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RESIDUAL ACCELERATOR AND CYTO-TOXICITY STUDIES OF NR SURGICAL GLOVES

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The quantification of residual accelerators present in natural rubber surgical gloves belonging to six commercial brands available in Indian market was carried out. The total accelerator leached into acetonitrile from these gloves was estimated using UV-Visible Spectroscopy. Separation of the glove extract components, followed by specific accelerator identification and subsequent quantification was achieved by HPLC method. HPLC studies have shown that the major accelerator used in all the glove brands was the zinc diethyl dithiocarbamate (ZDEC). The quantification by UV analysis showed that the total residual accelerators varied from 926 to 2725 $\mu g g^{-1}$. The quantification of the specific ZDEC peak by HPLC revealed that ZDEC content varied from 873 to 2616 $\mu g g^{-1}$ among the pre-powdered surgical gloves. The quantification of bioavailable accelerator from different brands showed that more than 14 per cent of the total residual accelerator was available in 'sweat equivalent' aqueous extracts. The cytotoxicity studies of the aqueous extract of the glove brand against L-929 cell line using MTT assay revealed severe toxicity with less than 20 per cent cell viability at various dilutions. The direct contact assay of the glove sample showed severe cytotoxicity of grade 4. The study highlights the need for methodologies to reduce or eliminate the residual accelerators to improve the biocompatibility of NR surgical gloves in Indian market.

Key words: Cytotoxicity, Gloves, HPLC, Residual accelerator

INTRODUCTION

Natural rubber (NR), Neoprene, Nitrile or Vinyl based disposable gloves are often worn by health care professionals as hygiene and contamination protection measures. In the market of disposable gloves, NR gloves (powdered, powder free and surgical) leads the segment with a total share of 66 per cent, followed by nitrile (28%) and vinyl (6%) as per the International trade data, 2015 (https://www.topglove.com). Both NR and synthetic gloves have its own advantages and limitations. The NR gloves have the advantages of high durability due to high tensile strength and puncture resistance. The NR gloves are just like a 'second skin' with best comfort, fit, flexibility and memory. In addition, the excellent touch sensitivity and dexterity, superior donnability and wet/dry grip and broad chemical resistance further makes them preferable over synthetic gloves specifically for long and complicated surgical procedures. Economically they are of lower cost than nitrile and neoprene and environmentally friendly due to its biodegradability. Their market lead is

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certainly attributed to the best balance of price versus performance. The consumers desire to go with greener and healthier products and the world need to shift to renewable technologies and sustainable economy is expected to certainly drive NR glove industry to still better position.

The pertinent issues of NR latex gloves can be broadly divided into three areas *viz.* powder residues and its associated problems, latex allergy due to latex proteins (Type-I allergy) and chemical allergy due to residual chemicals (Type IV allergy) (Heese *et al.*, 1991; Nettis *et al.*, 2002; Geier *et al.*, 2003). The above three issues finally results in a reduction of the global share of NR latex gloves in competition to synthetic counterparts. In the period of 2011 to 2016 the global share of powdered latex gloves reduced significantly from 55 to 40 per cent and the powder free latex gloves decreased from 26 to 19 per cent, whereas the share of nitrile increased sharply from 11 to 32 per cent during the same period. (https://www.topglove.com). The most propagated/highlighted issue for latex gloves is the latex allergy or the Type-I allergy which is specific to NR latex gloves. The issues related to powder residues and the chemical allergy or the Type IV allergy due to residual chemicals in gloves is common for both NR latex and synthetic gloves. Adverse reactions following exposure to latex products may be categorized into three types as Irritant Contact Dermatitis (ICD), Allergic Contact Dermatitis (ACD) also known as delayed type hypersensitivity/Type IV allergy, and the most highlighted Latex allergy/Type-I allergy leading to immediate hypersensitivity reactions like contact urticaria, angioedema, allergic rhinitis, asthma, or anaphylaxis. The Irritant Contact Dermatitis (ICD) is not a true allergic response while the other two namely ACD is a T cell mediated and latex protein allergy is an IgE antibody mediated allergic responses. The immediate-type hypersensitivity (Type-I allergy) is caused by natural proteins inherent to the rubber tree, which remain on the finished natural rubber products (Carrillo *et al.*, 1998; Charous *et al.*, 2002).

Chemical allergy is more common than latex protein allergy and was identified in the 1930s. Chemical allergy represents approximately 30 per cent of occupationally-induced skin diseases thereby making it a significant occupational hazard (Chen *et al.*, 2004). Chemical allergy is the second largest occupational disability reported to Occupational Safety and Health Administration, USA (OSHA). Occupational skin disease (OSD) is the second most common occupational problem for the general practitioners in Australia (Hendrie *et al.*, 2003). A survey of UK National Health Service (NHS) staff showed that 43 per cent had symptoms of irritant contact dermatitis or allergic contact dermatitis, while 10 per cent showed latex hypersensitivity (Johnson *et al.*, 1997). Today, the growing impact of chemical allergies is clear, considering that 30 per cent of glove-related reactions are chemical allergies, whereas 17 per cent are latex allergies. Over 80 per cent of reported glove-associated allergic contact dermatitis is attributable to chemical accelerators (Heese., 1991) Chemical accelerators commonly used in manufacture of gloves are thiurams, dithiocarbamates, thiazoles and diphenylguanidines. It has been well established that dithiocarbamates show a higher degree of allergenicity and cytotoxicity when compared to other rubber additives (Kaniwa *et al.*, 1986; Kaniwa *et al.*, 1988; Nakamura *et al.*, 1990; Knudsen *et al.*, 1993; Knudsen *et al.*, 1996; Knudsen *et al.*, 2000). The chemical involved penetrates the

skin, resulting in vesiculation, erythema, swelling, cracking and itching of the skin at the site of contact. This dermatitis frequently extends beyond the area of contact. The response is delayed rather than immediate, usually occurring in 6 to 48 hours after initial contact, although symptoms can last up to 4 days. Continued exposure may lead to chronic dermatitis manifested as dry, irritated, cracked, pruritic skin with erythema.

For avoiding these allergy problems, many alternatives are generated like radiation or peroxide vulcanized natural rubber lattices and recent invention of UV curable latex by Ansell gloves and other accelerator free gloves. The radiation/ peroxide vulcanized latices are free from sensitizing accelerators; they suffer from a number of limitations such as poor aging resistance, and low tactile sensitivity. The use of synthetic latex and synthetic latex products is another alternative. Synthetic latex products, though free from allergenic proteins has the same problem of residual accelerators and also suffer from serious disadvantages which include (i) higher modulus than NRL products, (ii) poor barrier integrity (iii) carcinogenic monomers and (iv) release of toxic gases during disposal by incineration. The use of less bioavailable accelerators in glove manufacture has been highlighted but again with lesser acceptance by the industries. The application of extensive post and pre-vulcanisation leaching during manufacturing process has been practiced by glove industries, but with significant quantities of chemicals still remaining within the gloves.

Considerable progress has been made for NRL products to reduce the risks from protein and glove powder through concerted research and strict regulations. The US FDA has decided to ban powdered latex gloves in medical use (http:// www.fda.gov). The specifications of total extractable protein (ASTM D 5712-99), total antigenic protein (ASTM D 6499-16) and the residual glove powder (ASTM D 6124-01) have been provided in American (ASTM) standards and also in International (IS) and European standards. But in the case of residual chemicals, the risks associated are not addressed properly or suppressed due to the incomplete and inconsistent data concerning the bioavailability of the accelerator residues in gloves to the end user (Elizabeth *et al.*, 2005) and the unavoidable dependence of the industry on sulphur/ accelerator based cure systems. So far, there is no standard issued by the American Society for Testing and Materials (ASTM) or International Organization for Standardization (ISO), European Standards or Indian Standards for the maximum allowable limit for the residual dithiocarbamates in latex surgical gloves or synthetic gloves. In this scenario, it would be of great significance to identify and quantify the residual accelerator present in NRL medical gloves. Therefore, it was attempted to understand the current scenario of residual accelerators in surgical gloves in Indian market, develop suitable analytical method to quantify the total residual accelerator and identify the specific type of the accelerators released into acetonitrile and aqueous extracts from surgical gloves and evaluate the cytotoxicity of the aqueous extract.

MATERIALS AND METHODS

Ten commercially available surgical gloves made of natural rubber latex, belonging to six brands ('A' to 'F') were procured from local market and were coded as given in Table 1. Out of the ten surgical gloves, eight pre-powdered gloves (brand 'A' to 'F') and two powder free gloves of

Table 1. **Details of the brands of NRL surgical gloves used in the study**

Sl. No.	Sample code	Year of manufacture	Glove type
1	A	2016	Pre-powdered
2	B	2016	Pre-powdered
3	C	2016	Pre-powdered
4	D	2016	Pre-powdered
5	E	2016	Pre-powdered
6	F 14	2014	Pre-powdered
7	F 15	2015	Pre-powdered
8	F 16	2016	Pre-powdered
9	F (PF)	2016	Powder free
10	B (PF)	2016	Powder free

brand 'F' and 'B' were used in the study. Among the pre-powdered gloves, brand 'F' manufactured in consecutive years of 2014, 2015 and 2016 were procured for the study and labelled as F14, F15 and F16 respectively. HPLC grade acetonitrile from Spectrochem Ltd., India, cuprous sulphate pentahydrate from SISCO Pvt Ltd., India, zinc diethyldithiocarbamate (ZDEC), zinc dimethyldithiocarbamate (ZDMC), tetramethyl thiuramdisulphide (TMTD) from Sigma Aldrich, USA and HPLC grade water from Merck, India were used in the study. All other chemicals used were of laboratory grade.

Optimisation of the preparation of copper-dithiocarbamate complexes from standard ZDEC solution

About 12.11mg of ZDEC (M.wt. 361.93) was quantitatively transferred into a 25mL standard flask. It is made up to the mark using acetonitrile and shaken well for uniform concentration and labelled as stock solution. From this stock solution, 25 mL each of 32, 16, 8 and 4 μgmL^{-1} of standard ZDEC solutions were prepared in acetonitrile by serial dilution. The optimisation of the conversion of ZDEC into copper-dithiocarbamate complexes was carried out using a 100 mM ammoniacal aqueous solution of copper (II) sulphate (24.9 mg of $CuSO_4$. $5H_2O$ in 1mL of 1.6% ammonia solution). A 5 mL each of the standard ZDEC solution (32µg/mL) was taken in 4 different capped bottles and the volume of ammoniacal copper sulphate solution was varied from 10, 20, 30 and 40 µL. After 30 minutes, the extent of complex formation was monitored from the absorbance at 430 nm using UV-VIS spectroscopy.

Preparation of calibration curve for UV quantification

A 20 µL of aqueous ammoniacal copper sulphate solution was pipetted out into four bottles having 5 mL each of the standard ZDEC solution 32, 16, 8 and 4 μgmL^{-1}, respectively. Bottles were tightly capped, stirred and allowed to stand for half an hour for complex formation. A graph was plotted with concentration of ZDEC solution along X-axis and absorbance of the corresponding copper dithiocarbamate complexes at 430 nm along Y- axis.

Quantification of total residual accelerator from glove brands using UV method

About 1g of each glove sample was accurately weighed and quantitatively transferred into air tight bottles. Acetonitrile (10 mL) was pipetted out to each bottle, capped tightly and kept in a shaking incubator for 2 hours at 120 rpm. Then 0.5mL of acetonitrile extract was pipetted out from each bottle and mixed with 4.5 mL of fresh acetonitrile. To this 20 µL of ammoniacal copper (II) sulphate solution was added and the mixture was then kept for half an hour

to obtain the copper-dithiocarbamate complex. The concentration of the total residual accelerator (μg/mL) from each glove sample was calculated by substituting the absorbance value of the corresponding copper –accelerator complex at 430 nm in the UV calibration curve equation. From this, the amount of total residual accelerator present in the glove sample was calculated.

$$\text{Amount of total residual accelerator by UV method (μg/g)} = \frac{\text{Conc. of total residual accelerator} \times \text{dilution factor (100 mL)}}{\text{Weight of the glove sample (g)}} \quad -[1]$$

Development of HPLC protocol for elution of the residual copper-accelerator complex

The HPLC system consisted of two Waters 510 pumps, a pump control module and 7725 Rheodyne injector (Waters, U.S.A). The presence of copper complexes of dithiocarbamates was detected using a variable wavelength UV detector. The detection wavelength used was 320 and 430 nm. Separation was achieved on a 5 μm particle size, 4.56 mm (ID) x 250 mm (L) (Waters) reverse phase C-18 column. Two buffer solutions, Buffer A (95% HPLC grade water and 5% acetonitrile) and Buffer B (95% acetonitrile and 5 % water) were degassed and used for the HPLC analysis. The column was equilibrated with buffer A for 30 minutes at a flow rate of 1 mL/min. The manual injection of 15 μL of copper- diethyl dithiocarbamate complex prepared from a standard solution of 32 μg/mL of ZDEC was injected into the HPLC column through the injection port after centrifugation of the solution at 5000 rpm for 5 minutes. Different isocratic elution conditions with buffer A to buffer B ratio of 10:90, 20:80, 30:70 and 40:60 were attempted to find out the optimal buffer condition for elution, at a flow rate of 1.0 $mlmin^{-1}$ for 30 minutes.

Identification of the accelerator type in glove brands using high performance liquid chromatography (HPLC)

High performance liquid chromatography was used to identify the type of accelerator. The copper complexes of standard compounds of ZDEC, ZDMC and TMTD, were separately prepared using 20 μL of ammoniacal copper sulphate solution. The retention time of each of the copper-complex was analysed using HPLC. The comparison of the retention time of the standard copper-accelerator complexes to the retention time of copper-accelerator complexes of glove extract of the different glove brands at 430 nm was used for the identification of the accelerator type.

Preparation of calibration curve for HPLC quantification using standard solutions of ZDEC

Standard solutions of ZDEC in acetonitrile (ranging from 8 μg/mL to 64 μg/mL) were prepared in a 25 mL standard flask. 5mL of each concentration is pipetted out into a capped bottle and 20 μL ammoniacal copper sulphate solution is added. It was allowed to stand for half an hour to obtain complex formation. The solution was centrifuged at 5000 g for 5 minutes. From each solution 15μL was injected and peak area analysed through the integration of the HPLC profile. A graph is plotted using concentration of ZDEC along X-axis and peak area along Y-axis to obtain calibration curve.

Quantification of residual ZDEC extracted from glove brands using HPLC

The procedure for the extraction of the residual accelerator from different glove brand, conversion of the accelerator to its copper complex and the HPLC elution of the copper-accelerator complex were done as per the above methods. The HPLC elution profile of each glove brand is generated. The area of the peak around 7.4 minutes is obtained from each HPLC profile. The substitution of the peak area into the HPLC calibration curve equation gives the corresponding concentration of the residual ZDEC accelerator in each glove brand from which the residual ZDEC content in each glove brand was calculated using the following equation.

$$\text{Amount of the residual ZDEC accelerator in glove by HPLC method } (\mu g/g) = \frac{\text{Conc. of residual ZDEC from HPLC peak area } (\mu g/mL) \times \text{dilution factor (100 mL)}}{\text{Weight of the glove sample (g)}} \quad - [2]$$

Quantification of the bioavailable accelerator in 'sweat equivalent' aqueous extract

An aqueous buffer equivalent to sweat extract was prepared by mixing 0.5 per cent NaCl, 0.1 per cent lactic acid and 0.1 per cent urea in 100 mL of distilled water and the pH adjusted to 6.5 using 1 per cent ammonia solution. About 1g of each glove sample was accurately weighed and quantitatively transferred to a bottle. 10 mL of aqueous sweat equivalent buffer was pipetted out to the bottle, capped tightly and kept in a shaking incubator for 24 h at 120 rpm. The aqueous layer was separated and mixed with 20 mL × 3 of dichloromethane (DCM) in a separating funnel. The total DCM layer was collected and evaporated to dryness and the residue reconstituted in 10 mL of acetonitrile. 0.5 mL acetonitrile extract was pipetted out and mixed with 4.5 mL of fresh acetonitrile. This was mixed with 20 µL of ammoniacal copper (II) sulphate solution. The mixture was then kept for half an hour to obtain the copper-dithiocarbamate complex. From the absorbance value at 430 nm obtained from UV-VIS profile of each glove extract, and its substitution in UV calibration curve, the concentration of bioavailable accelerator in aqueous buffer extract of the gloves (µg/mL) can be calculated. From this, the amount of total bioavailable accelerator present in the glove sample can be calculated using equation 1.

MTT cytotoxicity assay of the aqueous extract of the glove

American type cell culture (ATCC) Strain L-929 was used for the study. The culture medium used was the minimum essential medium supplemented with Foetal Bovine serum. Extract was prepared by incubating 6 cm^2 of the glove brand 'F15' in 1ml culture medium with serum at 37 ± 1 °C for 24 ± 2 h. After 24 h the extract was diluted with culture medium to get 50, 25 and 12.5 per cent. Cells cultured in normal medium were considered as cell control. Equal volume (100 µl) of various dilution of test samples, extract of negative control, cell control and positive control were placed on sub confluent monolayer of L-929 cells. After incubation of cells with various concentration of test sample and controls at 37 ± 1 °C for 24 ± 2 h, extract and control medium was replaced with 50 µl of 3-(4, 5-dimethyl thiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (1mg / ml in medium without supplements), wrapped with aluminium foil and were incubated at 37 ± 2 °C for 2 h. After

Table 2. **The scoring grade for the direct contact assay**

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 0.33 cm
4	Severe	Zone extending farther than 0.33 cm beyond specimen

discarding the MTT solution, 100 µl of Isopropanol was added to all wells and swayed the plates. The colour developed was quantified by measuring absorbance at 570 nm using a spectrophotometer. The data obtained for the test samples were compared with the cell control.

Cytotoxicity analysis by direct contact method of the glove with cell lines

An in vitro cytotoxicity test using 'direct contact' method was performed using the glove brand 'F15' as per ISO10993-5. The culture medium from the L-929 monolayer was replaced with fresh medium. Test samples, negative controls (polyethylene film) and positive controls (stabilised PVC disc) in triplicate were placed on the cells. After incubation at 37 ± 1 °C for 24 to 26h. the reactivity were graded as 0, 1, 2, 3 and 4 based on zone of lysis, vacuolization, detachment and membrane disintegration as per Table 2.

RESULTS AND DISCUSSION

In response to the growing concern over the allergic reactions to NRL products, regulatory agencies such as the FDA, EEC, and ASTM have put forward essential requirements/specifications with regard to

Table 3. **ASTM specification for surgical gloves**

No	ASTM specifications	NR Latex	Neoprene	Nitrile	Vinyl
1.	Physical properties				
	Tensile strength, MPa				
	(Before /After ageing)	24/18	14/14	14/14	11
	Elongation, %				
	(Before /After ageing)	750/650	500/500	500/400	300
	500 % Modulus MPa	5	NS	NS	NS
2	Pinholes (ASTM D 5151-92)	Nil	Nil	Nil	Nil
3	Powder content (powder free) mg/glove				
	(ASTM D 6124-01)	2	2	2	2
	Powder content (powdered) mg/dm2				
	(ASTM D 6124-01)	15	10	10	10
4	Extractable protein µg/dm^2 (ASTM D 5712-99)	200	NA	NA	NA
5	Antigenic protein µg/dm^2 (ASTM D6499-16)	10	NA	NA	NA

NA- Not Applicable, NS-Not Specified

Acceptable Quality Limit of pinholes, protein content and powder levels in surgical and examination medical gloves made from NR latex and synthetic rubber (Polychloroprene, nitrile and vinyl). The manufacturer is expected to meet these minimum standards while claiming conformity of the product with the quality certification by regulatory agencies. The ASTM specifications for surgical gloves are given in Table 3. It is evident that the threshold level of chemicals has not been specified in the ASTM specification. A method for determining the total extractable accelerators in the finished medical gloves has been provided by ASTM agencies (ASTM D7558-09). The above ASTM method has the limitations that it cannot distinguish between thiuram and dithiocarbamates (*eg.* TMTD *vs* ZDMC) in the glove extract and also cannot identify the specific type of accelerator among the various structural homologues within a thiuram (*eg.* TMTD *vs* TETD) class or a dithiocarbamate (*eg.* ZDMC *vs* ZDEC) class of accelerators. Hence the present study evaluates both the total residual accelerator released into acetonitrile by UV spectroscopy and the identification of specific accelerators and its subsequent quantification by HPLC method. The study further quantifies the bioavailable accelerators released from the glove samples into sweat equivalent aqueous extract and the cytotoxicity of the aqueous extract of glove samples.

Preparation of copper-*bis*-diethyldithiocarbamate complexes from standard ZDEC solution

The preparation of copper bis diethyl dithiocarbamate complex from zinc dithiocarbamate complex was carried out in two stages. In the first step preparation of ammoniacal copper sulphate solution was carried out as follows. The copper ion in the aqueous solution exists predominantly as $[Cu(H_2O)_6]^{2+}$. This complex ion imparts a characteristic pale blue colour to the solution. Reactions of the hexa aqua copper ions with ammonia solution are complicated by the fact that the ammonia can have two quite different functions. It can act as a base (in the Bronsted-Lowry sense) and also as a ligand (Lewis base). In the present study a 100 mM ammoniacal copper sulphate solution is prepared using 1.6 per cent aqueous ammonia solution. The molar ratio between copper ion and ammonia is approximately 1:10. During the initial addition of ammonia solution it act as a base and the clear, light blue, aqueous solution of copper (II) sulphate changes to a powdery, light blue precipitate of copper (II) hydroxide. This occurs through the following steps. Since ammonia is a weak base, hydroxide ion forms

$$NH_3(aq) + H_2O(l) \Longleftrightarrow NH_4^+(aq) + OH^-(aq);\ pKb = 9.25$$

The hydroxide ion reacts with the hexa aqua copper (II) ion to form the insoluble compound, copper(II) hydroxide tetrahydrate:

$$[Cu(H_2O)_6]^{2+}(aq) + 2\ OH^-(aq) \Longleftrightarrow Cu(OH)_2 * 4\ H_2O(s) + 2\ H_2O$$

Further addition of ammonia solution causes the copper ion to go back into solution as a deep blue copper ammonia ion complex. When ammonia acts as a ligand, it is acting as a Lewis base. Ammonia replaces four of the six water ligands around the central metal ion to give the soluble tetraammine diaqua copper (II) ions complex. It's formation is shown in Figure 1 where the maximum absorbance of copper ammonium complex is obtained at 610nm. This ligand exchange reaction involves equilibrium and its stability is very high as evident from the high stability constant (Kc).

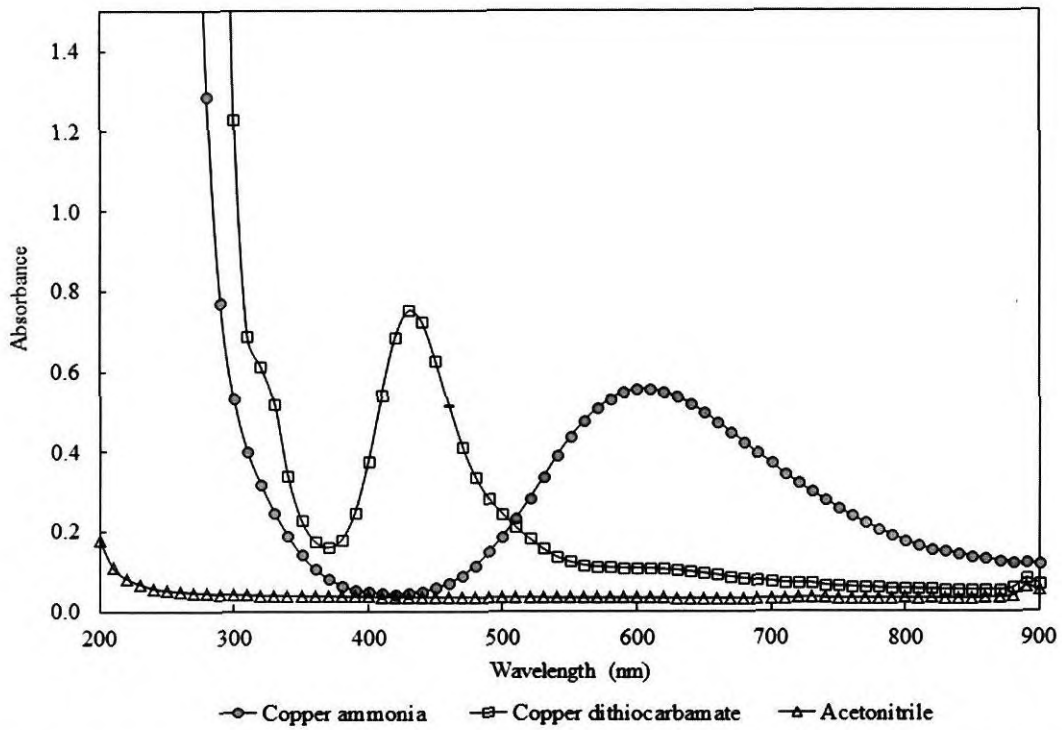

Fig. 1. UV-Visible Spectra of copper-*bis* diethyl dithiocarbamate complex and ammoniacal copper sulphate solution

$[Cu(H_2O)_6]^{2+}(aq) + 4\ NH_3\ (aq) <==> [Cu(NH_3)_4(H_2O)_2]^{2+}(aq) + 4\ H_2O(l)$; Kc = 1.2 X 10^{13}

In the next stage the treatment of ZDEC solution with ammoniacal copper complex results in the formation of copper dithiocarbamate complex. The driving force for the reaction is the chelate effect of the dithiocarbamate ligand and the higher stability of the copper dithiocarbamate complex compared to the corresponding zinc dithiocarbamate complex. The formation of the complex can be studied

Table 4. **The mole ratio between ZDEC and copper (II) ions from copper ammoniacal complex solution**

Vol. of 32 μgmL^{-1} ZDEC solution (mL)	Moles of ZDEC (μmol)	Volume of copper complex Solution (μL)	Moles of Cu^{2+} (μmol)	Ratio b/w ZDEC:Cu^{2+}
5	0.442	10	0.997	1:2.2
5	0.442	20	1.994	1: 4.51
5	0.442	30	2.991	1: 6.76
5	0.442	40	3.988	1: 9.0

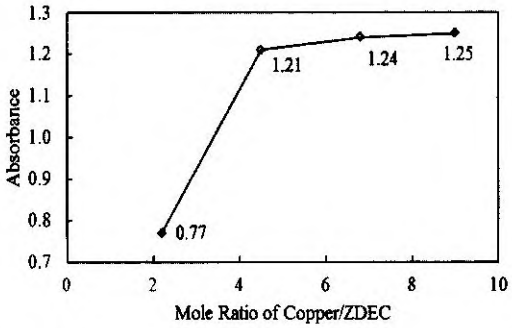

Fig. 2. Influence of ratio between ZDEC and copper (II) ion on the formation of copper-bis diethyl dithiocarbamate complex.

using the UV-VIS spectroscopy as the copper dithiocarbamate complex has strong absorbance with λ_{max} at 430 nm in the UV spectrum. The ammoniacal copper sulphate has λ_{max} at 610 nm and the solvent acetonitrile doesn't absorb in the above regions as shown in Figure 1.

$[Cu(NH_3)_4(H_2O)_2]^{2+}$(aq) + [Zn (diethyl dithiocarbamate)$_2$] <==> [Cu (diethyl dithiocarbamate)$_2$] + [$Zn(NH_3)_4$]$^{2+}$ + 2 H_2O

In the second stage, the optimisation for the quantitative conversion of ZDEC to copper *bis*-diethyldithiocarbamate complex was studied at room temperature. For this, the number of moles of ZDEC (0.442 µmol) was kept fixed and the number of moles of copper ion was increased by increasing the volume of ammoniacal copper solution (100 mM) from 10, 20, 30 and 40 µL. The formation of copper-dithiocarbamate complex is obtained from the absorbance at 430 nm in the UV spectrum of the resulting copper-complex solution. The mole ratio between ZDEC and copper (II) ions from copper ammoniacal complex solution is tabulated in Table 4. The influence of the mole ratio between ZDEC and copper (II) ion on the formation of copper-bis diethyl dithiocarbamate complex is given in Figure 2. The copper-dithiocarbamate complex formation reaches the maximum at a ratio of 1:4 between ZDEC and copper (II) ion during the ligand exchange reaction between ZDEC and ammoniacal copper complex solution. Further increase in the copper complex doesn't increase the copper-dithiocarbamate complex formation.

Quantification of total residual accelerators in gloves by ultraviolet-visible (UV-VIS) spectroscopy

Quantification of residual accelerator using UV has been carried out in two stages. In the first stage, calibration curve for UV quantification was prepared. The absorbance of copper dithiocarbamate complex formed from standard ZDEC solution in acetonitrile was used for the determination of calibration curve. The ZDEC solutions of 32, 16, 8, and 4µg/mL in acetonitrile were treated with ammoniacal copper complex and the absorbance at 430 nm was measured with UV-VIS spectroscopic technique. A calibration curve is plotted with concentration of ZDEC along X-axis and absorbance of copper complex at 430 nm along Y-axis (Fig. 3). The calibration

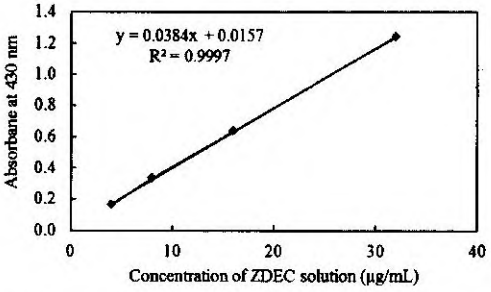

Fig. 3. Calibration curve for UV quantification of total residual accelarator in gloves

Table 5. **Effect of extraction time of different brands of glove samples**

Sl. No.	Brand used	Extraction time (h)	Absorbance at 430 nm
1	A	2	1.046
2	A	24	1.043
3	B (Powder Free)	2	0.445
4	B (Powder Free)	24	0.446

curve is a perfect straight line which means that the concentration range of 4 to 32 μgmL^{-1} of ZDEC solution follows a perfectly linear conversion to its copper – *bis* diethyl dithiocarbamate complex with the concentration of ammoniacal copper solution used in the study. This is also evident from the R^2 value of 0.9997 obtained in the regression analysis of the curve. Hence the relationship of absorbance of copper-dithiocarbamate complex = 0.0384 × Concentration of ZDEC solution (μgmL^{-1}) + 0.157 is obtained. This was used for the determination of the unknown ZDEC equivalents of the total accelerator (thiurams and dithiocarbamates) leached into the glove extract (μgmL^{-1}), provided the absorbance of the corresponding copper- accelerator complex is measured.

In the second stage, the amount of residual dithiocarbamates and thiuram (total residual accelerators) in the gloves was determined by extracting the gloves in acetonitrile. This was followed by a single step conversion of the zinc complex in acetonitrile to its copper complex

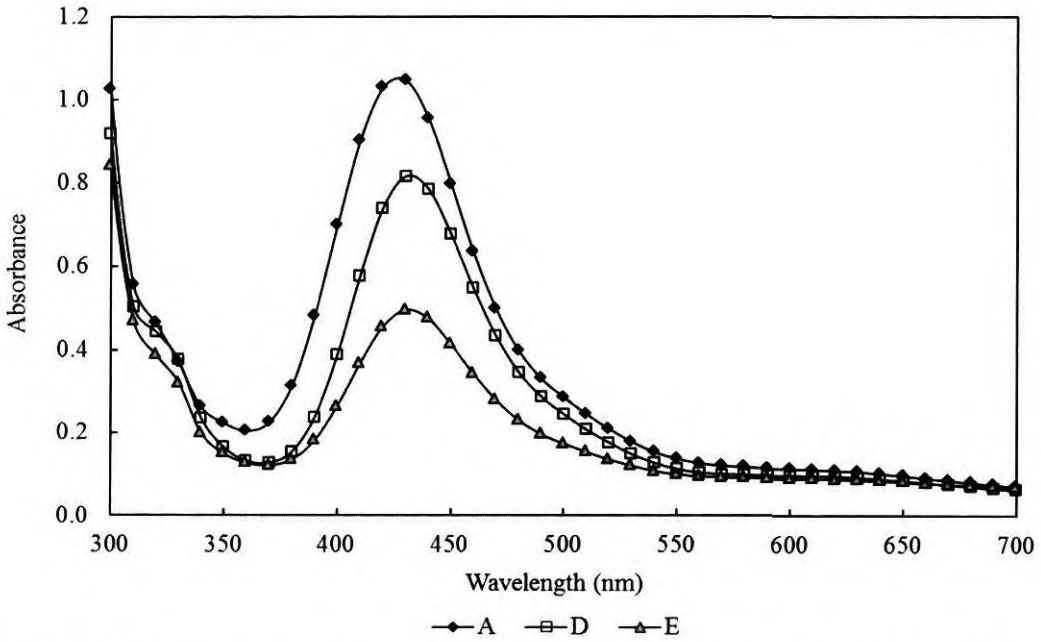

Fig. 4. UV-VIS spectrum of copper complex of total residual accelerator of glove brand 'A', 'D' and 'E'

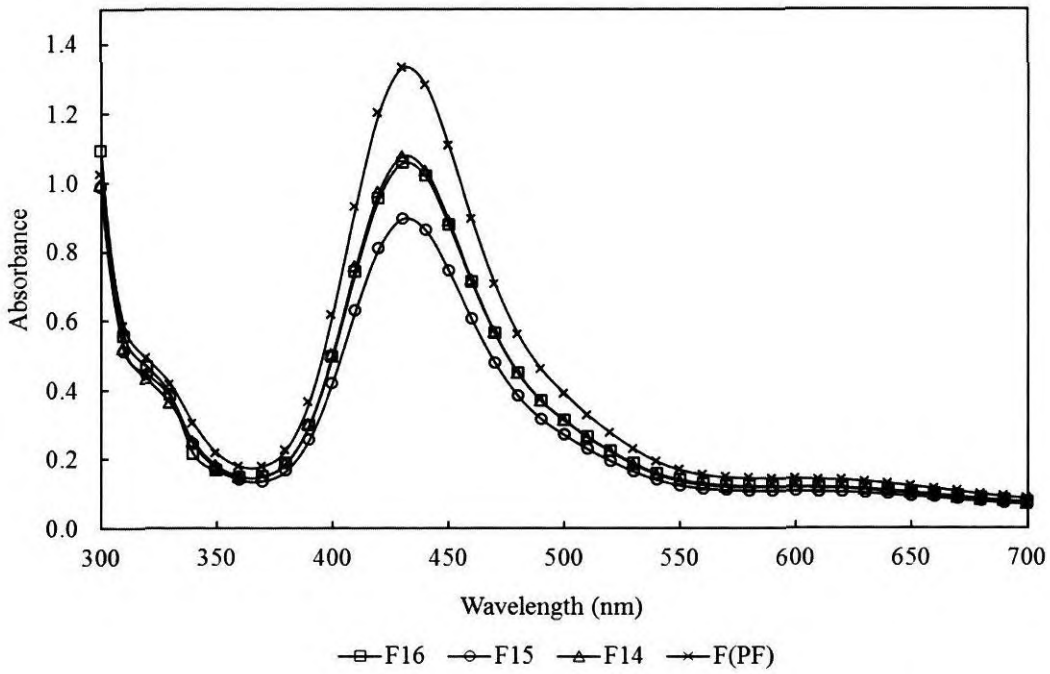

Fig. 5. UV-VIS absorbance spectra of copper complex of total residual accelerator of glove brand 'F 16', 'F 15', 'F 14' and 'F'(powder free)

preparation with a concentrated aqueous ammoniacal copper solution and its direct UV quantification. Previously acetonitrile has been employed as a solvent for the extraction of latex products in order to determine the level of residual accelerators (ASTM D7558-09). But in the ASTM protocol, cobalt complexes of the dithiocarbamate accelerators have been prepared, whereas in the present study, the copper complexes of the accelerators have been prepared. The advantage of copper complex over cobalt complex has been previously reported (Abraham *et al.*, 2007). But Abraham *et al.*(2007) reported a multi-step procedure where dichloromethane (DCM) was used for extraction of residual accelerators from gloves. The extract was treated with a dilute aqueous ammoniacal copper sulphate solution to make the copper dithiocarbamate complex. The copper complex present in the DCM layer was separated using aqueous/DCM phase separation. Then DCM was by removed by rotorary solvent evaporation. The residue was reconstituted in DCM, followed by the UV analysis of the copper complex. In the present method acetonitrile extraction of gloves, followed by a single step conversion of the zinc complex to its copper complex with concentrated aqueous ammoniacal copper solution was carried out. Under the experimental condition the copper complex did not precipitate out and hence its direct UV measurement could be carried out. As acetonitrile being a less volatile solvent than

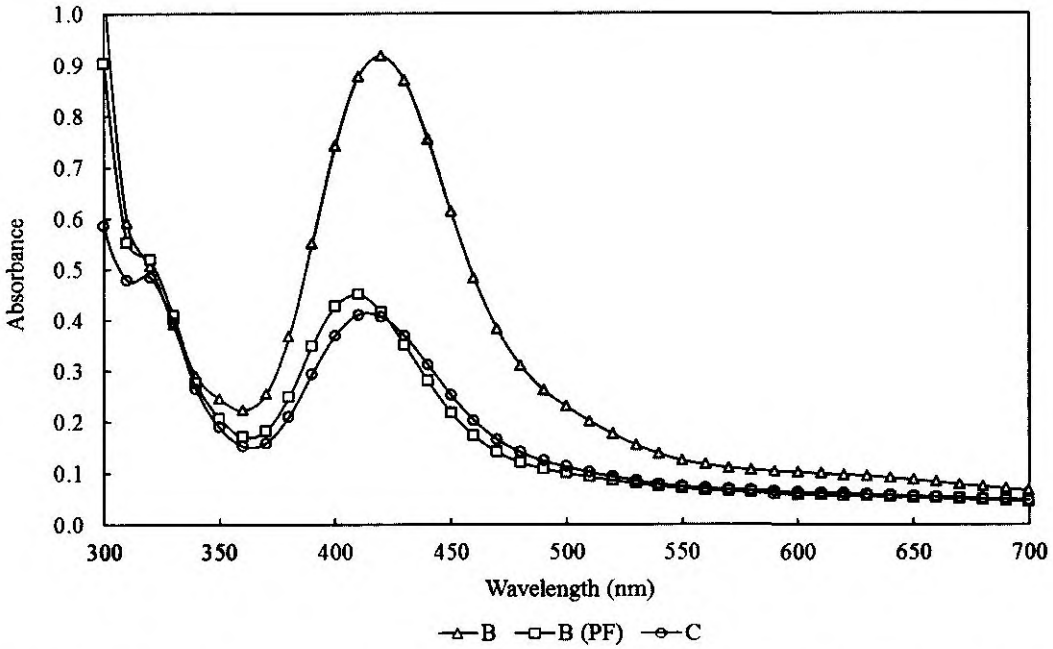

Fig. 6. UV-VIS absorbance spectra of copper complex of total residual accelerator of glove brand 'B', ' B' (powder free) and 'C'

DCM and a single step was employed, the quantification becomes more accurate, environment friendly (non-chlorinated) and simple (user-friendly).

An extraction time of 2 h was used in the study as like the ASTM method. In order to confirm the complete extraction, two glove brands (A and B) were subjected to continuous extraction for 2 hours and 24 hours with shaking. The acetonitrile extracts of the gloves were converted to corresponding copper-accelerator complexes. The absorbance of copper-dithiocarbamate complex at 430 nm after 2h and 24 h extraction time remains comparable as given in Table 5 for both glove brands.

The UV spectrum obtained for the extracts of different brands of commercial

Table 6. **Total residual accelerator ($\mu g g^{-1}$) from different glove brands by UV spectroscopy**

Brands	Absorbance at 430nm	Concentration of ZDEC equivalents ($\mu g mL^{-1}$)	Total amount of residual accelerator ($\mu g g^{-1}$)
A	1.050	26.93	2687
B	0.868	22.19	2203
C	0.378	09.43	926
D	0.820	20.94	2085
E	0.502	12.66	1259
F 14	1.069	27.43	2725
F 15	0.891	22.79	2276
F 16	1.056	27.09	2703
F (P F)	1.339	34.46	3298
B (P F)	0.355	08.84	880

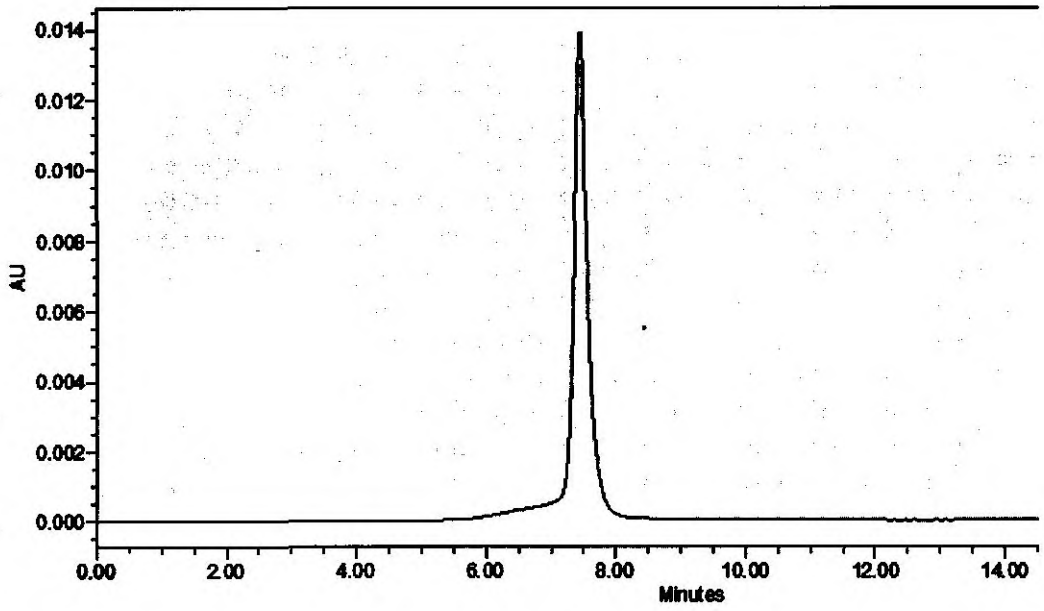

Fig. 7. HPLC profile of copper-bis-diethyl dithiocarbamate prepared from standard ZDEC under the given elution condition

surgical glove samples are plotted with wavelength against absorbance of corresponding copper complexes (Fig. 4, 5, 6).

The total residual accelerators in ten surgical gloves belonging to six commercial brands were quantified from the corresponding UV spectrum. The absorbance obtained at 430 nm is the absorbance of total copper-accelerator complexes formed, from both thiurams and dithiocarbamates, if both are present in the glove extracts. The substitution of absorbance of each glove sample into the UV calibration curve equation gives the concentration of the residual accelerator solution obtained from each glove sample. This is used to determine the total amount of residual accelerator present in the glove sample using equation 1 and has been tabulated in Table 6.

The amount of total residual accelerators varied from the lowest amount of 926 μgg^{-1} (brand C) to the highest amount 2725 μgmL^{-1} (brand F14) among the eight pre-powdered surgical gloves. The powder free surgical gloves under study showed residue of 880 μgg^{-1} (brand B) and 3278 μgg^{-1} (brand F). The analysis showed that same glove brand 'F' manufactured in three consecutive years of 2014, 2015 and 2016, showed comparable values of 2725, 2276 and 2703 μgg^{-1}, respectively.

Optimisation of HPLC protocol for elution of copper-accelerator complex

For the quantification and identification of accelerators in glove extracts, the elution condition of HPLC was optimised with two buffer systems of buffer A (5% acetonitrile in HPLC water) and buffer B (95% acetonitrile

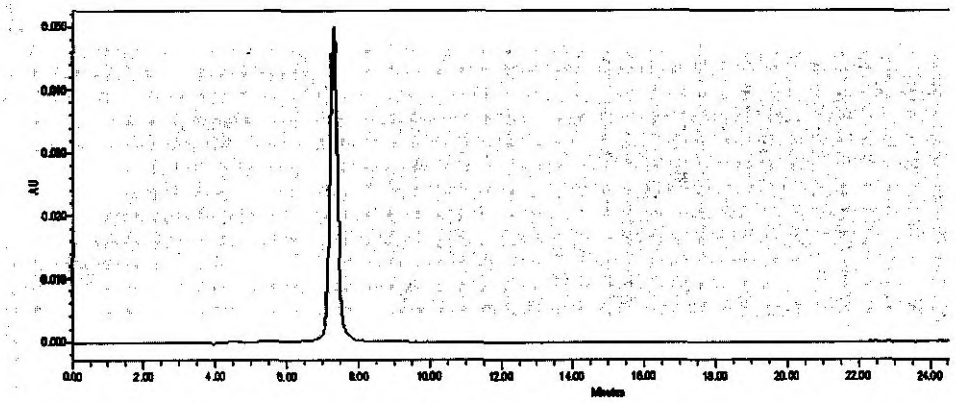

Fig. 8. HPLC profile of copper complex of glove extract of brand B at 430 nm

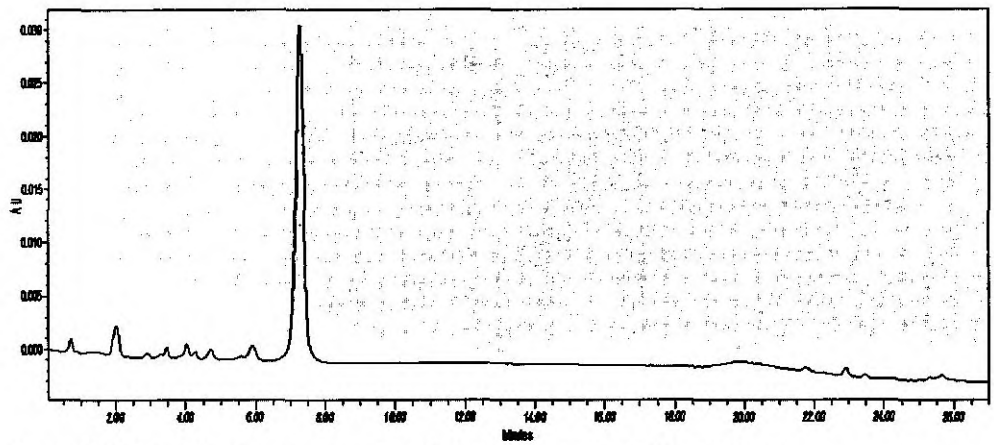

Fig. 9 HPLC profile of copper complex of glove extract of brand E at 430 nm

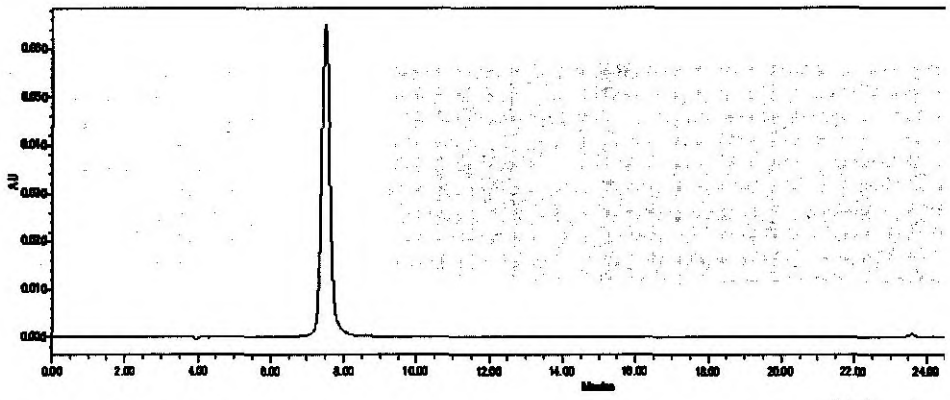

Fig. 10 HPLC profile of copper complex of glove extract of brand F14 at 430 nm

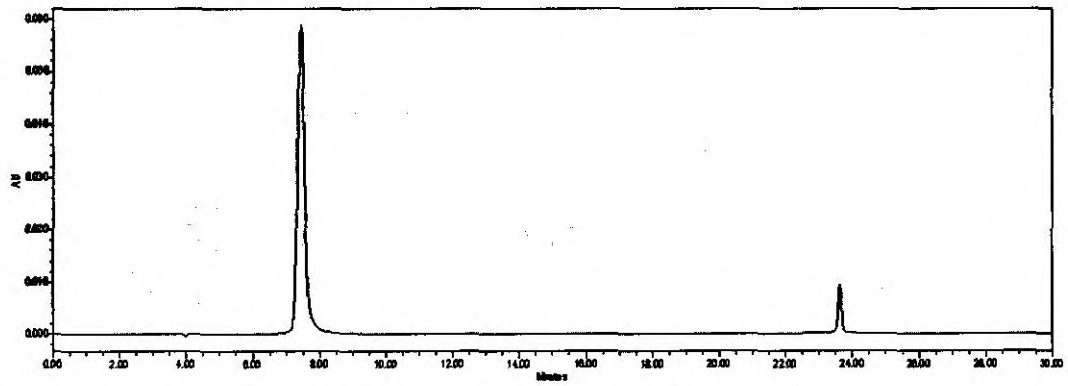

Fig. 11. HPLC profile of copper complex of glove extract of brand F 15 at 430 nm

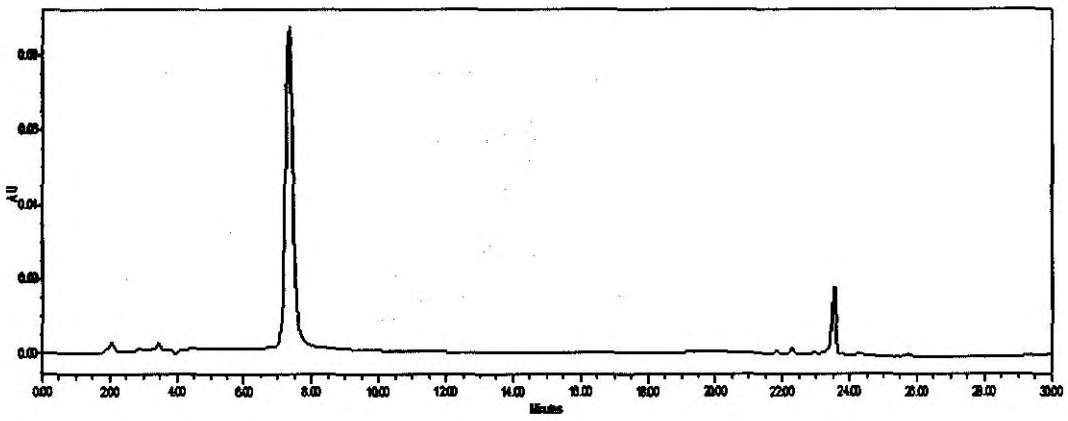

Fig. 12. HPLC profile of copper complex of glove extract of brand F16 at 430 nm

in HPLC grade water) at a flow rate of 1 mL/ minute and a UV-Visible detector. Different isocratic elution conditions of combination of buffer A and buffer B in the ratio of 10:90, 20:80, 30:70, 40:60 were tried. At 40 per cent buffer A and 60 per cent buffer B, the copper bis diethyl dithiocarbamate complex eluted with a retention time around 7.4 minute. Other isocratic conditions resulted in retention time less than 7 minutes such as 2.33min, 4min *etc.* Hence the isocratic elution condition as 40 per cent A and 60 per cent B was chosen for the present study.

Identification of the major residual accelerator using HPLC

The most frequently used accelerators in glove industry are zinc dithiocarbamates and zinc mercaptobenzothiazoles. Some manufactures use thiuram in spite of it being prohibited. The identification of residual accelerator type in each glove extract is done by comparison with the retention times of HPLC profiles of standard accelerators. The HPLC profile of the copper complex of standard solution of ZDEC elutes with a retention time of 7.485 minutes as shown in

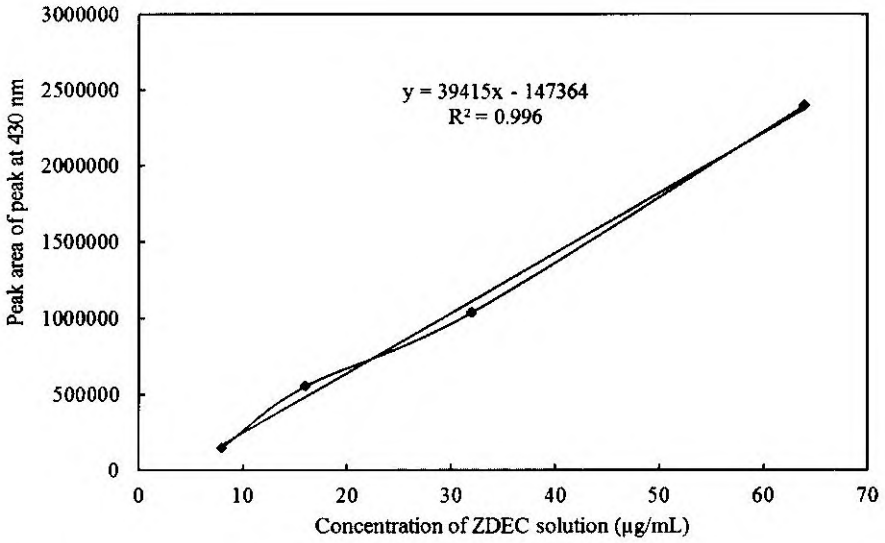

Fig.13.Calibration curve for HPLC quantification of ZDEC accelarator in gloves

Figure 7. The HPLC profiles at 430 nm of copper complexes of the extracts of the entire ten glove brands were measured. The representative HPLC profiles of the most prominent brands in Indian market are given in Figures 8 to 12 and the retention time of the major peak in each profile is tabulated in Table 7 (HPLC profiles of brand A, C, D, F(PF) and B(PF) were similar to Figure 8 and hence data not shown). The

Table 7. **Residual ZDEC accelerator (µg/g) from different glove brands by HPLC analysis**

Glove Brand	Retention Time (minute)	Peak Area of HPLC peak at 430 nm	Concentration of ZDEC solution ($\mu g mL^{-1}$)	Residual accelerator ($\mu g g^{-1}$)
A	7.510	767598	20.43	2038
B	7.545	690882	18.76	1862
C	7.495	237056	8.89	873
D	7.382	1009945	25.70	2558
E	7.474	498181	14.57	1449
F14	7.488	1039059	26.33	2616
F 15	7.441	890648	23.11	2307
F 16	7.463	997845	25.44	2538
F (Powder free)	7.468	1049947	26.57	2543
B (Powder free)	7.443	255359	9.29	925

Table 8. **Residual accelerator in sweat equivalent aqueous extract of gloves**

Glove brand	Total residual accelerator by UV ($\mu g g^{-1}$)	Bioavailable residual accelerator by UV ($\mu g g^{-1}$)	Percentage of *bioavailable accelerator* (%)
F15	2276	318.5	14.0
B	2203	313.3	14.2
F14	2725	395.1	14.5

Table 9. **Direct contact assay of the glove**

No	Sample	Grade	Reactivity
1	F14	4	Severe
2	Negative control	0	None
3	Positive control	4	Severe

analysis of the extracts shows that the major peak elutes in between 7.4 to 7.5 minutes for all the surgical gloves under study which is same as that of the standard ZDEC peak. The comparison of the retention time confirms that the major peak in the glove extract was that of copper-bis-diethyl dithiocarbamate complex and led to the identification of ZDEC (dithiocarbamate) as the major residual accelerator in all the glove brands. In the brand 'F15' and 'F16', along with the major ZDEC peak, a characteristic peak around 23 minutes was observed, which was notably absent in 'F14'. The brand E shows multiple peaks along with the major ZDEC peak. The identification of peaks other than that of

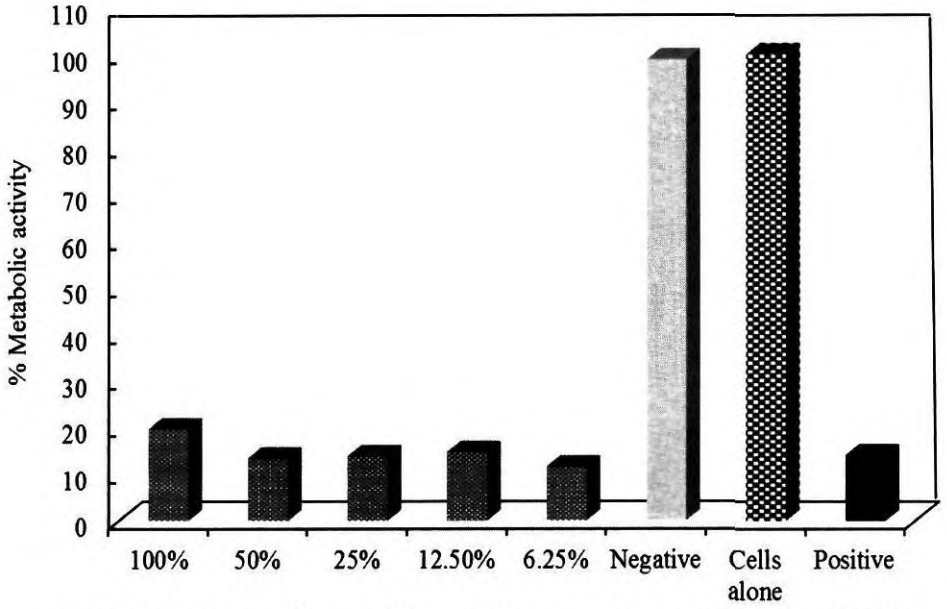

Fig. 14. Cytotoxicity of different dilution of the aqueous extract of glove brand 'F14' in comparison to the positive, negative and cell controls

ZDEC copper complex in the HPLC profile of each of the glove brand is currently under progress.

Preparation of calibration curve for HPLC quantification

The ZDEC solutions of 64, 32, 16 and 8 μgmL^{-1} in acetonitrile was treated with ammoniacal copper complex and the area of the peak at 430 nm is measured with HPLC integration technique. A calibration curve (Fig. 13) is plotted with concentration of ZDEC solution along X-axis and peak area of corresponding copper complex at 430 nm along Y-axis. The calibration curve is a perfect straight line which means that the concentration range of 8 to 64 μgmL^{-1} of ZDEC solution follows a perfectly linear conversion to its copper – dithiocarbamate complex with the concentration of ammoniacal copper solution used in the study. This is also evident from the R^2 value of 0.996 obtained in the regression analysis of the curve. Hence the relationship of peak area of copper-dithiocarbamate complex = 39415 × concentration of ZDEC solution (μgmL^{-1}) - 147364 can be used for the determination of unknown ZDEC concentration of the glove extract, provided the peak area of its copper-accelerator complex formed is measured at 430 nm.

Quantification of residual ZDEC from glove brands using HPLC method

The preparation of the copper complex of the glove extract is same as that of UV-VIS analysis. A 15 µL of copper accelerator complex is injected to HPLC system with UV detector and the retention time of the major peak and its peak area was obtained. Using the calibration curve equation the amount of residual ZDEC accelerator obtained by acetonitrile extraction is determined. From the area of the ZDEC peak in HPLC profiles, the concentration of residual ZDEC is obtained using the HPLC calibration curve equation. It's substitution in equation-2, gives the residual ZDEC accelerator content in ten surgical glove samples and are given in Table 7. The amount of ZDEC accelerator varied from the lowest amount of 873 μgg^{-1} (brand C) to the highest amount 2616 µgg-1 (brand F 14) among the eight pre-powdered surgical gloves. The powder free surgical gloves under study showed residue of 925 μgg^{-1} (brand B) and 2543 μgg^{-1} (brand F). The analysis of same glove brand 'F' manufactured in three consecutive years of 2014, 2015 and 2016, showed comparable values of 2616, 2307 and 2538 μgg^{-1} respectively by HPLC analysis

Quantification of bioavailable accelerator leached in aqueous extract from gloves

The residual accelerator leached into the aqueous 'sweat equivalent' extracts of the prominent glove brands F14, F15 and B were quantified by UV spectroscopy (bioavailable accelerator) as previously reported (Abraham *et al.*, 2007) with a minor modification. An aqueous buffer with composition equivalent to sweat extract was prepared and the glove samples were placed into the buffer. The aqueous extract was separated from the glove samples and the residual accelerator was partitioned from the aqueous to the DCM layer. In the modified protocol the DCM was evaporated and the residue re-constituted in acetonitrile. The copper complex of the residual accelerator was prepared as described earlier and quantified by UV spectroscopy. The residual accelerator available in the aqueous extract (bioavailable) and its percentage with respect to the total residual accelerator (acetonitrile extract) of each glove brand is

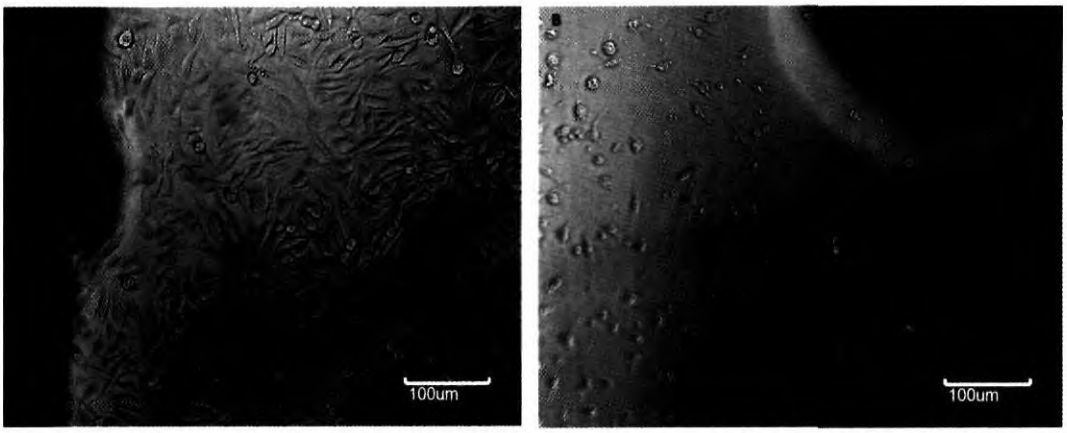

Fig. 15. L-929 cell lines after direct contact with polythene (negative control) (A) and L-929 cells after direct contact with the brand 'F14'(B)

given in Table 8. This is quiet high when compared to the available accelerator limit of 0.1 per cent (w/w) of the total accelerator specified for latex gloves by Nottingham University for safe glove selection. (https:// www.Nottingham.ac.uk).

Cytotoxicity of the glove sample against L929 cell lines

The cytotoxicity of the glove brand F14 was evaluated by two methods (MTT assay and Direct contact assay). L-929 is an established and well characterised mammalian cell line that has demonstrated reproducible results for cytotoxicity assays. The MTT assay was performed to measure the metabolic activity of cells to reduce yellow colored tetrazolium salt 3-(4, 5-Dimethyl thiazol -2-yl)-2, 5-diphenyltetrazolium bromide to purple colored formazan. The extracts of the glove samples in the aqueous cell culture medium were diluted to different dilutions from 100 to 6.25 per cent using the same culture medium. Each dilution was treated with the confluent L-929 cell lines . The viability of cell lines was measured from the MTT assay. The MTT assay of L-929 cells after contact with 100, 50, 25, 12.50 and 6.25 per cent extract of glove brand 'F14' showed 19.79, 13.54, 13.95, 14.79 percent metabolic activity respectively (Fig. 14). Positive control showed 14.11 per cent and negative control showed 98.68 per cent and the cell alone showed 100 per cent metabolic activity. In all the dilutions studied, the cell viability was between 10 to 20 per cent. The severe cytotoxicity of the extract is evident from the fact that even up to dilution of 6 per cent of the neat extract; the high cytotoxicity was still retained.

The cytotoxicity was also measured by the direct contact assay. The test samples were directly placed above a layer of confluent L-929 cell line for 24 h and the microscopic observation of the changes in the cell line morphology after the treatment period was obtained. The grading of cytotoxicity as described in the method section based on the visual changes has been carried out (Table 9). The cell line image after direct contact assay with the glove sample 'F 14' clearly shows sever cytotoxicity with complete loss in cell

morphology (B in Fig. 15). The control with polythene after direct contact with L-929 cell lines shows intact cell morphology (A in Fig. 15). The results have been tabulated in Table 9 and a grade of 4 has been obtained for the brand F14 like the positive control of PVC, indicating severe toxicity whereas the negative control of polythene shows the grade of 0 indicating the non-toxic nature.

CONCLUSIONS

The key to managing allergies and adverse glove reactions in healthcare professionals lies in education and awareness programs, correct recognition and appropriate action. In this study, the quantification of residual accelerators present in ten surgical gloves belonging to six commercial brands (coded as 'A' to 'F') available in Indian market were analysed. Out of ten gloves, eight were pre-powdered surgical gloves and two were powder free surgical gloves. The accelerator release into acetonitrile from these gloves was estimated using UV-VIS spectroscopy and HPLC method. Using UV-VIS spectroscopy, the release of total amount of residual accelerator (both thiurams and dithiocarbamates) was obtained from the copper-accelerator peak at 430 nm. Using HPLC, separation of the specific accelerators by the reverse phase column, followed by its chemical identification and subsequent quantification of residual ZDEC accelerators were carried out. The major conclusions drawn from the study are

1. By UV-spectroscopy, the amount of total residual accelerators varied from the lowest amount of 926 μgg^{-1} for the brand C to the highest amount of 2725 μgg^{-1} in brand F14, among the pre-powdered surgical gloves. The powder free surgical gloves under study showed residue of 880 μgg^{-1} for the brand B (PF) and 3278 μgg^{-1} for brand F (PF). The analysis of glove brand 'F' manufactured in three consecutive years of 2014, 2015 and 2016, showed comparable values of 2725, 2276 and 2703 μgg^{-1} respectively.

2. The identification of the major residual accelerator in all the gloves was achieved by HPLC analysis. From the studies done on surgical gloves it is observed that Zinc diethyl dithiocarbamate (ZDEC) is the major residual accelerator found in every brand.

3. The residual ZDEC accelerator content in the surgical gloves under study was quantified by HPLC. The amount of ZDEC accelerators varied from the lowest amount of 873 μgg^{-1} for the brand C to the highest amount 2616 μgg^{-1} for brand F14, among the pre-powdered surgical gloves. The powder free surgical gloves under study showed residue of 925 μgg^{-1} for brand B (PF) and 2543 μgg^{-1} for brand F (PF). The analysis of glove brand 'F' manufactured in three consecutive years of 2014, 2015 and 2016, showed comparable values of 2616, 2307 and 2538 μgg^{-1} respectively.

4. The study on the bioavailable residual accelerator showed that about 14 per cent of the total residual accelerator in the glove was released into the sweat equivalent aqueous buffer which is significantly high.

5. The cytotoxicity studies of the glove sample showed severe toxicity in MTT assay even up to six per cent dilution of the aqueous extract. The direct contact assay of the glove sample showed a severe toxicity of grade four. Both cytotoxicity assays indicate the severe toxicity of the commercial glove sample.

The presence of relatively high amount of residual accelerators in surgical gloves

and its high bioavailability has to be seriously viewed and methodologies to reduce or eliminate the residual accelerators have to be worked out in future to further improve the biocompatibility of surgical gloves in Indian market.

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REFERENCES

Abraham, E.K., Ramesh, P., Joseph, R. (2007). Determination of zinc diethyldithio- carbamate released into artificial sweat from natural rubber latex vulcanizates by HPLC. *Journal of Chromatography Science*, **45**(1): 1-5.

ASTM D 5151-92. (1992). Standard test method for detection of holes in medical gloves.

ASTM D 5712-99. (1999). Standard test method for the analysis of aqueous extractable protein in natural rubber and its products using the modified lowry method.

ASTM D6499-16. (2016). Standard Test Method for The Immunological Measurement of Antigenic Protein in Natural Rubber and its Products.

ASTM D 6124-01. (2001). Standard test method for residual powder on medical gloves.

ASTM D7558-09. (2009). Standard Test Method for Colorimetric/Spectrophotometric Procedure to Quantify Extractable Chemical Dialkyldithiocarbamate, Thiuram, and Mercaptobenzothiazole Accelerators in Natural Rubber Latex and Nitrile Gloves.

Carrillo, T., Cuevas, M., Munoz, T., Hinojosa, M., and Moneo, I. (1986). Contact urticaria and rhinitis from latex surgical gloves. *Contact Dermatitis*, **15**: 69-72.

Charous, B.L., Blanco, C., Tarlo, S., Hamilton, R.G., Baur, X., Beezhold, D.H., Sussman, G.L., and Yunginger, J.W. (2002). Natural rubber latex allergy after 12 years: Recommendations and perspectives. *Journal of Allergy and Clinical Immunology*, **109**: 31-34.

Chen, H.H., Sun, C.C., and Tseng, M.P. (2004). Type IV hypersensitivity from rubber chemicals: A 15-year experience in Taiwan. *Dermatology*, **208**: 319-325.

Elizabeth, K. A., Ramesh, P. Joseph, R., Mohanan, P.V., Remakumari, V.M. (2005). Release of Dithiocarbamates into Sweat from Natural Rubber Latex Surgical Gloves. *Rubber Chemistry and Technology*, **78**(4): 674-681.

Geier, J., Lessmann, H., Uter, W., and Schnuch, A. (2003). Occupational rubber glove allergy: Results of the information network of departments of dermatology (IVDK), 1995-2001. *Contact Dermatitis*, **48**: 39-44.

Heese, A., van Hintzenstern, J., Peters, K.P., Koch, H.U., and Hornstein, O.P. (1991). Allergic and irritant reactions to rubber gloves in medical health services: Spectrum, diagnostic approach, and therapy. *Journal of the American Academy of Dermatology*, **25**: 831-839.

Hendrie, L., Driscoll, T. (2003). Work-related presentations to general practitioners in Australia. *The Journal of Occupational Health and Safety* -Australia and New Zealand, **19**: 133-43.

http://www.fda.gov/downloads/AboutFDA/ReportsManualsForms/Reports/EconomicAnalyses/UCM491783.pdf, Banned Devices; Proposal to Ban Powdered Surgeon's Gloves, Powdered Patient Examination Gloves, and Absorbable Powder for Lubricating a Surgeon's Glove, Docket No. FDA-2015-N-5017.

https://www.nottingham.ac.uk/safety/documents/gloves-selection.pdf

https://www.topglove.com.my/pdf/TG%20presentation.pdf

Johnson G. (1997). Time to take the gloves off ? *Occupational Health (London)*, **49**: 25-28.

Kaniwa, M.A., Ikarashi, Y., Kojima, S., Nakamura, A., Usuda, T. and Shibata, K. (1988). Chemical