

## EVALUATING THE TOXICITY OF REACTIVE DYES AND DYED FABRICS WITH THE HEPA-1 CYTOTOXICITY TEST

Kaisa Klemola<sup>1</sup>, John Pearson<sup>2</sup>, Atte von Wright<sup>3</sup>, Jyrki Liesivuori<sup>4</sup>, Pirjo Lindström-Seppä<sup>5</sup>

University of Kuopio, P.O.Box 1627, FIN-70211 Kuopio, Finland  
Savonia University of Applied Sciences, Piispankatu 9, 70100 Kuopio, Finland  
University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, UK

<sup>1</sup>Department of Textiles: e-mail: kaisa.klemola@designkuopio.fi

<sup>1</sup>Institute of Applied Biotechnology: kaisa.klemola@designkuopio.fi

<sup>2</sup>School of Art & Design: e-mail: j.s.pearson@hud.ac.uk

<sup>3</sup>Institute of Applied Biotechnology: e-mail: atte.vonWright@uku.fi

<sup>4</sup>Department of Pharmacology and Toxicology; e-mail: jyrki.liesivuori@ttl.fi

<sup>5</sup>Faculty of Medicine; e-mail: pirjo.lindstrom-seppa@uku.fi

### Abstract

*We investigated the cytotoxicity of reactive dyes and dyed fabrics using an in vitro hepa-1 cytotoxicity test. Hepa-1-mouse cells were exposed to three monochlorotriazinyl dyes: yellow, red and blue with different concentrations. The hepa-1-mouse cells were also exposed to water extracts of dyed fabrics. After 72 hours exposure, the viability of the cells was detected by measuring the protein content of the cells. The mean inhibitory concentration IC50, which shows the sample concentration when the protein content is 50%, was compared to the total protein content of the non-exposed cells. The inhibitory concentration IC20 value, which shows the sample concentration when the protein content is 80%, was also measured. The IC20 value shows the limiting value of low toxicity. The values measured showed high toxic effects of the dyes. The blue dye was shown to be the most toxic, although the red dye showed toxicity at the lowest concentrations. Whereas the pure dyes showed toxicity under low concentrations, the dyed fabrics showed no toxicity. The hepa-1 cytotoxicity test and the spermatozoa motility inhibition test supported each other, giving similar results. Both tests can be used when studying the toxicity of textile substances.*

### Key words:

*textiles, fabric extract, reactive dye, hepa-1 cells, cytotoxicity, spermatozoa*

### Introduction

A wide range of chemicals is used in the manufacture of textiles. The toxicity of many of these chemicals is known, but limited data are available on biological effects and on the toxicity of fabrics containing these chemicals. An *in vitro* test capable of detecting the combined effects of chemicals on textile products could give useful information for the development of less toxic textile products. Different textile processes can produce different levels of toxicity in products. For studying the overall toxicity Hepa-1 (Hepa-1c1c7) mouse hepatoma cell line and other cell lines can be used.

Textile dyes form a large group of textile chemicals and comprise over 8,000 different compounds with almost 40,000 commercial names [20]. The textile industry utilises mostly reactive dyes, which are used in dyeing cellulose fibres: cotton accounts for about 40% of world fibre production [26].

Reactive dyes have good wet-fastness, resulting from the conversion of soluble substances in the dye bath into relatively insoluble compounds within the fibres by the formation of covalent bonds between hydroxyl groups in cellulose and reactive groups on the dye molecules. The dyes have bright colours and the dyeing process is simple [22].

Reactive dyes have good technical characteristics but they have been found to cause adverse effects on workers in textile factories and on the environment. Wastewaters and land in an industrial area in India were studied to assess the possible genotoxic health risk and environmental genotoxicity due to

textile industry effluents by Mathur *et al.* [16]. After the study, water and land were found to be seriously spoiled. The toxicity was not caused only by textile dyes but by a large number of different textile chemicals. The study showed the importance of safe working in order to prevent industry workers and the environment becoming exposed to this wide range of potential harmful chemicals, including reactive dyes.

Allergic dermatoses and respiratory diseases are known to be caused by reactive dyes [5,7,15,17,24]. Contact dermatitis and asthma were also studied by Thoren *et al.* [21]. Park *et al.* have shown textile industry workers exposed to reactive dyes to have changes in their immunoglobulin levels [18]. Previous studies have also suggested increased risks of colon and rectum cancers; however, these cancers relate mostly to dyes for synthetic fibres [4]. Gonzales *et al.* stated that workers in the textile industry have a two fold risk of contracting bladder cancer compared to workers in other industries such as aviation, agriculture and construction [6].

In addition, work conducted by Keneklis [9] showed mutagenicity caused by some textile dyes. Wollin *et al.* showed several azo dyes to have genotoxicity when studied with HaCaT cells which are human keratinocytes [25]. Birhanli *et al.* [2] have used the frog embryo teratogenesis assay-Xenopus (FETAX) to establish that some reactive dyes have teratogenic potential. Rannung *et al.* [19] showed the mutagenicity of denim fabrics to be dependent on the quality of the denim fabric: many azo dyes were shown to be mutagenic either before or after metabolic activation. The study highlighted the importance of analysing the finished fabrics in addition to the pure chemicals. On the other hand, according to Kaur *et al.* [8], 11 out of 13 commercial azo dyes were not mutagenic.

The hepa-1 mouse hepatoma cell line (INVITTOX protocol number 112) has been used in studying the toxicity of different compounds and materials [10], complex mixtures like paper products [13], laboratory animal beddings and feeds [23] and fly ash samples from combustion processes [11]. Kopponen *et al.* [12] used hepa-1 mouse cells to investigate dyes and fabrics. The toxicity of vat, azo and naphthol, reactive and sulphur dyes was studied. The sample fabrics, which contained chemicals other than dyes, were dyed and finished under commercial conditions and the IC50 values were calculated for each sample. Reactive blues and sulphur black were found to be the most toxic. The results supported the hypothesis that the toxicity of a fabric extract cannot be predicted directly from the toxicity of the dye itself. The results emphasised that the hepa-1 cytotoxicity test is a useful addition to the other tests when investigating the toxicity of textile materials and textile chemicals.

Andersson [1] has studied toxicity in building materials exposed to moisture damage using spermatozoa cells. Klemola *et al.* have studied the toxicity of reactive dyes by analysing their effect on boar semen (paper submitted to AutexRJ). The spermatozoa motility inhibition test has been found to be useful for screening toxic samples and it may be a useful addition to the hepa-1 cytotoxicity test when studying the toxicity of textile chemicals and materials.

The environmental label of eco textile standard Öko-Tex-100 sets limiting values for the amounts of chemicals allowed in fabrics. The list of chemicals includes pesticides, heavy metals and other chemicals which can remain after the textile process [28]. However, the standard does not require any common biological tests to detect any adverse effects of the material due to the chemicals that they may contain.

Chemical Safety Data Sheets 2001/58/EY also give information about the toxicity of harmful chemicals [3]: these refer to irritation tests for rats, rabbits and guinea pigs. Environmental studies have also been undertaken by measuring DOC and COD values and toxicity studies on fish, microbes and water fleas. Reactive dyes have been studied, but there are minimal data about any adverse effects of these dyes when present in fabrics. However, these types of study do provide important information about the safety of the products.

The aim of the present study was to investigate three reactive dyes using the hepa-1 hepatoma cell line. These three reactive dyes represent the typical components used when mixing different basic colours. The effects of these dyes when present in dyed fabrics were also tested. The mean inhibitory concentration (IC) values for the dyes were calculated. The IC50 value shows the sample concentration at which the total protein content of the sample is 50 % of the total protein content of non-exposed cells. The IC20 value shows the sample concentration at which the total protein content of the sample is 80 % of the total protein content of non-exposed cells. Therefore the IC20 value

represents the concentration when the chemical has the lowest toxic value. These three reactive dyes have been studied by Klemola *et al.* using the spermatozoa motility inhibition test, which was found to be a suitable method for screening samples for further studies. It is proposed that the spermatozoa test together with the hepa-1 cytotoxicity test can give useful information about the toxicity of textile dyes and dyed fabrics. The aim of this study was to evaluate this hypothesis.

## **Materials, methods and procedures**

### **Materials**

The three reactive dyes used in this work were monochlorotriazinyl dyes: Drimarene red CL-5B (RR241), Drimarene yellow CL-2R (RY176) and Drimarene blue CL-2RL (CI number unknown); (Clariant Ltd. Switzerland). Extracts were taken from cotton fabrics (see below) which had been dyed with each of these dyes and the extracts were tested using the hepa-1 cytotoxicity test.

The samples of dye powder were dissolved in the solution of  $\alpha$ -MEM; concentrations of the dye were during the exposure: 1250 $\mu$ g/ml, 600 $\mu$ g/ml, 300 $\mu$ g/ml, 150 $\mu$ g/ml, 80 $\mu$ g/ml and 40 $\mu$ g/ml (0,1250%, 0,06%, 0,03%, 0,015%, 0,008% and 0,004% respectively).

Fabric samples were prepared using commercial plain weave bleached cotton fabrics typically used for sheets. The fabrics were gently washed without soap before dyeing and 10g samples were taken for dyeing with each of the three dyes using the following recipe: 400ml H<sub>2</sub>O; 0,3g reactive dye; 50g Na<sub>2</sub>SO<sub>4</sub>/1l H<sub>2</sub>O and 20g Na<sub>2</sub>CO<sub>3</sub>/1l H<sub>2</sub>O. Dyeing continued for 60 minutes at 55 °C. Sodium carbonate was not added until ten minutes after dyeing had commenced. After dyeing, the fabrics were rinsed in cool and warm water and kept in pure boiling water for 10 minutes.

The dyed fabrics were shaken for two hours in sterilised water using a liquor ratio of 1g fabric/20ml H<sub>2</sub>O at 20°C. The samples were incubated for 18 hours at 37°C and shaken again before centrifugation for 5 min at 4500 rpm and sterile filtration before exposure to the cell lines. Samples of dye extracts from the fabrics for exposure were made by adding medium compounds ( $\alpha$ -MEM).

2,4-dinitrophenol was used as a positive control at three different concentrations (0,5mg/ml, 0,05mg/ml and 0,005mg/ml DMSO). In this study, the mean value of each concentration in a culture plate is called a sample. Every plate had 2-4 independent samples of each concentration. In a culture plate the contents of protein between independent wells of the same concentration were less than 10%.

### **Methods and procedures**

Cultured hepa-1 mouse hepatoma cells in 96 well culture plates (200 $\mu$ l capacity) were exposed to 1:1 dilution series of samples for 72 hours. The method used to assess the potential toxicity of the dyes being studied was modified INVITTOX (protocol number 112). Cell viability was measured by assaying the total protein content in the cultures. IC<sub>50</sub> and IC<sub>20</sub> values, quoted as  $\mu$ g/ml of the dye, were calculated from the dose-response curves: IC<sub>20</sub> values show the lowest toxicity and IC<sub>50</sub> values show the toxicity value. All results were compared to the control samples of non-exposed cells in the medium solution. For the fabric extracts, the results were given as percentages of protein content compared to the total protein content of non-exposed cells. The limiting value of the toxicity of the fabric extracts was set as 80% protein in the sample compared to total protein of non-exposed cells.

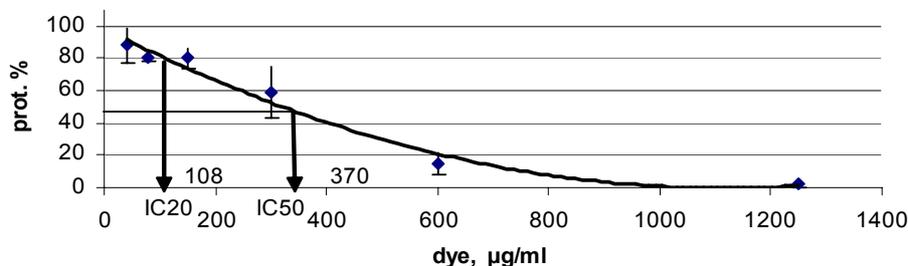
Standard deviation and CV values were calculated for all results to assess their reliability.

## **Results**

The blue and red dyes were found to be more toxic than the yellow dye, the IC<sub>50</sub> values being as follows: 392 $\mu$ g/ml (yellow dye); 370 $\mu$ g/ml (red dye) and 361 $\mu$ g/ml (blue dye). IC<sub>20</sub> values were much lower: 176 $\mu$ g/ml, (yellow dye); 108 $\mu$ g/ml (red dye) and 158 $\mu$ g/ml (blue dye). If the concentration values after the test are the same as or lower than inhibitory concentration values, the material can be regarded as toxic (Figures 1-3). These results show that the fabric extracts were not toxic since protein concentrations were over 80% for all three dyes (Figure 4).

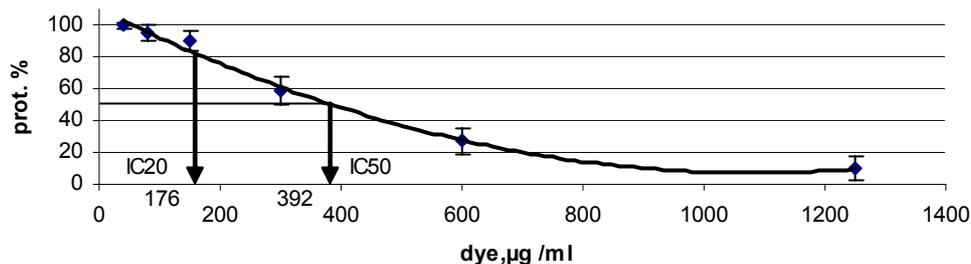
Coefficient of variation (CV) values for the samples of low dye concentration were below 10%; CV values for the samples of high dye concentrations ranged from 9% to 46%. The fabric extracts showed CV values between 10-17%. The positive control (2,4-dinitrophenol) showed CV values between 11-18% for low concentrations of the control. The toxic concentrations of the positive controls had protein values between 2-25% compared to the non-exposed cells.

**cytotoxicity, red**



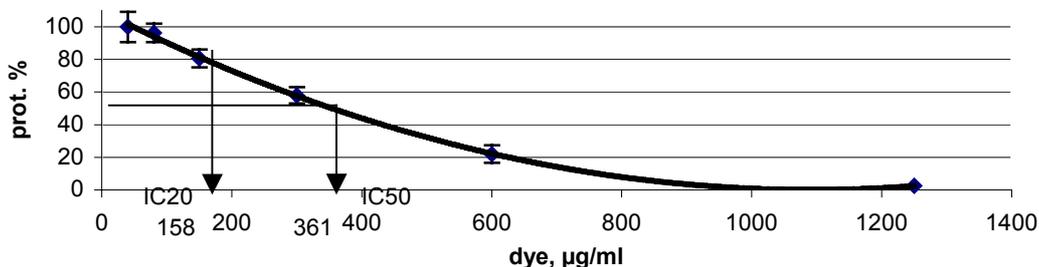
**Figure1.** IC50 and IC20 values for the red dye:  $(y = 7927, 3x^2 - 1771,1x + 98,61;$   
 $R^2 = 0,979;$  the number of samples in different concentrations:  $n = 4-6)$

**cytotoxicity, yellow**

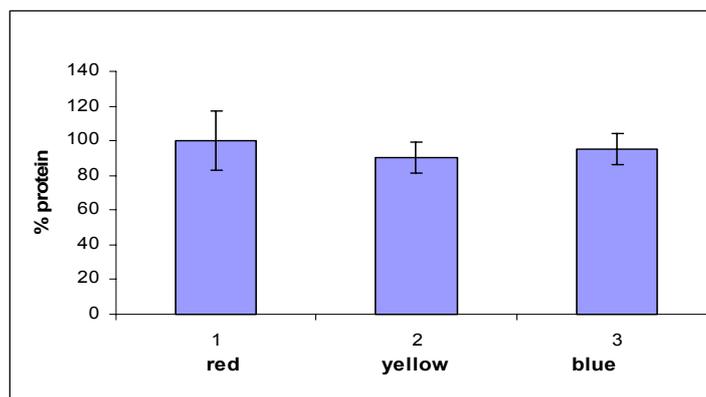


**Figure2.** IC50 and IC20 values for the yellow dye:  $(y = 8801x^2 - 1903, 6x + 110,16;$   
 $R^2 = 0,9928;$   $n = 4-7)$

**cytotoxicity, blue**



**Figure3.** IC50 and IC20 values for the blue dye:  $(y=9185,3x^2 - 2004,4x + 109,49;$   $R^2=0,9989;$   $n= 2-4)$



**Figure 4.** The protein content (%) of fabric extract samples compared to that of non-exposed cells. The number of independent samples, n=4.

## Discussion

Textile dyes are mixtures of chemicals which can contain salts, calcium stearate, carboxymethyl cellulose (CMC) and other unknown chemicals (personal communication: Esa Mäkelä, Clariant Ltd., Finland). In this study, the dyes represent a mixture of different chemicals. It is therefore not possible to know the exact concentration of the dye itself in the powder form of the commercial dyestuff and there could be other components in this mixture that could also be toxic.

The mean IC<sub>20</sub> value for the red dye was 108 µg/ml and the IC<sub>50</sub> value was 370 µg/ml. The red dye therefore has toxic effects when the concentration is 108 µg/ml or higher. By comparing the IC<sub>20</sub> values it can be seen that the red dye is more toxic than the yellow and the blue dyes. The mean IC<sub>20</sub> value for the yellow dye was 176 µg/ml and the IC<sub>50</sub> value was 392 µg/ml. For the blue dye, the corresponding values were: IC<sub>20</sub> was 158 µg/ml and IC<sub>50</sub> was 361 µg/ml. Comparing the IC<sub>50</sub> values of the three dyes, it can be seen that the yellow dye is the least toxic while the blue dye is the most toxic.

Kopponen *et al.* [21] have used hepa-1 cells and tested textile dyes and fabrics for toxicity. The dyes were not the same as those used in this study and the preparation of the fabric extracts was different from the method used in this study. However, according to the IC<sub>50</sub> values found in their work, the blue dyes were also found to be the most toxic. In this study, the IC<sub>20</sub> values showed that the red dye had a toxic effect at the lowest dye concentration and the IC<sub>50</sub> values showed that the blue dye had the most toxic effect.

The Chemical Safety Data Sheets show the blue and red dyes to be more toxic than the yellow dye. In these Safety Data Sheets, the LD<sub>50</sub> value (the single dose of a material expected to kill 50% of a group of test animals) for the yellow dye is 5000 mg/kg, higher than the 2000 mg/kg values for the red and blue dyes. According to toxicity tests using activated sludge, the toxicity of the blue dye measured as EC<sub>50</sub> (the molar concentration of an agonist, which produces 50% of the maximum possible response for that agonist): was higher than 100 mg/l. Using the OECD 209 method, 1984, the red and yellow dyes had IC<sub>50</sub> values higher than 1000 mg/l. Using the OECD 203 method (an acute fish toxicity test using *salmo gairdneri* and *oncorhynchus mykiss*) the values of LC<sub>50</sub> (the concentration of a chemical which kills 50% of a sample population) were found to be higher than 100 mg/l [3].

The spermatozoa motility inhibition test also showed that the blue dye was the most toxic after 72 hours exposure. In this test, IC<sub>50</sub> values were calculated after 24 and 72 hours exposure. The values after 72 hours exposure were clearly lower than those shown by the hepa-1 cytotoxicity tests; the results from the spermatozoa test after 72 hours exposure were: for the yellow dye, 60 µg/ml; for the red dye, 46 µg/ml; the blue dye was toxic and it was not possible to calculate an IC<sub>50</sub> value. In terms of sensitivity to potentially toxic chemicals, the spermatozoa cells have not the same metabolic ability as hepa-1 cells [14,27]. However, after 24 hours exposure, the results of the spermatozoa motility inhibition test showed similarities to the IC<sub>20</sub> values from the hepa-1 cytotoxicity test. After 24 hours exposure using the spermatozoa test, the IC<sub>50</sub> values were: for the yellow dye, 135 µg/ml; for the red

dye, 124µg/ml; and for the blue dye, 127µg/ml; the equivalent IC20 values from the hepa-1 test were: for the yellow dye, 176µg/ml; for the red dye, 108µg/ml; and for the blue dye, 158µg/ml. The results of the spermatozoa test after 24 hours exposure and the IC20 values of the hepa-1 cytotoxicity test supported each other in terms of the yellow dye having the highest IC20 value and the red dye having the lowest IC20 value in both tests.

It is important to compare both the IC50 and the IC20 values when using the hepa-1 cytotoxicity test. The IC20 value shows the lowest toxic concentration of the sample, but the IC50 value gives extra information. The red dye caused toxic effects even in low concentrations, but in higher concentrations the blue dye was clearly the most toxic.

The fabric extracts were not toxic when tested using hepa-1 cells. The dye and the textile fibre molecules form covalent bonds with each other. Textile dyes are also hydrolyzable and thence lose reactivity. This could be the reason for the non-toxicity of fabric extracts. The spermatozoa test also showed fabric extracts to be non-toxic.

## Conclusions

The hepa-1-cytotoxicity test can be used in studying textiles: together with the spermatozoa test, it can give useful information about the toxicity of textile chemicals and materials. These tests can be especially useful when developing textiles for instance for allergic people. The tests could also provide information about the purity of different processes, waste waters and the environment. These tests are currently used for studying, for example, paper products. One possibility is to utilize these tests in the area of textile production. However, these tests cannot fully replace tests for evaluating contact allergy, mutagenicity, carcinogenicity and acute toxicity. The development of eco labelling adds further complications; however, this work clearly shows that cell tests can be a good addition to other methods for assessing the toxicity of dyes in solution or in fabrics.

## References:

1. Andersson M.; *Bacterial Diversity and Toxicity in Air, Indoor Environment and Foods*. Gummerus Oy, Saarijärvi 1999.
2. Birhanli, & A.; Ozmen; M. *Evaluation of toxicity and teratogenicity of six commercial textile dyes using the frog embryo teratogenesis assay - Xenopus*. *Drug and Chemical Toxicologies*, 2005, 28(1), 51-65.
3. *The Chemical Safety data Sheets after 2001/58/EY; Drimarene yellow CL-2R (RY176), Drimarene blue*
4. De Roos, A.J.; Ray, R.M.; Gao, D.L.; Wernli, K.J.; Fitzgibbons, E.D.; Ziding, F.; Astrakianakis, G.; Thoma, D.B.; & Checkoway, H.; *Colorectal Cancer Incidence Among Female Textile Workers in Shanghai, China: A Case-cohort Analysis of Occupational Exposures*. *Cancer Causes and Control*, 2005, 16 (10), 1177-1188.
5. Estlander, T.; *Allergic dermatoses and respiratory diseases from reactive dyes*. *Contact Dermatitis*, 1988, 18 (5), 290 - 297.
6. Gonzales C.A.; Riboli E.; & Lopez-Abente G.; *Bladder Cancer among Workers in the Textile Industry: Results of a Spanish Case-Control Study*. *American Journal of Industrial Medicine*, 1988, 14 ( ), 673-680.
7. Hatch K. L.; & Maibach H.I.; *Textile dye dermatitis*. *Journal of the American Academy of Dermatology*, 1995, 32 (4), 631-639.
8. Kaur, A., Sandhu, R.S. & Grover, I.S.; (1993). *Screening of azo dyes for mutagenicity with Ames/Salmonella assay*. *Environmental and Molecular Mutagenesis* 22, 188-190.
9. Keneklis, T.; *Fiber reactive Dye Toxicological Profiles*. U.S. Consumer Product Safety Commission, Washington, D.C., 1981, Contact No. CPSC-C-81-1110, p.271.
10. Kopponen, P., Sinkkonen, S., Poso, A., Gynther, J. & Kärenlampi, S.; (1994). *Sulfur analogues of polychlorinated dibenzo-p-dioxins, dibenzofurans and diphenyl ethers as inducers of CYP1A1 in mouse hepatoma cell culture and structure activity relationships*. *Environmental Toxicology and Chemistry* 13, 1543-1548.
11. Kopponen, P., Välttilä, O., Talka, E., Törrönen, R., Tarhanen, J., Ruuskanen, J. & Kärenlampi, S.; (1994). *Chemical and biological 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in fly ash*

- from combustion of bleached kraft pulp mill sludge. *Environmental Toxicology and Chemistry* 13, 143-148.
12. Kopponen P.; Asikainen M.; Törrönen R.; Klemola K.; Liesivuori J.; & Kärenlampi S. *In Vitro Analysis of Textile Dyes and Extracts of Dyed/Finished Fabrics. Atla*, 1997, 25, 539-546.
  13. Kärenlampi, S., & Törrönen, R.; (1990). Induction of cytochrome P4501A1 in mouse hepatoma cells as a short-term bioassay. *ATLA* 17, 158-162.
  14. Mann T.; & Lutwak-Mann C.; *Male reproductive function and semen*, p 10 - 250. Springer Verlag, Heidelberg, 1982.
  15. Manzini, B.M.; Motolese, A.; Conti, A.; Ferdani, G.; & Seidenari, S.; (1996). Sensitization to Reactive Textile Dyes in Patients with Contact Dermatitis. *Contact Dermatitis* 34 (3), 172-175.
  16. Mathur, N.; Bathnagar, P.; Nagar, P.; & Bijarnia, M.K.; Mutagenicity assessment of effluents from textile/dye industries of Sanganer, Jaipur (India): a case study. *Ecotoxicology and Environmental Safety*. 2005, 61 (1), 105-113.
  17. Nilsson, R.; Nordlinder, R.; Wass, U.; Meding, B.; & Belin, L.; *Asthma, Rhinitis, and Dermatitis in Workers Exposed to Reactive Dyes. British Journal of Industrial Medicine*, 1993, 50 (1), 65-70.
  18. Park, H.S.; Lee, M.K.; Kim, B.O.; Lee, K.J.; Roh J.H.; Moon, Y.H.; & Hong C-S.; *Clinical and Immunologic Evaluations of Reactive Dye-Exposed Workers. Journal of Allergy and Clinical Immunology*, 1991, 87(3), 639-649.
  19. Rannung, U., Bramstedt, H.; & Nilsson, U.; (1992). The presence of genotoxic and bioactive components in indigo dyed fabrics: a possible health risk? *Mutation Research* 282, 45-53.
  20. Sundquist, J.; (1985). *Tekstiiliväriaineet. Kemia-Kemi*, 11,937-944
  21. Thoren, K.; Meding, B.; Nordlinder, R.; & Belin, L. (1980). *Contact Dermatitis and Asthma from Reactive Dyes, Contact Dermatitis*, 15 (3) 186.
  22. Trotman, E.R.; *Dyeing and chemical technology of textile fibres. Charles Griffin & Co Ltd, Worcester* 1984.
  23. Törrönen, R.; Pelkonen, K.; & Kärenlampi, S.; (1989). Enzyme-inducing and cytotoxic effects of wood-based materials used as bedding for laboratory animals. Comparison by a cell culture study. *Life Sciences* 45, 559-565
  24. Wilkinson, S.M.; & McGechaen, K.; *Occupational Allergic Contact Dermatitis from Reactive Dyes. Contact Dermatitis*, 1996, 35 (6), 376 - 378.
  25. Wollin, K.M.; & Gorlitz, B.D.; *Comparison of genotoxicity of textile dyestuffs in Salmonella mutagenicity assay, in vitro micronucleus assay, and single cell gel/comet assay. Journal of Environmental Pathology, Toxicology and Oncology*. 2004, 23 (4), 267-278.
  26. <http://www.utexas.edu/centers/natstat/data/1x0,002.pdf>. *Worldwide Textile Production, 1980-2003*.
  27. Zeng Y.; Clarke E.; & Florman H.M.; *Sperm membrane potential: Hyperpolarisation during capacitation regulates zona pellucida-dependent acrosomal secretion. Dev. Biol.* 1995, 171, 554-563.
  28. *Öko-Tex Standard 100, Textilveredlung* 32 (1997), Nr 7/8.

▽△