteins coated to the solid phase*

The solid phase may be prepared by the laboratory itself or may be purchased in commercial kits. We used the ImmunoCAPs of the Pharmacia CAP system. The sensitivity of the Pharmacia CAP-system is about 65 to 85 % depending on the severity of clinical symptoms in latex allergic patients. However this indicates that not all latex allergens are present on the ImmunoCAP.

2. *The serum pool of latex allergic patients*

It is known, that the different risk groups of latex allergic patients may react to different latex proteins, which serve as 'marker proteins'. Therefore the response of the RAST inhibition depends on the composition of the serum pool.

3. *The latex protein used for the standard curve*

The standard protein may be prepared from latex fluid, from glove extracts or a mixture of both. We used a dilution series of a lyophilized low ammoniated latex fluid from Malaysia.

RAST inhibition is not suitable for a standard procedure

At the moment it is not possible to standardize these three components. Hence result from different laboratories are not comparable, which renders the RAST inhibition unacceptable for a standard procedure.

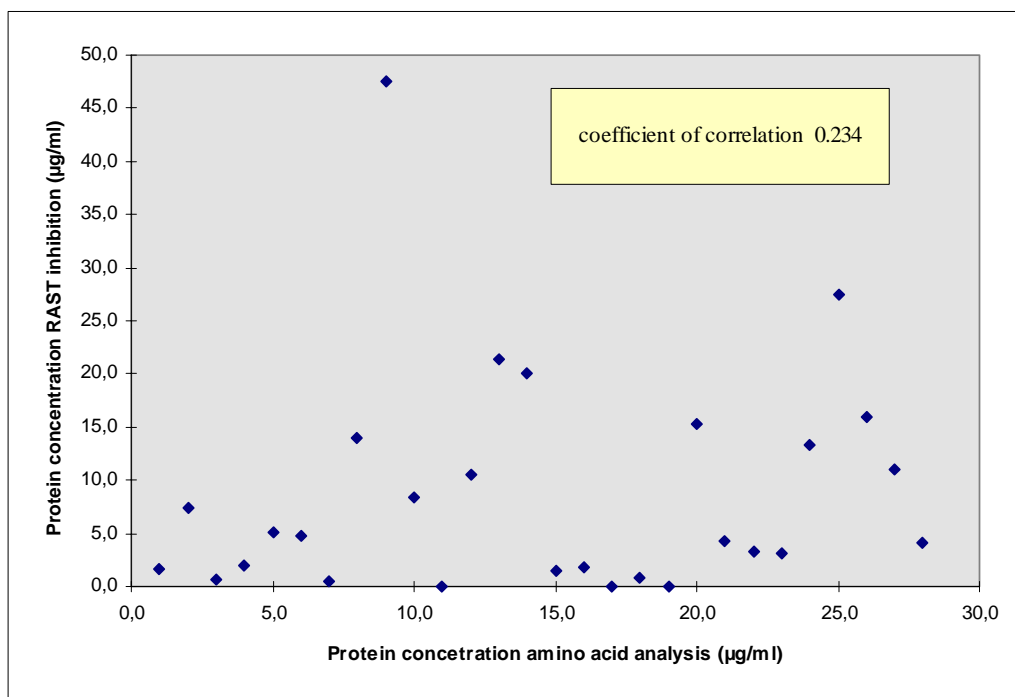


Figure 13 Correlation of the allergen concentration measured by RAST inhibition and the protein concentration measured by the amino acid analysis in 15 different glove extracts (TRIS pH 8.2).

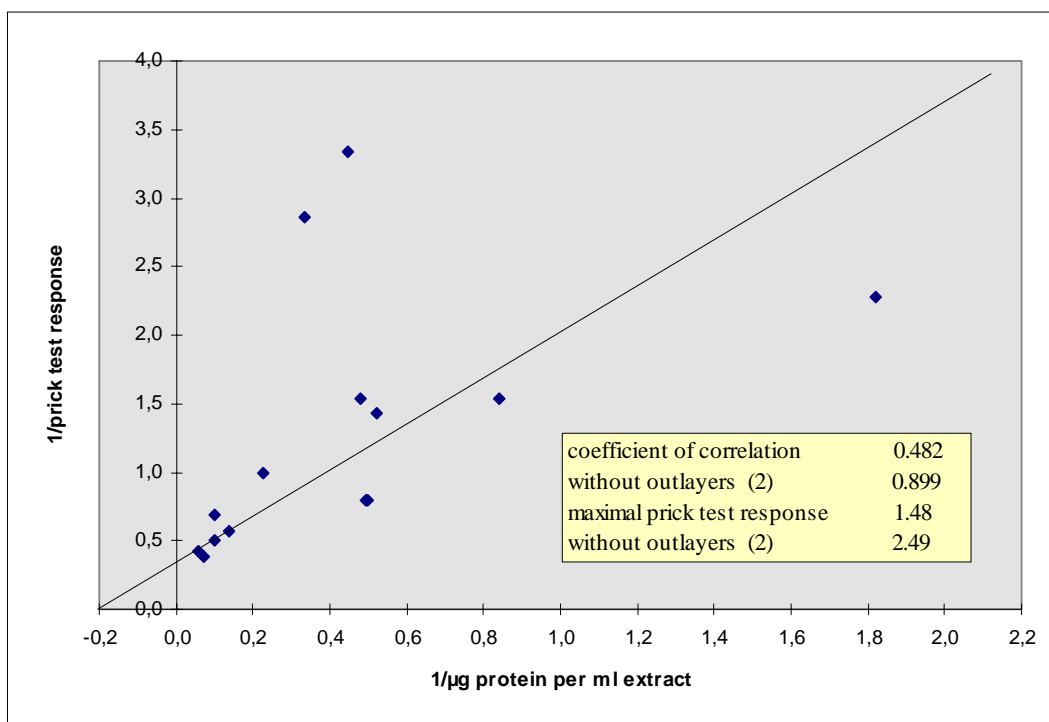


Figure 14 Double reciprocal plot of the mean prick test response in 17 different latex allergic volunteers versus the allergen concentration in 15 different glove extracts measured by RAST inhibition.

2.1.2.6 Latex Elisa for Antigenic Protein (LEAP)

Method

The LEAP is an immunologic method for the determination of latex proteins (1). In the first step of this assay the latex proteins are absorbed onto the inner layer of polystyrene microtiter plates. Subsequently the unreacted sites are blocked by bovine serum albumin and the immobilized latex proteins are detected with a polyclonal rabbit anti latex protein antibody followed by an enzyme conjugated goat anti-rabbit IgG antibody. The assay was obtained from Guthry Research Institute (Sayre, Pennsylvania, USA) and performed as recommended by the manufacturer.

Separation of high and low molecular weight protein

The extracts (4 ml) were filtered through the filter units (exclusion limit 3000 D and 10000 D, *Centricon*, Amicon, Witten Germany) by centrifugation at 2000 g. The filtrates (low molecular weight) were subsequently measured by different methods. The retained (high molecular weight) material (about 150 : 1) was redissolved in the extraction buffer to a total volume of 4 ml prior to measurement.

Results

The antigen concentrations measured by the LEAP did not show any correlation to both, the results of the amino acid analysis (figure 15) and to the prick test response in latex allergic volunteers (figure 16). However these results were against the assumption that the LEAP should be more specific for antigenic latex proteins than the applied chemical methods.

The insufficient binding of low molecular weight proteins onto the surface of polystyrene plates (the first step of this assay) probably is one main reason for these results. For further support of this assumption high and low molecular weight material in glove extracts was separated by ultrafiltration.

Selected latex gloves with a concentration of high and low molecular weight latex protein each above the detection limit ($> 1 \text{ : g/ml}$ using the amino acid analysis) were used for this study.

As shown in tables 11 and 12 these glove extracts contained considerable amounts of low molecular weight protein: 32.6 % to 70.8 % of the total protein was lower than 10 kD and 18,4 % to 58.3 % lower than 3 kD. Figures 17 and 18 clearly showed that these low molecular weight latex proteins (or peptides) are of allergological relevance. The mean prick test response in latex allergic volunteers revealed an equal dependency on the concentration of both, the low molecular weight protein and the total protein. However the $< 3 \text{ kD}$ fraction was slightly less reactive than the $> 3 \text{ kD}$ fraction.

Tables 13 and 14 clearly indicate that the LEAP is less suitable for the determination of low molecular weight protein compared to high molecular weight proteins.

The LEAP assay is not suitable as standard method

Due to lack in the determination of small proteins and peptides the method is not suitable for the determination of allergenic proteins in latex gloves. No correlation to the mean prick test response in latex allergic volunteers could be found.

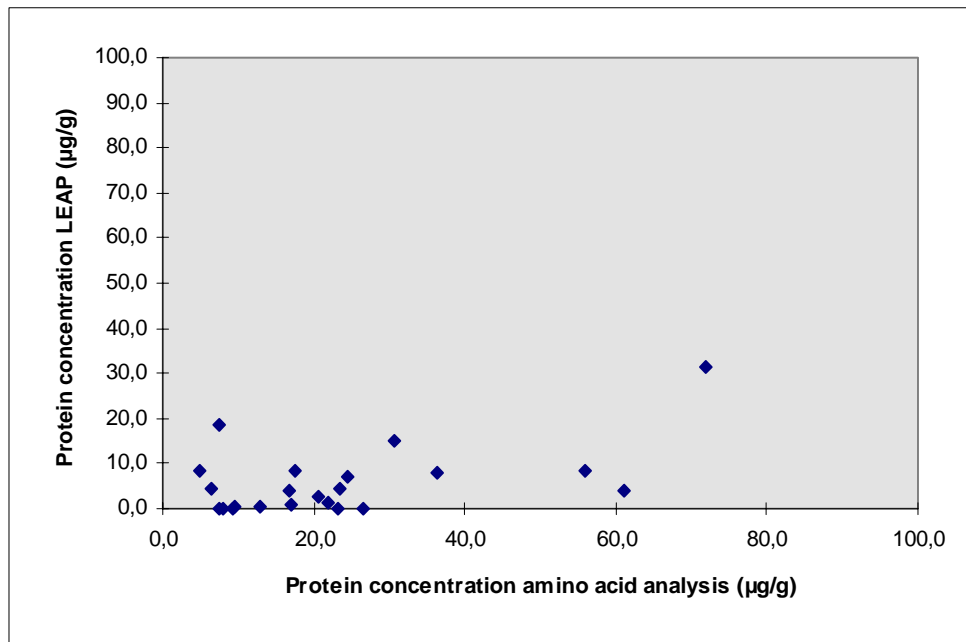


Figure 15 Correlation of the allergen concentration measured by LEAP and the protein concentration measured by the amino acid analysis in 24 different glove extracts (TRIS pH 8.2). Two gloves with very high protein concentrations in both methods were not depicted (AAA 319 and 192, LEAP: 1030 and 149, respectively)

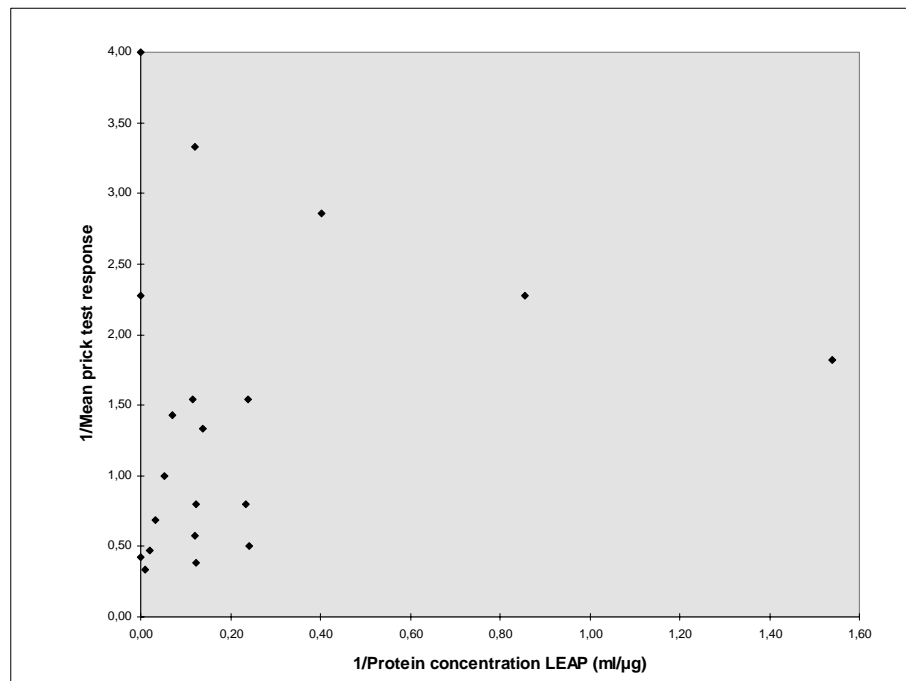


Figure 16 Double reciprocal plot of the mean prick test response in 16 different latex allergic volunteers versus the allergen concentration in 24 different glove extracts measured by LEAP.

Table 11 Protein fractionation of 12 glove extracts by Centricon units with an exclusion limit of 3 kD. The protein concentrations were measured by the amino acid analysis.

Glove	total protein (: g/ml)	< 3 kD		> 3 kD	
		: g/ml	% of sum	: g/ml	% of sum
4 A	38.1	14.6	38.3	23.5	61.7
5 A	55.7	21.2	38.1	34.5	61.9
6 A	36.1	19.2	53.2	16.9	46.8
9 B	55.6	10.2	18.4	45.4	81.7
12 C	214	55.5	25.9	159	74.1
14 E	91.9	20.3	22.1	71.6	77.9
15 E	267.8	66.8	24.9	201	75.1
21 G	61.3	22.1	36.1	39.2	63.9
25 I	38.8	22.6	58.3	16.2	41.8
26 K	445	189	42.5	256	57.5
27 K	287	164	57.1	123	42.9
30 K	285	86.9	30.5	198	69.5

Table 12 Protein fractionation of 19 glove extracts by Centricon units with an exclusion limit of 10 kD. The protein concentrations were measured by the amino acid analysis.

Glove	total protein (: g/ml)	< 10 kD		> 10 kD	
		: g/ml	% of sum	: g/ml	% of sum
3 A	16.0	7.9	49.4	8.1	50.6
7 B	14.8	5.3	35.8	9.5	64.2
8 B	42.8	22.1	51.6	20.7	48.4
9 B	61.7	38.7	62.7	23.0	37.3
10 B	26.1	10.8	41.4	15.3	58.6
12 C	193	122	63.4	70.5	36.6
14 E	93.2	46.0	49.4	47.2	50.6
15 E	355	167	47.0	188	53.0
31 K	13.3	8.4	63.2	4.9	36.8
32 L	16.4	8.9	54.3	7.5	45.7
21 G	72.5	40.0	55.2	32.5	44.8
22 H	25.9	10.4	40.2	15.5	59.8
23 H	23.0	12.5	54.3	10.5	45.7
26 K	506	358	70.8	148	29.2
25 I	36.8	23.9	64.9	12.9	35.1
27 K	194	63.3	32.6	131	67.4
28 K	30.4	13.5	44.4	16.9	55.6
30 K	343	189	55.1	154	44.9
31 K	13.4	8.4	62.7	5.0	37.3

Table 13 Protein concentration (: g/ml) in four different glove extracts measured by amino acid analysis and LEAP: Comparison of fractions larger and smaller 10 kD.

glove	< 10 kD		> 10 kD		sum	
	HPLC	LEAP	HPLC	LEAP	HPLC	LEAP
3 A	2.03	0	1.98	0.69	4.01	69
7 B	1.33	0	2.40	0.06	3.73	6
9 B	13.2	0.24	7.82	1.36	21.0	160
26 K	76.2	4.34	32.8	14.3	109	18.6

Table 14 Protein concentration (: g/ml) in four different glove extracts measured by amino acid analysis and LEAP: Comparison of fractions larger and smaller 3 kD.

glove	< 3 kD		> 3 kD		sum	
	HPLC	LEAP	HPLC	LEAP	HPLC	LEAP
3 A	1.8	0	1.1	0.36	2.9	0.36
7 B	3.5	0	3.6	0	5.3	0
9 B	7.4	0.19	16.2	0.66	47.2	0.85
26 K	38.1	0.52	68.1	21.8	106	22.3

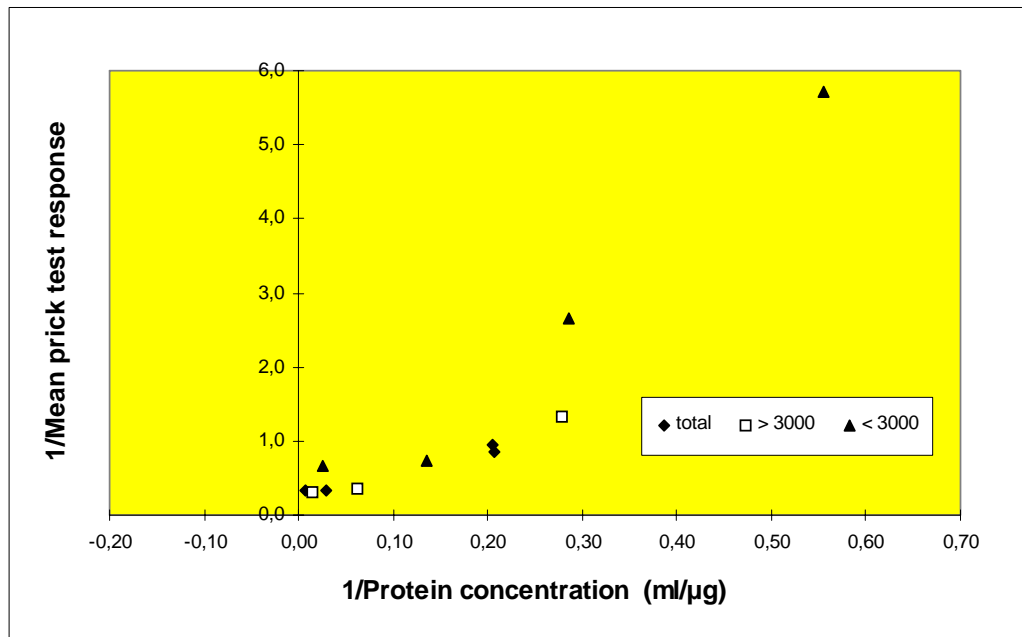


Figure 17 Double reciprocal plot of the mean prick test response with four latex glove extracts and the corresponding fractions (molecular weight < 3 kD and > 3 kD) in 16 latex allergic volunteers versus the protein concentrations (: g/ml) measured by the amino acid analysis.

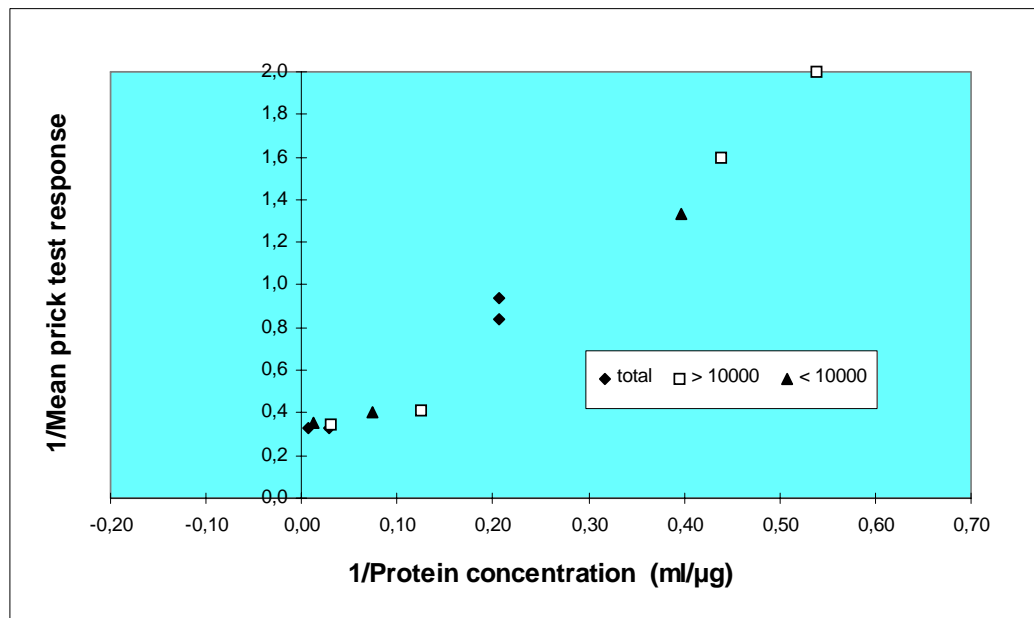


Figure 18 Double reciprocal plot of the mean prick test response with four latex glove extracts and the corresponding fractions (molecular weight < 10 kD and > 10 kD) in 16 latex allergic volunteers versus the protein concentrations (: g/ml) measured by the amino acid analysis.

2.1.2.7 Immunoblotting

In our studies (2.1.2.6) large amounts of the allergological relevant proteins in latex gloves have been shown to be of low molecular weight (< 10 kD and < 3 kD). Therefore we did a lot of experiments in order to separate low molecular weight proteins or peptides even in the range below 3 kD and finally were successful using TRIS-TRICINE gels. However all experiments with gloves failed in visualizing individual bands of proteins or peptides below 10 kD. The reason is that glove extracts contain aside from some originally small proteins (i.e. hevein) a large amount of breakdown products derived from larger proteins by alkaline (NH₃) hydrolysis during both, the storage of the rough latex milk and the manufacturing process. These breakdown products with different molecular weight and amino acid composition appeared as a bulk of stainable material in the lower part of the TRIS-TRICINE gels. This effect was simulated in the experiment shown in figure 19. A low ammoniated latex serum was incubated for one to ten days at 50 °C (lines 0 to 10 in figure 19). It was impossible to identify individual proteins with molecular weight lower than 10 kD in the samples which lost the proteins with a higher molecular weight (lines 7 to 10 in figure 19). Additionally we found that such small peptides were less stainable with Coomassie blue (figure 19) and even with silver nitrate compared to proteins of higher molecular weight. The total protein content (measured by the modified Lowry method) did not change during this experiment but the total stainable material on the gel disappeared mainly on day 9 and 10. Blotting experiments of such low molecular weight proteins were additionally unsuccessful because those peptides only incompletely bound to the blotting membranes (nitrocellulose or polyvinylidene) and most of them were lost during the blotting process. Since these small products of protein hydrolysis even in the 3 kD range have been shown to carry main parts of the allergological information (see 2.1.2.6) the immunoblotting has been turned out to be an unsuitable method for identifying proteins derived from latex gloves.

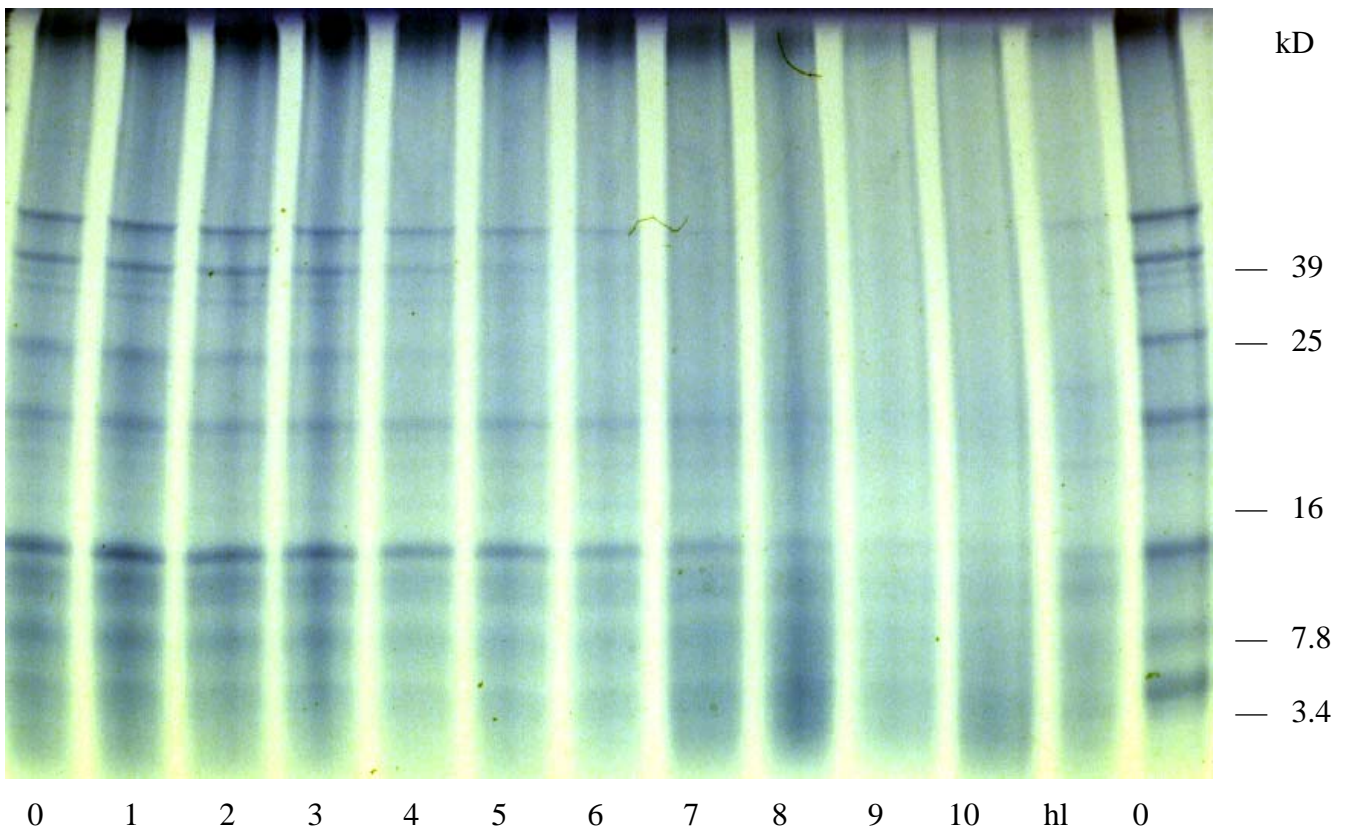


Figure 19 SDS polyacryl amide gel electrophoresis of an aged low ammoniated latex serum. The serum was derived from a low ammoniated latex fluid from Malaysia which was centrifuged at 50000 g. The serum was incubated for 0 to 10 days at 50 $^{\circ}$ C. hl is a high ammoniated latex serum stored refrigerated for about 12 month.

2.1.3 Development of an extraction procedure for the allergological relevant proteins in latex gloves

The extraction procedure should optimally reflect the physiological conditions of glove use. Up to the beginning of our study gloves were usually cut into small pieces and subsequently extracted with double distilled water. However, this method does not fulfil the requests for an optimal extraction procedure and has at least five disadvantages:

6. The proteins were extracted not only from the original glove surface but additionally from the cut surface. The extracted amount of latex protein depends on the size of the cut pieces.
7. Depending on the individual glove brand the cut pieces of gloves often stick together and hence impair the extraction procedure as well as the reproducibility of the results.
8. The relative high extraction volume used in the cut glove method lowers the sensitivity of the determination of proteins to 20 : g/g, if the modified Lowry method is used.
9. It is impossible to distinguish between the inner and outer side of gloves, which is important from the allergological point of view and in case may be indispensable for an optimization of the leaching process of gloves.
10. Using distilled water the pH-value of the glove extracts varies from 4.0 to 10.6 and does not reflect physiological conditions.

2.1.3.1 Double glove method

Keeping these disadvantages of the cut glove method in mind we created the double glove method, which comprises the simultaneous extraction of two gloves, one at the inner side and the other one at the outer side (fig. 20). Unlike the cut glove method the extraction is limited to the original glove surface. To improve the contact of the glove surfaces the inner glove is filled with a dye solution, which additionally mimics the conditions during glove use.

A differentiation of the protein concentration on the inner and outer glove surface is possible by turning the inner glove inside-out (extraction of the inner side) or by turning the outer glove inside-out (extraction of the outer side). Since the extraction volume is reduced to 25 ml per glove the sensitivity is increased to 10 : g/g.

2.1.3.2 Buffer system

At the beginning of our study we decided to use a buffer system in order to establish a pH-independent extraction procedure for powdered and unpowdered gloves, where differences in the pH often occur (table 15). Since the most common powdered gloves usually induce an alkaline pH on the skin (table 16) a TRIS-HCl buffer system at pH 8.2 was chosen to reflect the physiological pH during glove use as well as possible. In addition this TRIS-HCl buffer did not interfere with the amino acid analysis and thus was superior to other buffer salts (e.g. phosphate).

2.1.3.3 Time and temperature

Several investigators reported the amount of extractable protein to be definitely dependent on the temperature. However, we could never confirm this statement within our experiments (figure 21). One possible reason for this discrepancy is the loss of water out of non-covered flasks during an increase in temperature. This, however, may pretend higher amounts of extracted protein. A second reason for these controversial opinions on the influence of temperature on the amount of extractable protein may originate in a different efficacy of the applied extraction methods (figures 22 and 23).

In our experiment we compared the double glove method with the single glove method and the cut glove method in view of the fraction of protein extracted during a given time scale. A complete protein extraction (100 %) was assumed after 24 hours extraction time. Remarkably, the double glove method exclusively revealed an almost complete protein extraction within two hours (99.6 ± 5.0). In contrast the single glove method (a single glove was filled with the extraction medium) and the cut glove method only revealed $75.5 \% \pm 17.9 \%$ and $68.3 \% \pm 13.0 \%$ of the total extractable protein within the same time, comprising a much higher standard deviation. Using the latter methods an increased temperature may accelerate the protein extraction but, however, does not increase the amount of extractable protein. Due to an optimized surface contact between the glove and the extraction medium the double glove method has been shown to be more effective, less dependent on temperature and thus superior to the other methods in the extraction of proteins from latex gloves.

The allergological relevance of these glove derived TRIS extracts (double glove method) was thoroughly investigated in latex allergic volunteers by a comparison of their prick test response to different latex gloves and the corresponding extracts (figure 24). The high correlation of 0.963 clearly documents an identical allergological potency of the investigated gloves and their extracts.

2.1.3.4 Harmonisation of the CEN method and the ASTM method

The introduction of our new developed extraction procedure into the European standard EN 455-3 caused some problems. These were mainly due to differences to the ASTM method which secondary demanded the manufacturers to fulfil two standards assuming a world wide distribution of their products. The working group 3 of CEN TC 205 therefore aimed at harmonizing the CEN and the ASTM method. The results of this effort are shown in table 17. A minor change of the pH of the extraction buffer from pH 8.2 to 7.4 (which the Americans assumed to be more physiological) did not considerably change the amount of the extracted latex protein and therefore was accepted by us (figure 25; the amount of extracted protein at pH 7.4 was 89 % of that extracted at pH 8.2; the correlation was 0.984).

The harmonized extraction procedure was introduced into the European standard prEN 455-3

The very simple, reproducible and most physiological double glove method for the extraction of allergological relevant proteins is used in the European standard EN 455-3 and planned to be used in the ISO 12243-2.

Table 15 PH-values of the watery extracts of powdered and unpowdered medical gloves for single use. The gloves were filled with 50 ml of freshly produced ultra pure water (UHQ, Elgastat), closed as described in the double glove method and extracted for 1 h at ambient temperature shaking on a horizontal shaker at 200 rpm.

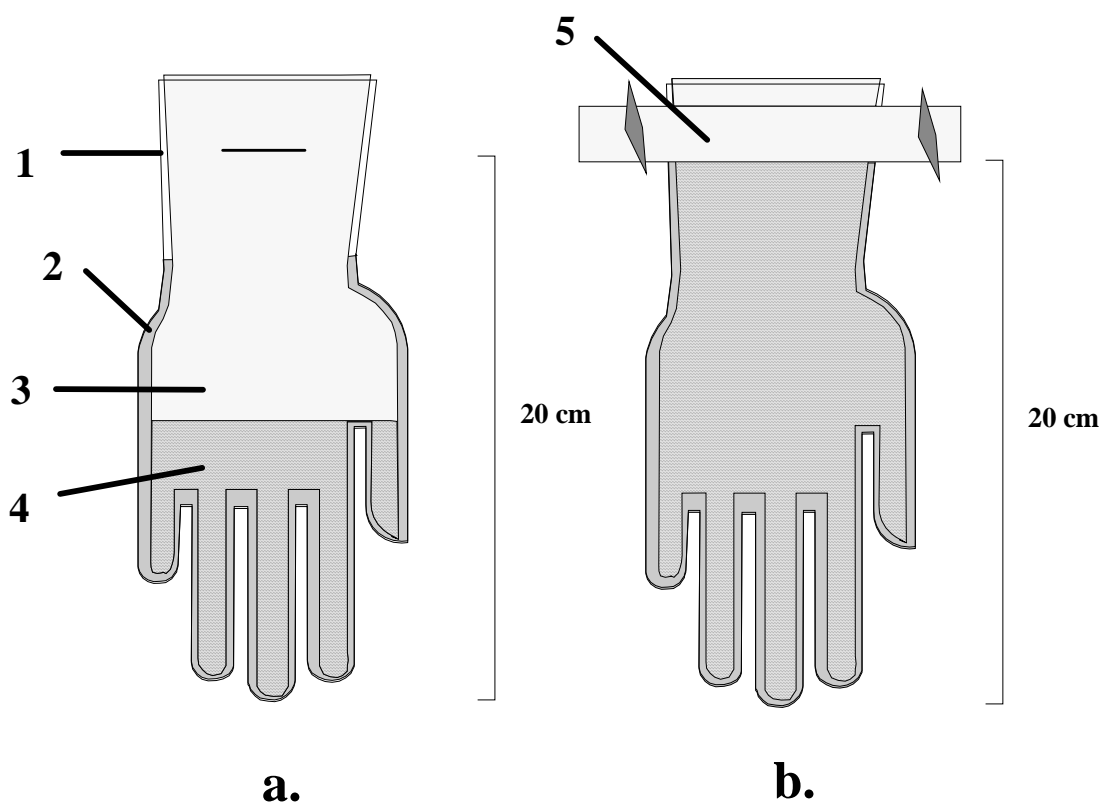
Glove name	Distributer	Lot-no.	Material	Powder	pH-values
Regent biogel OP	LRC products	89H07N6	Latex	no	3.28
Einmal-HS	Dahlhausen	-	Vinyl	no	4.49
Sempermed pf	Semperit	10C5TC15	Latex	no	4.98
peha taft pf	Hartmann	2723797P	Latex	no	5.64
Supra	Safeskin	01A5054A	Latex	no	5.65
Absogel	Ampri	850309	Latex	no	5.73
Triflex pf	Baxter	-	Latex	no	5.91
Gentle Skin Anatom	Meditrade	30331212	Latex	no	6.05
Regent Neotech	LRC products	8800060	Neoprene	no	6.07
Gentle Skin	Meditrade	60109343	Latex	no	6.11
Durafit	Safeskin	A41341169A	Latex	no	6.13
Satin Plus	Safeskin	A5116E7372	Latex	no	6.22
Sempermed Ultra	Semperit	VQ5B9	Latex	no	6.22
Exam Glove	Safeskin	A5116E5610	Latex	no	6.34
N-Dex	Roth	66096	Nitrile	no	6.4
No Powder	Ansell	506802403	Latex	no	6.52
Flexam pf	Baxter	8876 Cat-Nr.	Latex	no	6.67
NuTex	Ansell	506802203	Latex	no	6.8
mean:	5.85		median:	6.12	
Duraprene	Baxter	K5C016	Neoprene	yes	6.12
SIE Latex	Sänger	-	Latex	yes	6.96
Sensicare	Becton Dickinson	4F112R	Vinyl	yes	6.99
Gammex op	Ansell	506802503	Latex	yes	7.22
Sensi Touch	Ansell	506802303	Latex	yes	7.25
Sempermed Derma	Semperit	VQ5D5	Latex	yes	7.25
Micro touch	Johnson&Johnson	137538	Latex	yes	7.39
Sempermed Senso	Semperit	UP5B1	Latex	yes	7.51
Conform op	Ansell	506802603	Latex	yes	7.52
Sempermed Classic	Semperit	VQ5B9	Latex	yes	7.8
Neutralon	Johnson&Johnson	A-03254	Latex	yes	7.81
Maxxus	Johnson&Johnson	7431312	Latex	yes	8.29
peha taft sterile	Hartmann	54504701	Latex	yes	8.65
Manusoft	Beese	940410	Latex	yes	8.72
DermaPrene	Ansell	504759503	Neoprene	yes	8.74
TouchNTuff	Ansell Edmont	-	Nitrile	yes	8.76
Triflex	Baxter	-	Latex	yes	9.2
Reference	Meditrade	-	Latex	yes	9.26
Examination glove	Baxter	8856	Latex	yes	9.78
Manex Neoderm	Beiersdorf	3335-0001	Latex	yes	10.4
mean:	8.08		median:	7.81	

Table 16 Changes of the cutaneous pH on the back of the hands of 20 volunteers after donning a powdered and an unpowdered glove. The pH was measured before and immediately after a 30 min glove exposure using an electrode suitable for surfaces. The volunteers did not use any skin care products for at least 24 h before glove use.

Volunteer	Powdered glove		Unpowdered glove	
	Before glove use	Immediately after 30 min glove use	Before glove use	Immediately after 30 min glove use
1	4.8	7.3	5.5	5.5
4	6.5	7.9	6.2	5.8
7	5.9	8.7	4.9	5.8
8	5.7	8.3	4.7	5.4
10	5.6	8.9	5.6	6
11	5.2	8.6	5.4	5.7
15	5.6	9.2	6.2	5.6
16	4.5	7.6	4.6	5.8
18	4.7	6.9	4.3	5.1
20	4.5	9.3	5	5.6
21	4.6	6.1	4.3	5
24	5.6	8	5.6	5.6
25	6.6	8.3	6.1	5.8
27	5.6	7.7	5.6	5.9
30	4.4	5.6	4.4	4.9
31	5.2	7.6	5	5.4
33	6.7	8.5	5.6	5.8
34	4.3	7.6	4.4	5
35	5.9	7.6	nd	nd
36	6.2	8.1	6.2	6.1
mean	5.4	7.9	5.2	5.6
sd	0.7	0.9	0.6	0.3
CV	13.8	11.3	12.2	6
max	6.7	9.3	6.2	6.1
min	4.3	5.6	4.3	4.9

Table 17 Harmonization of the CEN and ASTM method for the extraction of proteins from latex gloves

Parameter	CEN method	ASTM method	Harmonized method
Extractant	TRIS buffer pH 8.2	distilled water	TES buffer pH 7.4 ± 0.2
Method	Double glove	Cut glove	Double glove
Time	2 hours	2 hours	2 hours
Temperature	Ambient	37 °C	25 ± 5 °C



1. Outer glove (glove 1)
2. Extraction buffer
3. Inner glove (glove 2)
4. Dye solution
5. Glove clip

Figure 20 Double glove extraction for the determination of proteins in medical gloves for single use.

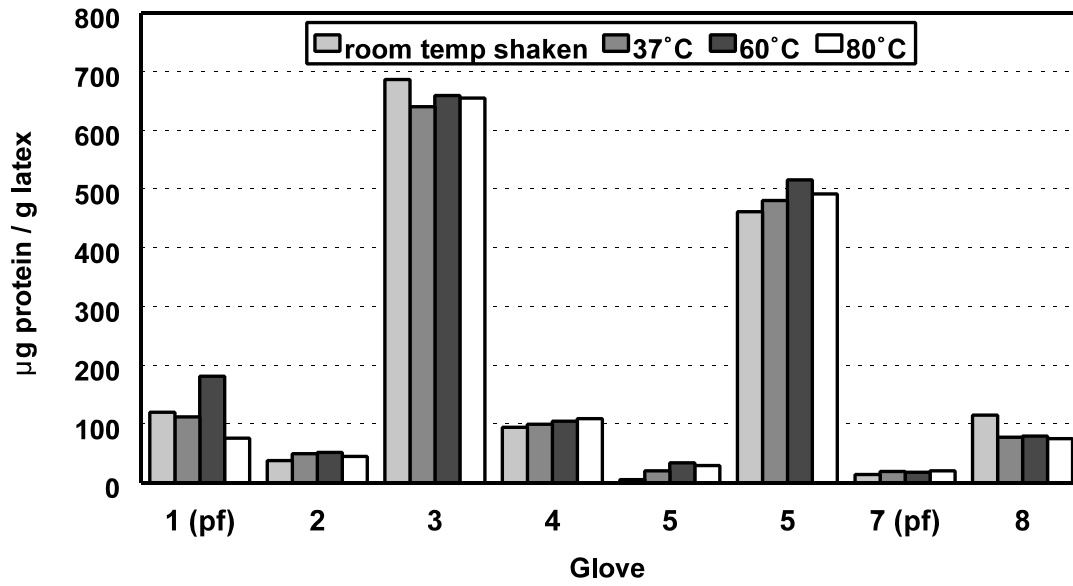


Figure 21 Effect of temperature on the amount of extracted protein in eight different gloves (two powder free gloves, pf). The double glove method with TRIS buffer pH 8.2 was used in all cases.

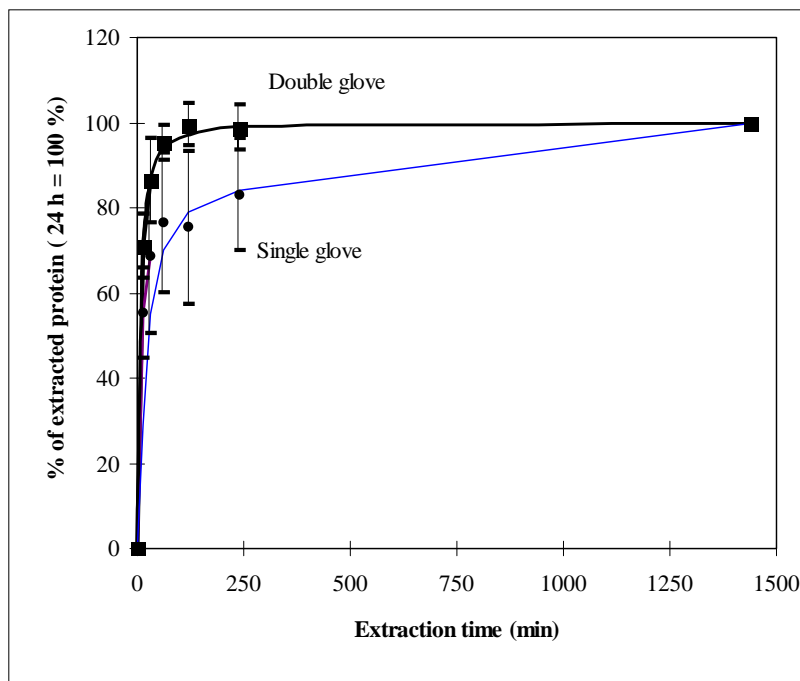


Figure 22 Time dependency of the double glove and the single glove extraction. Five different gloves (two powder free and three powdered gloves) with protein concentrations between 43.5 and 786 : g/g were extracted. The values given, are the mean fractions (%) from all 5 gloves (\pm sd) of the protein extracted within 24 h.

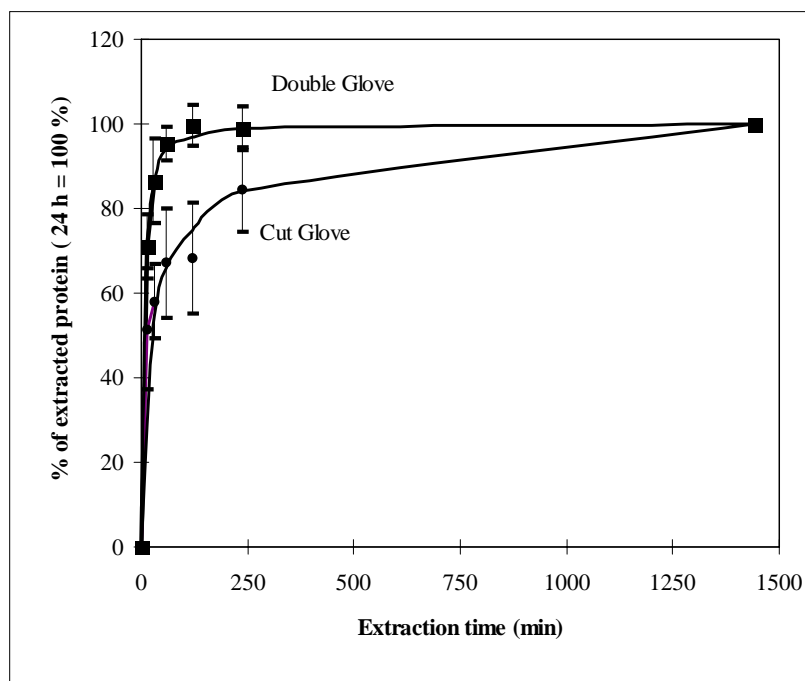


Figure 23 Time dependency of the double glove and the cut glove extraction. Five different gloves (two powder free and three powdered gloves) with protein concentration between 43.5 and 786 : g/g were extracted. The values given, are the mean fractions (%) from all 5 gloves (\pm sd) of the protein extracted within 24 h.

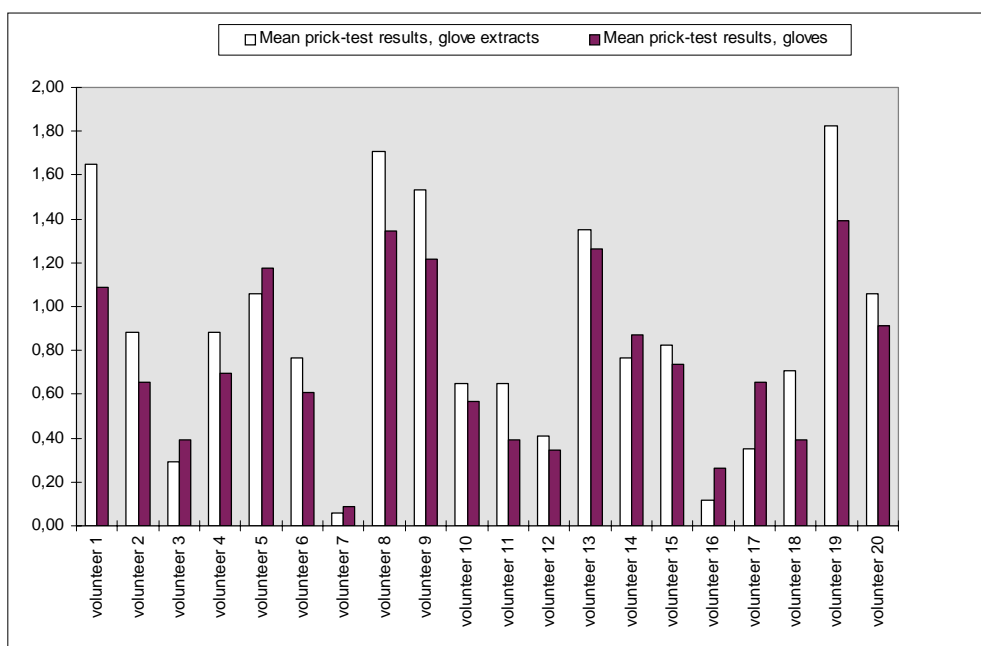


Figure 24 Comparison of the mean prick test responses to latex material and the corresponding extracts (double glove method, pH 8.2) of 17 gloves in 20 different volunteers. The coefficient of correlation was 0.963.

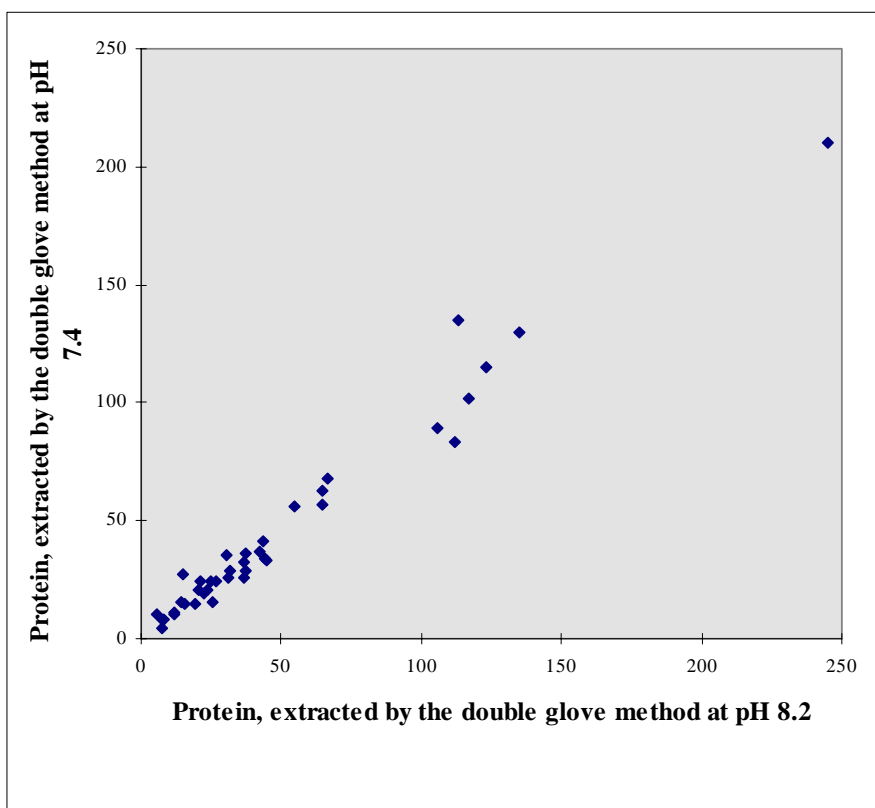


Figure 25

Comparison of the extraction

of latex gloves at pH 8.2 and pH 7.4 with the double glove method. The coefficient of correlation was 0.984 and the slope was 0.89.

2.1.4 Cleaning up procedures

Two main groups of chemicals usually interfere with the Bradford and the modified Lowry method, surface active substances and accelerators. Four procedures were tested for their efficiency to remove these chemicals from glove extracts:

1. Detergent absorber gel
2. Washing of the precipitated protein
3. Dialysis
4. Ion exchange membranes

2.1.4.1 Detergent absorber gel

The detergent absorber gel consists of hydrophobic small beads which have a large inner surface and therefore are able to bind large amounts of detergents (4). With respect to the distributor's information this gel is known to enable an almost complete extraction of detergents without any loss of protein. In order to verify this statement solutions of 5 different surfactants (1mg/ml TRIS buffer) with and without ovalbumin (10 : g/ml) were incubated with the absorber beads (10 mg/ml, 2 h at room temperature) under slight agitation. The absorber gel was removed by a disposable filter unit (0.2 : m, low protein binding, Millipore, Eschbach).

The determination of ovalbumin was performed by the Bradford and the modified Lowry method. Following the gel treatment the modified Lowry method revealed the correct values for the ovalbumin concentration (table18), but however there was an unspecific blue colour development in the ovalbumin free controls which was reproducible inexplicable. Using the Bradford method, which is more sensitive to surfactants, no convincing effects of the absorber gel could be found either especially not in the presence of SDS and Tween 20. The unspecific colour response in the protein free controls which was likewise documented in the Bradford method finally convinced us to cancel further experiments with this very expensive absorber gel.

Table 18 Influence of a detergent absorber gel on the results of the determination of ovalbumin in the presence of different surfactants. All values are recorded in : g/ml.

ovalbumin concentration	Bradford method				Modified Lowry method			
	0		10 : g/ml		0		10 : g/ml	
Absorber gel treatment	no	yes	no	yes	no	yes	no	yes
CHAPS	1.8	3.9	20.6	10.3	0.2	4.4	12.4	9.9
Nonidet P40	> 100	2.7	> 100	11.0	0	4.8	7.0	9.8
SDS	30.7	43.7	31.7	45.1	0	4.6	7.7	9.9
Triton X 100	> 100	3.2	> 100	10.6	0	5.1	5.7	10.1
Tween 20	> 100	12.7	> 100	31.0	2.7	6.3	19.9	10.4
no surfactant	0	1.4	10.6	8.9	0	4.2	10.1	9.8

2.1.4.2 Washing of the precipitated protein in the modified Lowry method

Washing the pellet of precipitated proteins is a known method to remove non protein materials. Detergents and accelerators which are known to interfere with the modified Lowry assay usually are soluble in organic solvents. Since acetonitrile is often used for the deproteinisation of plasma samples it was chosen for our experiments directed towards washing the protein pellet (TCA, PTA, DOC) during the first step of the modified Lowry method.

Although this method initially revealed encouraging results and was proven to be effective for ovalbumin in the presence of detergents, it failed to be applicable on glove extracts (table 19). Possibly the fraction of low molecular weight protein was partly lost by this procedure.

Table 19 Effects of washing the protein pellets with acetonitrile. The protein pellets produced by the precipitation with TCA, PCA and DOC in the modified Lowry method were resuspended in acetonitrile and centrifuged at 2000 g. The resulting pellets were dissolved in NaOH and the protein was afterwards determined as described in the modified Lowry method. The results were compared to the protein concentrations measured by the amino acid analysis.

Glove	Modified Lowry (: g/ml)		Amino acid analysis (: g/ml)
	Normal procedure	Washed pellet	
1	21.2	9.9	17.6
2	5.8	< 2.0	3.2
3	26.1	< 2.0	2.4
4	6.0	< 2.0	7.3
5	46.0	29.4	45.1
6	47.0	44.3	28.5
7	86.0	53.1	38.5
8	84.0	53.1	45.6
9	4.2	< 2.0	5.8
10	24.1	6.5	1.8
11	8.8	< 2.0	4.8
12	4.7	< 2.0	8.9

2.1.4.3 Dialysis

Dialysis is a very common method to remove low molecular weight material from proteins. It is usually performed by semipermeable membranes with an exclusion size of 10000 D. However, in our investigations by gel electrophoresis (see below) most of the extracted proteins from latex gloves were shown to be of low molecular weight (2000 to 10000 D). According to our prick test results in latex allergic volunteers (see LEAP method 2.1.2.6) these low molecular weight proteins were proved to be of allergological relevance. Therefore membranes with an exclusion size of 1000 D (Spectrapore, Carl Roth GmbH, Karlsruhe) were used in our experiments.

Following our initially less successful experiments on dialysis with distilled water or buffer (see report 2) 15 % n-propanol was added to the dialysis medium to remove surface active material by lowering the dielectric constant of the solution. Hence an increase in the critical micellar concentration of detergents was to be expected. As shown in table 20 this addition of propanol to the dialysis medium proved to be effective in some but unfortunately not in all gloves.

Table 20 Cleaning up of four examples of glove extracts by dialysis with distilled water and in the presence of 15 % n-propanol. The protein concentrations were measured both by the modified Lowry method and the amino acid analysis.

		before dialysis	after dialysis dd-H ₂ O	after dialysis 15 % n-propanol
glove 1	mod. Lowry	47.4	39.3	13.4
	Amino acid analysis	17.0	16.1	16.1
glove 3	mod. Lowry	84.1	41.5	20.0
	Amino acid analysis	18.6	25.0	20.7
glove 5	mod. Lowry	79.3	66.0	44.4
	Amino acid analysis	15.6	14.2	15.1
glove 14	mod. Lowry	260	245	260
	Amino acid analysis	60.0	80.9	74.5

2.1.4.4 Ion exchange membranes

A new generation of ion exchange membranes arranged in cartridges was assumed to be a simple method for the cleaning up of proteins. We used four different types of these membranes (Sartorius, Göttingen):

1. A strong acidic cation exchanger (sulphonic acid)
2. A weak acidic cation exchanger (carboxyl)
3. A strong basic anion exchanger (quaternary ammonium)
4. A weak basic anion exchanger (diethylamine).

If cation exchange membranes were used, glove extracts were produced with sodium acetate buffer (pH 4.5) in order to get positively charged proteins. In the case of anion exchange membranes glove extracts were prepared with TRIS-HCl, pH 8.2 in order to get negatively charged molecules. The binding of proteins to these membranes requires the salt concentration to be as low as possible. Hence, a twofold and a fivefold dilution of the extracts with dd water was performed. Twenty ml of the twofold dilution and 40 ml of the fivefold dilution were loaded onto the membranes. Following a 20 ml washing the proteins were eluted by 0.5 M NaCl in the corresponding buffer, by 0.1 M NaOH or by 0.1 M HCl.

The results of two gloves of high protein content (333 and 650 : g/g, with the same results in the modified Lowry and the amino acid analysis) are shown in table 21. The only acceptable recoveries were obtained with basic membranes and sodium chloride used for the elution.

In a second series of experiments 14 different gloves were fractionated under the optimal conditions established in the previous series: strong basic membrane; application with 0.025 M TRIS HCl buffer pH 8.2; elution with 0.5 M NaCl. Hence, the cleaning up was very successful in 4 gloves (table 22) from the same manufacturer whose gloves usually revealed false high protein concentrations in the modified Lowry method. Based on our HPLC measurements most of the other tested gloves, however, lost 25 to 70 % of the applied protein during this cleaning up procedure. This loss of protein included both not retained protein (possibly peptides not charged under these conditions) and not eluable protein in various ratios.

Table 21 Protein retained at and eluted from four different ion exchange membranes using different buffers for application and elution. The values are expressed as % of applied protein.

application buffer	25 mM sodium acetate pH 4.5		10 mM sodium acetate pH 4.5	
elution with	0.5 M NaCl	0.1 M NaOH	0.5 M NaCl	0.1 M NaOH
weak acidic	9,2	9,2	5.2	5.9
strong acidic	4,6	7,3	1.3	7.0
application buffer	25 mM TRIS HCl pH 8.2		10 mM TRIS HCl pH 8.2	
elution with	0.5 M NaCl	0.1 M HCl	0.5 M NaCl	0.1 M HCl
weak basic	77.1	5.9	63,0	5,9
strong basic	90.2	7.0	56,9	3,1

Table 22 Cleaning up of proteins from 14 different gloves by a strong basic anion exchange membrane. Glove extracts (0.05 M TRIS HCl, pH 8.2) were diluted to 0.025 M salt concentration by dd water. 20 ml were applied to the membranes and eluted with 5 ml of NaCl (0.5 M in TRIS buffer, pH 8.2). Values are given in : g/ml extract.

Glove	Modified Lowry		Amino acid analysis		Cleaning effect on mod. Lowry	Recovery (%) (based on amino acid analysis)
	applied	retained and eluted	applied	retained and eluted		
1	50.5	5.19	6.62	6.48	excellent	98
2	65.8	5.78	5.12	5.15	excellent	100
3	35.5	10.9	8.73	8.95	excellent	100
4	46.9	7.81	9.50	8.79	excellent	93
5	39.2	29.6	59.9	45.3	not necessary	75
6	75.7	62.8	126	91.9	not necessary	73
7	279	200	415	272	not necessary	66
8	611	172	146	89.9	partially	67
9	9.10	8.37	15.4	7.5	not necessary	49
10	63.0	42.8	78.7	50.2	not necessary	64
11	105	14.7	59.9	17.4	low recovery	29
12	597	373	913	708	not necessary	76
13	350	161	293	223	not necessary	76
14	1606	530	1327	811	not necessary	61

The Cleaning up of glove extracts is not generally possible

Cleaning up procedures may be successful in some but not in all gloves especially if the propanol dialysis and the ion exchange membranes are used. Since glove ingredients differ so much a general applicable cleaning up procedure for glove extracts could not be established within our studies. However, in view of figure 10 manufacturers are endeavoured to lower or eliminate differences in the protein concentration determined by the amino acid analysis and the modified Lowry method. Hence, cleaning up procedures for glove extracts probably are unnecessary for future applications of the modified Lowry method.

2.1.5 Inter-laboratory test on the determination of proteins in latex gloves

During the work for this study and for the working group 3 of CEN tasking group 205 we organized three inter-laboratory tests. One of these was part of this project and will be described in detail, the two others will be shortly summarized, because they are important for the final evaluation of the study.

2.1.5.1 Comparison of the CEN-method and the ASTM-method for determination of proteins in latex glove 1995 (CEN task 1995)

Due to a task from CEN/TC 205 WG 3 we started a inter-laboratory test in 1995 to compare the two methods from CEN and ASTM. At this time the double glove extraction was just an idea and not proven to be useful. The validation experiments described above were not done in 1995. The differences of both methods are summarized in table 23.

Table 23 Differences in the methods for the determination of proteins in latex gloves distributed by the CEN and the ASTM.

	CEN	ASTM
Extraction procedure	Double glove	Cut glove
Extraction medium	Buffer pH 8.2	dd water
Extraction time	1 h	2 h
Extraction temperature	Ambient	37 °C
Modified Lowry	minor differences not significant	
Sensitivity	10 : g/g	20 : g/g

Because we need for this round robin 650 gloves in a very short time, we were not able to sent the same batch to every laboratory.

The mean value measured in eight laboratories by the CEN method were 136 ± 14 : g/g and 218 ± 13 : g/g for the two batches of gloves. The measurement by the ASTM-method revealed for the same batches 158 ± 53 : g/g and 193 ± 24 : g/g.

For further analysis the results were expressed in % of the mean measured by all participants with one method.

Whereas the results obtained by the CEN methods (table24) revealed very good inter-laboratory precision with a coefficient of variation of 12.5 % measured over all values, the ASTM method (table 25) showed a very high coefficient of variation of 31,4 %. Similar results were seen in the intra-laboratory precision, the coefficient of variation varied from 4.6 to 14.1 in the CEN method and from 2.1% to 22.2 % in the ASTM method.

The CEN method is more precise than the ASTM

The results showed clearly that the CEN method was more precise than the ASTM procedure. The reason seemed to be the more precise description of the extraction procedure by the CEN method .

Table 24 Results of the protein determination by 8 different laboratories in Europe in one brand of gloves using the CEN method. Protein concentrations are expressed in % of the mean in order to normalise the results of two different batches of gloves.

Analysis	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8		
1	9919	84,17	131,47	97,49	110,05	106,21	104,37	111,38		
2	112,12	91,36	101,92	84,20	110,05	119,94	93,64	103,13		
3	108,87	78,02	99,71	76,07	109,31	103,92	93,83	107,71		
4	111,75	94,33	86,41	96,01	108,57	101,11	99,10	104,05		
5	103,77	84,47	121,13	84,94	108,57	105,39	96,44	101,76		
6	132,13	99,64	118,17	83,46	98,97	101,92	96,85	93,05		
7	103,84	91,07	121,86	80,50	111,52	102,15	90,71	96,71		
8	104,51	78,96	85,67	62,78	102,66	89,88	98,13	108,63	mean of all	100,0
9	104,14	80,32	108,57	7386	113,00	105,39	94,10	9305	sd of all	12,5
10	113,52	98,29		103,40	102,66	96,61	105,19	10817	cv (%) of all	12,5
mean	109,4	88,1	108,3	84,3	107,5	103,3	97,2	102,8	mean of means	100,1
sd	8,7	7,5	15,2	11,5	4,3	7,3	4,4	63	sd	89
cv (%)	8,00	8,57	14,06	13,69	4,00	7,03	4,56	6,08	cv (%)	8,9

Table 25 Results of the protein determination by 8 different laboratories in Europe in one brand of gloves using the ASTM method. Protein concentrations are expressed in % of the mean in order to normalise the results of two different batches of gloves.

Analysis	Lab 1	Lab 2	Lab 3	Lab 4	Lab 9	Lab 10	Lab 5	Lab 6		
1	146,05	50,29	102,87	104,77	85,72	106,68	103,73	95,62		
2	144,78	38,80	94,61	118,74	88,26	107,31	105,90	66,67		
3	160,02	50,04	89,53	61,59	80,01	111,76	108,07	121,46		
4	147,32	58,61	105,41	63,50	92,07	123,19	102,03	100,79		
5	165,73	48,45	95,88	67,94	86,36	114,30	104,04	75,98		
6	163,19	54,10	103,50	92,07	93,34	120,65	102,39	87,35		
7	159,38	58,17	104,14	68,58	106,04	109,22	103,16	101,30		
8	191,13	50,48	95,25	80,64	86,99	118,11	102,80	116,29	mean of all	1000
9	179,07	51,88	105,41	73,02	87,63	118,74	103,73	112,67	sd of all	314
10	165,10	57,91	104,14	76,20	88,90	126,36	100,22	85,80	cv (%) of all	314
mean	162,18	51,87	100,08	80,71	89,53	115,63	103,59	96,48	mean of means	1000
sd	13,94	5,63	5,40	17,91	6,49	6,48	2,15	17,72	sd	294
cv (%)	8,59	10,85	5,39	22,19	7,25	5,61	2,08	18,37	cv (%)	294

2.1.5.2 Inter-laboratory test for the evaluation of the modified Lowry method and the extraction procedure (current study)

The nine laboratories which participated in this inter-laboratory test are listed in table 26.

The glove extraction was performed by the double glove method using TRIS buffer pH 8.2 at room temperature with 2 hours agitation. All measurements were done by the modified Lowry method as described in ANNEX 1 using the commercial kit from BioRad laboratories.

The participating laboratories were invited to a workshop in Vienna (10. /11. April 19967) where the whole procedure was shown and performed under supervision. Laboratory 2, 3, 5, and 7 participated in this workshop.

Calculations, nomenclature and abbreviations were used according to DIN ISO 5725 (April 1988)

Table 26 Participants of the final inter-laboratory test of the current study

Nr.	Laboratory	Address
1	Department of Dermatology University of Erlangen	Hartmannstraße 14 D-91052 Erlangen
2	Cytotest Cell Research GmbH	In den Leppsteinswiesen 19 D-64380 Roßdorf
3	Tun Abdul Razak Research Center	Brickendonbury Hertfort SG13 8NL, UK
4	London International Group	206, Cambridge Science Park, Milton Road Cambridge CB4 4GZ, UK
5	Austrian Inst. of Biomedical Material Engineering (ÖIBW)	Arsenal Objekt 213 Franz-Grill-Straße 5 A-1030 Vienna
6	Johnson & Johnson Medical GmbH	Oststraße 1 D-22806 Norderstedt
7	Semperit GmbH	Triester Bundesstraße A-2632 Wimpassing
8	Drägerwerk AG	Moislinger Allee 53/55 23542 Lübeck
9	Laboratoire d'Evaluation des Matériels Implantables (LEMI)	Mrs Marie-Francoise Harmand Technopole Montesquieu F-33650 Martillac

This round robin consisted of three parts:

1. Repeatability and reproducibility of the modified Lowry test with a prepared solution of ovalbumin and a prepared glove extract.
2. Repeatability and reproducibility of the hole procedure with one glove brand.
3. Reproducibility of the hole procedure with three gloves brands.

2.1.5.2.1 *Repeatability and reproducibility of the modified Lowry test with a prepared solutions*

of ovalbumin and a prepared glove extract

Intra-day precision

Dry ice shipped sterile samples of an ovalbumin solution and a glove extract prepared in our laboratory had to be measured in eight triplicates (together 24 samples each) within one series with one calibration curve. The real concentration of the ovalbumin solution (10.8 : g/ml) was determined by the absorption at 280 nm, the concentration of the glove extract was 56.2 : g/ml measured by the amino acid analysis.

The results are summarized in figure 26 and 27. Since the wavelengths and the path lengths for the photometric measurements differed from one laboratory to the other a direct comparison of the extinction values was not possible. Therefore all further results within the inter-laboratory test were based on the values calculated by calibration curves.

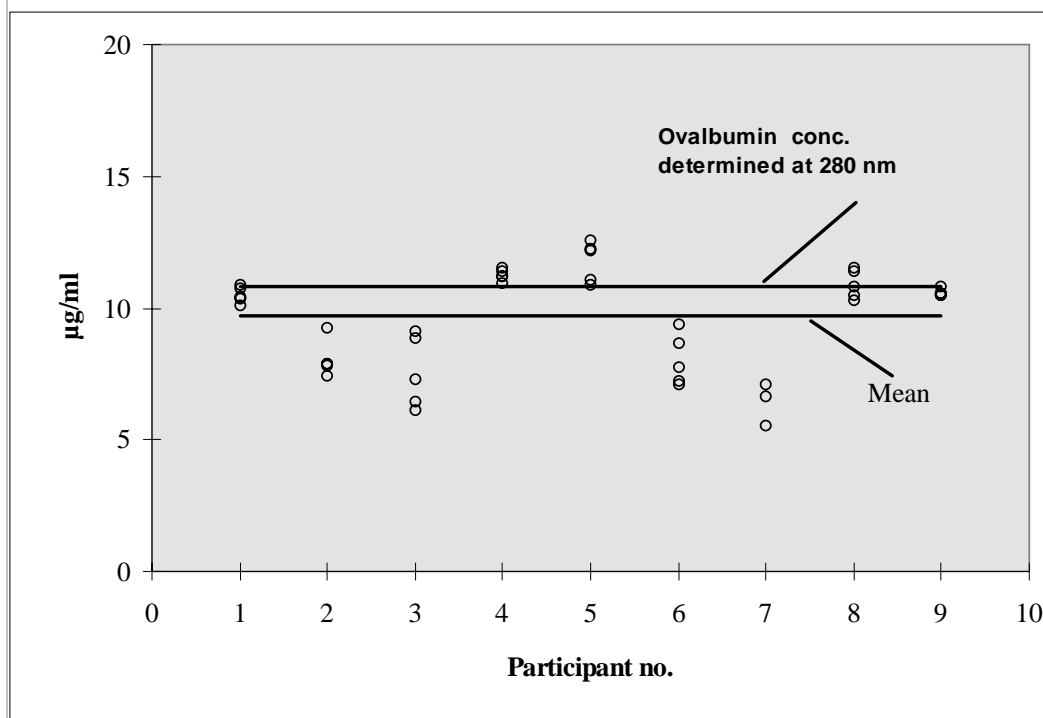
Repeatability

Three laboratories (2,3 and 7) achieved bad repeatabilities between 10% and 28 %, whereas the repeatability of the other six laboratories were below 10 % and often below 5 %. One possible reason for bad repeatabilities may be a wrong thawing of the frozen samples A. Complete resolving of frozen proteins (e.g. ovalbumin) often needs a 15 minutes heating to 45 °C, as described in the protocol (ANNEX 1). Especially in laboratory 5 this probably was the reason for the high coefficients of correlation. However, laboratory 3 had the highest coefficient of variation in both cases (28.4 and 19.9 %). Additionally the mean results of this laboratory were significant lower than those of the other laboratories throughout the whole inter-laboratory test. This may be a problem of the standard used or of the high variability throughout the whole test. Therefore the results of laboratory 3 were not further used in the final evaluation of this inter-laboratory test.

Reproducibility

The reproducibility of the measurement of the prepared ovalbumin solution (figure 26) was surprisingly low with a coefficient of variation of 18.5 %. However the mean (9.5 : g/g) and the median (10.5 : g/g) of all measurements (excluding participant 3) were very close to the concentrations determined by the extinction at 280 nm (10.6 : g/g). These results suggest difficulties in resolving the frozen ovalbumin solution.

The reproducibility of measurements of the prepared glove extract (figure 27) was much better revealing a coefficient of variation of 9.6 %. The mean protein value measured by the modified Lowry was about 14 % higher than that one measured by the amino acid analysis. However, this is a common phenomenon which is usually due to interfering substances. Participant 3 was clearly identified as an outlier by Dixon's test and therefore was excluded again for further evaluations.

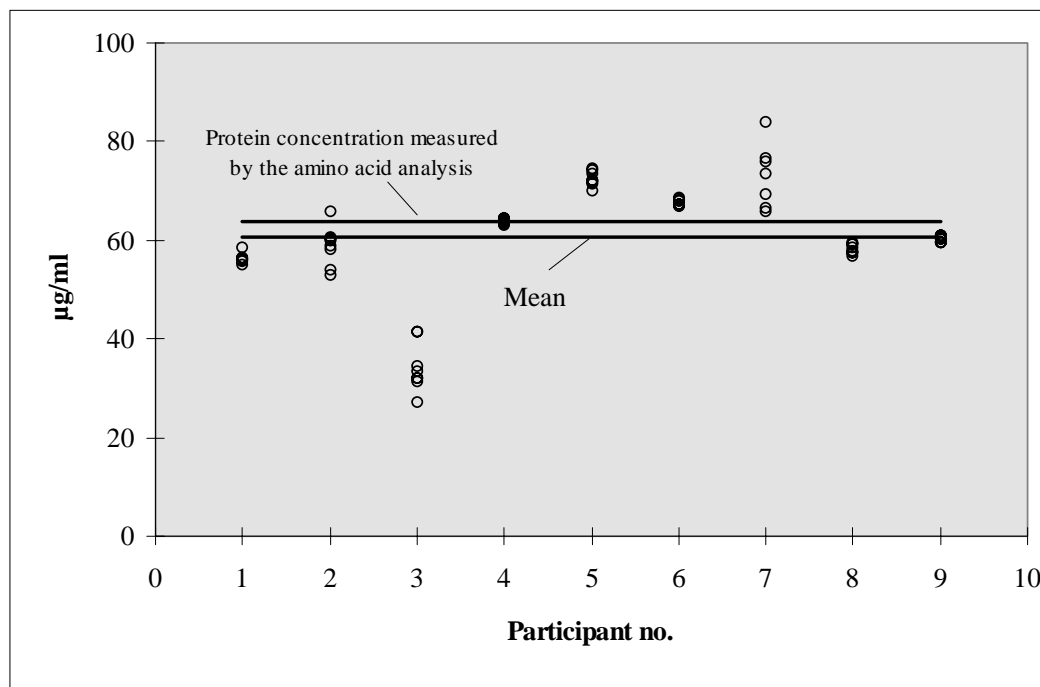


Participant	Mean (µg/ml)	SDev (µg/ml)	CV (%)
1	10,52	0,42	3,99
2	8,05	0,80	9,95
3	7,56	2,15	28,41
4	11,26	0,29	2,60
5	11,78	0,86	7,34
6	8,03	0,97	12,03
7	6,43	1,47	22,81
8	10,89	0,58	5,32
9	10,58	0,51	4,79
Mean	9,5	0,9	10,8
Median	10,5	0,8	7,3
S_R (µg/ml)	1,8		
CV (%)	19,2		
without participant 3			
Mean	9,7	0,7	8,6
Median	10,5	0,7	6,3
S_R (µg/ml)	1,8		
cv (%)	18,5		

Fig

ure 26

Repeatability and reproducibility of the protein determination in a prepared solution of ovalbumin (10.8 : g/ml) measured in one series at the same day.



Participant	Mean (µg/ml)	S _r (µg/ml)	cv (%)
1	56,32	1,19	2,11
2	58,81	7,81	13,28
3	34,18	6,79	19,85
4	63,88	0,70	1,10
5	72,56	2,29	3,15
6	67,58	1,33	1,96
7	73,14	10,59	14,48
8	58,32	1,14	1,96
9	60,24	0,65	1,08
Mean	60,6	3,6	6,6
Median	60,2	1,3	2,1
S_R (µg/ml)	11,0		
CV (%)	18,1		
without participant 3			
Mean	63,9	3,2	4,9
Median	62,1	1,3	2,0
S_R (µg/ml)	6,1		
CV (%)	9,6		

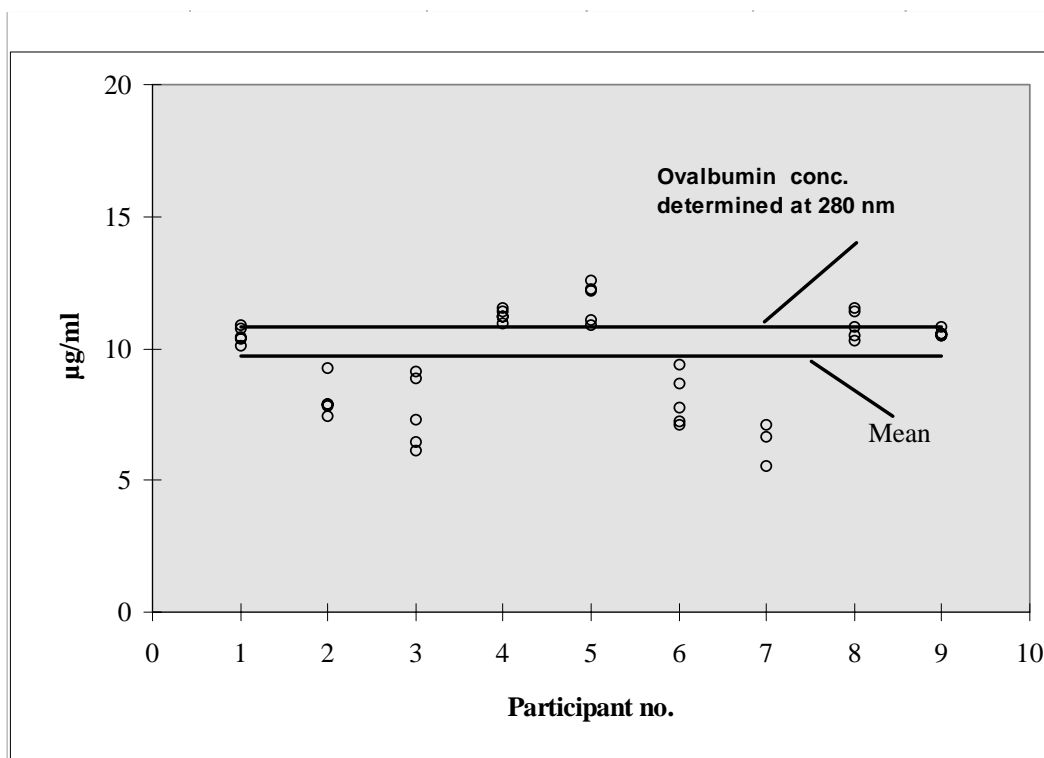
Figure 27 Repeatability and reproducibility of the protein determination in a prepared glove extract measured in one series at the same day.

Inter-day precision

The same prepared solutions used in 2.1 were taken for the inter-day precision. At five different days three samples of the ovalbumin solution and three samples of the prepared glove extract had to be measured. The results are shown in figures 28 and 29. The inter-day precision was very similar to those from the intra-day precision. Ovalbumin caused more problems than the glove extract. Participant 3 could again be shown to be an outlier by Dixon's test.

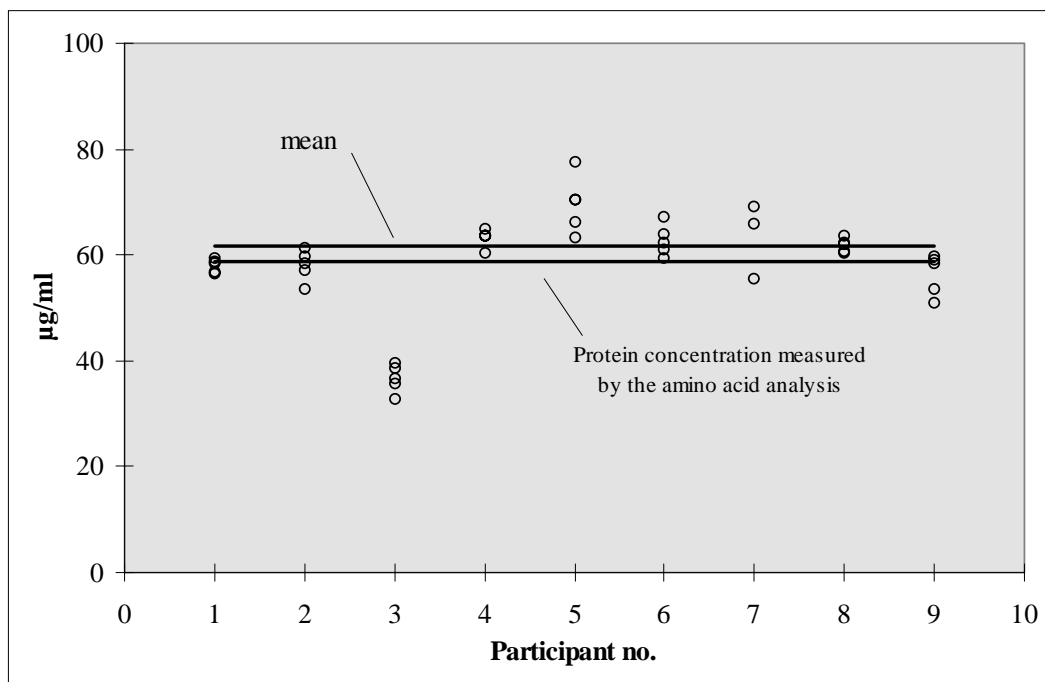
Repeatability and reproducibility of the modified Lowry method are acceptable

Although the Lowry method is known to be not very stable, it could be shown, that under standardized conditions using commercial kits this method is useful revealing coefficients of variation in repeatability and reproducibility below 10 %. The known problem of resolving frozen proteins (e.g. ovalbumin) however impaired the results produced with the prepared ovalbumin solution.



Participant	Mean (µg/ml)	S _r (µg/ml)	CV (%)
1	10,52	0,42	3,99
2	8,05	0,80	9,95
3	7,56	2,15	28,41
4	11,26	0,29	2,60
5	11,78	0,86	7,34
6	8,03	0,97	12,03
7	6,43	1,47	22,81
8	10,89	0,58	5,32
9	10,58	0,51	4,79
Mean	9,5	0,9	10,8
Median	10,5	0,8	7,3
S_R (µg/ml)	1,8		
CV (%)	19,2		
without participant 3			
Mean	9,7	0,7	8,6
Median	10,5	0,7	6,3
S_R (µg/ml)	1,8		
cv (%)	18,5		

Figure 28 Repeatability and reproducibility of the protein determination in a prepared ovalbumin solution measured at five different days.



Participant	Mean (µg/ml)	S _r (µg/ml)	CV (%)
1	57,99	1,32	2,27
2	58,04	4,17	7,19
3	36,65	4,67	12,75
4	63,22	1,72	2,71
5	69,63	11,95	17,17
6	62,85	2,85	4,53
7	63,56	7,38	11,62
8	61,50	1,62	2,63
9	56,78	3,64	6,40
Mean	58,9	4,4	7,5
Median	61,5	3,6	6,4
S_R (µg/ml)	8,7		
CV (%)	14,7		
without participant 3			
Mean	61,7	4,3	6,8
Median	62,2	3,2	5,5
S_R (µg/ml)	3,9		
cv (%)	6,3		

Figure 29
Repeatability and reproducibility of the protein determination in a prepared glove extract measured at five different days.

2.1.5.2.2 Repeatability and reproducibility of the whole procedure with one glove brand (glove A)

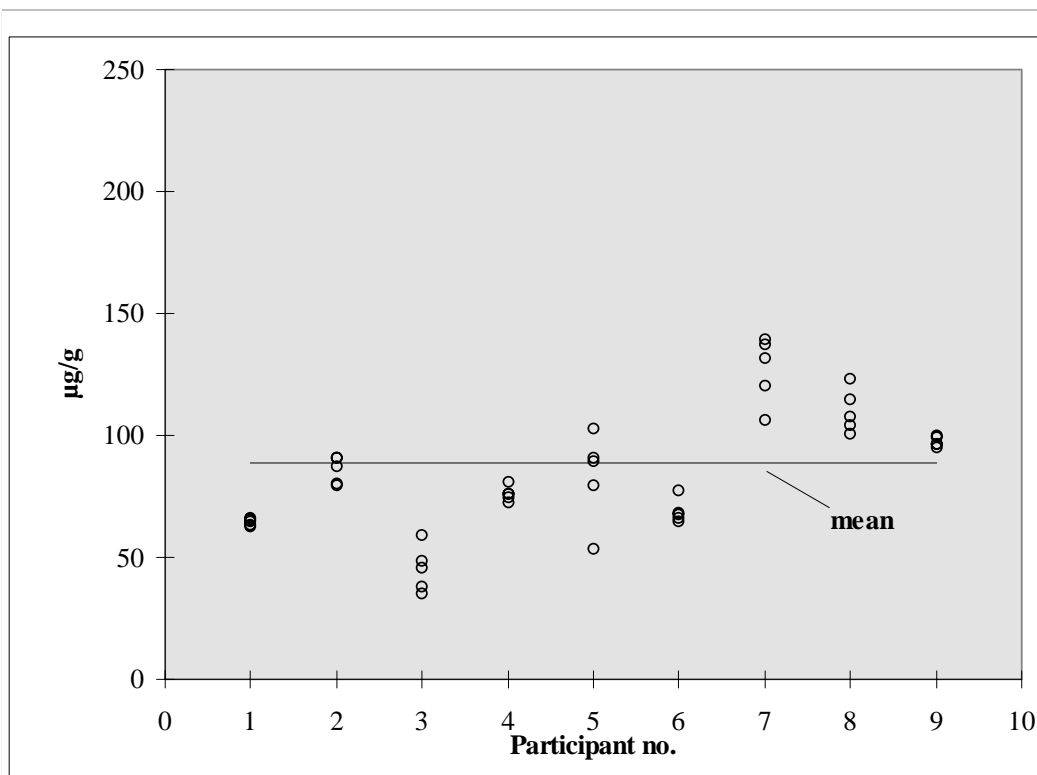
The gloves from one lot were extracted five times within one day and measured within one series (intra-day, figure 30). Additionally gloves from the same lot were extracted once a day, at five different days (inter-day, figure 31). All extracts were measured in triplicates using the modified Lowry method.

The results revealed a good repeatability with coefficients of variation below 10 % (except participants 3 and 5) and were thus comparable to the results concerning the prepared extract (figure 27). In view of the reproducibility however, the coefficient of variation was twice as high as that one found with the prepared extract. This indicated, that the extraction procedure is very repeatable within one laboratory, but some details performed in a different way may lead to an additional error of about 10 % to 15 %.

An unusual result was, that the inter-day precision was superior to the intra-day precision. Obviously this depends on the number of measurements which are to be done at one day. Performing a measurement with cuvettes (most of the laboratories did this) a long time interval between the first and the last sample may cause differences in the results.

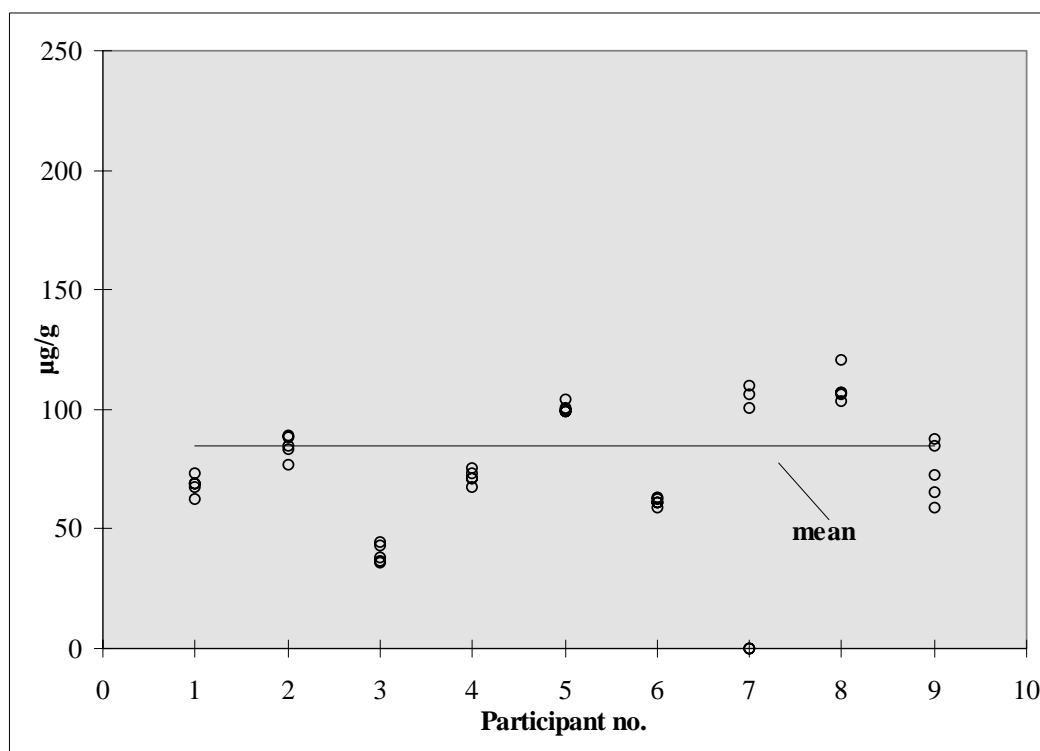
Excellent repeatability and acceptable reproducibility

In summary the repeatability of the whole method is excellent and its reproducibility is acceptable. This excellent repeatability shows that it is possible to produce gloves with very constant protein concentrations. The relative high variation in the reproducibility should be avoidable by practical training of the technicians.



Participant	Mean (µg/g)	S _r (µg/g)	cv (%)
1	64,45	1,35	2,10
2	86,22	5,68	6,61
3	45,49	9,57	21,04
4	76,06	3,05	4,01
5	81,73	18,74	22,54
6	67,97	4,78	6,94
7	126,41	13,64	10,72
8	110,12	8,85	8,03
9	97,39	2,15	2,20
Mean	84,0	7,5	9,4
Median	81,7	5,7	6,9
S_R (µg/ml)	23,2		
cv (%)	27,7		
without participant 3			
Mean	88,8	7,3	7,9
Median	84,0	5,2	6,8
S_R (µg/ml)	20,0		
cv (%)	22,5		

Figure 30 Repeatability and reproducibility of the whole procedure (extraction and modified Lowry) measured in one series at the same day. Five extractions were measured in triplicates each. Glove A



Participant	Mean (µg/g)	S _r (µg/g)	cv (%)
1	68,28	4,07	5,95
2	85,77	4,94	5,84
3	39,72	3,84	9,69
4	71,70	3,08	4,29
5	100,62	2,05	2,04
6	61,47	1,74	2,83
7	105,53	4,83	4,58
8	108,79	6,80	6,25
9	73,90	12,40	16,78
Mean	79,5	4,9	6,5
Median	73,9	4,1	5,8
S_R (µg/ml)	21,5		
cv (%)	27,0		
without participant 3			
Mean	84,5	5,0	6,1
Median	79,8	4,5	5,2
S_R (µg/ml)	17,2		
cv (%)	20,3		

Figure 31 Repeatability and reproducibility of the whole procedure (extraction and modified Lowry) measured at five different days. Five extractions were measured in triplicates each. Glove A.

2.1.5.2.3 *Reproducibility of the whole procedure with three glove brands (glove B, C, D)*

Three different brands of gloves were extracted three times in each laboratory and measured in triplicates.

Glove B (figure 32): The mean coefficient of variation of all laboratories was 20.2 %, which is very high compared to glove A (7.9 % and 6.1 %, respectively). These results clearly indicate that apart from the modified Lowry and the extraction procedure the variability of the protein content of the gloves within the same batch is an additional important factor for the assessment of the protein concentration measured in a glove. Nevertheless the reproducibility in glove B is about the same that one found in glove A. This indicates a random distribution of the gloves with different concentrations among the laboratories.

Glove C (figure 33): This glove revealed unexpected high values⁴ of protein concentration which were not measurable with the routine method. The extract had to be diluted prior to the modified Lowry or it had to be redissolved after precipitation in more NaOH-solution. Nevertheless the repeatability is superior to glove B but worse than in glove A. Mainly the three aforementioned laboratories revealed difficulties in the repeatability. The reproducibility was quite the same as in glove A and B.

Glove D (figure 34): In glove D repeatability and reproducibility were very low. The coefficient of variation of the reproducibility was more than 30 %. This was mainly due to the laboratories 6 and 8, which revealed a good repeatability in this and other experiments. The results indicates a not randomly distributed variability in the protein concentration in glove D. Excluding the results from laboratory 6 and 8 the coefficient of correlation of the reproducibility reached 20 %, according to the normal value in our gloves.

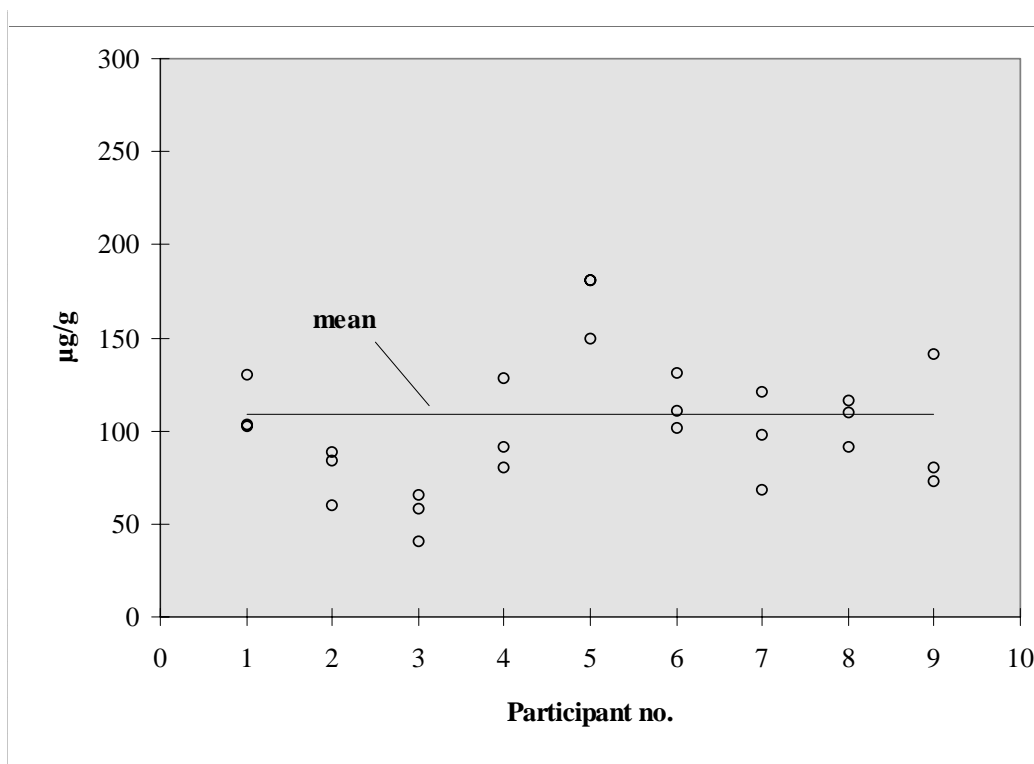
95 % of the values are expected to be in the range of mean \pm 28 %

The reproducibility of the whole procedure including glove extraction and modified Lowry revealed a coefficient of correlation of about 20 % if the protein content in the gloves of one lot did not differ more than 20 % and seemed to be independent of the protein concentration (between 800 and 46 : g/g). Higher variations indicate large differences in the protein content of the gloves. Assuming a coefficient of variation of 20 % the values are to be expected to lie within mean \pm 28 % (see DIN ISO 5725) on condition of a 95 % probability (table 27).

Table 27 Range expected to contain 95 % and 99 % of the values with the indicated mean assuming a coefficient of variation of 20 % for reproducibility.

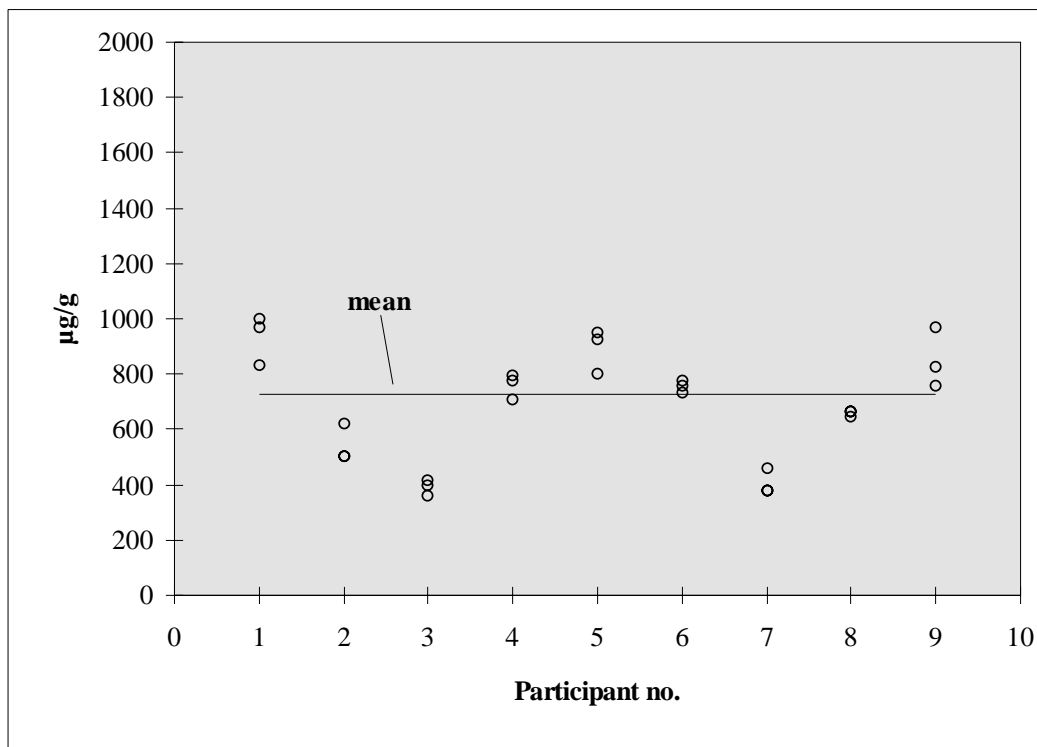
Mean protein concentration	Probability of 95 %	Probability of 99 %
10 : g/g	7.2 - 12.8 : g/g	6.4 - 13.6 : g/g
20 : g/g	14.4 - 25.6 : g/g	12.8 - 27.2 : g/g
50 : g/g	36 - 64 : g/g	32 - 68 : g/g
100 : g/g	72 - 128 : g/g	64 - 136 : g/g
200 : g/g	144 - 256 : g/g	128 - 272 : g/g
500 : g/g	360 - 640 : g/g	320 - 680 : g/g

⁴ Due to the increased number of extractions (as a CEN task in the last minute), we used another lot of gloves as originally tested. The lot tested revealed constant protein concentrations of about 250 : g/g.



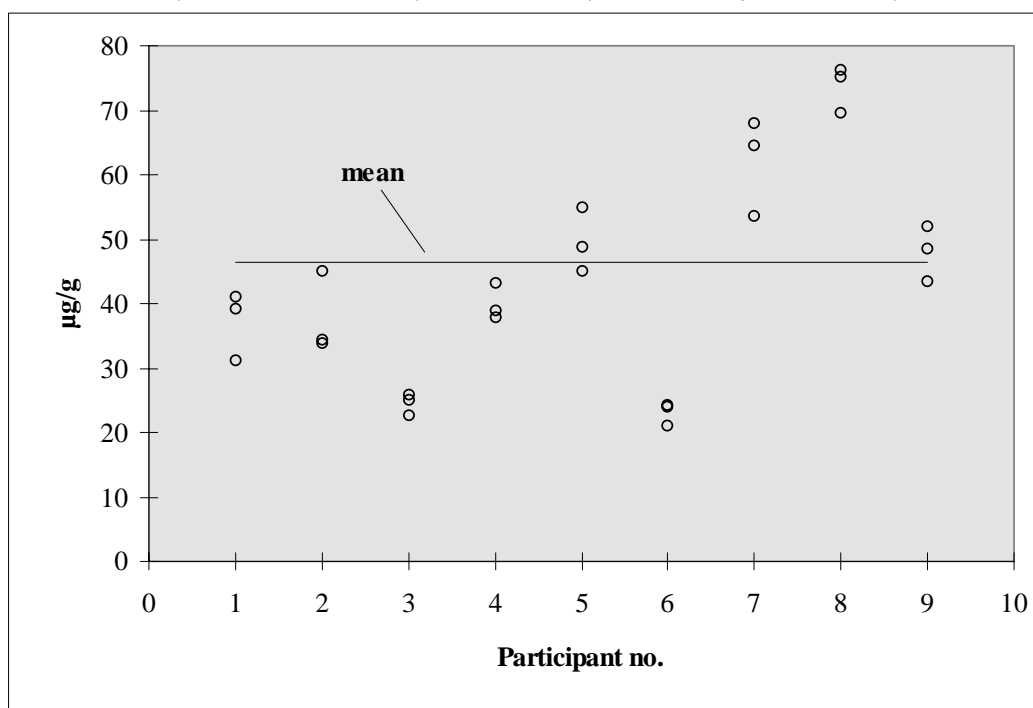
Participant	Mean ($\mu\text{g/g}$)	S_r ($\mu\text{g/g}$)	cv (%)
1	111,95	15,68	14,01
2	77,40	15,49	20,02
3	55,12	12,77	23,26
4	99,89	25,26	25,29
5	170,34	17,97	10,55
6	114,29	15,27	13,37
7	95,51	26,50	27,75
8	105,97	13,28	12,54
9	98,08	37,57	38,31
Mean	103	20,0	20,6
Median	99,9	15,7	20,0
S_R ($\mu\text{g/ml}$)	29,4		
cv (%)	28,5		
without participant 3			
Mean	109	20,9	20,2
Median	103	16,8	17,0
S_R ($\mu\text{g/ml}$)	25,5		
cv (%)	23,3		

Figure 32 Reproducibility of the whole procedure (extraction and modified Lowry), three extractions measured in triplicate, each. Glove B



Participant	Mean (µg/g)	S _r (µg/g)	cv (%)
1	934,48	91,30	9,77
2	562,48	68,16	12,52
3	389,83	29,14	7,47
4	760,51	45,76	6,02
5	892,70	79,20	8,87
6	754,52	22,56	2,99
7	404,10	46,68	11,55
8	658,16	12,10	1,84
9	850,51	106,51	12,52
Mean	690	55,7	8,2
Median	755	46,7	8,9
S_R (µg/ml)	190		
cv (%)	27,5		
without participant 3			
Mean	727	59,0	8,3
Median	758	57,4	9,3
S_R (µg/ml)	167		
cv (%)	23,0		

Figure 33 Reproducibility of the whole procedure (extraction and modified Lowry), three extractions measured in triplicate, each. Glove C



Participant	Mean (µg/g)	S _r (µg/g)	cv (%)
1	37,12	5,22	14,07
2	37,78	6,40	16,95
3	24,67	1,70	6,93
4	40,02	2,82	7,05
5	50,21	5,04	10,15
6	23,06	1,71	7,40
7	62,02	7,43	11,98
8	73,64	3,48	4,72
9	47,94	4,23	8,83
Mean	44,1	4,2	9,8
Median	40,0	4,2	8,8
S_R (µg/ml)	15,5		
cv (%)	35,3		
without participant 3			
Mean	46,5	4,5	10,1
Median	44,0	4,6	9,5
S_R (µg/ml)	14,8		
cv (%)	31,8		

Figure 34 Reproducibility of the whole procedure (extraction and modified Lowry), three extractions measured in triplicate, each. Glove D

2.1.5.3 Inter-laboratory test comparing the ASTM method and the CEN method with regard to the extraction of proteins and the modified Lowry assay(CEN task)

All laboratories which participated in the inter-laboratory test of the current study were invited for a supplemented test induced by the CEN task. Five of these measured all the additional samples whereas seven laboratories only measured a part of the additional samples excluding inter day variation of the 'cut glove method' with glove A. In addition to the inter-laboratory test of our study the 'cut glove extraction' was tested using both, double distilled water and TES buffer pH 7.4, respectively.

The results are shown in table 28 and 29, where the mean variations are listed. The double glove method (at pH 8.2 or 7.4) was superior to the cut glove method (pH 7.4 or dd water) and revealed the lower coefficients of variation in both, repeatability and reproducibility.

Table 28 Reproducibility of the different methods used in the inter-laboratory test induced by CEN. The values are given as mean \pm R (95 % / 99 %) of all values are within the given range; among 20 / 100 values one is expected to be out of the given range).

Glove	95 % probability				99 % probability			
	Double glove pH 8.2	Double glove pH 7.4	Cut glove pH 7.4	Cut glove dd water	Double glove pH 8.2	Double glove pH 7.4	Cut glove pH 7.4	Cut glove dd water
A intra-day	88.8 \pm 28.0	89.1 \pm 20.6	101 \pm 45.9	104 \pm 55.0	88.8 \pm 36.1	89.1 \pm 20.6	101 \pm 45.9	104 \pm 55.0
A inter-day	84.5 \pm 24.1	88.4 \pm 32.9	85.3 \pm 28.0	109 \pm 68.3	84.5 \pm 31.1	88.4 \pm 32.9	85.3 \pm 28.0	109 \pm 68.3
B	109 \pm 35.7	84.2 \pm 13.2	94.8 \pm 42.6	60.0 \pm 25.1	109 \pm 46.1	84.2 \pm 13.2	94.8 \pm 42.6	60.0 \pm 25.1
C	727 \pm 234	644 \pm 248	846 \pm 323	852 \pm 493	727 \pm 302	644 \pm 320	846 \pm 323	852 \pm 493
D	46.5 \pm 13.0	38.6 \pm 14,8	34.9 \pm 9.8	34.6 \pm 17,8	46.5 \pm 16.8	38.6 \pm 14,8	34.9 \pm 9.8	34.6 \pm 17,8

Table 29 Reproducibility and repeatability of the different methods used in the inter-laboratory test induced by CEN. The values are given as the mean coefficient of variation in %.

Glove	Repeatability				Reproducibility			
	Double glove pH 8.2	Double glove pH 7.4	Cut glove pH 7.4	Cut glove dd water	Double glove pH 8.2	Double glove pH 7.4	Cut glove pH 7.4	Cut glove dd water
A intra-day	9.4	5.4	7.8	9.7	22.5	16.5	32.5	37.8
A inter-day	6.5	8.9	14.9	20.7	20.3	26.6	23.4	44.8
B	20.2	25.5	34.2	26.3	23.3	11.2	32.1	29.8
C	8.3	8.5	12.1	7.2	23	27.2	27.3	41.3
D	10.1	13.5	16.7	29.6	20	27.5	20.1	37.1
Mean	10.9	12.4	17.1	18.7	21.8	21.8	27.1	38.2

2.2 Quantification of extractable accelerators

Residues of vulcanisation accelerators and probably also their decomposition products (free amines) contained in medical disposable gloves made of natural rubber latex possess an allergic potential and can cause a delayed allergic reaction of type IV. According to the contract, analytical procedure for the estimation of extractable accelerator residues including suitable extraction procedure had to be developed. The entire analytical procedure (extraction and quantification of accelerators in the extract) should give results which are a measure of the allergic potential of the glove, i.e., the analytical results should relate to the content of the bioavailable accelerator residues.

To obtain a good correlation of the analytical results with the response in sensitized patients, the extraction method must simulate the transport of accelerator from the latex film into the skin. This is a special requirement, not common in known analytical procedures dealing with the quantification of vulcanisation accelerators in vulcanised and/or non-vulcanised rubber compounds. Usually the extraction of analytes had to be as complete as possible because the total content of the analyte in the substrate is of interest.

From this reason, the main emphasis of the project work was the harmonising of analytical results (which depend on the yield of the intended incomplete extraction) with the clinical ones. In the search for the suitable extraction medium

- nearly complete insolubility of accelerators in water and liquids similar to sweat and
- the supposed mechanism of the direct accelerator transport from the hydrophobic latex film into some hydrophobic parts of the skin

had to be taken into account. The suitability of the extraction and analytical procedure had to be tested in an inter-laboratory test with industrial laboratories in the final stage of the project.

In the planning stage of the project, 16 substances listed as No. 1-16 in Table 30 were selected as relevant from the point of view of their allergic potential. However, in course of the search for representative glove brands it has been realised that some accelerators are not used in the glove production. On the other side, based on information provided by glove producers, other, originally not known substances were recognised as evidently or possibly used and have therefore been added to the selection afterwards. These "new substances" are indicated in the first column of Table 30.

The following findings were published in specialized literature, gained as producers' information, or have been found out in course of the own experimental work. They are essential for the understanding of the importance and properties of particular substances or classes of substances listed in Table 30 as well as for the assessment of the reliability of a producer's claim concerning the compounds used in production:

- a) In the glove production, latex formulations are further developed and modified relatively fast, but gloves impacted by these changes are further sold under old trade names and the package and labelling remain the same. Therefore, the changed latex formulation is not evident to the user.
- b) Thiurams (THs) are known to be strong allergens. Since the problem of latex allergies has been recognized as a serious one, the use of THs in the glove production is declining.
- c) With one exception, only PTT occurs in known latex formulations for the glove production. It has been confirmed in this project that PTT is converted into the corresponding ZDC during the latex processing. The conversion seems to be complete if the present analytical procedure is used for the quantification of PTT residues.
- d) The above exception is one glove brand whose producer claims that at least one thiuram (evidently not PTT) is a constituent of the latex formulation. Unfortunately, it was not stated by the producer what type of thiuram is used in this glove production.
- e) In the vulcanisation process, THs react with ZnO and are converted into corresponding zinc-dithiocarbamates (ZDCs) for the most part (if not completely).
- f) Because of this reaction, the claims of the glove producers concerning accelerators used in the production of particular gloves possess only restricted reliability.
- g) In some gloves, different accelerators have been found than those claimed by the producer.

- h) In some glove brands produced without use of THs, traces of THs corresponding with used ZDCs can be found by the analytical procedure developed in this project; i.e., also the conversion of ZDCs in THs seems to be possible under particular conditions.
- i) Like the conversion of THs into ZDCs, some other accelerators, applied as sodium or triethylammonium salts (e.g. Setsit[®] 104) or as pure organic material (e.g. MBT not complexed with metal ions or PPD), react obviously with ZnO present in the latex formulation and form corresponding zinc-derivatives. Not all possible parent substances (zinc-free or containing other ion than Zn²⁺) are listed in Table 30 because they are probably not present in the finished product.
- j) Simple secondary amines (substances No. 13-16) were recognised as decomposition products of corresponding accelerators. The estimation of free amines could be important from two points of view
- they could be allergologically relevant
 - if the direct identification of these substances in the latex film would be simple and fast, it could help in drawing first conclusions about the type of TH or ZDC used in the production

With respect to the above paragraph (a), all investigations concerning particular glove brand were carried out on gloves of the same lot number.

The conversion of THs into ZDCs (and probably also vice versa) as well as the conversion of MBT and MBTS into ZMBT mentioned above in paragraphs (e), (h) and (i) can be understood if the close relationship between the relevant chemical structures (see Fig. 35) is considered.

Table 30: Substances of interest

No.	Abbrev.	Substance	Claimed for use in gloves	Accelerator group
1	TMTD	tetramethylthiuram disulfide		TH
2	TMTM	tetramethylthiuram monosulfide		TH
3	TETD	tetraethylthiuram disulfide		TH
4	TBTD	tetrabutylthiuram disulfide		TH
5	PTD	dipentamethylenethiuram disulfide		TH
6	ZDMC	zinc-dimethyldithiocarbamate	yes	ZDC
7	ZDEC	zinc-diethyldithiocarbamate	yes	ZDC
8	ZDBC	zinc-dibutyldithiocarbamate	yes	ZDC
9	ZEPC	zinc-ethylphenyldithiocarbamate		ZDC
10	ZPC	zinc-pentamethylendithiocarbamate	yes	ZDC
11	MBT	mercaptobenzothiazole		MB
12	MBTS	dibenzothiazyl disulfide		MB
13	DMA	dimethylamine		amine
14	DEA	diethylamine		amine
15	DBA	dibutylamine		amine
16	PP	piperidine		amine
new substance	ZDNC	zinc-diisononyldithiocarbamate		ZDC
new substance	ZMBT	zinc-mercaptobenzothiazole	yes	MB
new substance	PTT	dipentamethylenethiuram tetrasulfide	yes	TH
new substance	PPD	piperidine-pentamethylenedithiocarbamate	yes	DC
new substance	MBI	mercaptobenzimidazole		(antioxidant)

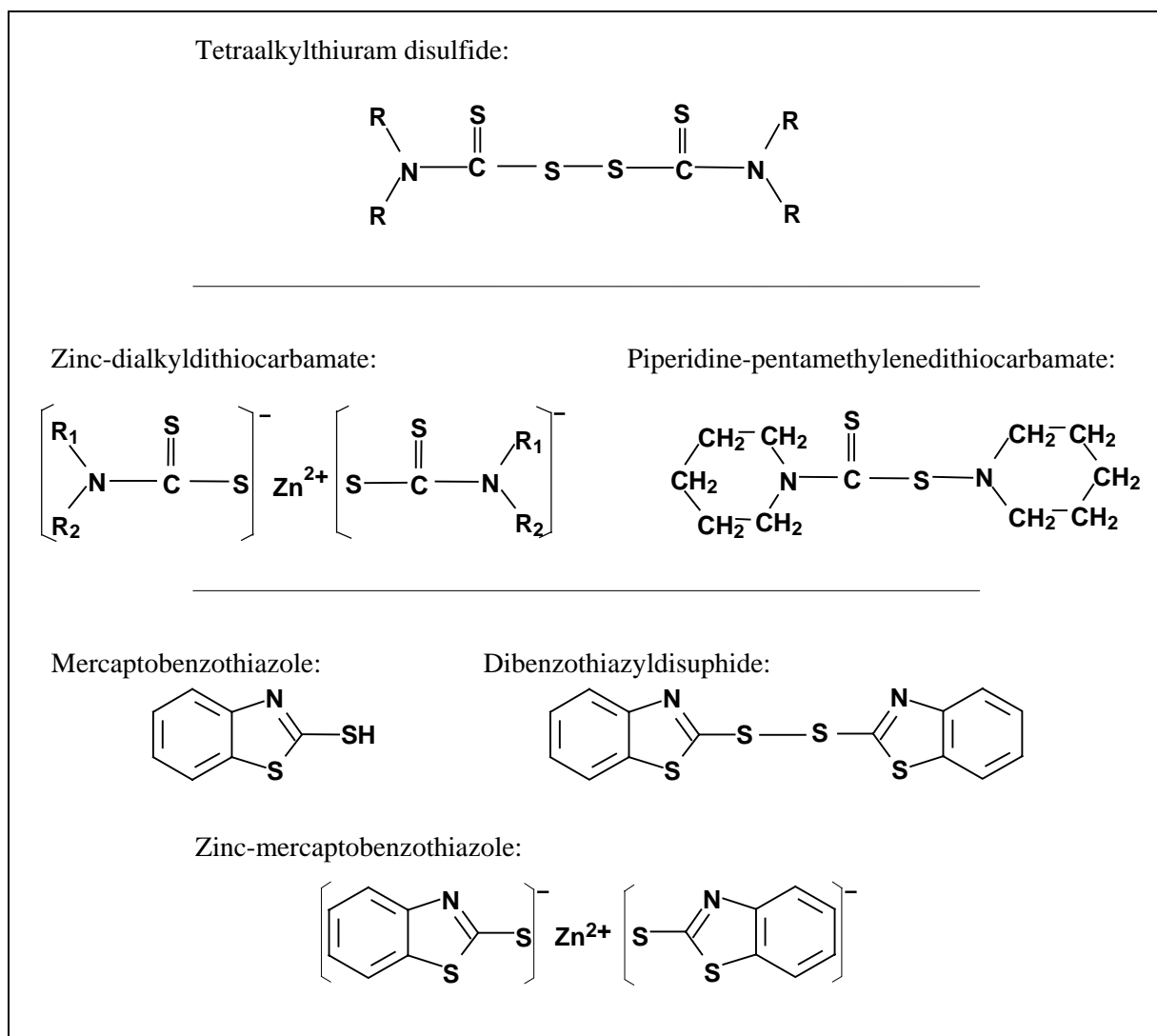


Fig. 35: Chemical structure of the most important accelerators

2.2.1 Assessment of the suitable method for the determination of accelerators

As stated above the extraction procedure is an inseparable part of the entire procedure for the determination of bioavailable accelerator residues in medical gloves. Nevertheless, in the first stage of the project the general analytical procedure was established without consideration of the glove extraction. In this part of the work, solutions of standard substances were used instead of real glove extracts. Four principally different methods were examined in this phase in accordance with the working program and with following findings:

1. *High performance liquid chromatography* (HPLC) is – generally speaking – a very efficient separation procedure and has been reported as suitable for the analysis of accelerator mixtures. This method was originally expected to be suitable for both qualitative and quantitative analysis of accelerators in glove extracts.
2. *Gas chromatography* (GC) is also an effective separation procedure and especially in the combination with *mass-selective detection* (MS). It is – again generally speaking – well suitable for both qualitative and quantitative analysis of multicomponent mixtures. The utilization of GC for the analysis of vulcanisation accelerators is not new. However, the most substances which have been of interest in this project are non-volatile and cannot be analysed by GC without conversion into

volatile reaction products (derivatization). It was evident already at the start of this work that only amino groups bound in the accelerator molecule will be detected by GC after derivatization. The central part of the accelerator molecule, containing sulphur and eventually also zinc (and making the difference between thiurams and dithiocarbamates), get lost in the course of derivatization. From this reason GC cannot distinguish between THs and ZDCs, but the method was expected to be suitable for both qualitative and quantitative analysis of amino groups bound in the accelerator molecule.

3. *Thin layer chromatography* (TLC) was originally proposed for screening test only. This method is less time-consuming and cheaper than HPLC if only qualitative analysis is of interest. It can also be helpful in the mobile phase tuning for HPLC. The finding of proper spray reagents for the development of TL-chromatograms and utilization of characteristic colour reactions of THs and ZDCs were the necessary prerequisite to enable the distinction between these two accelerator groups in TLC.
4. *Photometry* (UV-VIS) was proposed as a part of the screening procedure for the fast qualitative analysis of glove extracts (colour reactions without previous separation of analysed substances by means of chromatographic methods). In addition, UV spectroscopy was to be utilized in finding out suitable wavelength for the detection of particular substances in HPLC.

As the development of the extraction procedure progressed, experiments with respect to the analytical procedure were carried out not only with standard solutions, but also with glove extracts. The laboratory work for the development of the analytical procedure were based on following steps performed in ÖIBW:

- development of the HPLC-method for a simultaneous qualitative and quantitative analysis of thiurams, mercaptobenzothiazoles and metal-complexes of dithiocarbamates
- development of the procedure for the conversion of the unstable zinc-dithiocarbamates into more stable metal-complexes suitable for HPLC
- modification of the HPLC-method by introducing a clean-up-step based on solid-phase-extraction
- development of the high performance thin layer chromatography (HPTLC) as the more suitable method for the qualitative analysis of the glove extract
- development of the derivatization procedure for non-volatile accelerator residues as a part of the GC-analysis
- development and calibration of the GC-estimation of alkylamino-substituents of relevant thiurams, dithiocarbamates and mercaptobenzothiazoles (GC-FID and GC-MS)
- experiments with respect to the direct GC-estimation of free amines by means of GC with a specific nitrogen/phosphor-detector
- examination of colour reactions of particular accelerators as possible screening procedure for the quick qualitative test of glove extract
- experiments with respect to the applicability of thin layer chromatography (TLC) as screening procedure
- analysis of 35 glove brands with respect to the content of the extractable accelerator residues
- establishment of the preparation procedure for glove extracts used in clinical study and providing of the clinical study with both latex film samples and glove extracts
- inter-laboratory test concerning the developed procedure

The above steps are not listed chronologically and there were always more themes in work at the same time. Feedbacks from all experiments were always utilized in investigations performed later.

A number of experiments were, with extreme effort, carried out for the development of the HPLC-method, at the beginning with individual substances and later with mixtures and glove extracts. Although the HPLC-apparatus is not common in the majority of industrial laboratories, a single procedure for both qualitative and quantitative analysis was considered to be a big advantage as these experiments were performed. However, the entire procedure necessary for the accelerator analysis turned out to be considerably time consuming. In addition, serious technical problems reducing the reliability of this method and increasing costs, caused evidently by number of contaminants in glove extracts, became apparent as number of glove extracts were analysed.

A suitable alternative to HPLC has been found in HPTLC & GC-combination, and thereafter HPLC was dropped from the procedure protocol submitted for standardization. Similarly the originally intended use of photometry in the screening procedure (without chromatographic separation) was renounced as fast HPTLC-technique with sufficient selectivity was developed and suitable spraying reagents were found. Finally, the direct estimation of free amines (substances no. 13 to 16, as well as the last three substances in Table 30) by means of gas chromatography with specific nitrogen-sensitive detection (NPD) has been found as not reasonable and has been skipped, too.

The work leading to this decision is described in section 2.2.1.1, 2.2.1.3 and 2.2.1.4 in detail. As a consequence of the rejection of HPLC, GC-NPD and photometry, these three methods are not involved in the standard procedure protocol described in ANNEX 2. The other methods (HPTLC and GC), which are part of the standard procedure, are thoroughly described in ANNEX 2, and the relevant sections 2.2.1.2 to 2.2.1.4 are therefore kept short.

2.2.1.1 High performance liquid chromatography (HPLC)

The Hewlett-Packard 1050 series liquid chromatograph with a deuterium lamp double beam photometer-detector, equipped with 250 x 4 mm original-Hewlett-Packard stainless steel or (later) and also 250 x 4 mm PEEK-column (product of Panosch, Vienna), both columns packed with Spherisorb ODS-2 5 μ m, has been used in all experiments. Along with experiments performed for the development of the new procedure, a procedure proposed by ASTM was tested, too. This procedure differs from the procedure developed within this project with respect to:

- extraction technique (solvent, time, temperature, preparation of the glove sample)
- treatment of the extract
- HPLC-conditions

Solutions of standard substances and HPLC-grade water and methanol were used as the mobile phase in preliminary experiments for the development of the new procedure. These experiments confirmed results of other authors, who have shown that the direct estimation of ZDCs by HPLC does not work properly. The low stability of ZDCs which undergo a metal exchange reaction with metallic parts of the analytical instrument, is obviously the main reason for this complication. The replacement of the steel column by a PEEK-column, as recommended in the literature, did not solve this problem; many peaks in the chromatogram showed a strong tailing if ZDCs were analysed.

In the next step ZDC standard substances were treated with Co(II)-salts to get more stable complexes suitable for the HPLC-analysis. It was an attempt to reproduce reported good HPLC-separation of co-dithiocarbamates mixtures. However, the co-complexes gave more peaks in the chromatogram than the number of components in the original carbamates mixture, e.g. binary mixtures gave four peaks. Moreover, it has been found that THs also react with CoCl₂ forming coloured reaction products. A redox reaction of Co(II)-salt with accelerators seems to be responsible for this behaviour.

Later, Ni-complexes of ZDC mixtures have been found to give less complicated chromatograms than Co-complexes (binary ZDC mixtures gave only three peaks after they were treated with Ni-salts). Unlike cobalt(II)-salts, nickel(II)-salts cannot be oxidized, and nickel salts do not form coloured reaction products with THs and MBs. Nickel sulphate has been assessed as a suitable reagent for the conversion of ZDCs into more stable (for the HPLC-analysis more suitable) complexes. A good separation of different carbamates has succeeded using PEEK-column, giving narrow symmetrical peaks after treatment with NiSO₄. Later, stainless steel column also proved to work if the ZDCs are converted to Ni-complexes. Even more, the stainless-steel column from HP was more stable than PEEK-column from Panosch.

In the following step, standard solutions of individual substances in chloroform were used

- to find out the optimum conditions for the conversion of Zn-dithiocarbamates into Ni-complexes
- to optimise the HPLC-separation of individual substances
- to find out optimal wavelength in the detection of individual substances
- to find out retention times of individual substances (qualitative analysis)
- to gain calibration curves of individual substances (quantitative analysis)

Ni-complexes of dithiocarbamates form yellow-green solutions in chloroform. Their UV-spectra show absorption maxima at about 320 nm. Therefore, if HPLC-analysis is run with respect to the estimation of ZDCs, it is advantageous to perform the detection at 320 nm. This wavelength is also suitable for MBT, whereas the optimum wavelength for the detection of THs, ZMBT and MBTS is 275 nm (see also Table 40 in section 2.2.1.4). Conditions listed in Table 31 were found to be an optimum for the HPLC-separation and quantitative estimation of extractable accelerator residues in this stage of the work.

Table 31: Optimum HPLC-conditions

mobile phase	0-4 min	75% methanol + 25% water (HPLC-grade)
	4-10 min	linear gradient up to 95% methanol + 5% water, developed over 6 minutes
injection volume	20 μ l	
column temperature	room temperature	ca. 25°C
flow	0,75 ml/min	
detection	UV	at 275 nm and 320 nm

Retention times of individual substances (R_t) given in Table 32 were found at these conditions (dithiocarbamates are listed as Zn-salts in this Table, however they were analysed as Ni-complexes in reality). With the exception of PTT single peaks, in no case were reproducible R_t -values found; therefore, a time range is given for every analysed substance in Table 32. This time range means that the R_t -values drift within the given interval as the performance of the column changes (see below).

It is not possible to distinguish between ZMBT and MBT due to comparable R_t -values of both substances. This fact seems to be without importance because the basic structure of the organic residues is the same in both ZMBT and MBT. In addition, it is supposed that ZMBT is formed from MBT in the course of the latex processing.

A special problem concerning the qualitative analysis has been faced as dipentamethylenethiuram tetrasulfide (PTT), originally not considered as a current accelerator in medical gloves but later claimed as used in latex formulations, was analysed by HPLC. As indicated in Table 32 seven peaks within the first 30 minutes were found as the available sample of PTT was analysed. The melting point of the examined PTT-sample (126°C) was not the same as referred (117°C). The available sample of PTT of technical grade was obviously a mixture of more individual substances.

Experiments performed with mixtures of standard substances (conc. about 100 ppm) have shown that the number of peaks increases if two or more THs or Ni-dithiocarbamates are contained in the solution. Thus, two different ZDCs, transformed into Ni-complexes, form three peaks. Similarly, two THs mixed together and analyzed by HPLC give a chromatogram with three peaks. The formation of the substance giving the third peak is time-dependent. The height of this peak increases in the time period of some hours if the reaction mixture stands at room temperature. For the same reason, a mixture of three THs or ZDCs gives a chromatogram with 6 peaks; four ZDCs form 10 peaks. MBTS shows the same effect, i.e. more peaks than individual substances in the mixture if it is mixed with thiuram(s). A mixture of one Ni-dithiocarbamate and one thiuram gives only two peaks; however, the phenomenon has not been studied thoroughly in all possible mixtures.

Table 32: Retention times R_t of individual substances and optimum detection wavelength

THs		MBs and MBI			ZDCs *)	
$\lambda=275$ nm	R_t [min]	$\lambda=320$ nm	$\lambda=275$ nm	R_t [min]	$\lambda=320$ nm	R_t [min]
TMTM	4,4 - 5,0		ZMBT	3,5 - 4,4	ZDMC	6,7 - 6,9
TMTD	4,9 - 5,9	MBT		3,8 - 4,6	ZDEC	12,4 - 13,5
TETD	9,9 - 10,3		MBTS	16,2 - 17,9	ZPC	15,0 - 16,0
TBTD	17,6 - 18,7				ZEPC	14,3 - 14,4
PTD	10,9 - 11,8				ZDBC	20,0 - 23,0
PTT	7 peaks	MBI		4,0	ZDNC	--- **)

*) all ZDCs have been analysed as Ni-complexes

**) no peak after 30 minutes

The problem of the formation of recombination peaks in accelerator mixtures has also been faced in the analysis of an accelerator mixture sold under the trade name Setsit[®] 104. According to the claim of one glove producer, it is used in glove production and it consists of

- sodium-diisobutyldithiocarbamate
- sodium salt of mercaptobenzothiazole
- triethylammonium-dimethyldithiocarbamate

As the CHCl_3 -solution of Setsit[®] 104 was treated with the buffered solution of NiSO_4 and subjected to HPLC-analysis, three peaks with the below listed retention times were found. Supposing that dithiocarbamates in Setsit[®] 104 react with the Ni-salt and that free MBT is formed from its sodium salt under the reaction conditions, retention times in Table 32 and Table 34 can be employed for the evaluation of HPLC-analysis of Setsit[®] 104. Thus, retention times of single peaks of Setsit[®] 104 correspond with the constituents indicated below:

- $R_{t1} = 4,37$ min. probably MBT
- $R_{t2} = 14,64$ min. probably the recombination product of diisobutyldithiocarbamate with dimethyldithiocarbamate
- $R_{t3} = 19,49$ min. probably diisobutyldithiocarbamate,

However, the peak corresponding with the dimethyldithiocarbamate alone was not found. Therefore, a reliable qualitative HPLC-analysis fails in case of Setsit[®] 104 similarly because this method is not reliable in all multicomponent mixtures.

The first standard procedure in the sample preparation and HPLC-analysis of glove extracts was established irrespective of these complications. It consisted of:

- glove extraction with acetone (see section 2.2.2)
- transfer of substances extracted from gloves into a chloroform solution
- treatment of the CHCl_3 -solution with NiSO_4 , dissolved in a phosphate buffer (30 min., pH = 5,6; Ni^{2+} -concentration 20 mmol/l)
- washing of the CHCl_3 -solution with water
- separation of the CHCl_3 -solution from the watery phase and freezing down to approx. -20°C
- filtration of the frozen CHCl_3 -solution (next day; membrane filter, pore size 0,45 μm)
- (eventually dilution of the CHCl_3 -solution with methanol, particularly in cases of some special studies with more concentrated solutions of standard substances)
- HPLC-analysis under conditions listed in Table 31

As stated above, the procedure proposed by ASTM was also tested at the beginning of the project. According to the available working paper of ASTM concerning the estimation of accelerator residues in medical gloves, HPLC-conditions listed in Table 33 apply and

- only ZMBT, MBT, TMTD and ZDEC are taken into consideration,
- there is no conversion of ZDCs in other metal complexes involved in the method and
- gloves shall be cut in small pieces and extracted using a phosphate buffer of pH = 6,5.

Table 33: HPLC-conditions according to ASTM

mobile phase	linear gradient from 60/40% water/acetonitrile to 100% acetonitrile developed over 20 minutes
injection volume	20 μ l
column temperature	approx. 25°C (room temperature)
flow	0,75 ml/min
detection	UV at 254 nm

HPLC-grade water and acetonitrile were used as solvent and as the mobile phase. Standard substances of ZPC and ZDBC were dissolved (concentration about 10 ppm) in 60/40% water/acetonitrile and analyzed by HPLC. At the same time, gloves produced using the above accelerators were extracted as described in the ASTM-paper, and extracts were analyzed. Peaks found in chromatograms of standard substances (retention times 3,21 and 3,26 min., respectively) have not been found in the chromatogram of the glove extract.

In contrast to this finding, gloves extracted according to the procedure described in the section 2.2 (acetone extraction), treated with NiSO₄-solution and analyzed by HPLC under conditions listed in Table 31 have shown accelerators claimed by the producer in most cases. Under these conditions, accelerator residues in glove extracts have been found at retention times given in Table 32.

Falling column performance (an increase of retention times) and increasing pressure were observed as more and more HPLC-runs were carried out also with glove extracts. Finally, after the work with standard substances was finished and great number of routine analyses of glove extracts were performed, the column clogged and had to be refilled frequently. Moreover, in this stage of the work, a cross-check by RFA showed that the conversion of ZDCs into Ni-complexes does not run quantitatively. The voluminous the amino group in the Zn-dithiocarbamate, the lower was the yield of the nickel complex.

It is substantiated in section 2.2.2 why acetone has been selected as the optimum extraction solvent. However, it extracts obviously not only accelerator residues but also other soluble constituents of the latex film, e.g. sulphur, waxes and paraffines, antioxidants, latex proteins, surface active substances and some polymeric materials. It was found that these contaminants were not completely removed in the sample treatment procedure and caused the obstruction of the column. Therefore, a special clean up procedure has been developed and has been applied since that time in the preparation of samples for the HPLC (see section 2.2.3 for the description of the clean-up). Simultaneously, the treatment of extracts with Ni-salt has been modified to increase the efficacy of the complex forming. The new optimized procedure is the final result of this part of the work. It consisted of:

- a) glove extraction with acetone (see section 2.2.2)
- b) treatment of the acetone extract (5 ml) with 1%-NiSO₄-solution in a phosphate buffer, pH = 5,6 (1,5 ml, shaking at room temperature for 15 min)
- c) clean-up procedure for the elimination of contaminants (section 2.2.3)
- d) evaporation of solvents at max. 40°C (P ≤ 0,6 bar, rotavapor)
- e) dissolving of the solid residue in 0,5 ml chloroform
- f) methanol is filled up to 1 ml
- g) freezing down of the solution to approx. -20°C
- h) filtration of the frozen solution (next day; membrane filter, pore size 0,45 μ m)
- i) HPLC-analysis under conditions listed in Table 31

Applying this new procedure and using a fresh refilled column, retention times (R_t) of individual substances and recombination peaks in binary mixtures listed in Table 34 and recovery rates (R) given for the examined standard substances in Table 35 were found.

Table 34: Retention times R_t [min] of individual substances (bold type on the diagonal line) and some products of binary recombinations (an "X" means: no reaction or formation of the new substance has been observed in this case)

	TMTM	TMTD	TETD	PTD	TBTD	ZMBT	MBT	MBTS	ZDMC	ZDEC	ZPC	ZEPC	ZDBC
TMTM	3,6												
TMTD		4,0	5,1	5,5	11,3	X							
TETD		5,1	7,1	7,9	13,0	X		10,7					
PTD		5,5	7,9	7,5	13,4	X		11,4					
TBTD		11,3	13,0	13,4	15,9	X		14,5					
ZMBT		X	X	X	X	3,6		X		X	X		X
MBT							3,6	X					
MBTS			10,7	11,4	14,5	X	X	13,8					
ZDMC									5,4	7,6	8,6		13,2
ZDEC						X			7,6	10,5	11,2		14,2
ZPC						X			8,6	11,2	11,8		14,6
ZEPC												12,4	
ZDBC						X			13,2	14,2	14,6		17,0

Table 35: Recovery rates R [%] in the HPLC-analysis of accelerators ("new method" with clean-up by means of solid phase extraction)

THs	Recovery [%]			ZDCs	Recovery [%]			MBTs	Recovery [%]	
TMTM	100			ZDMC	97	72	88	MBT		
TMTD	91	84		ZDEC	92	98		MBTS		
TETD	87	88	96	ZPC	77	75	57	ZMBT	77	72
PTD	90	90	96	ZDBC	75	84	84			
TBTD	54	45		ZEPC	85					

The difference of R_t -values in Table 32 and Table 34 shows the obvious uncertainty in the qualitative HPLC-analysis as caused by the instability of the column performance. The clean up procedure has improved the stability, but the successive obstruction of the column has not been completely eliminated. Also, if the clean up procedure is applied the column is clogged slowly and R_t -values listed in Table 34 drift (increase). Therefore, a periodical re-calibration is necessary to keep the reliability of the results. Nevertheless, the reliability of the results is in any case not the best, and the qualitative analysis (interpretation of the chromatogram) requires much experience.

For the estimation of repeatability (r), six standard CHCl_3 -solutions of individual substances in the concentration range between 18 $\mu\text{g/ml}$ and 26 $\mu\text{g/ml}$ were prepared after the treatment of acetone solutions of accelerators with nickel sulphate solution according to the standard sample preparation procedure described above. All solutions were analysed by HPLC two times and standard deviations (s_x) of each of the 12 analysis were evaluated. The repeatability (r) for 11 individual accelerators is listed in Table 36.

For the quantitative analysis, the HPLC system was calibrated using the external standard method. Stock standard solutions of relevant accelerators in acetone were prepared. TH-solutions were used as prepared, and ZDC-solutions were treated with NiSO_4 in phosphate buffer pH = 5,6 for 15 minutes. Thereafter, acetone and water were evaporated and the dry residue was taken up in 5 ml chloroform, washed with dist. water (3x) and filtered using a membrane filter with a pore size of 0,45 μm . Calibra-

tion standards were prepared at a minimum of four concentration levels by dilution of the stock standard solutions in the concentration range of 5 µg/ml to 500 µg/ml with methanol.

Table 36: Repeatability ($r = 2,8 \times s_x$) based on 12 measurements ("new method" with clean-up by means of solid phase extraction)

THs	r [µg/ml]	ZDCs	r [µg/ml]	MBs	r [µg/ml]
TMTM	4,8	ZDMC	2,4	MBT	
TMTD	1,6	ZDEC	2,2	MBTS	5,9
TETD	1,2	ZPC	2,6	ZMBT	
PTD	3,5	ZDBC	3,1		
TBTD	4,2	ZEPC	2,3		

A reasonable calibration function (response versus concentration) was set up only for each single carbamate, thiuram and mercaptobenzothiazole. A quantitative calibration of the HPLC method in cases of accelerator mixtures is connected with considerable uncertainties. The separation of a ternary ZDC-mixture is problematic due to the formation of combined Ni-complexes. As a consequence, it is difficult to evaluate a quantitative HPLC-analysis if accelerators were used in complex mixtures consisting of many components.

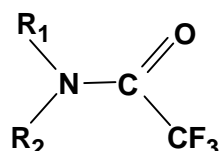
The "Within Laboratory Detection Limit" (WDL) were calculated on the basis of the upper confidence bound and the slope and intercept of linear calibration curves. The results are summarized in Table 37.

Table 37: Detection limits (WDL) for various accelerators in HPLC ("new method" with clean-up by means of solid phase extraction)

THs	WDL [µg /ml]	ZDCs	WDL [µg /ml]	MBs	WDL [µg /ml]
TMTD	0,2	ZDMC	3,0	MBTS	0,7
TETD	1,0	ZDEC	2,5	ZMBT	1,9
PTD	2,2	ZPC	2,3		
TBTD	0,9	ZDBC	3,0		

2.2.1.2 Gas chromatography (GC-MS and GC-FID)

As stated above THs, ZDCs and MBs are not volatile enough for direct analysis by means of GC. Voluminous, non-volatile molecules of accelerators have to be converted into more volatile reaction products prior to GC-analysis. A convenient procedure of this type has been found in a derivatization reaction of accelerators with trifluoroacetic acid anhydride (TFAA). In this reaction the volatile product shown below (dialkyl-trifluoroacetamide) is formed from the amino group $R_1R_2=N-$ bound in the accelerator molecule:



If free amines are contained in the latex film (formed from relevant accelerators by a decomposition reaction) they react with TFAA, too. Therefore, this technique would give false results with respect to the accelerator content if significant amounts of relevant free amines (decomposition products) were present in the latex film (see also section 2.2.1.3).

The gas chromatograph Hewlett-Packard HP 5890 with mass selective detection (GC-MS), equipped with a 30 m dimethyl silicon quartz capillary column was employed at the beginning of the work. Later, the above column was replaced by a 25 m dimethyl silicon column. Along with the GC-MS, the same type of apparatus but with flame ionisation detection (FID) and 15 m phenyl-methyl silicone silicon quartz capillary column has been used. GC-conditions found to be an optimum are listed in ANNEX 2 since this method has been adopted as the standard procedure.

2.2.1.3 Direct estimation of free amines (GC-NPD)

As the present project was proposed, it was supposed that free amines originating from accelerators (decomposition products) or added for particular purpose, e.g. as antioxidants, can have some allergic potential. It was further supposed that these substances can be found in latex gloves, and the relevant amines were put in Table 30. According to the working program, suitable method for the estimation of free amines in latex gloves had to be developed at first, and selected glove samples were to be examined second. The objective was to decide if this procedure should be incorporated in the standard protocol for the estimation of allergic potential of latex gloves, or if it should be skipped.

Gas chromatograph of the type Ai Cambridge, Model GC 94 with NP-detector, equipped with a 30 m quartz capillary column (35% phenyl-65% dimethylsilicon, 0,25 mm / 0,5 µm) has been used in all experiments. The optimum GC-conditions are listed in Table 38.

Table 38: GC-conditions applying to direct amine-estimation by GC-NPD (head space technique)

carrier	Helium, 80 kPa	
gas flow	about 1 ml/min	
injection	split to injection ratio:	1:10
injection quantity	1 µl	
detector	NPD	
oven program	isothermal 1:	80°C, 3 min
	ramp 1:	15°C / min
	isothermal 2:	120°C, 3 min
	ramp 2:	25°C / min
	isothermal 3:	150°C, 2 min
injection temperature	220°C	
detection temperature	220°C	
column	30 m quartz capillary column; 0,25 mm / 0,5 µm; 35% phenyl- 65% dimethylsilicone	

Retention times R_t of standard substances dissolved in cyclohexane (direct injection of the solution) and in chlorobenzene (head space technique) were estimated. These experiments have shown that decomposition products of accelerators, i.e. substances no. 13 to 16 in Table 30, can be quantified by GC-NPD; the relevant R_t -values are listed in Table 39. Under the above conditions, all four amines listed in Table 39 can be separated within 10 minutes; however, the separation of DMA and DEA is not perfect. Caused by the strong basic nature of secondary amines, considerable peak tailing has been observed. The problem of tailing becomes serious as the molecular mass of the amine increases. Due to this fact, the detection limit is disadvantageously high, especially in low volatile amines (e.g. DBA and PP) which are – unlike small volatile molecules of DMA or DEA – rather retained in the latex film and therefore of more importance from the point of view of the allergic potential.

Table 39: Retention times R_t of different amines in GC/NPD carried out under conditions listed in Table 38.

Amine	retention time [min]	Amine	retention time [min]
dimethylamine	2,6	dibutylamine	9,1
diethylamine	3,0	piperidine	5,9

For the quantification of free amines in gloves, the gloves were first cut into small pieces. About 1 g of the glove sample was weighed exactly in a head space vial, covered with 5 ml of cyclohexane to swell and closed gas-tight immediately. The vials were kept at 90°C for 60 min. before samples were taken for the GC-analysis. Free amines were extracted from the latex film at these conditions. However, the direct injection of the cyclohexane solution did not enabled the quantification of the amine content. Therefore, in the second variant of the free-amine-estimation, chlorobenzene was used for swelling the sample, and head space technique was utilized in the GC-analysis. The vials were kept at 90°C for 60 min. before samples of the gas phase were taken for the analysis.

No significant amounts of free amines were found in any of the gloves which were analysed according to above procedures. The lack of clinical evidence of allergological relevance of free amines in latex gloves and discouraging results of direct GC-analysis of selected gloves lead to decision to leave this way of glove examination out the standard protocol. Phenyleted phenylene-diamines, claimed to be constituent in some latex formulations, are detected and can be quantitatively estimated in the standard GC-method after they were derivatised with TFAA.

2.2.1.4 Photometry and colour reactions

Accelerators are known to form partially characteristic colour reaction products with various metal ions (after treatment with inorganic salts). Therefore, absorption characteristics and maxima of different accelerators (treated with inorganic salts as well as untreated) have been obtained by UV-VIS-spectrometry. An effort has been made to exploit the colour reactions in the screening method (see section 2.2.4). The objective was to find reagents which react specifically with one of the accelerator groups under formation of colored products and show no reaction with the other ones. A specific proof for freedom from thiurams (negative evidence of thiurams), not impaired by ZDCs and eventually also present MBs and other substances, was of particular interest.

The absorption maxima of different accelerators dissolved in CH₂Cl₂ in a concentration of about 0,001 % are listed in Table 40.

Table 40: UV-VIS absorption maxima of various accelerators

Accelerator group	Wavelength of the absorption maximum
TMTM; TMTD; TETD and TBTD	253-280 nm
ZDMC; ZDEC; ZEPC; ZPC and ZDBC	260-276 nm
MBT	328 nm
MBTS; ZMBT	274 nm

The wavelength differences of the absorption maxima among the three accelerator groups (ZDCs, THs and MBs) are not large enough for the sure distinction. Only the absorption maximum at 328 nm is positioned considerably higher and could be utilized for the identification of MBT or ZMBT in a glove extract.

As colour reactions were examined, accelerators were first dissolved in acetone or dioxane in concentrations of about 0,01% to 0,001% and shaken with aqueous solutions of suitable Cu²⁺ and Mn²⁺ salts. In contrast to acetone, 1,4-dioxane does not impair the important range of UV-VIS spectra between 260 nm and 340 nm. The absorption maxima of the reaction products are listed in Table 41. The wavelengths differences of these maxima are not large enough for the distinction between THs and ZDCs.

Table 41: Absorption maxima of reaction products of various accelerators with 1% solution of CuSO₄ in acetone and 2% solution of MnCl₂ in 1,4-dioxane

Accelerator	Wavelength of the absorption maximum	
	CuSO ₄	MnCl ₂
TMTM; TMTD; TETD and TBTD	422 - 424 nm	274 - 286 nm
ZDMC	434 - 436 nm	497 & 280 nm
ZDC	434 - 436 nm	497 & 325 nm
ZPC		497 & 327 nm
ZEPC	434 - 436 nm	485 & 336 nm
ZDBC	434 - 436 nm	496 & 324 nm

Mn-complexes formed from ZDCs show always an absorption maximum at approximately 500 nm, which would indicate the presence of carbamates. However, as stated above, it must be expected that carbamates are important constituents in nearly all latex formulations, and in gloves produced only with THs (and eventually MBs but no ZDCs), carbamates are formed from THs. This means that this absorption maximum will be found in nearly all extracts.

The colours of reaction products with other salts are listed in Table 42. If mixtures of accelerators are treated with these reagents without previous separation, e.g. by chromatographic methods (TLC, HPTLC), neither the examined salts as a single test nor in combinations allow estimation of what type of accelerator is present in the extract and what is not. Thus, these colour reactions, when used without previous separation, do not possess the necessary universality.

2.2.1.5 Thin layer chromatography (TLC)

Taking into consideration that colour reactions without previous separation of present accelerators, e.g. by means of TLC, cannot serve the purpose (see section 2.2.1.4), this technique was attempted as the first step of the screening procedure. TLC plates covered with silica gel without fluorescence indicator proved to be suitable. A mixture of n-hexane, toluene, ethyl acetate 30:58:12 (v/v/v) has been found to be an optimum eluent. Five different spray reagents were tested, partially in accordance with the findings summarized in section 2.2.1.4. However, as HPLC turned out to be unsuitable for both qualitative and quantitative analysis, a demand has been raised to increase the selectivity and sensitivity of the TLC. From this reason the method described above has been further developed. In particular, a switch-over to HPTLC has been made (see section 2.2.1.6).

Some other eluent mixtures were tested for optimization of the separation of ZDBC and TBTD in addition to the basic eluent composition [n-hexane / toluene / ethyl acetate in ratio 30 : 58 : 12 (v/v/v)]. The results of these experiments are summarised in Table 43. Using the eluent n-hexane / toluene 27 : 73, these two compounds were separated best. The disadvantage of this mixture is the low R_f value of ZMBT; the spot of this compound interferes with many impurities extracted from the glove. For the separation and qualitative analysis of MBI and amines like diethyl- and dibutylamine, an eluent mixture of n-hexane / toluene / ethyl acetate 33 : 33 : 33 showed best results, though carbamates and thiurams cannot be separated from each other.

Table 42: Colours of reaction products of various accelerators dissolved in acetone with inorganic salts dissolved in water

Accelerator	CoCl ₂	Ni(NO ₃) ₂	FeCl ₃
ZPC	green	light yellow	dark green
ZEPC	green	yellow	dark green

ZDNC	colourless	colourless	dark green
ZDEC	blue	yellow	dark green
ZDMC	green	yellow	dark green
ZDBC	blue	yellow	dark green
TMTD	blue	colourless	yellow
TETD	blue	colourless	yellow
TBTD	blue	colourless	yellow
TMTM	blue	colourless	yellow
MBT	blue	colourless	yellow
MBTS	blue	colourless	yellow
PPD	blue	colourless	intense yellow
ZMBT	colourless	colourless	colourless

Table 43: R_f -values (mean values)

substance	R_f -values			
	eluent composition : n-hexane: toluene: ethyl acetate (v/v/v)			
	26 : 70 : 4	0 : 10: 1	27 : 73 : 0	1: 1: 1
ZDBC	0,91	0,91	0,73	0,95
TBTD	0,84	0,85	0,51	0,95
ZDEC	0,72	0,75	0,43	0,92
TETD	0,54	0,71	0,16	0,92
ZDMC	0,54	0,69	0,29	0,90
TMTD, TMTM	0,35	0,55	0,14	0,88
ZPC	0,71	n.e.	0,42	0,92
PTD, PTT	0,55	n.e.	0,23	0,88
ZDINC	n.e.	n.e.	0,91	n.e.
ZEPC	n.e.	n.e.	0,44	n.e.
ZMBT	0,23	0,5	0,18	0,71
MBI	0	n.e.	0	0,41
DEA	0	n.e.	0	0,23
DBA	0	n.e.	0	0,53

n.e.: not estimated

The actual R_f -value depends on

- the quality of HPTLC- or TLC-plate
- the actual eluent composition (changes on each opening of the tank)
- the amount of substance (the higher the concentration, the lower the R_f -value)
- the amount and chemical character of impurities

The last point is very important for the practical analysis of glove extracts. Antioxidants are very often also present in glove extracts. They can be very disturbing because of their high concentrations and the similar R_f -value to ZDBC and TBTD. These antioxidants can be detected with spray reagent B.

2.2.1.6 High performance thin layer chromatography (HPTLC)

The difference between TLC and HPTLC is in the quality of plates. The common TLC-plates have a uniform layer of an adsorbent on it, whereas HPTLC are provided with a concentration zone in the start area. Further, the more expensive HPTLC-plates possess an active layer with optimised particle size distribution of the adsorbant. Owing to these features, the spots on HPTLC-plates are sharper and – if the concentration of the analyte in the solution is too low – the sufficient amount of the analyte

can be put on the start by multiple applying of the sample accompanied with only a moderate lost of the selectivity.

This modification of the original TLC-method is now employed as a part of the standard procedure (qualitative analysis) for the assessment of accelerator residues in medical gloves proposed for the standardization and, as such, thoroughly described in ANNEX 2. Simultaneously, it constitutes the screening procedure for the fast estimation of accelerator classes present in extracts.

2.2.2 Extraction procedure for accelerators

As a part of the assessment of bioavailable accelerator residues in medical gloves, the extraction procedure must fulfil the following basic requirements:

- It should be adequately efficient to ensure a sufficient sensitivity of the accelerator estimation.
- Solutions of accelerators in the extraction solvent should be stable.
- The extraction solvent must not impair the analytical procedure and alter its results.
- The procedure should enable the distinguishing between inner and outer surface of the glove, because differences regarding release of extractable substances can be expected depending on the examined surface, particularly in coated gloves.
- Extractable constituents of the latex film other than the substances of interest should be affected by the extraction medium as little as possible.
- The selectivity of the extraction procedure against particular types of accelerators should be comparable with the selectivity of the accelerator-uptake by the skin by direct glove contact under normal wear conditions.
- The same extraction procedure should be utilized in the preparation of extracts for patch tests (clinical examination); the extraction solvent must not impair these tests.

The ability of the extraction solvent to dissolve the substances of interest and its diffusibility with regard to the latex film matrix is decisive for the efficacy of the extraction. Standard substances were utilized in the study of the solubility of accelerators and the stability of solutions. Results of the solubility study are summarized in

Table 44. In these experiments, solvent and a specified amount of the accelerator giving the concentration indicating on the top of the

Table 44 was treated for 5 minutes in an ultrasonic bath at room temperature if the solid was not dissolved in reasonably short time on standing and occasionally shaking. It is indicated in

Table 44 whether another treatment (heating or longer treatment with ultrasound) was necessary to solve the particular accelerator. The solubility was assessed visually (clearing of the original turbid mixture). The result has been ascertained by the appropriate dilution of the original mixture in cases where lower solubility than in the head line is indicated in the table. Latex proteins were suspected to cause problems in the analysis procedure. Therefore, the solubility of proteins was also assessed using ovalbumine as a model.

Table 44: Results of the solubility tests (numerical results in % if not as µg/ml specified)

Subst.	Solvent and starting concentration											
	H ₂ O 0,1%	phosph. ¹⁾ buffer pH=6,5	CH ₃ CN 0,05%	MeOH 0,5%	EtOH 0,5%	i-PrOH 0,05%	n-hexane 0,1%	dioxane 0,05%	THF 0,5%	toluene 0,1%	acetone 1%	CHCl ₃ 1%
TMTM	–	n.e.	++	+	+	+	n.e.	+	++	++	++	++
TMTD	–	16 µg/ml	+	+/-	+/-	+	–	+	++	+	++	++
TETD	–	2,2 µg/ml	++	+	++	+	–	+	++	++	++	++
TBTD	–	n.e.	++	+	++	+	n.e.	++	++	n.e.	≤1	++
PTD		3,2 µg/ml	+	≤0,01	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	≤3,8	+
PTT	–	n.e.	<0,01	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	(+)	++

ZDMC	-	1,8 µg/ml	<0,025	<0,016	-	++/-	n.e.	++	-	n.e.	<0,05	<0,05
ZDEC	-	1,6 µg/ml	+	++	+/-	+	-	+	++	n.e.	≤0,5	++
ZEPC	-	n.e.	+	≤0,025	+	+	n.e.	++	++	n.e.	++	++
ZPC	-	0,6 µg/ml	<0,016	≤0,01	+	++/-	n.e.	+	++	+	++	++
ZDBC	-	-	+	<0,05	++	+	n.e.	++	<0,1	++/-	++/-	<0,15
ZDINC	n.e.	n.e.	+	≤0,02	n.e.	+	n.e.	n.e.	++	n.e.	n.e.	n.e.
MBT	-	n.e.	+	≤0,5	+	+	-	++	++	+	0,05	++
MBTS	-	n.e.	<0,01	<<0,01	++/-	++/-	n.e.	++	+	-	-	++
ZMBT	-	66 µg/ml	<0,01	<<0,01	<0,01	n.e.	n.e.	n.e.	<0,02	n.e.	80 µg/ml	80 µg/ml

- ¹⁾ numerical values estimated by means of HPLC
 ++ soluble in the given concentration on standing / shaking at room temperature
 + soluble in the given concentration after 5 min. treatment with ultrasound at room temp.
 (+) soluble after 30 min treatment with ultrasound at room temperature
 +/- soluble after heating
 - not (completely) soluble in the concentration given in the head of the table
 n.e. not estimated

Since common accelerators are practically insoluble in aqueous media, extraction media of this class (water, buffer solutions, artificial sweat) are evidently not suitable. This has also been confirmed experimentally in the HPLC-test carried out according to ASTM draft standard (see section 2.2.1.1). The transport of the accelerators from the latex film into the lipid parts of the skin takes place obviously through the direct contact of the film with the skin without previous solution of these lipophile substances in the sweat.

For most accelerators, chloroform (and similarly also methylene chloride) is the solvent with the highest dissolving ability. The disadvantage of these solvents is that the extracted latex film swells extremely. If the latex film is extracted under normal conditions (room temperature, no change of the solvent) with a appropriate amount of CHCl₃ to get a reasonable concentration of the extract, most of the solvent is absorbed by the swollen latex and only a smaller part is obtained as the extract. This phenomenon reduces the yield of the extraction procedure. Thus, no differentiation between inner and outer surface of the glove can be made if solvents of this extreme aggressivity against the latex film are used. Due to this fact, the bioavailability of accelerators under normal wear conditions cannot be simulated using chlorinated solvents as extraction medium.

Most of the relevant accelerators are sufficiently soluble in acetone. Only ZDMC, ZDBC, MBT and MBTS are considerably more soluble in chloroform than in acetone. ZMBT is only slightly soluble in acetone as well as in chloroform. Acetone does not swell the latex film as much as CHCl₃. Due to this fact acetone allows extraction of only one side of the glove. Being an organic solvent, it does not simulate human sweat, but it has been shown in the later stages of this project that it is a good simulation for the uptake of accelerators by direct skin contact under normal glove use conditions. Acetone also fulfils requirements concerning its suitability for the preparation of extracts for clinical studies (patch tests).

The solubility of accelerators in THF and dioxan is only slightly lower than in acetone and chloroform. THF is not suitable for HPLC, and both solvents are inconvenient due to their toxicity. In addition, there is no experience concerning these solvents in connection with patch tests, and extensive preliminary clinical tests would be necessary for this reason if these solvents would be used.

Lower solubility of accelerators in alcohols was the reason for rejection of alcohols as an extraction medium, although alcohols were suitable for HPLC analysis and patch tests (low toxicity). They are, however, disadvantageous in GC because of their reactivity in the derivatization reaction with TFAA. Lower solubility of accelerators in acetonitrile and its toxicity have been the reason for rejection of this solvent as an extraction medium. It is suitable for HPLC analysis, but it is disadvantageous in GC because of more impairing peaks. The toxicity of acetonitrile is a massive handicap for its use in patch tests.

The stability of THs dissolved in acetone is limited. Thus, the concentration of a thiuram solution (originally about 30 µg/ml) decreases typically by approx. 10 % after 48 h and by approx. 40% after two weeks standing at room temperature. TMTM is still less stable, ZDC-solutions in acetone are more stable. For this reason acetone solutions of accelerators and glove extracts in acetone should be processed as fast as possible.

The following procedures have been tested in connection with appropriate analytical method, i.e. ASTM-HPLC, self developed HPLC, HPTLC and GC-FID(MS).

- „cut-glove-method“ (method proposed in the ASTM working paper: extraction with 20 mM phosphate buffer pH = 6,5 to simulate sweat)
- „one-glove-method“ (one glove was filled with 100 ml of 20 mM phosphate buffer pH=6,5 and shaken for a total 120 min at 37°C)
- „double-glove-method“ (the experimental arrangement was similar to the double-glove method used in the protein analysis: the test specimen of known weight was extracted with acetone by shaking for a total of 120±5 minutes at room temperature)
- „finger-method“ (method derived from the double glove method to minimize the quantity of extraction medium and to optimize the handling)

The „finger-method“ is used in the standard procedure and, as such, this method is thoroughly described in ANNEX 2.

To check the recovery of the extraction step, the "finger method" was employed, and acetone solutions of accelerators not contained in extracted gloves (ZMBT, ZDMC, ZDBC) were used as extraction medium instead of pure acetone. In the case of ZMBT, recovery of approx. 40-60 % was found independent of the ZMBT-concentration in the extraction medium. The recovery of ZDMC was practically quantitative (due to the uncertainty of the analysis, recovery was more than 100% in some cases), whereas the recovery of ZDBC was 70 to 90 %.

2.2.3 Clean-up procedure for accelerator extracts

According to the optimized method, the conversion of ZDCs with NiSO₄ is carried out in acetone, i.e. immediately after the extract is obtained, and not (as in the original procedure) after the extract was transferred into CHCl₃. In the clean up procedure, which is used in the optimized conversion procedure, a Chromabond® C18 / 3ml / 500mg column is used. The column is conditioned with 5 ml methanol and subsequently with 5 ml distilled water. The acetone extract, mixed with NiSO₄-solution (0,65 ml) and 2 ml distilled water are put in the column, the column is washed with 6 ml dist. water and all liquid is sucked off of the bottom of the column until it is dry. Then the column is washed with 3 ml n-hexane, and finally the accelerators are eluted by CHCl₃. The elute is dried under vacuum, dissolved in 0,5 ml CHCl₃ and filled up to 1 ml with methanol. The solution is frozen down to approx. -20°C and filtered through a membrane filter (pore size 0,45 µm) before analysis by HPLC. A cross-check made by RFA confirmed that the conversion of ZDCs into Ni-complexes carried out in acetone solution is nearly quantitative. ZDNC, e.g., which does not form a Ni-complex in CHCl₃, is converted with a 93%-yield in acetone. ZMBT and MBTS do not react under these conditions and also THs form no coloured Ni-containing reaction products. On the other hand, in a mixture of TETD and ZDBC in acetone treated with NiSO₄ in phosphate buffer, TBTD and Ni-diethyldithiocarbamate are formed.

2.2.4 Routine recognition of important accelerator groups (screening procedure)

A screening method for the simple and quick distinction between ZDCs, THs and MBs in gloves had to be developed according to the working program. Effort made in the frame of this sub-task is de-

scribed in section 2.2.1.4 and 2.2.1.5. The work was interrupted temporarily when CEN TC 205/WG 3 established that glove producers have to declare all chemicals used in the production. Such declaration, if reliable, would have made the qualitative analysis unnecessary. However, qualitative analyses performed within this project on a number of gloves by HPLC have shown that this declaration is not reliable enough. In the meantime, HPLC has been rejected as the method for the qualitative analysis, and HPTLC, described in ANNEX 2, has been established for this purpose. It is as simple as TLC originally proposed for the screening method. Therefore, no other modification of TLC has been established for the screening procedure. If a quick qualitative analysis of the extract is required, a standard HPTLC-procedure should be carried out. It takes about two hours to extract the glove and to develop the HPTLC-plate.

2.2.5 Summary of the estimation of extractable accelerators in selected glove brands

Results of qualitative and quantitative analysis carried out on a selected group of gloves are summarised in table 45. The major part of the examination has been performed applying the "finger-method" for the extraction. The inner surface of the glove was examined in most cases, and it is indicated in table 45 if outer surface was also examined. The gloves are identified by ÖIBW-numbers (bold types), which differ in some cases from numbers given by participant 1 (FAU, co-ordinator) for the purpose of the protein analysis. Black marking in table 45 in the column "Claim" indicates that the particular accelerator is used in the latex formulation for the manufacturing of the given glove brand according to the producer's claim. If the particulate accelerator has been found in the extract by HPTLC, HPLC or GC, it is indicated with \oplus in table 45. The original ("old") procedure, without clean-up or utilising Ni^{2+} -treatment in chloroform solution, has been abandoned as the HPLC method has been further developed. The results gained by this "old" method – provided that it was possible to evaluate them quantitatively – are labelled with "c" in table 45; the results of the later analysis (with the clean-up procedure) are not specially labelled.

In table 45, quantitative results of the GC-analysis are labelled with "d" if both the particulate TH and the corresponding ZDC with the same amine-residues were found by the qualitative analysis (HPTLC). In such cases, the concentrations of the THs and corresponding ZDCs calculated from the found amount of the dialkyl-trifluoroacetamide differ a little due to the difference in the molecular mass of TH and the corresponding ZDC. THs and ZDCs with identical N-substituents are separated by a dotted line in table 45, whereas accelerators with different substituents are separated by a full line.

Table 45: Results of glove extract analysis carried out in ÖIBW

ÖIBW No	Subst.	Claim ^{a)}	HPTLC (qualit.)	HPLC [$\mu\text{g/g}$] [*]	GC (qualit.)	GC (quantitative analysis) ^{b)} [$\mu\text{g/g}$]			
1 (FAU=1)	ZDMC								
	ZDEC		⊕	⊕	⊕	360			
	ZPC		⊕	6650 ^c	⊕	1950			
	ZMBT								
2 (FAU=2)		A							
3 (FAU=3)	ZDEC		⊕	1140	⊕	490			
	PTT								
	ZPC		⊕	8600	⊕	2590			
	MBI								
4 (FAU=4)	ZDEC		⊕	⊕	⊕	875			
	PTT								
	ZPC		⊕	1150 ^c	⊕	5740			
	ZMBT		⊕	⊕	⊕	158			
	MBI		⊕	⊕	(⊕)	lower than detection limit			
5 (FAU=5)	ZDEC		⊕	⊕	⊕	420			
	PTT								
	ZPC		⊕	4703		1820			
	MBI								
6 (FAU=6)	ZDEC		⊕	⊕	⊕	968			
	PTT								
	ZPC		⊕	2560 ^c	⊕	2414			
	MBI		⊕						
7 (FAU=8)	TBTD		⊕	320	⊕	<5075 ^d			
	ZDBC		⊕	5900	⊕	<5915 ^d			
	ZMBT								
8 (FAU=7)	ZDBC				⊕	309			
	ZMBT		⊕	1600	⊕	257			
9 (FAU=10)	TBTD		⊕	<35	⊕	<502 ^d			
	ZDBC			380	⊕	<131 ^d			
	ZMBT		⊕	2100	⊕	589			
10 (FAU=9)	ZDBC		⊕	300	⊕	302	328	224 ^e	188 ^e
	ZMBT		⊕	600	⊕	356	413	546 ^e	432 ^e
11 (FAU=14)	TETD		⊕	59,5	⊕	<665 ^d			
	ZDEC		⊕	1320	⊕	<502 ^d			
	TBTD		⊕		⊕	175			
	ZMBT			2528	⊕	113			
	PPD								
12 (FAU=16)	TMTD			⊕	⊕	80			
	ZDEC			⊕	⊕	329			
	ZDBC		⊕		⊕	246			
	ZDiBC		⊕	⊕	⊕	221			
	ZMBT			⊕	⊕	113			
13 (FAU=18)	ZDEC		⊕	530 ^c	⊕	210 (i.s.)		394 (o.s.)	
	ZPC		⊕	190 ^c	⊕	175 (i.s.)		421 (o.s.)	

cont. see next page

Table 45 (cont.)

ÖIBW No	Subst.	Claim ^{a)}	HPTLC (qualit.)	HPLC [$\mu\text{g/g}$]*	GC (qualit.)	GC (quantitative analysis) ^{b)} [$\mu\text{g/g}$]		
14 (FAU=??)	ZDEC		⊕	⊕	⊕	140		
	ZDBC		⊕	6060 ^c	⊕	100		
15 (FAU=25)	ZDEC		⊕	3630 ^c	⊕	1940	1054 ^e	1137 ^e
	ZDBC		⊕	28300 ^c	⊕	2766	1633 ^e	1654 ^e
16 (FAU=26)	ZPC		⊕	1680	⊕	2418		
	ZDBC		⊕	3580	⊕	3675		
	ZMBT		⊕	1301	⊕	≤ 60		
17 (FAU=27)	ZPC		⊕	1349	⊕	1842		
	ZDBC		⊕	3270	⊕	2692		
	ZMBT		⊕	357	⊕	123		
18 (FAU=29)	ZPC		⊕	420	⊕	427	505	
	ZDBC		⊕	450	⊕	520	816	
	ZMBT			802	⊕	131		
19 (FAU=28)	ZPC		⊕	968	⊕	1427		
	ZDBC		⊕	295	⊕	3116		
	ZMBT			1303				
20 (FAU=17)	ZDiBC		⊕	⊕	⊕	156	280 (i.s.);	448 (o.s.)
	ZMBT			⊕	⊕		135 (i.s.);	0 (o.s.)
	PPD							
21 (FAU=19)	ZDEC		⊕	2800 ^c	⊕	5070		
22 (FAU=20)	ZDEC		⊕	2450 ^c	⊕	7771		
23 (FAU=21)	ZDEC		⊕	286	⊕	2691	2027	
	ZDBC		⊕	1740	⊕	4728	3722	
24 (FAU=30)	TETD		⊕		⊕	<1047 ^d	<858 ^d	<900 ^d
	ZDEC			⊕	⊕	<1278 ^d	<1047 ^d	<1098 ^d
	TBTD		⊕	⊕	⊕	1332	1098	557
	ZDBC							844
	ZMBT				70	⊕	111	140
25 (FAU=35)	ZDEC		⊕	4200	⊕	5179		
	ZDBC		⊕	3530	⊕	4033		
	ZMBT		⊕	400	⊕	119		
26 (FAU=32)	TETD		⊕	40 ^c	⊕	<4644 ^d		
	ZDEC		⊕	3150 ^c	⊕	<5672 ^d		
	TBTD		⊕	130 ^c	⊕	46		
	ZMBT		⊕	60 ^c	⊕	92		
27 (FAU=12)	ZDEC		⊕	600	⊕	1361		
	ZDBC		⊕	68	⊕	401		
	ZMBT				⊕	136		
28 (FAU=11)	TBTD	A		<70	⊕	<1409 ^{df}		
	ZDBC	A		<100	⊕	<1637 ^{df}		
	ZMBT							
29 (FAU=24)	TETD		⊕		⊕	<490 ^d		
	ZDEC			86	⊕	<599 ^d		
	TBTD		⊕	<76	⊕	<133 ^d	<151 ^d	
	ZDBC			50	⊕	<154 ^d	<175 ^d	

cont. see next page

Table 45 (cont.)

ÖIBW No	Subst.	Claim ^{a)}	HPTLC (qualit.)	HPLC [$\mu\text{g/g}$]	GC (qualit.)	GC (quantitative analysis) ^{b)} [$\mu\text{g/g}$]
30 (FAU=22)	ZDMC		⊕	760	⊕	183
	ZDEC		⊕	3102	⊕	6759
	ZDBC		⊕	402	⊕	930
31 (FAU=23)	ZDEC		⊕	3102	⊕	3668
	ZDBC		⊕	1642	⊕	3338
32 (FAU=15)	TETD		⊕		⊕	
	ZDiBC		⊕	240	⊕	433 362 333 (i.s.); 174 291 346 (o.s.)
	ZMBT				⊕	60 51 53 (i.s.); 2 53 36 (o.s.)
	MBTS			690		
33 (FAU=34)		A				
34 (FAU=??)	ZDMC		⊕	⊕	⊕	385
	ZDEC		⊕	440 ^{c)}	⊕	490
	ZDBC		⊕	21100 ^{c)}	⊕	770
35 (FAU=??)	ZDEC		⊕	4560 ^{c)}	⊕	2402
	ZMBT		⊕	98 ^{c)}	⊕	80

- *) Quantitative HPLC-results shall not be employed for the assessment of the glove quality; all values listed here shall only illustrate the work done in course of the method development
- a) Black marking = the accelerator has been claimed by the glove producer as used in the latex formulation; all claims are stated in the table, also in that case, if no analysis has shown that the given substance is present in the extract
gloves No. 2, 28 and 33 are **not made** of natural rubber latex
- b) i.s. = inner surface; o.s. = outer surface
- c) HPLC-analysis without clean up; Ni-treatment in CHCl_3
- d) Caused by the derivatization procedure, it is not possible to distinguish between THs and corresponding ZDCs in the GC-analysis. If both TH and ZDC with the same amine-residue are present in the glove extract, only the sum of both (calculated as one of them) can be estimated by means of GC. In such cases both results are labelled with ^d and given with the sign < (the numerical value is true only if the second result in this pair, i.e. concentration of the corresponding TH or ZDC, is zero). The GC-results must be established in connection with the qualitative analysis (performed by HPTLC) in such cases.
- e) Results from the ÖIBW-part of the validation procedure
- f) Only dibutylamine was found by GC (without reliable result for TBTD or ZDBC in HPTLC or HPLC)

Results in table 45 labelled with "^d" should be read as follows: The concentration indicated in the table, e.g. for TH, would be true only if the concentration of the corresponding ZDC would be zero, and vice versa. If the extract contains both accelerators, the true concentrations of both accelerators are lower than the values in table 45, and the results are therefore written with the sign < standing in front of the numerical value.

In the most cases ZDC is found but not the corresponding TH. This applies also for gloves which were made from latex formulations containing the THs according to the producer's claim. In such cases the ZDC-content is calculated on the base of the quantitative GC-estimation of the corresponding dialkyl-trifluoroacetamide, and the result in table 45 is not labelled with "^d". However, there are also gloves in the table 45 which contain THs (although only ZDCs are claimed), but not the THs corresponding with ZDCs claimed (see e.g. No 11, 24, 26 or 32). In table 45 there are, in many cases, great differences in quantitative results obtained by GC (FID or MS) and by HPLC [„old procedure“ (Ni-treatment in CHCl_3 , without clean up) and „new procedure“ (Ni-treatment in acetone, with clean up)]. The rejection of the HPLC as part of the standard procedure is substantiated elsewhere, the above discrepancies being some of the most important reasons. The great part of the HPLC-analyses

were carried out in the course of the method development as test runs, and their results were of preliminary nature. As HPLC-chromatograms are rather complicated and consist of number of peaks, a cross-sensibility check with other substances having similar retention times as relevant accelerators causing higher values in HPLC in comparison with GC is well possible. On the other hand, an incomplete conversion of ZDCs into Ni-complexes (found in the „old procedure“) can cause too low results in the HPLC-analysis. However, there is a general uncertainty concerning the yield of the conversion reaction in such cases (conversion of the standard in comparison to the conversion of the accelerator in glove extract) so that HPLC-results could be also higher than GC results from this reason. Since only the GC method has been selected for the standard procedure, only the GC-results in table 45 shall be employed for the assessment of the glove quality.

Gloves No 2, 28 and 33 are not made of natural rubber latex. No accelerators common in NR-latex gloves have been found in extracts from these glove brands as the analysis procedure developed within the frame of this project has been applied. Only in No. 28 di-n-butylamine has been found, which corresponds with TBTD and ZDBC, but there has been no evidence in the HPTLC and HPLC that these accelerators were in fact present in the extract.

2.2.6 Preparation and characterisation of extracts for patch tests

For the purpose of the clinical studies, always more extracts of one glove brand were added together. Combined extracts were characterised by a control analysis, dispensed in more PE-vials, and the solvent was evaporated. The results of the control analysis are summarized in Table 46 (last column). Dried extracts closed in PE-vials were sent to hospitals, the amount of the accelerators in every vial being reported. The number of single extracts combined in one „dried extract“ were dependent on the accelerator content in the particular glove brand. Possibly equal amounts of accelerators in every vial (about 2 mg) were intended. In the hospital, dried extracts were dissolved in a known amount of acetone to get a solution of known concentration and immediately used in the preparation of patches.

Table 46: Summary of extracts prepared for patch tests

Glove No. (ÖIBW *)	Number of vials prepared for patch tests	Approx. number of extracts in one vial	Amount of accelerators in the vial (sum) [mg]	Accelerators contained in the sample in µg per vial (results of the control analysis)**)
10	20	25	0,35	ZDBC: 204 ZMBT: 150
10 (p)	20	25	n.d.	not detectable
13	20	100	1,3	ZPC: 773 ZDEC: 790
13 (o)	20	100	1,0	ZDEC: 467 ZPC: 500
15	20	8	0,6	ZDEC: 267 ZDBC: 303
15 (p)	20	8	<0,03	ZDEC: <25 (detectable)
16	20	6	0,7	ZPC: 320 ZDBC: 372
20	20	10	0,09	ZDiBC: 93
20 (o)	20	100	0,8	ZDiBC: 762
23	20	10	0,8	ZDEC: 260 ZDBC: 518
24	20	21	0,6	TETD: 311 TBTD: 272
29	20	58	1,1	ZDMC: 123 ZDEC: 935
32	20	10	0,2	ZDiBC: 107 ZMBT: 70
32 (o)	20	50	0,6	ZDiBC: 642

*) (p) means: extraction by 20 mM phosphate buffer pH=6,9 according to ASTM extr. method

(o) means: outer surface of the glove (in all other cases inner surface was extracted)

***) + means: low concentration in the accelerator mixture (20 % or less)

++ means: middle concentration in the accelerator mixture (about 50%)

+++ means: high concentration in the accelerator mixture (80% or more)

2.2.6.1 Clinical evaluation of the chemical analysis on accelerators

The aim of this part of our investigations was the evaluation of the analytical data on accelerators in glove extracts (performed in Vienna) by comparison with the patch test response in volunteers with known type IV-allergies to accelerators from Erlangen and København.

Patients

Volunteers were recruited among patients of the contact clinics of Erlangen and København. Inclusion criteria were positive patch test reactions (according to ICDRG-criteria) to thiurams, mercaptobenzothiazoles or dithiocarbamates during the last two years. Volunteers should not be younger than 18 and not older than 65 years. Pregnant or lactating women and patients with ongoing severe eczema were excluded. They all signed a consent form accepted by the ethic commissions in Erlangen and København.

All together 50 volunteers were included into this study. In the first 8 volunteers the patch test conditions were checked up, including an equalization between the methods in Erlangen and København. From the main group of 42 volunteers two were excluded since their former positive reactions to accelerators could not be confirmed during this series of patch testing.

Testing material

Single substances

Volunteers were tested with the main accelerators known to be present in medical gloves for single use. (Table 29)

Table 29 Chemicals tested as single substances in petrolatum

Abbrev.	Substance	Concentration in petrolatum
TMTD	tetramethylthiuram disulfide	1 %
TMTM	tetramethylthiuram monosulfide	1 %
TETD	tetraethylthiuram disulfide	1 %
TBTD	tetrabutylthiuram disulfide	1 %
PTD	dipentamethylenethiuram disulfide	1 %
PTT	dipentamethylenethiuram tetra sulfide	1 %
ZDMC	zinc-dimethyldithiocarbamate	1 %
ZDBC	zinc-dibutyldithiocarbamate	1 %
ZPC	zinc-pentamethylenedithiocarbamate	1 %
MBT	mercaptobenzothiazole	2 %
MBI	dibenzothiazylsulfide	1 %
ZMBT	zinc-mercaptobenzothiazole	1 %

Gloves

Gloves for single use were selected so that different chemical compositions of the most commonly used derivatives of the accelerators were supposed to be represented. A total of 19 gloves from different manufacturers was selected. In 15 of them manufacturers had disclosed information on the content of accelerators (table 30).

Table30 Accelerators found by chemical analysis in Vienna and/or declared by the manufacturer in 19 gloves selected for patch test analysis. Values are given in : g/g latex, d = accelerator was declared by the manufacturer.

Glove	PTT	ZPC	TETD	ZDEC	TBTD	ZDBC	ZDiBC	TMTD	ZMBT	MBI
3	d	6.7		1.35 d						
9						0.66 d			0.96 d	
25				5.4 d		5.8 d				
15							0.8		0.14 d	
5	d	4.7		1.2 d						d
6	d	6.2		2.7 d						d
10					< 1.2	< 0.27 d			1.5 d	
11					< 3.4	< 4.1			d	
12				3.7		0.8			0.3 d	
16				0.9		0.5	0.4	0.4	0.3	
17							0.4 d		0.3	
18		0.4 d		0.6 d						
19				14						
27		4.7 d				5.6 d			0.3 d	
28		3.7 d				6.5 d			d	
32			< 15.6	< 15.7 d	0.1				0.2 d	
21				6.5		8.9				
24			< 1.6	< 1.6	< 0.3	< 0.3				
26		6.2 d				7.7 d			< 0.15 d	

Glove extracts

In order to compare the patch test results of the gloves with those of the extracts a total number of 14 extracts was prepared in Vienna (table 46)

Patch test technique

The skin was stripped with Tesa-Tape (3M, 2.5 cm) prior to the application of the test samples. One piece of tape was to strip the entire back (from shoulder to shoulder) from the neck up to the end of the test area. A second tape then was used in the opposite direction from the lower part of the test area to the neck. This procedure was repeated for three times, so that the whole test area was stripped for six times. The tape was pressed against the back, holding both ends, no further pressure was added.

The pure rubber chemicals were tested in petrolatum at a concentration of 1 %, except MBT which was tested at 2 % in petrolatum, using small Finn chambers (9 mm in diameter). Extracts were tested in small Finn-chambers using 15 : 1 on a filter disk. Pieces of gloves (2.5 x 2.5 cm) were applied flat onto the stripped back, fixed and partly covered by large Finn-chambers (11 mm diameter). Finn-chambers was fixed by Scanpore tape. After a two days application the test substances were removed and the reactions were read 1 h later (48 h) and at the next day (72 h). The scoring was carried out according to the ICDRG, considering only + or stronger reactions as positive.

Comparison of the patch test results with the chemical analysis

It was more difficult than expected to find patients with type IV allergies to rubber chemicals. Most of our former patients were allergic to thiurames, a class of accelerators which almost completely disappeared within the last years. Therefore 14 of 50 of our currently recruited volunteers had shown a positive patch test response to thiurames during former evaluations but unfortunately had become negative in the mean time due to the lack of further contact to thiurames.

Since the patch test response has to be evaluated for at least four days during which the volunteers have to report daily to the hospital it was not possible to repeat former patch tests without paying reimbursement to all patients. Therefore the whole panel of gloves was tested in all volunteers despite the risk of any angry back syndrome (unspecific reactions in the presence of too many positive reactions), which however happened in one of our patients. Eight of 50 volunteers were used to optimize and to adapt the test conditions in the two participating hospitals.

The remaining evaluable 25 volunteers were included in the statistical analysis (table 31).

In the 25 patients a total of 432 glove reactions were evaluable. The interpretation of these results had to be simplified, because of the differences in pattern of sensitivity in patients and the content of rubber additives in the gloves. Several studies have confirmed, that the reactivity to carbamates, without any concomitant reactivity to thiurams is extremely rare (Knudsen 96, Logan 89). We therefore decided not to distinguish between a thiuram derivative (mono, di or tetrasulfide) and the corresponding dithiocarbamate. For example volunteers with a positive patch test to either PTT, PTD or ZPC were thus expected to have positive patch test reactions to gloves where any of these derivatives had been detected by analysis. Similarly, patients reacting to either ZMBT, MBT and/or MBI were expected to react to gloves where at least one of these chemicals were present. TETD, TMTD and TMTM were not simultaneously tested with gloves. Hence the patch test results with the gloves, containing TETD or TMTD (3 gloves) were not further evaluable.

The patch test reactions to the gloves were thus classified as 'expected positive', 'expected negative', 'unexpected positive' and 'unexpected negative'. The distribution of these four categories is shown in figure 35. Based on these results, the chemical analytical test methods had a sensitivity of 71 % to predict positive reactions, and a specificity of 61 % to predict negative reactions. This sensitivity and specificity are quite similar to those calculated if the manufacturers information were considered.

Table 31 Positive patch test results of the 25 patients evaluable.

Patient	Centre	Sex	Age	Positive patch test to	
				glove number:	rubber chemicals
1	Erlangen	m	57	3,6,9,18,19,25,27,28	ZDMC, PTD
3	Erlangen	m	19	9,16,17,28	ZBMT
7	Erlangen	f	64	3,5,6,12,19,27,28,(25)	ZDEC
8	Erlangen	f	37	9,10,11,15,16,18,32	ZMBT, MBT
9	Erlangen	f	59	9,15,16,27,28	ZMBT, MBT, MBI
10	København	f	39	3,5,6,9,19,25,27,28,32	PTD, PTT, TBTD, ZDMC, ZPC, ZDEC, ZDBC
11	København	f	48	3,5,6,12,19,25,27,28	PTD
13	København	m	56	none	ZMBT
15	København	f	49	3,5,6,12,18,19,25,27,28	PTD, ZPC, ZDEC
17	København	m	49	3,5,6,9,12,15,17,18,19,25,27,28	PTD, ZDMC
18	København	f	21	3,5,6,12,21,25,26,27,28	PTD
23	København	f	22	3,5,6,12,19,25,27,28	PTD
24	Erlangen	f	31	3,5,6,12,19,21,24,25,26,27,28	PTD
26	Erlangen	f	35	25,18,34	ZMBT
27	Erlangen	f	33	3,5,6,15,19,21,25,26,27,28,32	ZDMC
28	Erlangen	f	56	3,5,6,10,12,15,18,19,21,24,25,26,27,28,32	PTD; ZDMC; ZDEC
31	Erlangen	m	31	3,5,6,12,18,19,21,25,26,27,28,34	PTD
32	Erlangen	m	26	3,5,6,9,12,18,19,24,25,26,27,32	ZDMC; PTD
33	Erlangen	f	26	18,34	ZMBT; MBT
35	Erlangen	f	33	3,5,6,10,12,15,18,19,21,24,26,27,28,32	PTT; ZDEC
40	Erlangen	f	36	3,5,9,10,11,16,17,19,21,24,26,26,27,32,34	PTD; PTT; TBTD; ZDMC; ZPC; ZDEC; ZDBC
41	København	m	25	3,5,6,9,10,12,15,16,17,18,19,21,24,25,26,27, 28,31,32	PTD; ZDMC; ZDEC
42	København	f	51	3,5,6,12,15,18,19,21,25,26,27,28,32	PTD; ZPC; ZDEC
44	København	f	19	15	ZMBT; MBT
45	København	f	38	9,15,10,26,31	ZMBT; MBT

Comparison of the patch test response to gloves and the corresponding acetone extracts in glove allergic volunteers

The volunteers was additionally tested with the acetone extracts of nine different gloves which were prepared as shown in table 46. Since not all patients were tested with the same glove extracts only 157 reactions were further evaluated. The results (table 32) revealed a strong correlation between patch test reactions to gloves and their corresponding extracts with $p < 0.005$ in the χ^2 -test .

Table 32 Patch test response of nine different gloves and their corresponding acetone extracts.

	Positive with glove	Negative with glove
Positive with extract	68	10
Negative with extract	22	57

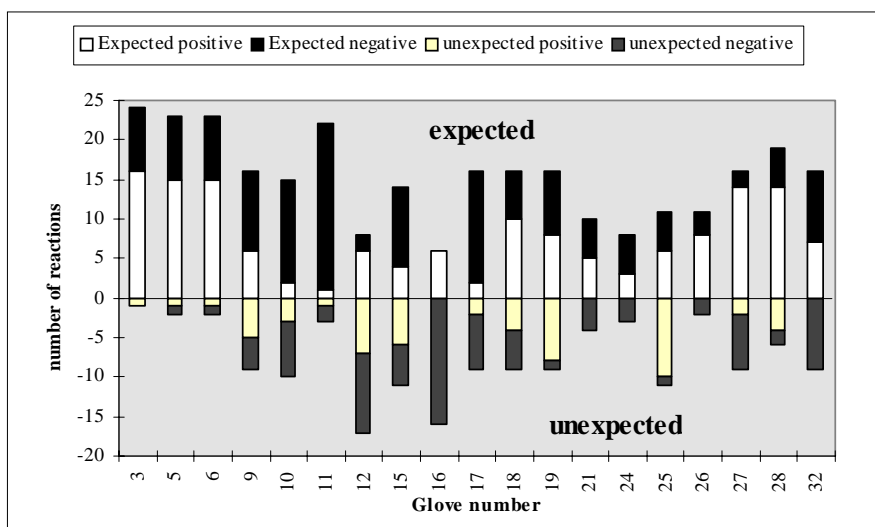


Figure 35 Distribution of the expected (+ scale) and unexpected (- scale) reactions with the different gloves tested in 25 glove-allergic volunteers.

Watery extracts

Nine of 25 glove-allergic volunteers were additionally tested with two watery extracts listed in table 46. The chemical analysis revealed no detectable amounts of any accelerator in glove 9 and only traces of ZDEC in glove 25. These results were in line with further experiments (table 15 ota) which clearly showed that none of the accelerators was soluble in water in measurable amounts. Surprisingly these watery extracts revealed positive in most of the evaluated volunteers, although no positive reaction was expected especially in view of the negative results with former acetone extracts (table 33).

Possible reasons for these unexpected positive results may be an unspecific irritant patch test response or the presents of some water soluble breakdown products in the evaluated glove extracts. However, these very interesting results, which may be important for a future improvement of the diagnostic procedures for type IV allergies to rubber chemicals, have to be confirmed by further investigations apart from the current project.

Table 33 Comparison of the patch test results of two watery extracts and the corresponding acetone extract found in 9 different glove-allergic volunteers.

		expected positive	expected negative	unexpected positive	unexpected negative
Glove 9	Acetone extract	0	5	0	4
	Watery extract	2	0	5	2
Glove 9	Acetone extract	0	6	0	3
	Watery extract	2	1	5	1

Conclusion

The aim of this part of our study was the selection and optimization of analytical procedures for the measurement of rubber chemicals (type IV allergens) in gloves. These analytical procedures should reflect the allergological potency of the gloves as best as possible. Due to the large number of different chemicals used in gloves this task was much more complex than the development of an optimal method for the determination of latex proteins. In view of the allergologic significance the establishment of an appropriate extraction procedure was very problematic and time-consuming. Watery (buffered) extracts seemed to be most physiological (although the lipid layer of the skin may participate in the transport of water-insoluble accelerators) but however, proved to be unsuitable for the chemical analysis. Methylene chloride in reflux, a common method for the determination of accelerators, was considered to be unsuitable from allergiological point of view, since it extracts the total amount of accelerators which is not fully available on the glove surface and therefore only partly relevant.

The acetone extraction as presented above proved to be quite suitable for the determination of allergological relevant concentrations of accelerators in gloves. The sensitivity of 71 % and the specificity of 61 % in comparison to the patch test results were within the same range as the patch test responses to glove pieces used for the evaluation of type IV allergies to gloves.

The high patch test response to watery extracts found in our study may be important for accelerators in gloves, but however needs further confirmation by additional experiments apart from our project.

2.2.7 Inter-laboratory test on the methods for the determination of accelerators

The detailed description of the method given in ANNEX 2 has been submitted to all participants. Additionally, information presented to the participants as overheads and discussed in the theoretical part of the workshop are listed below:

- GENERAL SCHEME and SUCCESSION OF SINGLE STEPS
- GENERAL NOTES
- SCHEME OF THE ANALYSIS OF STANDARDS
- SCHEME OF THE ANALYSIS OF EXTRACTS
- SAMPLES AND STANDARDS
- SCHEME OF THE TREATMENT OF EXTRACTS FOR GC- AND HPTLC-ANALYSIS
- Description of the EXTRACTION STEP
- Description of the CALIBRATION STEP
- Description of the HPTLC-ANALYSIS
- Description of the GC-ANALYSIS

After the theoretical part of the workshop, all attendants intending to participate in the exercise practised all procedures necessary for performing the analysis (glove extraction, HPLC, GC, evaluation of the results).

The samples submitted for the inter-laboratory test and their intended purpose are described in Fig. 36 (copy of the overhead). All extracts were submitted as „dry extracts“, i.e. after evaporation of acetone, in closed plastic vials and had to be dissolved immediately prior the start of the work in a known amount of solvent. For all results which were to be reported, detailed forms were submitted to the participants; these forms are not included in this report.

The results of the exercise are presented in Fig. 37 through Fig. 46. In the light of this evaluation, ZMBT seems to be rather difficult to estimate. In fact, some quantitative results are higher as they should be if the solubility of the accelerator in the extraction solvent is concerned. These higher results are thought to be due to a migration of ZMBT on the latex film surface and its rinsing off in the course of the extraction procedure (as undissolved solid). In such a case, small differences in the further handling of the extract can lead to enormous differences in the result of the analysis.

The inter-laboratory test concerning accelerators has been evaluated as follows:

- (1) The evidence for or against the proposed procedure is relatively low because of the small number of participants and self-willed reduction of the work programme by two of them
- (2) Nevertheless, with some exception, most results were always in the same range in both extracts and gloves as far as the analysis were performed (it can be well seen in the summary-diagrams plotted for ZDBC/ZDiBC; ZMBT (only partially) and ZDEC/TETD)
- (3) The accordance of the results obtained by the Lab No. 1 and Lab No. 3, which both ran the full program, was relatively good with the exception of
 - extract Y and glove A1&A2 (found amounts of ZDiBC),
 - extract Z and Glove B (qualitative analysis with respect to ZDMC) and finally
 - glove D (found amounts of ZMBT)
- (4) If also Labs No. 2 and 4 are involved in the evaluation, the above mentioned exceptions concern particularly:
 - Labs No. 2 and No. 4 did not find any ZMBT in extracts S and X, whereas Labs No. 1 and No. 3 did
 - Lab No. 2 found as low of an amount of ZDiBC in extract Y as Lab No. 3, and at the same time in this extract Lab No. 4 found as high of an amount of ZDiBC as Lab No. 1
 - Lab No. 2 found twice the amount of ZDBC found by others in extracts S and X and still more in extract Y

- Only Lab No. 1 found ZMBT in glove A1&A2 (Lab No. 2 did not analyse any gloves and Labs No. 3 and 4 did not find any ZMBT in glove A1&A2)
- Similarly, as in the case of extract Y, Lab No. 4 found as high of an amount of ZDiBC in gloves A1&A2 as also Lab No. 1 did

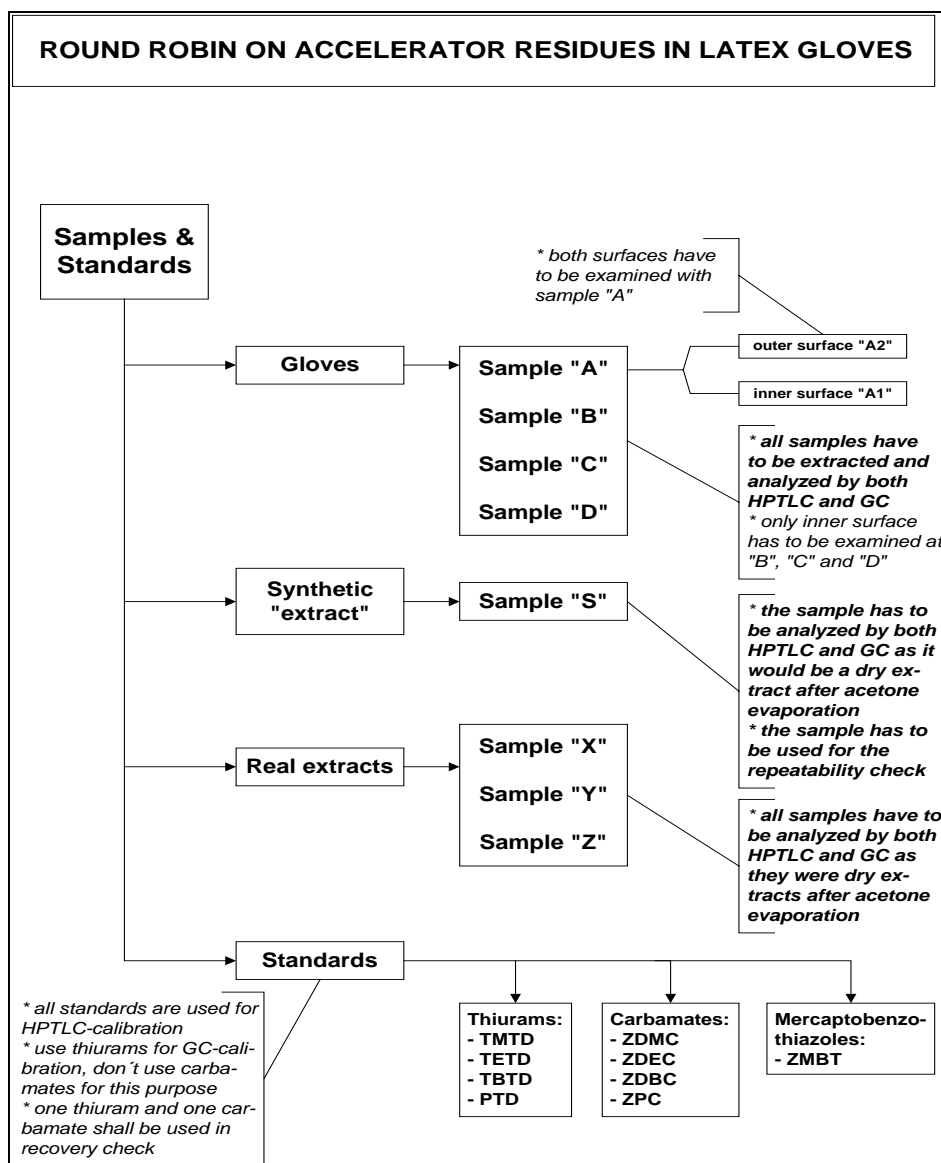


Fig. 36: Samples submitted for the inter-laboratory test

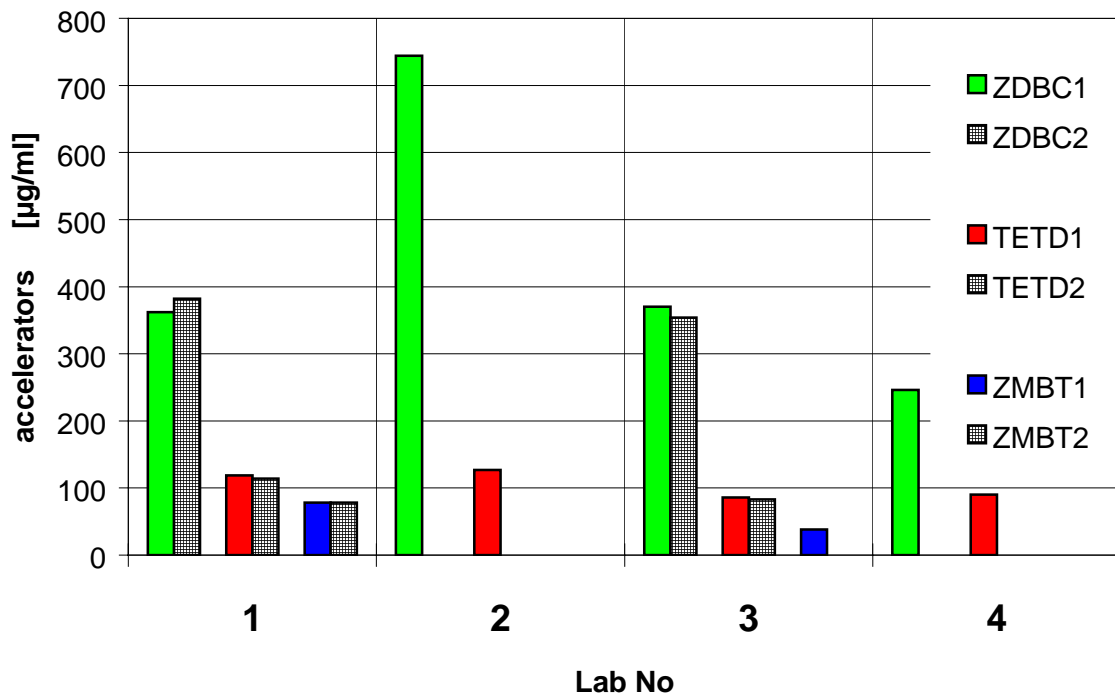


Fig. 37: Results of the inter-laboratory test - Extract S

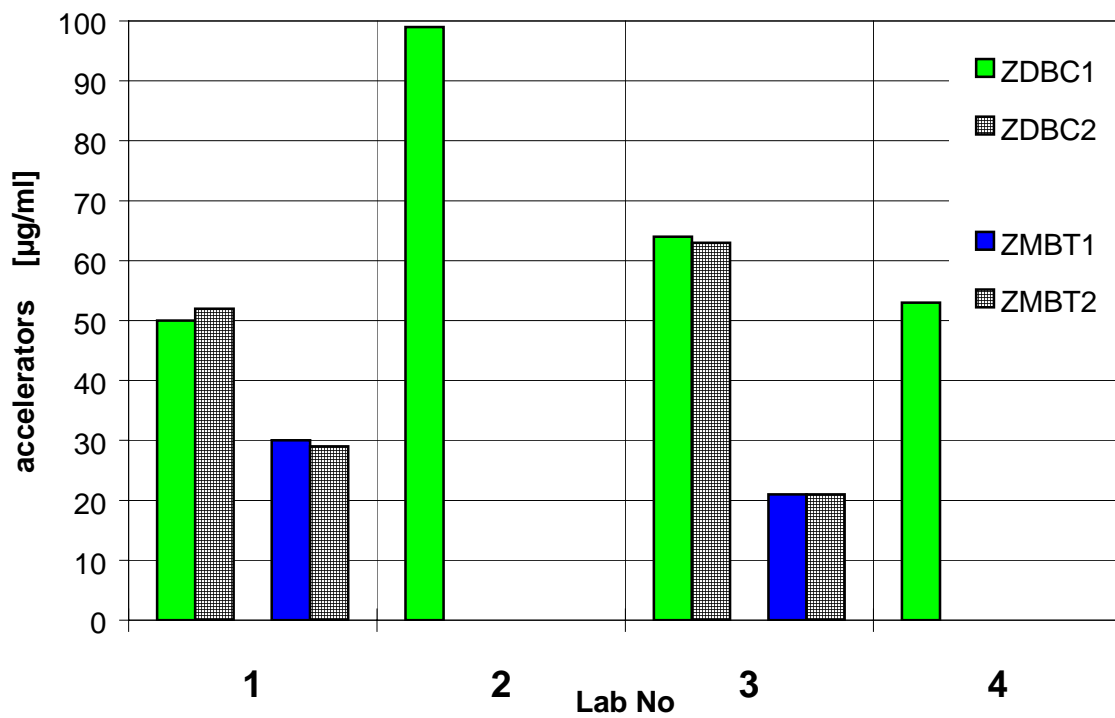


Fig. 38: Results of the inter-laboratory test - Extract X

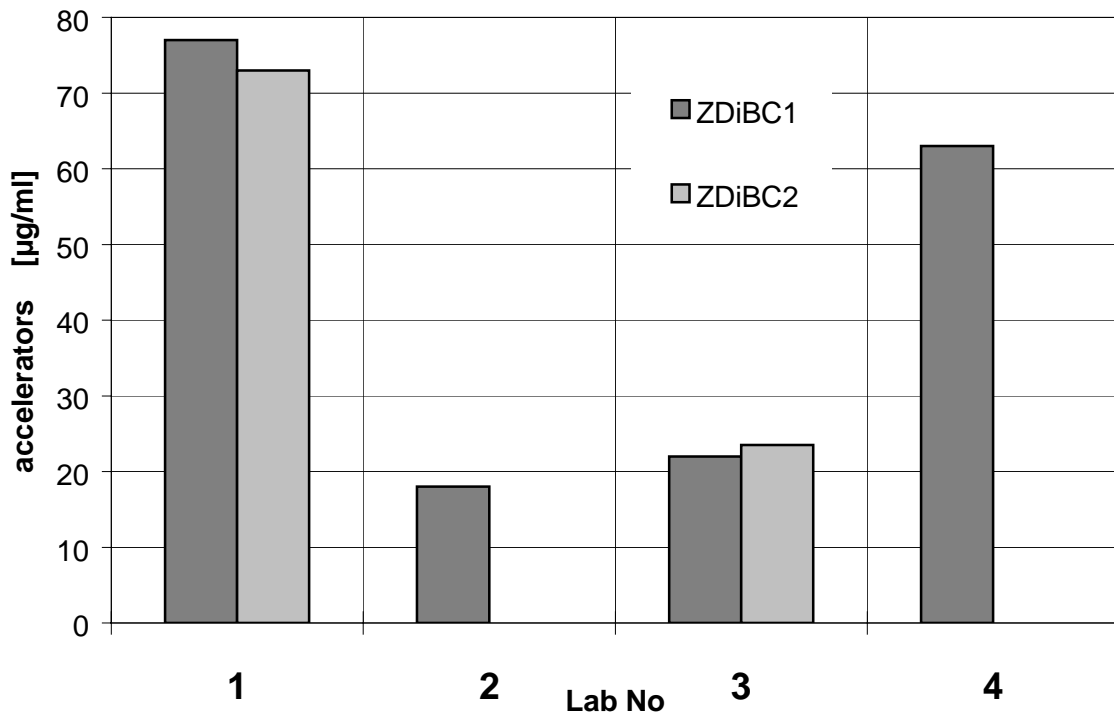


Fig. 39 Results of the inter-laboratory test - Extract Y

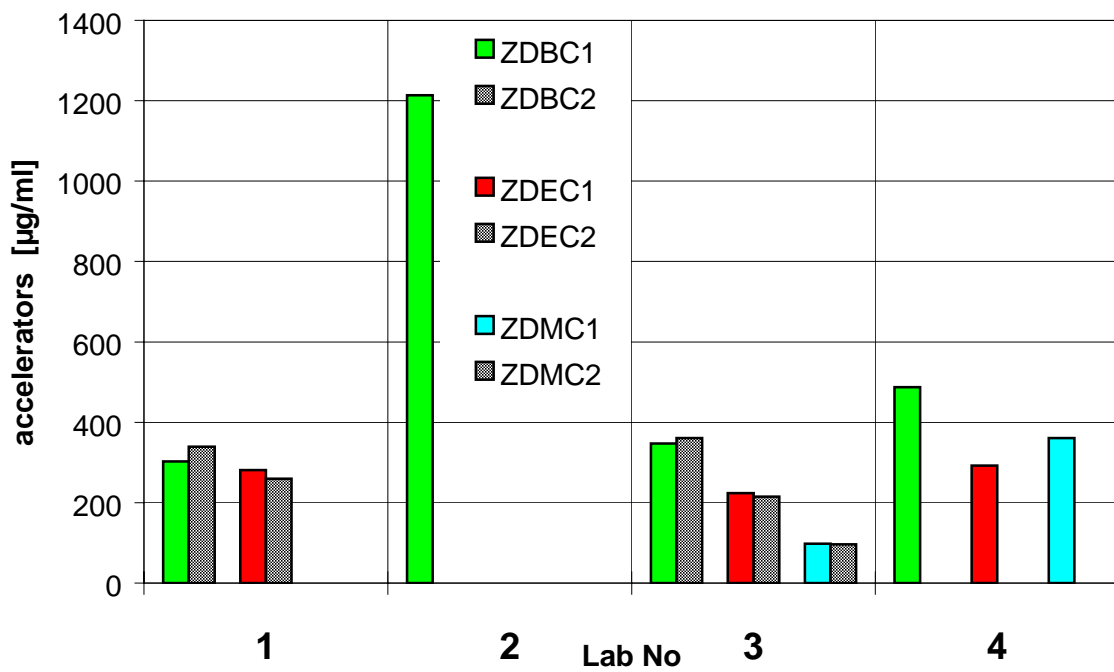


Fig. 40: Results of the inter-laboratory test - Extract Z

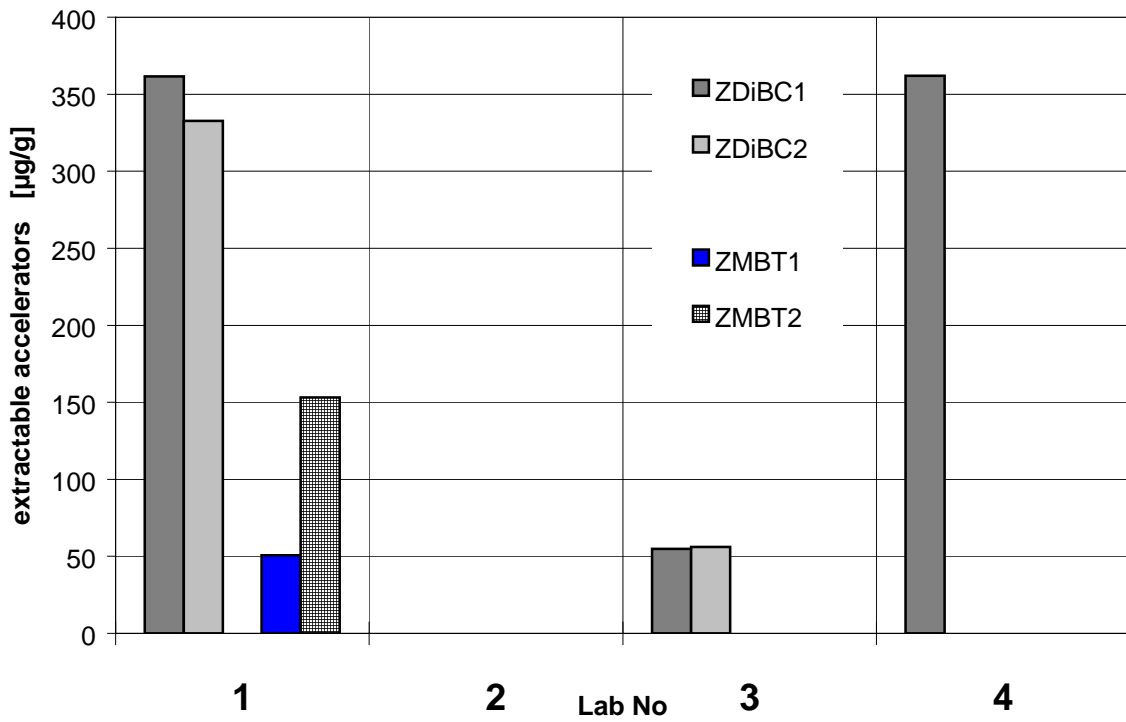


Fig. 41: Results of the inter-laboratory test - Glove A1

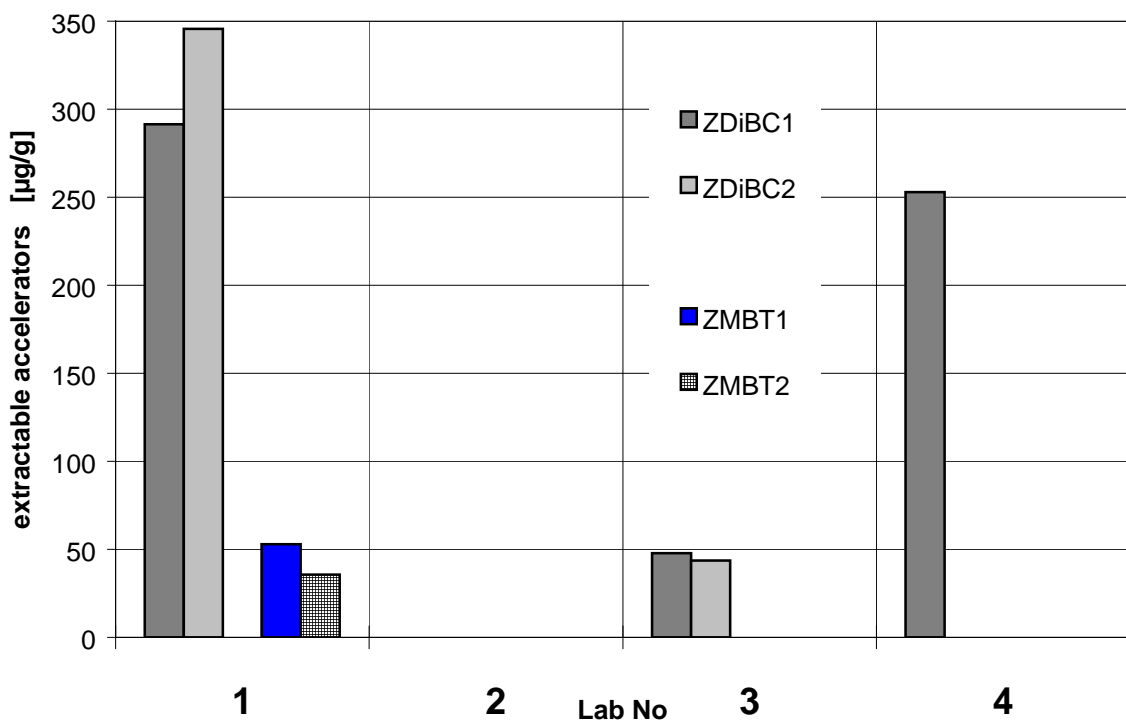


Fig. 42: Results of the inter-laboratory test - Glove A2

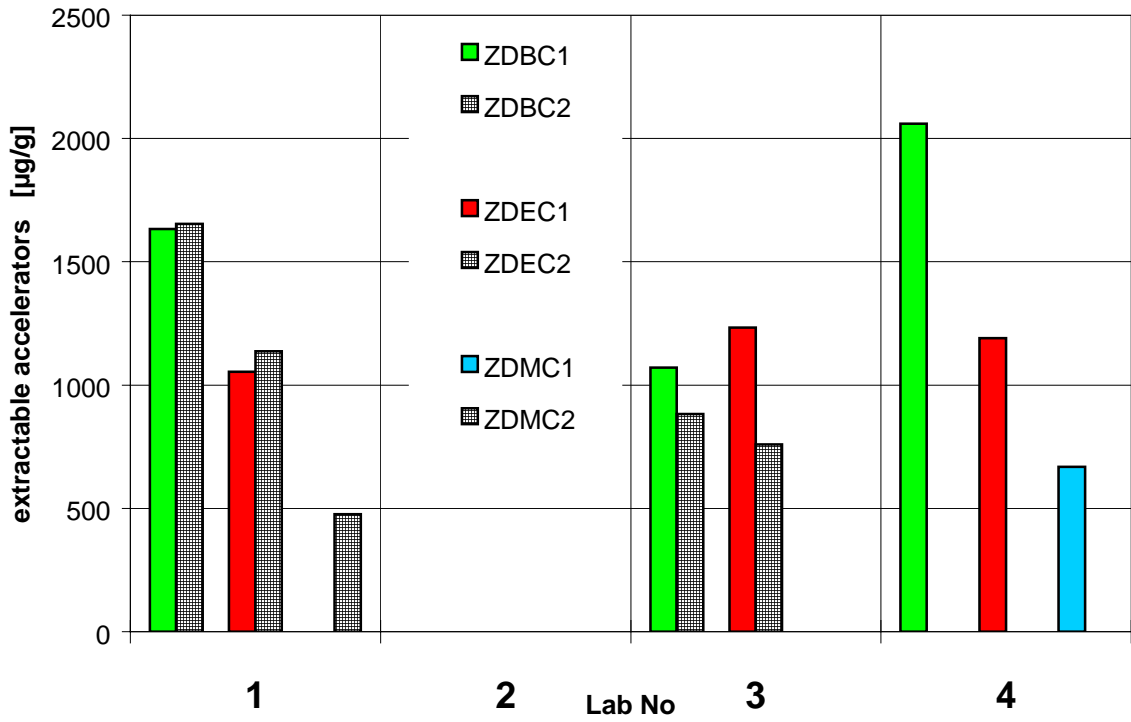


Fig. 43: Results of the inter-laboratory test - Glove B

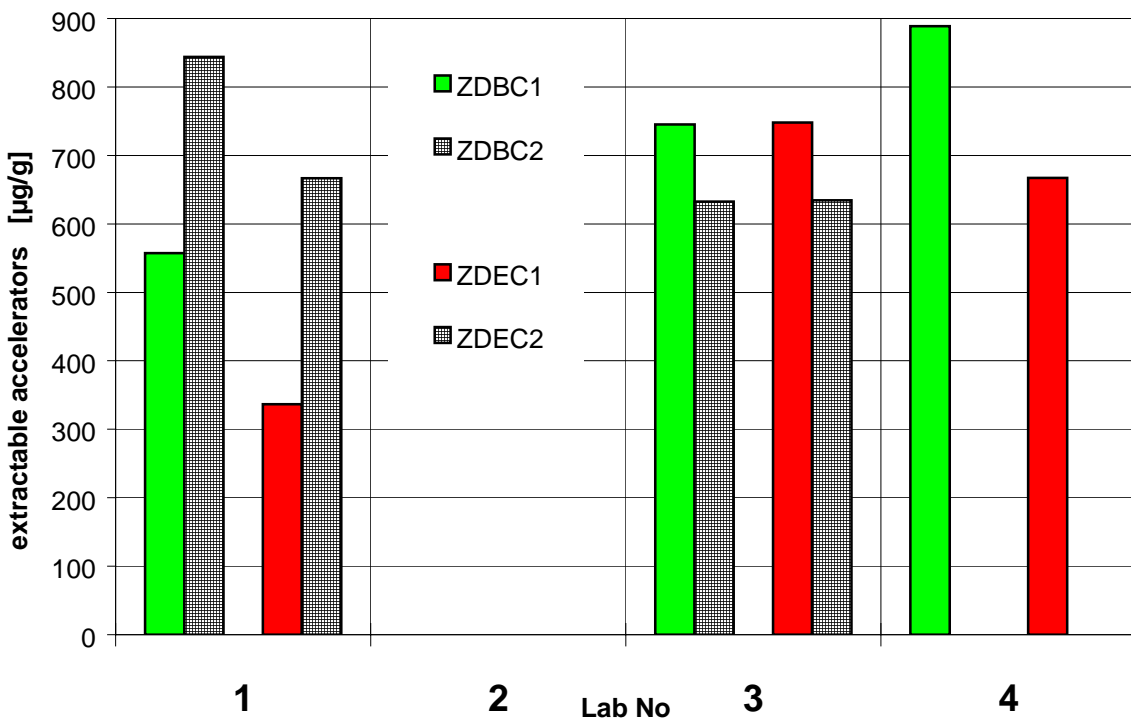


Fig. 44: Results of the inter-laboratory test - Glove C

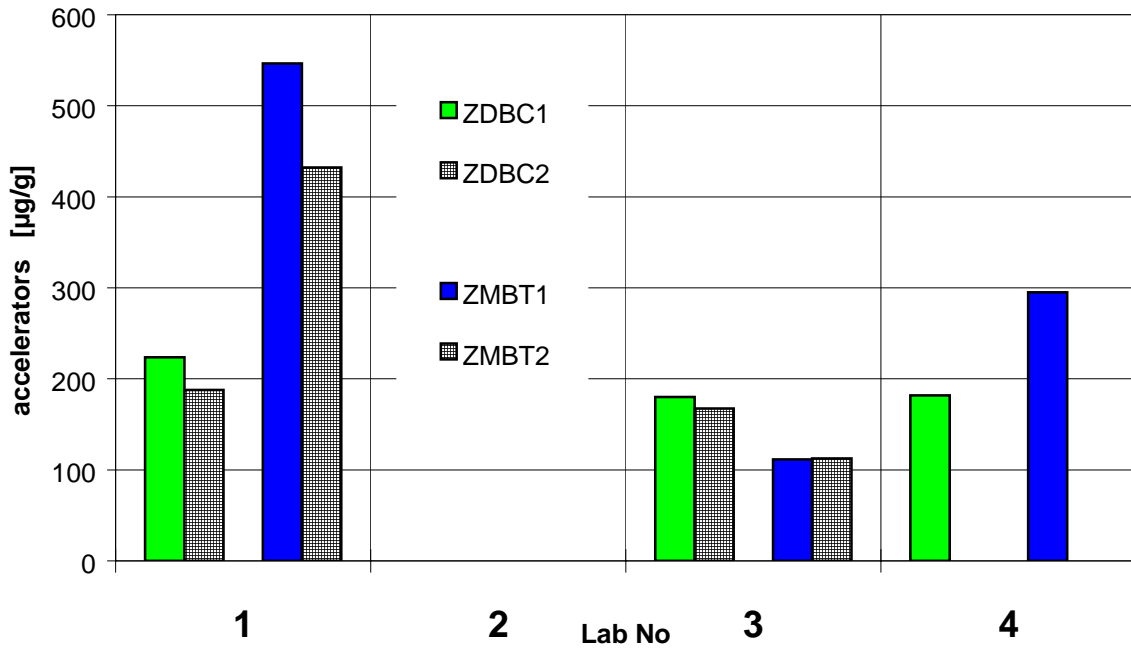


Fig. 45: Results of the inter-laboratory test - Glove D

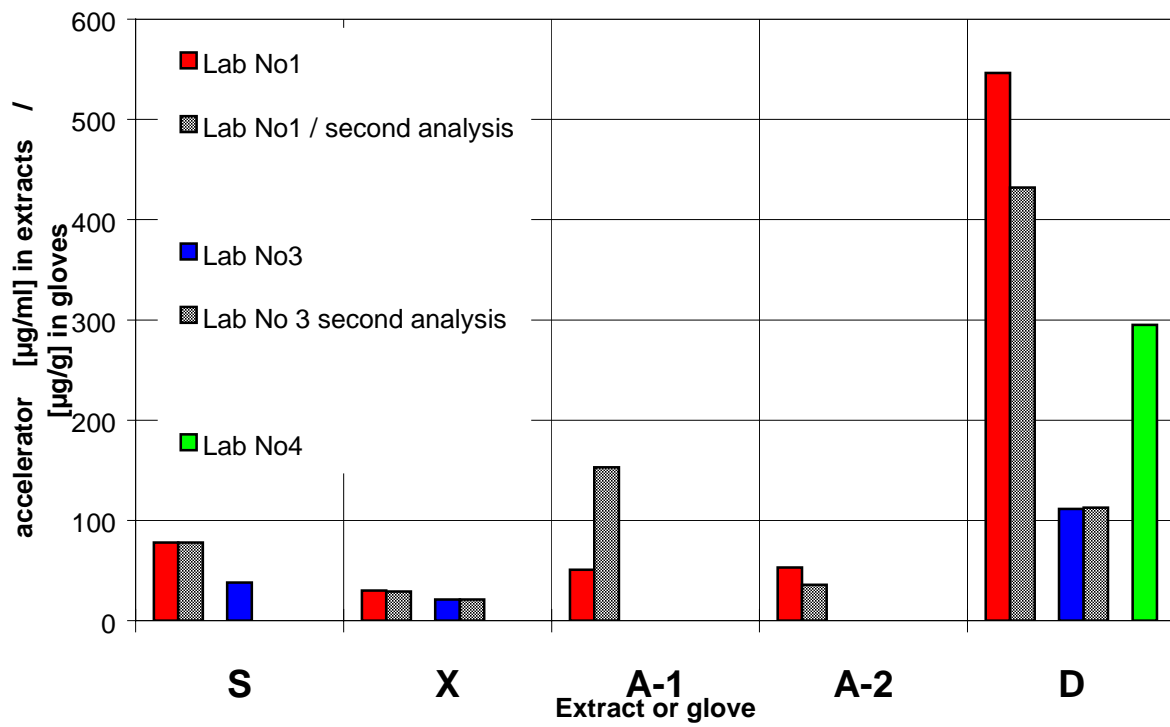


Fig. 46: Results of the inter-laboratory test - Estimation of ZMBT in extracts and gloves

2.3 Establishment of methodological procedures and their validation

Based on previous described experimental work in the analytical laboratory and on the results of the clinical tests, standard procedure protocols regarding the estimation of latex proteins and accelerator residues in medical gloves made of natural rubber latex were proposed for the adoption by CEN (involving in prEN 455-3)

2.3.1 Drafts of procedure protocols for the adoption by CEN

2.3.1.1 Quantification of latex proteins

The standard procedure protocol for the quantification of latex proteins in single-use medical gloves, proposed for involving in prEN 455-3 and in summer 1997 adopted by CEN TC 205/WG 3, is described in Annex A.

2.3.1.2 Quantification of accelerators

The standard procedure protocol for the qualitative estimation of extractable (bioavailable) residues of vulcanisation accelerators and their quantification in medical gloves for single use, proposed for involving in prEN 455-3, is described in ANNEX 2.

This procedure has not been adopted by CEN TC 205/WG 3 for the integration into prEN 455-3 in the meeting held in May 1997. The main reasons are listed below:

- There is a great difference between the risk caused by latex proteins and caused by chemicals (vulcanisation accelerators) as allergies of Type IV are not dangerous to life.
- At present the number of allergies of Type IV is not increasing as fast as the number of allergies of Type I because glove manufacturers are changing latex formulation and eliminate thiurams, which are known to be stronger allergens than the alternative accelerators (dithiocarbamates and mercaptobenzothiazoles).
- At the time of the decision for or against the adoption, the proposed procedure had not yet been validated (whereas the procedure for the quantification of latex proteins passed two round robins organised by the project team and had been discussed and harmonised with ASTM before it was adopted by CEN).
- In contrast to the procedure for the quantification of latex proteins, the proposed procedure for the accelerator analysis was quite new for attendants of the CEN-meeting in May 1997; its adoption was rejected until more experience was gathered.
- The proposed procedure is complicated and expensive if statistically relevant results are required. The costs of multiple analysis cannot be reduced by simple means as, e.g., the use of microtitre plates in the quantification of latex proteins.

3 CONCLUSION

1. The determination of extractable proteins in latex gloves.

(Department of Dermatology, University of Erlangen)

The known methods for the determination of proteins were designed to measure protein as the main component in biological fluid. In contrast, in latex gloves traces of protein (ppm) have to be measured aside large amounts interfering substances.

We tested six different methods (chemical and immunological) and found the amino acid analysis to be the best method with respect to the allergological correctness.

Because this method was not suitable for the European standard we could show an acceptable correlation to the allergological data by the modified Lowry method.

A main and important part of the method was the extraction of the water soluble proteins from latex gloves. A new method was developed, the double glove method, which reproducibility and repeatability were superior to the other methods tested (cut glove, single glove).

The extraction procedure was harmonized with the American standard procedure with respect to the pH, the temperature and the extraction time.

Three inter-laboratory test were performed in order to evaluate the modified Lowry method and to compare different extraction methods (cut glove, single glove, double glove, dd water, buffer). The best reproducibility and repeatability were found in glove extracts obtained by the double glove method with TRIS buffer pH 8.2 or TES buffer pH 7.4..

On the basis of this study both, the modified Lowry method and the extraction procedure were introduced into the European standard EN 455-3..

2. The allergological evaluation of the method for the determination of proteins in latex gloves.

(Department of Dermatology, University of Erlangen)

At the beginning of our study we did not know how to evaluate the allergological potency of a glove. We therefore planned three possible methods, the prick test in latex allergic volunteers, the glove use test with latex allergic volunteers and the immunoblotting of glove extracts with sera from latex allergic patients.

The prick test response correlated surprisingly good to the results of the chemical analysis of extractable proteins from latex gloves with a perfectly clear mathematical meaning.

In contrast the immunoblotting was not possible with glove extracts (due to the high amount of small peptides) and the glove use test was very time and volunteer consuming.

Therefore we focussed on the prick test in latex allergic patients which was very successful in the evaluation of the allergological potency of latex gloves.

3. The determination of extractable accelerators and related compounds in disposable gloves.

(Österreichisches Institut für biomedizinische Werkstofftechnik Wien)

The problems were quite the same than in the determination of proteins. The known methods were not evaluated by the allergological response.

A suitable extraction method, using acetone was developed, and several methods for the qualitative and quantitative determination of the accelerators were evaluated (GC, HPLC, HPTLC, colorimetric).

Due to the high variability of the results obtained by HPLC this method is not suitable for a standard procedure, although it is a method for qualitative and quantitative determination of all accelerators tested.

GC is the best method for the quantitative determination of accelerators, but it is not possible to distinguish between dithiocarbamates and thiurames, because the thiurame structure is lost during derivatisation.

HPTLC methods were developed for qualitative determination and screening and were necessary to interpret the results obtained by HPLC (i.e. to distinguish between thiurames and dithiocarbamates).

Due to the complex problem (high number of possible allergens and complicated analytical procedures) and due to the decreasing allergological relevance of the currently used accelerators this

methods were not introduced into the European standard but was evaluated to be useful for a standard procedure. CEN decided to oblige the manufacturer to declare all allergological relevant ingredients on request.

A final Inter-laboratory test could not be performed in the planned volume because only four laboratories could participate the part concerning accelerators..

4. The allergological evaluation of the methods for the determination of accelerators and related compounds.

(Department of Dermatology, University of Erlangen and Department of Dermatology, University of København)

The allergological evaluation of the methods developed in Vienna was very complex and difficult. The main problem was to recruit suitable volunteers to get an almost complete spectrum of adverse reactions to the actual accelerators. Most of the patients in our hospitals were allergic to thiurames which are used decreasingly in latex gloves due to their allergological potency. Therefore it was not possible to find enough volunteers to get statistically relevant results for single substances. Hence, we combined the results of accelerator groups with the same aliphatic side chains.

The extraction procedure should be as physiological as possible. Due to high amounts of unspecific positive reactions watery extracts were not suitable for the allergological evaluation of gloves. The extraction of the accelerators with acetone by the finger method was the most suitable method.

The sensitivity of the determination of the acetone extractable accelerators was nearly the same as the diagnostic sensitivity using the patch test with glove pieces. Hence, the method is a suitable tool for the determination of the allergological potency of gloves concerning the type IV reactions to accelerators.

ANNEX 1

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ANNEX 2

DETERMINATION OF ACCELERATOR RESIDUES IN DISPOSABLE MEDICAL LATEX GLOVES

O Introduction

In the production of NR-latex gloves, vulcanisation accelerators such as thiurams, dithiocarbamates and/or mercaptobenzothiazoles are necessary for a proper vulcanisation of latex. Substances of this type (mostly a mixture of them) are always put into the latex compound. Not all added accelerators decompose in the vulcanisation process, and some of them remain in the finished product. Several accelerator residues can be produced from others during the vulcanisation process so that the declaration of the glove producer regarding accelerators used in the production is not sufficient for the knowledge of the real composition of the accelerator residues in the finished product.

In contact with human skin the accelerator residues can lead to allergic reactions (delayed type of allergy). The lower the content of the accelerator residues in the glove, the lower the allergologic potential of the glove. Yet only accelerators which migrate from the latex film into the skin cause first sensibilization and later allergic reaction. Therefore, if an analytical method for the estimation of the accelerator residues is to be developed in order to determine the allergological potential of gloves, the extraction method, which simulates the migration of the analyte into the skin, is crucial for the correlation between the analytical result and allergological response of a sensitized glove user.

The present extraction method, in combination with the qualitative and quantitative analysis described below, has shown a good correlation between analytical results and allergological response in a clinical test performed in two hospitals [Lit. 1]. According to this procedure, gloves are extracted with acetone under defined conditions. High performance thin layer chromatography (HPTLC) is used for the qualitative analysis. The quantitative determination of accelerators in extracts is carried out by means of gas chromatography (GC-FID). The result of the analysis is given in $\mu\text{g/g}$ latex. The method is suitable for the quantitative determination of accelerators with contents of $10 \mu\text{g/g}$ to $2000 \mu\text{g/g}$ latex.

1 SCOPE

This annex of prEN 455-3 describes

- extraction (sample preparation from the latex glove) and
- analytical methods (including calibration and precision data)
for the qualitative and quantitative determination of accelerator residues of the following types:
 - alkylthiurams
 - zinc-alkyldithiocarbamates
 - mercaptobenzothiazolesin a finished latex product, i.e. medical gloves.

The method does not distinguish 2-mercaptobenzothiazole (MBT), zinc-mercaptobenzothiazole (ZMBT) and 2,2'-dibenzothiazylsulfide (MBTS), as each of these accelerators may be produced from the others during the vulcanisation process. Only ZMBT can be found in glove extracts.

If the qualitative analysis shows that thiurams and dithiocarbamates derived from the same amines are present, the quantitative analysis does not allow a separate determination of thiurams and dithiocarbamates because the quantitative analysis is based on amine residue derivatives. If, however, the qualitative analysis has shown that a particular accelerator type is present, e.g. TBTD, and the corresponding dithiocarbamate, i.e. ZDBC, is not, a quantitative assertion of the result of the GC-analysis by stoichiometric conversion is possible.

2 PRINCIPLE OF ANALYSIS

The accelerators are extracted from one surface of the sample (latex film) by means of acetone in an ultrasonic bath. The extracts are transferred into chloroform without any delay, and the chloroform solutions are used for a further qualitative analysis carried out by high performance thin layer chromatography (HPTLC) and for quantitative analysis carried out by gas chromatography (GC-FID).

Note: Liquid chromatography (HPLC) could be used instead of HPTLC, but past experience has shown that HPTLC is sufficiently feasible and subject to less difficulties than HPLC. Problems in HPLC-analysis of vulcanisation accelerators are e.g.: the instability of zinc-dithiocarbamates, exchange reactions and recombinations of different ligands resulting in increased number of peaks if zinc-salts are converted into more stable complexes with other metals as well as a successive column obstruction. These problems have yet to be solved.

2.1 Thin layer chromatography

Chloroform solutions of the extractable accelerators are applied to a high performance thin layer chromatography plate, and the plate is developed with a suitable eluent. The separated accelerators are detected using spray-reagents and allocated by comparing the R_f -value and color of the TLC spots obtained from the solution of the sample with those obtained from the standard solutions prepared and analyzed under equal analysis conditions.

2.2 Gas chromatography

Dry chloroform solutions of extractable accelerators are derivatized by means of trifluoroacetic anhydride, and the trifluoroacetamides formed are analysed by gas chromatography with e.g. flame ionisation detection. Qualitative confirmation is achieved either by mass selective detection or by analysis with a column of another polarity. Quantitative estimation is carried out by calibration with external standards.

3. REAGENTS

3.1 Analytes

The following accelerators and other related substances can be comprised by the method and are in this case necessary for the calibration:

3.1.1 Thiurams:

- tetramethylthiuram monosulfide, TMTM M = 208,41 g/mol
- tetramethylthiuram disulfide, TMTD M = 240,48 g/mol
- tetraethylthiuram disulfide, TETD M = 296,60 g/mol
- tetrabutylthiuram disulfide, TBTD M = 408,84 g/mol
- pentamethylthiuram disulfide, PTD M = 320,62 g/mol
- pentamethylthiuram tetrasulfide, PTT M = 384,76 g/mol

3.1.2 Zinc-dialkyldithiocarbamates

- zinc-dimethyldithiocarbamate, ZDMC M = 305,87 g/mol
- zinc-diethyldithiocarbamate, ZDEC M = 361,99 g/mol
- zinc-dibutyldithiocarbamate, ZDBC M = 474,23 g/mol
- zinc-diisobutyldithiocarbamate, ZDiBC M = 474,23 g/mol
- zinc-pentamethylenedithiocarbamate, ZPC M = 386,01 g/mol
- zinc-ethylphenyldithiocarbamate, ZEPC M = 458,07 g/mol
- zinc-diisononyldithiocarbamate, ZDiNC M = 750,79 g/mol

3.1.3 Mercaptobenzothiazoles ¹

- 2-mercaptobenzothiazole, MBT M = 167,27 g/mol

¹) Only ZMBT can be found in glove extracts.

- 2,2'-dibenzothiazyl disulfide, MBTS M = 332,52 g/mol
- zinc-mercaptobenzothiazole, ZMBT M = 397,91 g/mol

3.1.4 Others (accelerators and/or antidegradants with possible allergologic potential)

- 2-mercaptobenzimidazole, MBI

Note: Some of these reagents are only available in technical grade without any detailed specification.

3.2 Chemicals

- (1) acetone p.a.
- (2) chloroform >99%, stabilised with ethanol
- (3) 2-propanol p.a.
- (4) ethanol p.a.
- (5) trifluoroacetic anhydride, TFAA >99%
- (6) 2,6-dichloroquinone-4-chlorimide
- (7) copper sulfate
- (8) dithizone (diphenylthiocarbazone)
- (9) n-hexane p.a.
- (10) toluene p.a.
- (11) ethyl acetate p.a.

3.3 Solutions

3.3.1 Stock solutions of standards in chloroform in a known concentration of approx. 1000 mg/l

100 (\pm 1) mg TMTD, TETD, PTD, TBSD, MBT, ZEPIC, ZDiNC and diphenylamine are each weighed with an accuracy of \pm 0,1 mg in a 100 ml volumetric flask and dissolved in approx. 80 ml chloroform [3.2 (2)] by shaking or in the ultrasonic bath. Chloroform is then added up to the reference mark. The exact concentrations in mg/l shall be calculated. The procedure shall be repeated in order to obtain a second standard stock solution.

Note: These standard stock solutions can be stored up to 4 weeks in the dark at + 5°C.

3.3.2 Stock solution of MBI in chloroform in a known concentration of approx. 50 mg/l

25 (\pm 1) mg MBI with an accuracy of \pm 0,1 mg are weighed in a 500 ml volumetric flask and dissolved in approx. 450 ml chloroform [3.2 (2)] by shaking or in the ultrasonic bath. Chloroform is then added up to the reference mark. The exact concentrations in mg/l shall be calculated. The procedure shall be repeated in order to obtain a second standard stock solution.

Note: This standard stock solution can be stored up to 4 weeks in the dark at + 5°C.

3.3.3 Spray-reagent A

0,05% (g/g) dithizon [3.2 (8)] in ethanol [3.2. (4)]

3.3.4 Spray-reagent B

0,5% (g/g) 2,6-dichloroquinone-4-chlorimide [3.2. (6)] in 2-propanol [3.2 (3)]

3.3.5 Spray-reagent C

1% (g/g) copper sulfate [3.2 (7)] solution in deionised water.

4 APPARATUS

4.1 Ultrasonic bath 30-50 kHz

4.2 Test tubes

- (1) Approx. volume 50 ml (\varnothing 20 mm, length 15 cm)

(2) Approx. volume 100 ml (Ø 30 mm, length 20 cm)

4.3 Rotary evaporator

4.4 High performance thin layer chromatography plates

Note: The following HPTLC-plates have shown to be suitable: Merck-HPTLC-plates 10 x 10 cm, silica gel 60, without fluorescence indicator, with a 10 x 2,5 cm concentration zone, used without any activation at elevated temperature. The following eluent has been found suitable when the above plates are used: mixture of n-hexane [3.2 (9)], toluene [3.2 (10)], and ethyl acetate [3.2 (11)], in the ratio (V/V/V) 30:58:12 Suitable test conditions need to be found if other plates are used

4.5 Micropipettes or microliter syringes, ranging from 10 µl to 100 µl

4.6 Developing tank for thin layer chromatography

4.7 Squeeze bottles for spray-reagents

4.8 Gas chromatograph

A gas chromatograph equipped with a capillary column and e.g. a flame ionization detector is used. It should be able to separate each analyte and also separate the analytes from possible interferences. Alternatively, a gas chromatograph with a mass selective detector can be used. This type of detector is also useful when retention times of particular analytes have to be confirmed. Suitable analysis conditions need to be determined for the equipment available.

Due to the soilage of the samples and the non-volatile byproducts present in the sample after derivatization (zinc containing substances), the injection system shall be cleaned regularly. The use of a deactivated precolumn is recommended.

Note: The following conditions have been found to be suitable:

Column.....	25 m x 0.25 mm ID; 100% methyl silicone, 0.25 µm film thickness
Temperature programme	40°C isothermal 4 min 20°C/min up to 280°C
Run time	25 min
Carrying gas	helium 70 kPa
Injector.....	250°C, split 1:10
Detector.....	FID 280°C

Having analyzed various samples under above analysis conditions, no interferences between the peaks of the analytes were observed.

4.9 Microliter syringe 10 µl

4.10 Microliter syringe or digital piston pipette 50 µl

4.11 Microfilter

Microfilter with PTFE-membrane, Ø 3 mm, pore size 0,45 µm

4.12 Glass vials

Borosilicate glass vials, volume 2 ml, with screw- or crimp-caps and PTFE-faced septa

4.13 Graduated pipettes

Graduated pipettes 0,5 ml, 1,0 ml, 2,0 ml, 3,0 ml, 4,0 ml and 5,0 ml

4.14 Drying cabinet

Adjusted at $75^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for the derivatization procedure (GC) resp. at $60^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for the drying of HPTLC-plates after spraying. A water bath adjusted at $75^{\circ}\text{C} \pm 5^{\circ}\text{C}$ can be used alternatively for the derivatization procedure.

Note: The apparatus listed above do not include the standard laboratory equipment, non critical aids and common glassware.

5 SAMPLES

5.1 Glove extracts

Cut $5\text{ cm} \pm 1\text{ mm}$ of the middle finger off a glove and weigh it with a precision of 0,1 mg with a precision balance. Put this part of the glove finger tightly over a dry 50 ml test tube [4.2 (1)] as though the tube was a finger. If the inner surface of the glove is to be examined, the inside of the glove shall be turned outwards before it is put on the glass tube. Put the test tube with the latex sample into a 100 ml test tube [4.2 (2)] containing $10 \pm 0,1\text{ ml}$ of acetone [3.2 (1)] so that most of the latex film surface is in contact with the solvent but the upper edge of the cut finger glove does not submerge in the acetone. Seal the large test tube with a suitable lid and submerge it in a room temperature ultrasonic bath [4.1] for 30 minutes.

After this step is finished, transfer the liquid into a 50 ml round bottom flask, rewash the test tubes with the latex sample with $2 \times 5\text{ ml}$ of acetone and combine the resulting solutions with the extract in the flask. Evaporate the solvent in a rotary evaporator [4.3] completely without any unnecessary delay, and dissolve the dry residue in 2,0 ml of chloroform [3.2 (2)].

The above extraction step shall be carried out at least twice for each glove sample (using two different gloves of the same lot), and at least two extracts for each glove sample shall be analyzed subsequently.

5.1.1 Treatment of samples for HPTLC-analysis

Chloroform solutions obtained according to 5.1 can be applied onto the HPTLC-plates without further treatment. If the concentration of analytes turns out to be insufficient, it can be increased by evaporating chloroform in a stream of nitrogen.

5.1.2 Treatment of samples for GC-analysis

Put 500 μl of the chloroform solution [5.1] in a 2 ml glass vial [4.12] and add 50 μl TFAA [3.2 (5)] to this solution. Close the vial with a septum and a cap and store it at 75°C in a water bath or drying cabinet [4.14] for 30 minutes.

If the reaction mixture is turbid after derivatization, the suspension shall be filtered through a microfilter [4.11] using a syringe and simultaneously refilled into another glass vial [4.12].

5.2 Blank for gas chromatography

The same procedure as in 5.1 and 5.1.3 is applied, only the acetone is used without a latex sample.

5.3 Standard solutions

5.3.1 Standard solutions for HPTLC

Stock solution [3.3.1 od 3.3.2] is used. Depending on the concentration range required, 1 μl to 20 μl can be applied to the start positions of the plate.

5.3.2 Standard solutions for GC

5.3.2.1 Standard solutions in a concentration range between 50 mg/l and 500 mg/l

Using a graduated pipette [4.13], 0,5 ml, 1,0 ml, 2,0 ml, 3,0 ml, 4,0 ml and 5,0 ml of the stock solution [3.3.1] are each pipetted into 10 ml volumetric flasks, and chloroform is added up to the reference mark. The exact concentrations in mg/l shall be calculated. The standard solutions are then derivatized as described in 5.1.3.

The procedure shall be repeated in order to obtain a second series of standard solutions.

5.3.2.2 Standard solutions in a concentration range between 5 mg/l and 50 mg/l

Using a graduated pipette [4.13], 0,5 ml, 1,0 ml, 2,0 ml, 3,0 ml, 4,0 ml and 5,0 ml of the stock solution [3.3.1] are each pipetted into 100 ml volumetric flasks, and chloroform is added up to the reference mark. The exact concentrations in mg/l shall be calculated. The standard solutions are then derivatized as described in [5.1.3].

The procedure shall be repeated in order to obtain a second series of standard solutions.

5.3.2.3 Standard solutions of MBI in a concentration range between 10 mg/l and 50 mg/l

Using a graduated pipette [4.13], 2,0 ml, 4,0 ml, 6,0 ml, 8,0 ml and 10,0 ml of the stock solution [3.3.2] are each pipetted into 10 ml volumetric flasks, and chloroform is added up to the reference mark. The exact concentrations in mg/l shall be calculated. The standard solutions are then derivatized as described in [5.1.3].

The procedure is repeated in order to obtain a second series of standard solutions.

5.4 Samples for recovery check

50 (\pm 1) mg each of TMTD, TETD, PTD, TBTD, MBT, ZEPC, ZDiNC and diphenylamine are weighed with a precision of \pm 0,1 mg into a 100 ml volumetric flask and dissolved in approx. 80 ml of acetone [3.2 (1)] by shaking or in an ultrasonic bath [4.1]. Then acetone is filled up to the reference mark. The exact concentrations in mg/l shall be calculated.

100 μ l of the standard solution in acetone (prepared as described above), i.e. an amount corresponding to approx. 50 μ g analyte, is added to the extraction solvent, and the procedure is carried out according to 5.1.

6 ANALYTICAL PROCEDURES

6.1 Thin layer chromatography

Mark the end of the run at approx. 1 cm underneath the upper edge of the plate [4.4] and the starter positions approx. 1,5 cm above the lower edge of the concentration zone, with a distance of 1,5 cm between two spots and at least 0,5 cm distance to the side edge of the plate before applying the samples.

Different extracts and standard solutions may be applied using micropipettes and/or microliter syringes [4.5] to one plate [4.4], and the concentrations may be varied by the application of different volumes (1 μ l to 100 μ l). It is necessary to apply at least one standard solution [3.3.1] (5 μ l) to at least one position in order to be able to correct the possible fine quality differences of the plates and any deviation in the developing procedure.

Put the plate into the developing tank [4.6]. Ensure that the starter positions are above the level of the eluent.

Remove the plate from the developing tank with tweezers as soon as the eluent front has reached the upper marking. After the eluent has evaporated at room temperature, spray the plate with spray-reagent A [3.3.3] using squeeze bottles [4.7]. After drying at room temperature, the carbamates and

the thiurams can be identified through their differing colours (see Table 2) and according to their R_f -values determined by means of standard solutions.

Spray the plate thereafter with spray-reagent B [3.3.4] and dry it for approx. 3 min. at 60°C in the drying cabinet [4.14]. The assignment of the spots to carbamates, thiurams and mercaptobenzothiazoles is done according to their colours in line with Table 2 and with respect to their R_f -values estimated by means of standard solutions.

Spray-reagent C is useful in cases where confirmation of the identification of thiurams is necessary. This spray-reagent cannot be used in combination with the spray-reagent A; therefore, the HPTLC separation procedure shall be repeated with a new plate as described above if the spray-reagent C is used.

In order to achieve a proper vapor saturation of the TLC developing tank the tank [4.6], shall be filled with the eluent at least 30 minutes prior to the start of the analysis. Additionally, the walls of the developing tank shall be covered with a filter paper.

Note: HPTLC-plates need not to be activated at an elevated temperature prior to use if plates and eluent mentioned in note in 4.4 are utilized.

6.2 Gas chromatography

Baseline stability and linearity of the detectors shall be checked prior to the start of the tests. The GC-system shall be maintained at constant conditions during the analysis of the samples and standards. The individual analytes are identified by comparison of their retention times with the retention times of standard trifluoroacetamides, and the relevant peak areas are measured.

6.2.1 Analysis of extracts

The sample extracts derivatized according to 5.1.3 are injected into the gas chromatograph [4.8] using a microliter syringe [4.9]. At least one double injection of a sample shall be carried out.

6.2.2 Analysis of standard solutions

The blank [5.2] and standard solutions [5.3.2] are injected into the gas chromatograph. Every standard solution shall be at least double-injected.

6.2.3 Samples for recovery check

The solution prepared according to 5.4 is injected into the gas chromatograph. At least one double-injection shall be carried out.

6.2.4 Calibration

The peak areas of each analyte are obtained. The calibration curves are received by plotting the peak areas A_i against the concentration of analyte in the standard solution. The calibration for the two different ranges of concentration [5.3.3.1] and [5.3.3.2] shall be executed separately. The procedure shall be repeated with the second series of standard solutions.

The correlation shall be linear and the correlation coefficient shall be greater than or equal to 0,996. New standard solutions shall be prepared from the stock solutions and the calibration shall be repeated if the correlation coefficient is lower than 0.996. Linear calibration function (with A_i being the individual peak area and c_i being the individual concentration):

$$A_i = a(c_i [\mu\text{g/ml}] + b$$

is useful in the numerical evaluation of GC-data.

6.2.4 Confirmation

Carrying out the confirmation procedure ensures the correct identification of the analytes and absence of interferences. As far as the quantitative evaluation is concerned, it means that the results calculated according to 6.3 can be considered to be the correct values.

6.2.4.1 Confirmation by mass-selective detection

The extract samples, the blank, and the calibration standard solutions prepared according to 5.1 to 5.3 are examined yet again under (e.g.) below mentioned conditions (see the note). The identification of the analytes' derivatives is carried out by means of their mass spectrum.

Note: The following conditions have been found suitable:

Column.....	30 m x 0.25 mm ID; 100% methyl silicone 0,5 μm film thickness
Temperature program	40°C isothermal 4 min 20°C/min up to 280°C
Run time	25 min
Carrying gas	helium 70 kPa
Injector.....	250°C, split 1:10
Injection method.....	without split
Detector.....	mass spectrometer in scan-mode

6.2.4.2 Confirmation with the help of a column of another polarity

The extract samples, the blank, and the calibration standard solutions prepared according to 5.1 to 5.3 are analyzed a second time under alternative conditions (see note below). The analytes' derivatives are identified by their retention times.

Note: The following conditions have been found suitable:

Column.....	30 m x 0,32 mm ID; 14% cyanopropyl-phenylmethylsilicone, 1,2 μm film thickness
Temperature programme	40°C isothermal 4 min 20°C/min up to 280°C
Run time	25 min
Carrying gas	helium 100 kPa
Injector.....	250°C, split 1:10
Detector.....	FID

6.3 Evaluation of the data

6.3.1 HPTLC

The qualitative evaluation of the appropriate accelerator is carried out by means of the standard substances and by comparison of R_f -values and colours of the TLC-spots. For R_f -values see the note below. The colours of TLC-spots after spraying with reagents A, B, and C are described in Table 1 for orientation.

Tab. 1: Colours of TLC spots

Analyte	Spray-reagent A	Spray-reagent B	Spray-reagent C
all thiurams	pale yellow	dark brown	yellow-green
all Zn-dithiocarbamates	pink-violett	red-brown	red-brown
ZMBT	–	orange	brown
MBI	–	red	–

Note: If HPTLC-plates and eluent mentioned in 4.4 are used, approximate R_f -values given in Table 2 apply.

Table 2: Characteristic R_f -values when Merck HPTLC-plates and *n*-hexane/toluene/ethyl acetate (V/V/V 30:58:12) as eluent are used; corresponding thiurams and dithiocarbamates are placed in the same line.

Analyte	R_f	Analyte	R_f	Analyte	R_f
TMTM	0,35	ZDMC	0,44	ZMBT	0,27
TMTD	0,37			MBT	– ^{b)}
TETD	0,54	ZDEC	0,61	MBI	0,0
TBTD	0,79	ZDBC	0,83		
TiBTD	0,80	ZDiBC	– ^{a)}		
PTD	0,55	ZPC	0,58		
PTT	0,57				
		ZEPC	0,63		
		ZDiNC	0,90		

^{a)} Standard substance not available

^{b)} Cannot be detected with the spray-reagent A,B, or C.

6.3.2 Gas chromatography

6.3.2.1 Determination of analytes in the glove sample extracts

The mean value of a sample's peak areas obtained by double injection [6.2.1] is calculated and put into the assignable calibration straight line [6.2.4]. Based on the read concentration c_{Calib} , the calculation of the concentration of the analyte in the latex [$\mu\text{g/g}$] is carried out according to the following equation, with E being the weight of the extracted glove finger portion in g:

$$c_{\text{Analyte}} [\mu\text{g/g}] = 2 * c_{\text{Calib}} / E$$

Alternatively the evaluation can be carried out on the base of the calibration function [6.2.4] gained from the linear regression of the calibration data. The concentrations of analytes in the latex are then calculated according to the following equation, with E being the weight of the extracted glove finger portion in g:

$$c_{\text{Analyte}} [\mu\text{g/g}] = 2 * (A_{\text{Analyte}} - b) / (a * E)$$

The results of the evaluations of both extracts from one glove shall be combined.

6.3.2.2 Stoichiometric conversion to other accelerator types:

Stoichiometric conversion takes place on the basis of the following equations:

$$c_{\text{Carbamate}} = f \cdot c_{\text{Thiuram}} \quad \text{with } f = M_{\text{Carbamate}} / M_{\text{Thiuram}}$$

$$c_{\text{ZMBT}} = f \cdot c_{\text{MBT}} \quad \text{with } f = M_{\text{ZMBT}} / (2 \cdot M_{\text{MBT}})$$

6.3.2.3 Determination of analytes in recovery test samples

Determination of analytes takes place according to 6.3.2.1. The recovery R_{Analyte} is calculated according to the equation:

$$R_{\text{Analyte}} [\%] = 100 \cdot c_{\text{Analyte}} / c_{\text{Analyte,z}}$$

with $c_{\text{Analyte,z}}$ being the added calculated concentration of analytes and c_{Analyte} the concentration determined by GC.

The recovery of analytes ($R_{\text{Analyte}} [\%]$) should lie between 80% and 100%. In all other cases, procedure 5.4 has to be performed once more.

7 PRECISION

7.1 Validation

7.1.1 Repeatability and Reproducibility

Depending on the analyte-type, the evaluation of the precision experiments within one laboratory at a concentration of 400 µg/g showed the following repeatability [r]:

$$\text{Repeatability: } r = 30,8 - 62,0 \text{ } \mu\text{g/g latex}$$

The reproducibility [R] will be estimated by a round robin in the first half of 1997. It will be carried out by approx. 10 laboratories.

7.2 Detection limits (DL)

The detection limits within one laboratory were between 9,6 µg/g and 18,4 µg/g latex. Detailed data are summarized in Table 3.

Table 3: Detection limits

Analyte	DL [µg/g]	Analyte	DL [µg/g]	Analyte	DL [µg/g]
TMTD	12,8	ZDMC	16,4 *)	ZDiNC	13,6
TETD	10,8	ZDEC	13,2 *)	MBT	15,6
PTD	10,0	ZPC	12,0 *)	MBTS	15,6 *)
PTT	12,0 *)	ZDBC	14,0 *)	ZMBT	18,4 *)
TBTD	12,0	ZEPC	9,6		

*) obtained by stoichiometric recalculation.

Note: The detection limits (DL) within one laboratory were established according to DIN 32 645.

8 TEST REPORT

The test report shall include at least the following information:

- Name and address of the laboratory
- Identification of the glove sample
- Dates of tests
- Analytes found
- Quantitative results in $\mu\text{g/g}$ latex (mean values)
- Glove surface extracted for the analysis (inner surface or outer surface)
- Details on the samples, such as:
 - Origin of sample
 - Date and manner of acquisition of sample
 - Storage conditions
- Performance features of used analytical method
- Reasons for changes of the method, if applicable
- Details on the execution of the confirmation procedure, if applicable
- Reference to this standard
- Names of the staff responsible for the tests
- Date of the report

Literature:

1. EC-Research Project (Contract No. MAT1-CT 940060): „Determination of allergologically relevant compounds in disposable gloves, correlation of chemical, allergological and immunological data“, 2. Interim Report, March 1997