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Abstract

Disperse dyes are the most common allergens among textile dyes. It is not known whether the purified dyes, impurities in the commercial dyes, or metabolites are the actual sensitisers. Moreover, it is not known whether those disperse dyes that are now present in test series are actually used in textile dyeing today.

The aim of this thesis was A) to evaluate the significance of the impurities found in the commercial dyes Disperse Orange 1 and Disperse Yellow 3 and their potential metabolites from azo reduction regarding contact allergy; B) to investigate the sensitising capacity of Disperse Orange 1 and its metabolites and their cross-reactivity to Disperse Yellow 3, its metabolites, and PPD; and C) to determine whether eight disperse dyes, hitherto the most widely quoted as allergenic, are still used in textiles sold in various countries all over the world. Evaluation of the many published studies on contact allergy to disperse dyes used for dyeing textiles was also performed.

It was shown that the commercial dyes Disperse Orange 1 and Disperse Yellow 3 each contain at least one impurity acting as a sensitiser. Positive patch test reactions to Disperse Orange 1 and Disperse Yellow 3 were linked to positive reactions to some of their metabolites: p-aminodiphenylamine and 2 –amino-p-cresol, respectively. It was found that Disperse Orange 1 and p-aminodiphenylamine are strong sensitisers and cross-react with each other in the guinea pig maximisation test. PPD, 4-nitroaniline, 4-aminoacetanilide, 2-amino-p-cresol, or Disperse Yellow 3 did not show any cross-reactivity to them. Our observations did not directly support the metabolite theory, and the results regarding elicitation thresholds spoke against this theory. Available data in the medical literature indicated that positive patch test reaction prevalence rates to Disperse Blue 106 and 124, and Disperse Orange 3 were over 1% when screening dermatitis patients. From 121 analyzed items, Disperse Yellow 3, Disperse Blue 124 and 106 and Disperse Orange 1 were detected in three garments made in the European Union and India.

Key words: Disperse Orange 1, Disperse Yellow 3, metabolites, azo reduction, p-aminodiphenylamine, 4-nitroaniline, 2-amino-p-cresol, 4-aminoacetanilide, TLC, HPLC, guinea pig maximisation test.

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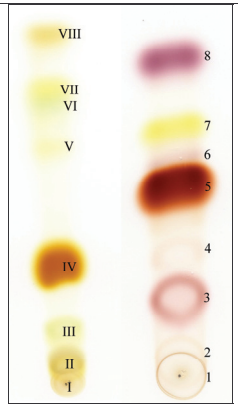
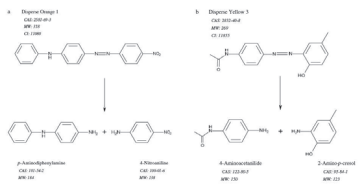
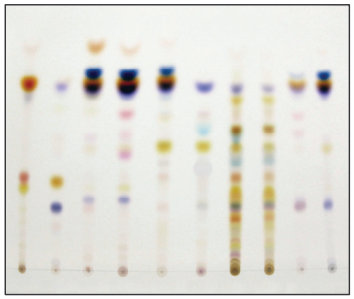
For my beloved grandparents
Viktorija (1921 – 2011) and Balys (1921-2009) Dragūnai

Thesis at a glance

	Objective	Method	Illustration	Main findings/Conclusions
Paper I **Textile dyes Disperse Orange 1 and Disperse Yellow 3 contain more than one allergen as shown by patch testing with thin-layer chromatograms**	To evaluate the significance of the impurities found in the commercial dyes DO1 and DY3 regarding contact allergy in patients with known sensitivity to them.	Ten patients allergic to DY3 and/or DO1 were tested with dilution series of commercial and purified DY3 and DO1, with the water-soluble part prepared from the commercial dyes, and with naphthalene sulphonate. A total of nine patients were additionally tested with TLCs made from the commercial DO1 and DY3 and with paper chromatograms made from the water-soluble part of these dyes.		Among positives to the TLCs made from DO1 and DY3, four of eight and two of three patients, respectively, were positive not only to the main but also to another mutual spot on the TLCs. Six out of eight patients, positive to DO1, and two out of six positive to DY3, were also positive to the water-soluble part of commercial DO1 or DY3, respectively. None of the patients reacted to naphthalene sulphonate. Results suggest that there are more relevant allergens in the fat- and in the water-soluble fractions of the commercial disperse dyes.
Paper II **Patch testing with the textile dyes Disperse Orange 1 and Disperse Yellow 3, some of their potential metabolites and simultaneous reactions to *para*-amino compounds**	To evaluate the hypothesis that the molecules of disperse dyes migrate onto the skin while one is wearing garments, are metabolized and degraded by commensal skin bacteria. These molecules penetrate the skin and induce sensitization.	Ten patients allergic to DY3 and/or DO1 were patch-tested with dilution series of purified dyes, 4-nitroaniline and PADPA in equimolar concentrations to purified DO1 in the dilution series, 4-aminoacetanilide and 2-amino-*p*-cresol in equimolar concentrations to purified DY3 as well as the ingredients of black rubber mix and PPD in the dilution series.		An overrepresentation of simultaneous positive reactions between DO1 and PADPA and DY3 and 2-amino-*p*-cresol, respectively, was seen. The elicitation thresholds for DO1 and DY3 were lower than for proposed metabolites and test reactions from all substances tested in equimolar concentrations had similar strength and developed with similar speed. Observations indicate that the observed simultaneous reactions are due to cross-reactivity.

	Objective	Method	Illustration	Main findings/Conclusions
Paper III **Are allergenic disperse dyes used for dyeing textiles?**	It is unknown whether disperse dyes, which are present in specific patch-test series, are in fact present in synthetic textiles on the market. The primary objective was to determine whether eight disperse dyes, hitherto most widely quoted as allergenic, are still used in textiles sold in various countries all over the world.	Synthetic textiles from 13 countries in Europe, Asia and Americas were analysed. The procedure used for dye identification was thin-layer chromatography. When there were matching spots from the textile extract and reference dye, HPLC was performed.		From 121 analyzed items, three showed positive results for some of the investigated disperse dyes. Four dyes in these items could be confirmed by HPLC. A light brown lady' tights manufactured and purchased in Italy, contained DY3, DB124 and DB106, and a set of black bra and panties purchased in India, contained DO1. The eight disperse dyes which are most frequently incriminated to be the cause of textile dye dermatitis are rarely used in textiles nowadays.
Paper IV **Contact allergy to disperse dyes in textiles – a review**	To evaluate published studies and reports on contact allergy to disperse dyes used for dyeing textiles.	Studies and case-reports published in PubMed during the last 22-year period (1990-2012) were reviewed.	PubMed	Available data indicates that positive patch test reaction prevalence rates at least to three dyes (DB106, DB124 and DO3) were over 1% in screening dermatitis patients.
Paper V **Sensitising capacity of Disperse Orange 1 and *p*-aminodiphenylamine and cross-reactivity to 4-nitroaniline, Disperse Yellow 3, its potential metabolites and *p*-phenylenediamine**	To investigate the sensitising capacity of DO1, PADPA and 1,4-nitroaniline and the cross-reactivity between them and DY3, 2-amino-*p*-cresol, 4-aminoacetanilide, and PPD.	The guinea-pig maximisation test.		It was found that DO1 and PADPA are strong sensitisers and cross-react with each other. Neither PPD, 4-nitroaniline, 4-aminoacetanilide, 2-amino-*p*-cresol, nor DY3 showed any cross-reactivity to DO1 or PADPA.

DO1, Disperse Orange 1; DY3, Disperse Yellow 3; DB, Disperse Blue; DO3, Disperse Orange 3; PADPA, p-aminodiphenylamine; PPD, *p*-phenylenediamine; TLCs, thin-layer chromatograms; HPLC, high-performance liquid chromatography.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis.

I. **Textile dyes Disperse Orange 1 and Disperse Yellow 3 contain more than one allergen as shown by patch testing with thin-layer chromatograms.**
Malinauskiene L, Zimerson E, Bruze M, Ryberg K, Isaksson M. *Dermatitis* 2011: 22: 335-343

II. **Patch testing with the textile dyes Disperse Orange 1 and Disperse Yellow 3 and some of their potential metabolites, and simultaneous reactions to para-amino compounds.**
Malinauskiene L, Zimerson E, Bruze M, Ryberg K, Isaksson M. *Contact Dermatitis* 2012: 67: 130-140

III. **Are allergenic disperse dyes used for dyeing textiles? .**
Malinauskiene L, Zimerson E, Bruze M, Ryberg K, Isaksson M. *Contact Dermatitis* 2012: 67: 141-148.

IV. **Contact allergy from disperse dyes in textiles – a review.**
Malinauskiene L, Bruze M, Ryberg K, Zimerson E, Isaksson M. *Accepted for publication in Contact Dermatitis, August 2012*

V. **Sensitising capacity of Disperse Orange 1 and its potential metabolites from azo reduction and their cross-reactivity pattern.**
Malinauskiene L, Zimerson E, Bruze M, Ryberg K, Isaksson M. *Submitted for publication in Contact Dermatitis*

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ABBREVIATIONS

ACD	Allergic contact dermatitis
Ac	Acetone
Aq	Aqueous
BRM	Black Rubber Mix
CAS	Chemical Abstract Service
C.I.	Colour Index
D	Disperse
DD	Disperse Dye
EU	the European Union
GPMT	Guinea Pig Maximisation Test
HPLC	High Performance Liquid Chromatography
PADPA	*p*-Aminodiphenylamine
Pet	Petrolatum
PET	Polyethylene Terephthalate, polyester
PPD	*p*-Phenylenediamine
TDM	Textile Dye Mix
TLC	Thin-Layer Chromatography
TLCs	Thin-Layer Chromatograms

CONTENTS

1. INTRODUCTION

The aim of the work presented in this thesis was to investigate contact allergy to disperse azo dyes. A general introduction to textiles and textile dyes is given here to provide the reader with a more in-depth knowledge of the topic.

BACKGROUND

Textiles

Textile (from Latin *textilis* "woven") originally referred only to woven fabrics, but nowadays refers to the material made from any filament by different methods (e.g., knitting, bonding, felting, etc) is named textile, fabric or cloth (1). It is speculated that the first clothes worn at least 70 000 years ago were probably made of animal skin (2). The primary functions of the textiles were protection and comfort. The dyed fibres of the flax plant from Dzudzuana cave in the Caucasus Mountains, located in the Republic of Georgia, are the oldest example of a fabric as they were dated to have existed more than 30 000 years ago (3). The fact that the fibres were dyed indicates that prehistoric humans were also interested in the exclusiveness and other functions of the textile, not only protection.

The nature of colour

Colour is a visual perceptual property of humans. It is produced by the visible wavelengths of electromagnetic radiation, which stimulate specific receptors- cones- in the retina of the eye. Visible wavelengths ("visible light") which humans can perceive are within 400-700 nm (4). The red, yellow, and blue, known as primary colours, can be combined in varying proportions to produce all other colours (5). Colour vision is not exclusive to human beings. Many animals also have this ability. Animals with two interacting cone types, such as most mammals other than old-world primates, have two-dimensional colour vision. Thus, studies of colour vision are important when studying evolution of species (6).

Colour of an object depends not only on the wavelengths but also on the surface of the object, ambient illumination, and colours of other objects nearby. So, perception of the colour is a complex process which depends on the object, the physical properties of the electromagnetic radiation (light), and the characteristics of the eye and brain that perceives the colour.

Different colour theories exist to explain why we see so many colours. In *On Colors* (*De Coloribus*) Aristotle (384-322 BC) describes his theory that all colours (yellow, red, purple, green and blue) are derived from mixture in different proportions of white and black (7). This theory remained the most influential one until Isaac Newton´s (1642-1727) experiments, where he first identified the light as the source of the colour sensation, which is entirely a physical event according to him (5).

Johann Wolfgang von Goethe (1642-1727) in his *Theory of Colors*, published in 1810, introduced the importance of the mechanics of human vision and how the brain process information. So the colour of the object, according to him, depends upon the object, light, and perception (8).

In the beginning of the 19th Century Thomas Young (1773-1829) proposed his trichromatic theory, based on the observation that any colour could be matched by a combination of three primary colours – red, green and violet (9). The German physiologist Ewald Hering (1834-1918) developed the opponent process theory of colour where six primary colours were coupled in three pairs: red–green, yellow–blue and white–black. Any receptor that was turned off by one of these colours was excited by its coupled colour (9). Ultimately the opponent and trichromatic approaches were merged into one by Leo M. Hurvich and Dorothea Jameson in 1960 (8). They showed that processes in the retina correspond to the trichromatic theory, while processing at the level of the lateral geniculate nucleus in which neurons send their axons up to the visual cortex corresponds to the opponent theory (10). A more recent and complex model is the retinex theory developed by Edwin H. Land in 1977, where lightness is recognized as a fundamental stimulus of colour (8). He explained the ability of humans to see essentially true colours despite a wide variation in the colour of the light illuminating objects (8).

In 1931, an international group of experts known as the Commission Internationale de l´Eclairage (The International Commission on Illumination, CIE) developed a mathematical colour model - XYZ colour space (CIELAB). It was the first widely accepted, international standard way of defining colour and is still the "gold standard" today. Observable colours are mapped out in space and assigned a set of three numbers to each in this model. This model is used in the colour industry to define colours of the dyes (9, 11).

Dye structure and colour

In 1876 German chemist Otto Witt proposed that dyes contain conjugated systems of benzene rings bearing simple unsaturated groups (e.g., –NO2, –N=N–, –C=O), which he called chromophores, and polar groups (e.g., –NH_2, –OH), which he named auxochromes. The term chromogen is used for specific chromophore-auxochrome combinations (11).

The colours of different dyes are due to the absorption of visible light by their compounds and are directly related to the molecular structure of the dye. Organic compounds absorb electromagnetic energy, but only those with several conjugated double bonds appear coloured by the absorption of visible light. Progressive absorption into the visible region gives orange (430–480 nm), red (480–550 nm), violet (550–600 nm), and blue (600–700 nm). Absorption at 400–450 and 580–700 nm gives green. Black objects absorb all visible light, and white objects reflect all visible light. Synthetic dyes tend to give brilliant colours and this is one of the reasons of their popularity because natural dyes give rather drab, diffuse colourations (11).

TEXTILE DYES

Dyes are any substance, belonging to a class of intensely coloured complex compounds, used to colour textiles, leather, paper, and other materials so that the colouring is not readily altered by washing, heat, light or other factors to which a material is likely to be exposed (11). Colouring substances can be classified as dyes and pigments. Dyes differ from pigments, which are finely ground solids dispersed in a liquid, such as paint or ink, or blended with other materials. Most dyes are organic compounds (i.e., they contain carbon), whereas pigments may be of inorganic as well as organic origin (11).

History of the dyes and dyeing

The oldest description of dyeing found in Europe is *The Papyrus Graecus Holmiensis*, which is also known as the Stockholm Papyrus (12) (Fig. 1). It dates from 300 AD and contains 154 craft recipes for dyeing written in Demotic Greek (13).

Figure 1. *Excerpt from Stockholm Papyrus with a dye recipe (12).*

105. Dyeing in Dark Blue.

Put about a talent of woad in a tube, which stands in the sun and contains not less than 15 metretes, and pack it in well. Then pour urine in until the liquid rises over the woad and let it be warmed by the sun, but on the following day get the woad ready in a way so that you (can) tread around in it in the sun until it becomes well moistened. One must do this, however for 3 days together.

Dyes known to the ancients came from shells, like Tyrian purple, from mucus of predatory tropical sea snails Murex, or insects, like cochineal from the insects' scale, but mostly from plants (12). Probably the oldest known dye is the blue dye, indigo, obtained in Europe from the leaves of the dyerswoad herb *Isatis tinctoria* in Europe and in Asia from the indigo plant, *Indigofera tinctoria* (Table 1).

Even by modern standards, some natural dyes (e.g., indigo, alizarin) are considered to have very good dyeing properties, but today logwood is the only natural dye used in the industry (11).

Table 1. *Some examples of the natural dyes used to dye textiles.*

Colour	Name	Source
RED	Alizarin	Roots of the madder plant (Rubia tinctorum), wild madder (Rubia peregrina L.), munjeet (Rubia cordifolia L.), ladies' bedstraw (Galium verum L.) and several species of Relbunium;
	Kermes	Insects *Coccus ilicis* (or *Kermes ilicis);*
	Cochineal	Insects: *Dactylopius coccus (Central America),* Porphyrophora polonica (Central Europe), lac (Kerria lacca Kerr).
YELLOW	Weld	Seeds, stems, leaves of *Reseda luteola*;
	Quercetin	North American oak bark, *Quercus tinctoria nigra* dried petals of *Carthamus tinctorius;*
	Safflower	Stigmas of *Crocus sativus;*
	Crocetin	Heather, Calluna vulgaris, dyer's broom, Genista tinctoria, common buckthorn, Rhamnus cathartica, chamomile (Anthemis tinctoria L.), Bog myrtile (Myrica gale L.).
BLUE	Indigo, woad	Indigo plant leaves, *Indigofera tinctoria L,* dyerswoad herb, *Isatis tinctoria*.
PURPLE	Tyrian purple	Molluscs: spiny dye-murex, *Murex brandaris,* rock-shell, Thais haemastoma, banded dye-murex (Hexaplus trunculus L.), Nucella lapidus L. (the North Atlantic countries), Purpura patula L. (Caribbean and Florida), Rapana bezoar L. and Thais clavigera Küster (Japan), Mancinella kieneri and Dicathais orbita (Australia). Lichens: Rocella tinctoria D.C., Rocella fuciformis Orchrolechia tartarea L.
BLACK	Logwood	Heartwood, *Haematoxylon campechianum L., Quercus cerris,* sumac (Rhus) species.

Extracts of heartwood of the logwood tree, *Haematoxylon campechianum*, is used mainly to dye silk and leather (12).

The formation of different colours was well known in old times, and inorganic metal salts (mordants, from Latin *mordere* - "to bite") were used for the retention of dyes on the desired material and also to vary colour shades (14).

In the middle of the 19th Century, the Industrial Revolution in Europe increased demands for readily available, inexpensive, and easily applied dyes. Increased use of coal and discovery of many new compounds in coal tar stimulated research in the organic chemistry. In 1856 the first commercially successful synthetic dye, mauve or mauveine, was accidentally discovered by British chemist William H. Perkin (1838-1907). Mauve had a short commercial lifetime, but its success catalyzed activities that quickly led to the discovery of better dyes and nearly 90% of industrial dyes were synthetic in the beginning of the 20th Century (12).

A few new dye types were introduced in the 20th Century, and major challenges were posed by the introduction of synthetic fibres, which continue to hold a major share of the world market. Today most dyes are made from coal tar and petroleum chemicals. The chemical structure of dyes is relatively easy to modify, so many new colours and types of dyes have been synthesized. It is estimated that today some 9 000 colourants with more than 50 000 trade names are used (11).

Short history of dyeing in Sweden

Dyed textiles have been found in Sweden from the beginning of the Migration Period (400-800 AD) (15). The oldest examples date from 500 AD and are from the burial of a chieftain in Högom in the north of Sweden which included textiles dyed with madder, *Rubia peregrina* L., and *Porphyrophora polonica*. Johan Linder´s *Svenska Färge-konsten* published in 1720 is the first Swedish book on dyeing (15). The 18th Century was a period of activity aimed at strengthening the domestic industries in Sweden and at the request of the government, Carl Linnaeus (1707-1778) recorded the existence and use of plants for dyes during his travels through Sweden - from Lapland in the north to Skåne in the South. According to tradition, the Swedish national flag, which is one of the oldest existing national flags of Europe, was dyed with woad and weld (15).

Short history of dyeing in Lithuania

The oldest dyed fabrics found in Lithuania in archaeological excavations of cemeteries, date from the first century AD (16). Most researched fabrics were dyed with a blue colour, but also red, yellow and black. Most of the plants, which were used at those times in Western Europe for dyeing, were not indigenous to Lithuania. The dyer's woad was a very rare plant in Lithuania, so devil's bit (*Succisa pratensis*) as well as water smartweed (*Polygonum amphibium*) could have been used for obtaining a

blue colour. Madder is not native of Lithuania, so red colour fabrics most probably were dyed using *Galium* genus plants that also give a red colour, e.g., lady's bedstraw (*Galium verum*), hedge bedstraw (*Galium mollugo*), sweet woodruff (*Galium odoratum*), or common cleavers (*Galium aparine*) (16).

Textile fibres

The selection of dyes depends on the chemical structure of the fibres in the fabric. Fibre molecules are polymeric chains of repeating units of five major chemical types (1):

- Proteins (e.g., wool, silk, milk).
- Polyamide (e.g., nylon, aramides, polyphthalamides) are synthetic analogues of proteins.
- Polyester (polyethylene terephthalate), or PET, is the main synthetic fibre, accounting for more than 50% of worldwide production of synthetic fibres.
- Acrylic fibres, made from polyacrylonitrile.
- Cellulose (e.g., cotton, bamboo, birch, pineapple, lyocell, rayon (viscose) or acetate rayon – semisynthetic cellulose).

Fibres are made by various spinning techniques that produce bundles of up to several hundred roughly aligned strands of polymer chains. It is common to blend different types of fibres, e.g., cotton and polyester. For the dyeing process, an important characteristic of fibres is their porosity. There are a huge number of microscopic pores aligned mainly on the longitudinal axis of the fibres such that there are roughly 10 million pores in a cross-section of a normal fibre. Upon immersion in a dye bath, the fabric absorbs the aqueous dye solution, and the dye molecules can move into pores that are sufficiently large to accommodate them. Then, depending on the fibre type and dye class, different chemical bonds can be formed between the dye molecule and the fibre (11).

Classifications of dyes

Dyes can be classified by the chemical structure or by the method of application. Classification according to the colour is compiled in the *Colour Index* (C.I.), which is edited by the Society of Dyers and Colourists and by the American Association of Textile Chemists and Colorists since 1924 (17). The fourth edition of the index lists more than 8 000 dyes used for textile fibres, plastics, printing inks, paints, and liquids. Each C.I. generic name covers all colourants with the same structure, but these are not necessarily identical products in terms of additive or impurity content. While there are thousands of C.I. generic names, each manufacturer can invent a trade name for a given colourant, and, consequently, there are more than 50 000

names of commercial colourants (11, 17). All this brings confusion in identification of the dyes used when it comes to the consumer.

Classification of the dyes according to dyeing techniques

Direct dyeing

Direct dyes are applied directly to the natural fibres from a hot aqueous solution of the dye. After treatments, frequently applied to the dyed material to improve wash-fastness properties, one should include chelation with salts of metals (usually copper or chromium), and treatment with formaldehyde or a cationic dye-complexing resin (11).

Disperse dyeing

Synthetic fabrics (acetate, PET, acrylic fibres) are dyed by immersion in an aqueous dispersion of insoluble dyes, in which the dye transfers into the fibre. The transfer mechanism is unclear, but it appears that the fibres loosen slightly to permit the dye's entry and, on cooling, revert to the original tightly packed structure. Dyeing at higher temperatures (120–220°C) under pressure avoids the need for carriers. Disperse dyes were originally developed for acetate rayon, but became the fastest growing dye class in the 1970s because of the rapid increase in world production of PET, which is dyed mainly using these disperse dyes (11).

Vat dyeing

Vat dyeing, an ancient dyeing method, implies the conversion from the soluble to an insoluble dye after transfer to the fibre. This process was traditionally done outdoors in large vessels or vats and, hence, was named vat dyeing. The term is still used for this procedure. An example of vat dyeing is the application of indigo onto a cotton fabric (11).

Azo dyeing

The fabric can be first treated with a solution of the coupling component and then placed in a solution of the diazonium salt to form the dye on the fabric. Alternatively, the fabric can be treated with a solution of the diazo component before diazotization, followed by immersion into a solution of the coupling component.

Reactive dyeing

Reactive dyeing directly links the dye to the fibre by formation of a covalent bond. The introduction of reactive dyeing in the mid-20th Century increased the spectrum of colours and dye types that could be used for cotton, since almost any chromogen can be converted to a reactive dye.

Sulphur dyes

The dyeing of cellulose fibres and its blends with synthetic fibres is the main application of sulphur dyes. These dyes are used for deeper, muted shades, such as black, dark blue, olive, brown, and green. Sulphur dyes contain S–S bridges in their molecule and their molecular weight is high. Sulphur dyes, like vat dyes, are fixed on the textiles by oxidation.

Mordant dyes

The mordant dyes (or metal-complex dyes) are preferred for deep colours in black, navy blue, and brown shades. For textile fibres, only the chromium, cobalt, and copper complex dyes achieve the desired technical effects. They represent the most important dye class for dyeing wool.

Solvent dyes

Solvent dyes are those that are basically insoluble in water, but can be dissolved in the different types of solvents. Solvent dyes also function as dyes for certain polymers, such as polyacrylonitrile, polystyrene, polymethacrylates, and polyester. They are also used to dye various kinds of oils, waxes, lubricants, plastics and fuels.

Basic dyes

Basic dyes ionise in solutions to give cations. The auxochrome group is an amine forming salts with acids. They are mainly applied to polyacrylonitrile fibres, but with slight modifications of the process they can be used for dyeing wool, silk, paper, and cotton. Bright colours are obtained when using these dyes, but the fastness, especially in light, is poor.

Acid dyes

Acid dyes are sodium (less often – ammonium) salts of a sulphuric, carboxylic or phenolic organic acid. They have a rather low molecular weight and are soluble in water. Acid dyes lack affinity for cellulose fibres. They can be used for dyeing leather and cosmetics. When dyeing, the ionic bonds with fibres' cationic sites accounts for fixation in the dyed material. They are resistant to sunlight and will not fade due to washing. Some acid dyes are used as food colourants.

Azoic (or naphthol) dyes

These are water-insoluble azo dyes mainly used for dyeing cellulose fibres, but sometimes also PET products. Azoic dyes are produced directly on or within the fibre by applying two chemically reactive water-soluble compounds to the fabric: a diazoic compound and a coupling component, 2-hydroxy-3-naphthanilide (Naphthol AS) under suitable conditions. The two components react to produce the insoluble azo dye. Thus excellent fastness properties are achieved. Today, azoics are used primarily for deep clear shades, like red and purple.

Classification of the dyes according to chemical structure

The Chemical Abstracts Service (CAS), a division of the American Chemical Society assigns identifiers to chemicals that have been described in the literature by giving them a CAS number, which is a unique numerical identifier (18). A dye may have a defined CAS number, but no C.I. number if the chemical formula of the dye is not known. Compilation of the two systems of the dyes classifications is given in Table 2.

Table 2. *Usage classification of dyes according to K. Hunger (11) with modifications by the author.*

Dye class according to application	Main substrates	Dye classes according to chemical structure
Acid	Nylon, wool, silk (also paper, inks, leather)	azo, antraquinone, triphenylmethane, azine, xanthenes, nitro, nitroso
Azoic	Cotton, rayon, cellulose acetate, PET	azo
Basic	Polyacrylonitrile, modified nylon, PET (also paper, inks)	cyanine, hemicyanine, diazahemicyanine, diphenylmethane, triarylmethane, azo, azine, xanthene, acridine, oxazine, anthraquinone
Direct	Cotton, rayon, nylon (also paper, leather)	azo, phthalocyanine, stilbene, oxazine
Disperse	PET, polyamide, acetate, acrylic (also plastics)	azo, anthraquinone, styryl, nitro, benzodifuranone
Mordant	Wool (also leather)	azo and anthraquinone
Reactive	Cotton, wool, silk, nylon	azo, anthraquinone, phthalocyanine, formazan, oxazine, basic
Solvent	Plastics, fuels, varnishes, lacquers, inks, oils, waxes	azo, triphenylmethane, anthraquinone, phthalocyanine
Sulphur	Cotton, rayon	indeterminate structures
Vat	Cotton, rayon, wool	anthraquinone, indigoids

Impact on the human health

Systemic effects

Toxicity (i.e., mortality, genotoxicity, mutagenicity and carcinogenicity) of the dyes has been investigated in numerous studies using various forms of life - from aquatic organisms (algae, bacteria, fish, etc.) to mammals.

The acute toxicity of dyes is generally low. The most acutely toxic dyes for algae and fish are cationic basic dyes and fish also seem to be relatively sensitive to many acid dyes compared to other animals. Mortality tests with rats showed that only 1% of

4461 commercial dyes tested had the median lethal dose (LD50) values below 250 mg/kg body weight (19). According to the authors, the risk of human mortality due to acute toxicity to the dyes is apparently very low.

Chronic effects of dyestuffs, especially of azo dyes, have been studied for several decades.

Most research is focused on the effect of food dyes, which usually are azo compounds. Furthermore, the effects of occupational exposure to dyestuffs in workers in dye manufacturing and dye-utilising industries have also received attention. Azo dyes in purified form are seldom directly mutagenic or carcinogenic, except for some azo dyes with free amino groups (19). However, reduction of azo dyes, i.e. cleavage of the dye's azo linkage(s), leads to formation of aromatic amines and several aromatic amines are known mutagens and carcinogens (20). In mammals, metabolic reduction of azo dyes is mainly due to bacterial activity in the anaerobic parts of the lower gastrointestinal tract. Various other organs, especially the liver and the kidneys, can also reduce azo dyes. After azo dye reduction in the intestinal tract, the released aromatic amines are absorbed by the intestine and excreted in the urine. The acute toxic risk of aromatic amines is carcinogenesis, especially bladder cancer. The carcinogenicity mechanism probably includes the formation of acyloxyamines through N-hydroxylation and N-acetylation of the aromatic amines followed by O-acylation. These substances bind to DNA and RNA, which induces mutations and tumour formation (21).

Food dyes. Upon their discovery, synthetic dyes rapidly replaced many metallic compounds used to colour foods. The advantages of the synthetic food dyes over natural colourants — such as brightness, stability, colour range and lower cost — were quickly appreciated, but recognition of their adverse effects was slower. A synthetic dye is permitted for dyeing food only if toxicological studies reveal no danger of toxic effects to the consumer. Food dyes are among the food additives that have been subjected to the most thorough toxicological examinations (22). However opinion remains widely divided on this issue, since few countries agree on which dyes are safe. For example, no synthetic food dyes are used in Norway and Sweden, whereas 16 are approved in the United Kingdom, although some of these dyes have been linked with adverse health effects. Some food dyes are banned in one country, but approved in others (e.g., the azo dye amaranth or Ponceau 4R is banned in the USA, but approved in Canada) (12).

The use of food additives in the European Union (EU) is regulated by a framework directive (Council Directive 89/107/EEC of 21 December 1988 on the approximation of the laws of the Member States concerning food additives authorised for use in foodstuffs intended for human consumption, as amended by Directive 94/34/EC) and a specific directive (European Parliament and Council Directive 94/36/EC of 30 June 1994) on colours for use in foodstuffs (23, 24). Approved dyes

within the EU listed in EEC Directives are assigned E numbers (Table 3). Most food dyes belong to the azo dye class.

Systemically ingested food dyes used in food and as colouring agents for drugs have been described to provoke urticaria and bronchial asthma (22, 25). In general there is a lack of data saying that routine exclusion of the dyed foods benefits most patients with asthma or urticaria except those very few individuals with proven sensitivity (22, 26, 27). Formation of the immunoglobulin E seems not to be involved in the reactions and non-allergic hypersensitivity mechanisms (e.g., mediated by the direct degranulation of the mast cells) are suspected as the cause (25).

There are reports of other skin diseases possibly caused by the food dyes in foods or medications. Orchard et al. reported a case of recurrent fixed drug eruption to tartrazine, present in cheese chips, in an 11 year-old girl proven by oral provocation tests (28).

Table 3. *Synthetic food dyes which are approved in the EU (excerpt from 94/36/EC, Annex 1) (24).*

E No.	Common name	CAS No.	Chemical class
E 102	Tartrazine	1934-21-0	Azo
E 104	Quinoline Yellow	8004-92-0	Quinophthalone
E 110	Sunset Yellow FCF	2783-94-0	Azo
E 122	Azorubine	3567-69-9	Azo
E 123	Amaranth	915-67-3	Azo
E 124	Ponceau 4R	2611-82-7	Azo
E 127	Erythrosine	16423-68-0	Xanthene
E 128	Red 2G	3734-67-6	Azo
E 129	Allura Red AC	25956-17-6	Azo
E 131	Patent Blue V	3536-49-0	Triarylmethane
E 132	Indigo Carmine	860-22-0	Indigoid
E 133	Brilliant Blue FCF	3844-45-9	Triarylmethane
E 142	Green S	860-22-0	Triarylmethane
E 151	Black PN (Brilliant Black BN)	2519-30-40	Azo
E 154	Brown FK	8062-14-4	Azo
E 155	Brown HT	4553-89-3	Azo
E 180	Litholrubine BK	5284-04-9	Azo

There are few reports on the systemic contact dermatitis-like symptoms in patients already sensitised to *p*-phenylenediamine (PPD). Sornin de Leysat et al. reported two patients with contact allergy to PPD presenting exacerbated eczema following the ingestion of an antihistamine tablet containing Sunset Yellow FCF (E 110) (29). The authors speculated that this could be caused by a cross-sensitivity between PPD and sulphanilic acid (4-aminobenzenesulphonic acid), which is a metabolite of the Sunset

Yellow FCF. A similar case report is published by Rogkakou et al. where Sunset Yellow FCF was suspected to cause a severe skin reaction in a patient positive to PPD and Disperse (D) Orange 3 (30).

Systemically ingested azo dyes (e.g., tartrazine (E 102), have been blamed for provoking attention deficit syndrome (ADD) in children (31).

Dermal effects

Allergic contact dermatitis

Contact allergy is caused by more than 4300 substances found in the environment (32). The Copenhagen Allergy study showed that as many as 15–20% of the population can be allergic to one or more chemicals from their environment (33). Allergic contact dermatitis (ACD) is the clinical manifestation of contact allergy. It develops at the site of contact with the allergenic compound in a sensitised individual when the dose at the exposed skin area exceeds the present sensitivity threshold of the individual (34).

Contact allergy is a delayed hypersensitivity (type IV according to Gell and Coombs) reaction, which is mediated by antigen-specific T-lymphocytes (34). Three phases of the ACD development are described. *In the afferent phase (sensitisation)* an individual is sensitised by skin contact with reactive chemicals (haptens) of low molecular weight (usually below 500-700) and high lipophilicity (log Po/w>1) which can penetrate into the stratum corneum (35, 36). Haptens must link to proteins in the skin to form a complete antigen before they can sensitise (37). Some substances are prohaptens, and must be metabolized in vivo to become antigenic, or pre-haptens, in chemical processes before entering the skin, e.g., oxidation in contact with air and light, give them electrophilic properties (37, 38). Proteins to which haptens react can be both soluble proteins and membrane-bound proteins present on the surface of the Langerhans cells (LCs). The amino acids cysteine and lysine, as well as histidine, methionine, and tyrosine are considered the most important electron-rich nucleophilic functional groups present in the skin proteins (39). Hapten binds proteins because they contain functional groups called nucleophiles which are negatively charged, and most haptens are electrophiles, so on interaction these groups can form covalent bonds (40).

LCs are the most important antigen-presenting dendritic cells located in the epidermis. They become activated after contact with the protein-hapten conjugate (antigen) and migrate via the afferent lymph vessels to the draining lymph nodes where they present the processed antigen to naive T-lymphocytes. Then formation of antigen-specific effector and memory T-lymphocytes clones may occur and thereafter these cells circulate in the blood and lymph vessels. It is supposed that the sensitisation phase in humans lasts about seven days. After repeated contact with the same hapten, memory T-lymphocytes are recruited to the site of contact, where

interactions between T-lymphocytes and antigen-presenting cells can take place directly in the epidermis, initializing the inflammatory process which results in the clinical manifestation of ACD (*the efferent phase – elicitation*). In humans it takes usually one to two days after contact with the allergen, although for some substances the elicitation phase may take more than two to three weeks before ACD develops (34, 35, 41, 42).

The resolution phase of ACD starts as early as 24 hours after allergen challenge (43). Various soluble mediators, derived from the same cells responsible for eliciting the reaction, and an expanding number of T regulatory cells play an active role in suppressing the response to the allergen.

Finally, desquamation of antigen-laden skin, cellular, or enzymatic degradation of the antigen with destruction of the antigen-presenting dendritic cells and other regulatory mechanisms contribute to the resolution of the allergic response (44, 45).

Contact allergy is chronic and usually lifelong (although temporal changes of the degree of the sensitivity can occur) and usually avoidance of the offending agent can prevent elicitation of ACD (34).

Regulations for textile dyes

Some of the dyes have the capacity to release certain aromatic amines which pose cancer risks. The prohibition on the use of certain azo dyes is laid down in Annex XVII to the Regulation (EC) 1907/2006 on the registration, evaluation and authorisation of chemicals (REACH), which is directly applicable to all EU Member States (46). The Regulation does not give a list with the names of colourants that are prohibited. This means that all azo dyes which do not release one of the 22 carcinogenic amines in an amount higher than 30 ppm for each amine are allowed to be used (46).

According to the EU regulations for eco-labelling of textile products, the manufacturer of the garments produced in or imported to the EU must either provide a statement of non-use of certain disperse dyes (DDs) listed as carcinogenic or allergenic, or provide a test report proving their colour fastness (47). The Oeko-Tex Association, a group of 14 textile institutes in Europe and Japan, has a list of allergenic dyes, which are forbidden in clothes certified with the ecological Oeko-Tex label (48) (Table 4). In the US textile products that are produced in accordance with the Global Organic Textile Standard (GOTS) may be sold as organic. GOTS prohibit the use of azo dyes releasing carcinogenic arylamine compounds and disperse dyes classified as allergenic (49, 50).

Table 4. *Disperse dyes classified as allergens and listed by the EU Commission and by Oeko-Tex.*

C.I. Generic Name	C.I. Structure Name	CAS
C.I. Disperse Blue 1[$]	C.I. 64 500	2475-45-8
C.I. Disperse Blue 3*[#]	C.I. 61 505	2475-46-9
C.I. Disperse Blue 7	C.I. 62 500	3179-90-6
C.I. Disperse Blue 26	C.I. 63 305	-
C.I. Disperse Blue 35[*]	-	12222-75-2
C.I. Disperse Blue 102	-	12222-97-8
C.I. Disperse Blue 106*[#]	-	12223-01-7
C.I. Disperse Blue 124*[#]	-	61951-51-7
C.I. Disperse Brown 1[$*]	-	23355-64-8
C.I. Disperse Orange 1	C.I. 11 080	2581-69-3
C.I. Disperse Orange 3*[#]	C.I. 11 005	730-40-5
C.I. Disperse Orange 37	C.I. 11 132	-
C.I. Disperse Orange 76	C.I. 11 132	-
C.I. Disperse Orange 149[$]	-	85136-74-9
C.I. Disperse Red 1*[#]	C.I. 11 110	2872-52-8
C.I. Disperse Red 11[#]	C.I. 62 015	2872-48-2
C.I. Disperse Red 17*[#]	C.I. 11 210	3179-89-3
C.I. Disperse Yellow 1	C.I. 10 345	119-15-3
C.I. Disperse Yellow 3[$*#]	C.I. 11 855	2832-40-8
C.I. Disperse Yellow 9*[#]	C.I. 10 375	6373-73-5
C.I. Disperse Yellow 23[$]	C.I. 26 070	6250-23-3
C.I. Disperse Yellow 39	-	-
C.I. Disperse Yellow 49	-	-

CAS, Chemical Abstract Service number; C.I., Colour Index number; -, there is no C.I. or CAS number for that dye; [$] listed by Oeko-Tex. [*]Present in the Textile Colours & Finish (TF-1000) series by Chemotechnique Diagnostics, Sweden, www.chemotechnique.se. [#]Present in the Textile & Leather dyes series by Trolab, Germany, www.hermal.com.

DISPERSE AZO DYES

Properties and use

DDs constitute a large dyestuff class, which accounts for 22 % of all dyes produced in the world (51). DDs have low solubility in water, so the dyes are mixed with a dispersing agent, e.g. lignin sulphonate, naphthalene sulphonate or lignosulphonate to make them soluble in water (11). DDs are currently used to dye cellulose acetate,

cellulose triacetate, synthetic polyamides, and to a lesser degree, polyacrylonitrile and polypropylene. They are not used to dye natural fibres (wool, silk, cotton) (11, 52). Their major application is clearly for dyeing polyesters, but since only a few individual dyes have satisfactory properties for all synthetic fibres, many dye producers offer separate lines for individual fibres as well as for various application methods (11).

Disperse azo dyes are characterized by the presence of one or more azo groups (-N=N-) in the chemical structure. Chemically they are divided into mono- and diazo types; then each of these classes are subdivided according to the diazo and the coupling components (11).

Depending on the fastness of the dyed fabric, these dyes can be removed by rubbing and/or by exposure to water (11, 53). There is only one single example of the presence of an azo group in a natural product - 4,4′-dihydroxyazobenzene in the fungus *Agaricus xanthodermus* (54). Therefore the azo dyes are all xenobiotic compounds.

Impact on the environment

All dyes do not bind to the fabric. Depending on the class of the dye, its loss in wastewaters could vary from 2% for basic dyes to as high as 50% for reactive dyes, leading to severe contamination of surface and ground waters from nearby dyeing industries (55). Many dyes are visible in clear water at concentrations as low as 1 mg/L, and wastewaters from the dye manufacturing plants and dye houses can contain 10–200 mg/L of the dyes (56).

Recent studies (56) have shown that azo dyes contribute to mutagenic activity of ground and surface waters polluted by textile effluents. Furthermore, their discharge into surface water leads to aesthetic problems, obstructs light penetration and oxygen transfer into the organisms living in the water, so aquatic life is affected negatively (55, 56). Due to poor water solubility (less than 1 mg/L), DDs have a low acute ecological impact, but they may cause long-term adverse effects because they are not easily biodegradable and are suspected to bioaccumulate (55). Thus, the removal of colour from textile effluents is very important.

As wastewaters are treated before leaving the plant, biological treatment using bacteria or fungi is the most common and most widespread technique used in effluent treatment, having been employed for over 150 years (57). Except for a few, the aromatic amines formed from decolourisation of azo dyes are recalcitrant to biodegradation under anaerobic conditions. These anaerobically decolourised effluents can still be hazardous, as many aromatic amines are toxic. Thus their removal, which requires aerobic conditions, is essential (57).

Metabolism

Oxidative metabolism of the azo dyes is catalyzed by different enzymes from the cytochrome P (CYP) family. They are located either in the inner membrane of mitochondria or in the endoplasmatic reticulum of cells, mainly in the liver, but also in the squamous epithelium and sebaceous glands of the skin although in lower levels (58). There are no studies exploring oxidation of the azo dyes applied onto the skin.

There is strong evidence that water-soluble azo dyes are metabolized by the intestinal microflora and by reductases, mostly present in the liver. Reductive cleavage of the azo linkages is probably the most toxicologically important metabolic reaction of azo compounds. Its occurrence in the liver has been regarded as a detoxification reaction, but it could also be used for obtaining an effective metabolite from some drugs, e.g. *Prontosil* (benzenesulfonamide), since azo reduction in vivo gives the potent antibacterial agent sulphanilamide (59).

Intestinal microflora and azo reduction. The reduction of azo dyes in the intestines of humans and other mammals has been known for a long time. Roxon et al. were the first to isolate a pure culture of *Proteus vulgaris* from the feces of rats and they studied the reduction of Tartrazine in 1966 (60). Nowadays several anaerobic bacterial strains, like *Escherichia coli, Coprococcus catus, Acidaminococcus fermentans, Fusobacterium prausnitzii, Bacteroides thetaitaomicron , Bifidobacterium infantis, Eubacterium biforme, Peptostreptococcus productus,* and *Citrobacter* species as well as the enteropathogen, *Shigella dysenteriae*, are isolated from human feces and demonstrated to reduce azo dyes (61).

The azo reductase in these bacteria is inducible by azo dyes. At least three different types of azo reductase enzymes were found to be produced by the different isolates. All azo reductases are produced constitutively and released extracellularly (61).

Skin microflora with azo reductase activity. The number of bacteria colonising the mucosal and skin surfaces exceeds the number of cells forming inside the human body. The number of skin bacteria approaches 10^{12}, and this population includes mainly Gram-positive bacteria, obligate aerobes (*Micrococcus*) or facultative anaerobes (e.g. *Staphylococcus* and *Corynebacterium*) (62, 63). In recent years it was demonstrated that human skin bacteria are able to split azo dyes into the corresponding aromatic amines (64). The gene of azo reductase from *S.aureus* was identified and cloned and was thus demonstrated that this azo reductase is able to decolourise azo dyes (65). Also in the experiment it was shown that skin bacteria, representing the genera *Staphylococcus, Corynebacterium, Micrococcus*, *Dermacoccus* and *Kocuria,* were able to reduce the azo dyes Methyl Red and Orange II by 74–100% in 24 h (66).

The prevalence of contact allergy

At least 26 DDs are described as contact allergens in the scientific literature published in 1991-2011.

The prevalence of DD contact allergy varies depending on the population and the dyes tested. In those studies in which patients appeared for routine patch testing and DDs were included (Table 5), prevalence values range from 0.4% (67, 68) to 6.7% (69). Prevalence values in patient populations known or most likely sensitised to DDs ranged from 5.5% to 100% (Table 6).

Available data indicates that the prevalence of positive test reaction at least to three dyes (D Blue 106, 124 and D Orange 3) is over 1% when screening dermatitis patients (Table 7). So according to Bruze et al., these DDs should be included in baselines series (110).

On the other hand, there are some DDs the prevalence rate of which in aimed testing is over 10% (D Blue 7, D Yellow 64, D Black 1 and D Black 2), but they are not included in the series of the commercial patch test manufacturers (e.g., Trolab or Chemotechnique), so relevant allergies to them might be missed if testing is limited to commercial series only.

There is a lack of data on patch testing with D Blue 26, D Blue 102, D Orange 37, D Orange 149, D Yellow 23 and D Yellow 49 which are listed by the EU Commission and by Oeko-Tex as allergens. It is also obvious that there are much more allergenic DDs than classified by these institutions, so Oeko-Tex labelling or the label "Made in the EU" does not mean that no allergenic dyes are used, as was also pointed out by Carozza and Nestle in their case report, where they describe ACD from "ecological" (i.e., not listed in Oeko-Tex standard) disperse textile dyes (105).

A recently published systematic review and meta-analysis of allergens responsible for ACD among children by Bonitsis et al. (111) also covers children's sensitivity to several DDs: D Blue 124 and 106, D Orange 3, D Red 1, and D Yellow 3. Here the positive reactions at least in 1% of tested children were found to be statistically significant only to D Blue 124.

The North American Contact Dermatitis group compared sensitivity to D Blue 106 in children and adults and did not find a significant difference (82): the prevalence rate was 2.1% in children versus 2.4% in adults. The Portuguese Contact Dermatitis group found a low prevalence of positive reactions to DDs – 0.3% of 327 tested children (74). Other studies were performed in Italy. They found that the most prevalent DD contact allergy in children is towards D Blue 106 and D Red 1, followed by D Blue 124, D Orange 3 and D Yellow 3 (77, 112, 113). Seidenari et al. describe sensitivity in 23 DD-positive children. In their study the most prevalent sensitisers were D Red 1 and D Orange 3 (73).

In the vast majority of studies where sex distribution of positive reactions to DDs was reported, there was an overrepresentation of women: from 56 to 100%. The high frequency of sensitisation to DDs in women reported in the studies may reflect the proportions of the investigated women in the study population. It might also be caused by women's tendency to wear tighter-fitting clothes, lace underwear or tights which are always synthetic. So whether women are more susceptible to sensitisation to DDs is not yet known.

Table 5. *Review of the literature on the prevalence and clinical relevance of disperse dye contact allergy in consecutively patch-tested dermatitis patients (screening patch testing).*

Ref.	Study period (publication year)	Country	Number of patient tested	Positive reactions, number (%)	Patch testing with the extract/textile	Clinical relevance according to the reference
(70)	1987-1991	Belgium	3336	28 (0.8)	Yes, 3 patients, not positive to dye allergens, were positive to textile	Not stated
(71)	1988-1990	Italy	2752	100 (3.6)	No	Not stated
(72)	1988-1990	Italy	576	19 (3.3)	No	8/19 (42%)
(73)	1990-1995	Italy	6203	236 (3.8)	No	Not stated
(67)	1991	Italy	569	2 (0.4)	No	Not stated
(74)	1992	Portugal	329	2 (0.6)	No	Not stated
(75)	1994	Portugal	78	2 (2.6)	Not stated	100%
(76)	1995-1999	Germany, Austria	1986	86 (4.3)	No	Current clinical relevance 70%
(77)	1996-2000	Italy	1098	51 (4.64)	Not stated	Past/current relevance 70%
(69)	1996-2000	Italy	6478	437 (6.7)	Not stated	371/437(85%)
(78)	1998	Italy	1012	31 (3.1)	Yes: 10/1012 (1%) 5/10 positive (50%) 2 only to cloth, not to the DDs	Not stated
(79)	1999-2003	Sweden	3325	50 (1.5)	Yes	Not stated

(80)	2001-2002	North America	4888	146 (3)	Not stated	45.5% possible relevance, 5.5% past relevance, 10.3% probable relevance
(81)	2001-2002	Germany, Austria, Switzerland	3041	40 (1.3)	Not stated	Not stated
(82)	2001-2004	USA	391	8 (2.1)	Not stated	47.6%
(83)*	2002	Israel	286	15 (5.2)	Not stated	68.7% current relevance, 9.4% past relevance, 21.8% no relevance
(84)	2003-2004	North America	5136	98 (1.9)	Not stated	Not stated
(85)	2003-2005	Germany	24980	337 (1.4)	Not stated	Not stated
(86)	2004-2005	Sweden Belgium	1780	35 (2)	Not stated	Not stated
(87)	2005-2006	North America	4454	94 (2.1)	Not stated	35.5% possible relevance, 7.5% past relevance, 20.4% probable relevance, 8.6% definite
(88)	2006-2008	Sweden, Belgium	2546	65 (2.6%)	Not stated	Not stated
(68)	2007-2008	Finland Italy United Kingdom	760 2938 9201	4 (0.5) 47 (1.6) 37 (0.4)	Not stated	Not stated
(89)	2008	USA	65	7 (4.6%)	Not stated	Probable/possible 71.4%
(90)*	2010	China	532	Volunteers 6/205 (2.9%): Eczema patients 13 /327 (4.0%)	No	2/6 (33%) volunteers – past relevance 6/13 (46%) eczema patients – past/present relevance.

DDs – Disperse Dyes; * including positive reactions to other classes of dyes.

Table 6. *Review of the literature on the prevalence and clinical relevance of disperse dye contact allergy in patients suspected or thought likely to have contact dermatitis caused by disperse dye allergy (aimed patch testing).*

Ref.	Study period (publication date)	Country	Number of patient tested	Positive reactions, number (%)	Patch testing with the extract/textile	Clinical relevance according to the reference
(70)	1987-1991	Belgium	159	28 (17.6)	Yes, 3 patients, not positive to dye allergens, were positive to textile	Not stated
(71)	1988-1990	Italy	198	134 (67.7)	No	Not stated
(72)	1988-1990	Italy	145	23 (15.9)	No	8/19 (42%)
(91)	1988-1992	Portugal	6	6 (100)	TLC results of the extracts from clothing were compared with reference dyes. In all 3 cases DDs to which patients were patch test-positive were detected	100%
(92)	1989-1994	USA	50	12 (24.0)	Yes, positive 5/12 (41.7%)	Not stated
(93)*	1991-1997	Israel	55	22 (40)	Yes	Present relevance 20/22 (90.9%)
(94)	1991	Italy	2	2 (100)	No	100%
(95)	1992	Japan	1	1 (100)	Yes. Dyes were obtained from the manufacturer and analyzed by TLC and mass spectrometry	100%
(96)*	1993-2006	Australia	2069	114 (5.5)	Yes (positive 12.8%)	100% in patients positive to extract/textile
(97)	1995	France	1	1 (100)	Yes	100%
(98)	1996-1999	The Netherlands	577	79 (13.7)	No	Relevant (probably) 75%

(99)	1997-1999	Canada	271	40 (18.0)	Yes, 11/271 (41%)	34/40 (85%) relevant, 6/40 (15%) unknown relevance
(100)	1996-2000	Italy	130	13 (10.0)	No	8/13 (61.5%) in sensitized only to DDs
(101)*	1998	Israel	103	30 (29.0)	No	100%
(102)	1998	Italy	1	1 (100)	Yes, positive. Dye detected by HPLC in the suspected garment	100%
(103)*	1999-2002	Israel	644	43 (6.7)	Yes 21/664 (3.2%) Positive 5/21 (23.8%)	Present relevance 81.4% Past relevance 6.8% No relevance 11.7%
(104)	2000	Portugal	5	5 (100)	Yes, 5/5 positive. TLC analysis revealed D Blue 106	100%
(105)	2000	Switzerland	1	1 (100)	Yes: positive Dyes obtained from manufacturer and analyzed by HPLC – not detected	Not stated
(106)	2001	Canada	2	2 (100)	Yes: negative in 1, positive in another patient	100%
(107)	2004	Australia	1	1 (100)	Yes	100%
(108)	2004	Australia	1	1 (100)	Yes	Yes
(109)	2011	Italy	1	1 (100)	Yes	100%

DDs – Disperse Dyes; TLC – thin-layer chromatography; HPLC – high performance liquid chromatography; * including positive reactions to other classes of the dyes; No – number.

Table 7. *Prevalence of each disperse (D) dye by study.*

Disperse dye	Concentration wt/wt, %	Aimed testing		Studies (No)	Screening		Studies (No)
		Number of patients positive/total tested	%		Number of patients positive/total tested	%	
D Blue 1	1.0% pet	1/19	5.3	2	NR		
D Blue 3	1.0% pet	14/1441	0.9	13	3/2682	0.2	3
D Blue 7	1.0% pet	2/12	16.7	3	NR		
D Blue 35	1.0% pet	30/1779	1.7	13	11/4135	0.3	3
	0.5% pet	NR			3/3325	0.1	1
	0.3% pet	NR			4/2376	0.2	2
D Blue 85	1.0% pet	31/1599	1.9	9	15/2682	0.6	3
D Blue 106	1.0% pet	342/2051	16.7	16	639/35334	1.9	13
	0.3% pet	NR			3/2049	0.2	1
	0.1% pet	NR			5/3325	0.2	1
D Blue 124	1.0% pet	376/2363	15.5	15	517/19964	1.7	14
	0.3% pet	NR			4/2049	0.2	1
	0.1% pet	NR			6/3325	0.2	1
D Blue 153	1.0% pet	7/1453	0.7	5	3/2682	0.2	3
D Red 1	1.0% pet	171/2266	7.5	17	236/30120	0.8	13
	0.5% pet	NR			6/3325	0.2	1
D Red 11	1.0% pet	0/24	0	2	NR		
D Red 17	1.0 % pet	64/1883	3.4	16	17/6511	0.3	5
	0.5% pet	NR			5/3325	0.2	
D Red 35	1.0% pet	0/1	0	1	NR		
D Orange 1	1.0% pet	34/1498	2.3	9	52/6184	0.9	4
	0.5% pet	NR			17/3325	0.5	1

D Orange 3	1.0% pet	244/2256	10.6	17	334/27899	1.2	12
	0.5% pet	NR			1/3325	0.03	1
D Orange 13	1.0% pet	11/810	1.4	5	1/2355	0.04	2
D Orange 76	1.0% pet	26/282	9.2	4	NR		
D Yellow 1	1.0% pet	2/40	5.0	1	NR		
D Yellow 3	1.0% pet	157/2265	6.9	18	218/28053	0.8	12
	0.5% pet	NR			8/3325	0.2	1
D Yellow 9	1.0% pet	26/1607	1.6	13	2/2355	0.06	2
D Yellow 39	1.0% pet	0/6	0	1	NR		
D Yellow 54	1.0% pet	6/131	4.6	2	NR		
D Yellow 27	1.0% pet	1/104	0.9	2	NR		
D Yellow 64	1.0% pet	1/5	20	1	NR		
D Brown 1	1.0% pet	22/1498	1.5	10	2/2355	0.06	2
D Black 1	1.0% pet	18/137	13.1	3	1/569	0.2	1
D Black 2	1.0% pet	4/6	66.7	1	NR		

NR – no reports found; No - number of studies; pet – petrolatum.

Seven studies reported figures for monosensitisation to a particular dye, but none of them, except one, reported clinical relevance of this finding or an association with positive reactions to other *para*-compounds (70, 71, 73, 77, 102, 108). The prevalence rate varied from 2.3 to 17.0%. The monosensitised patients most frequently had positive patch test reactions to D Blue 124, D Blue 106, D Red 1, D Orange 3, D Yellow 3, and D Red 17, but there was no association with positive reactions to PPD or clinical relevance reported. Only one case report by Foti et al. showed the monosensitisation to D Yellow 27 to be of clinical importance detecting the dye in the extract of the patient's clothes by HPLC (102). In almost all published case reports sensitisation to at least two DDs is reported. It seems that co-sensitisation to several dyes, usually of similar structure or having the same impurities exists, but possibly a particular sensitisation to one dye may be important. These DDs, to which monosensitisation is reported, are on the top of the most prevalent dye allergens. Some of them are shown to be strong allergens. This may imply that a positive reaction to one DD is more related to the strength of the allergen and show a general predisposition of the immune system to recognize a particular pattern of the structure

rather than the culprit allergen for the patient's dermatitis. The DD can act as a marker for a group sensitisation as is seen, for example, with the corticosteroid budesonide (114).

Clinical relevance of contact allergy

It is very important to know whether dyes to which a patient has a contact allergy are used in textiles. Indeed, in the majority of studies reporting positive reactions to DDs, clinical relevance is not stated, although it was shown that dyes to which patients are patch test positive infrequently are found in the suspected garment (115). As some of the DDs (e.g., D Blue 106) are potent sensitisers (116), it seems that sometimes clinical relevance of the positive patch test reactions may be overestimated. Not all papers provide fibre composition of the "culprit" textile because it might indirectly point out the possibility of the DDs to be present in the garment. As DDs are not used to dye all types of synthetic fibres and not used for wool or cotton, a positive patch test reaction to a disperse azo dye and such a type of textile could not be related etiologically. On the other hand, other substances could be present in the textile, which cause positive patch test reactions, and in this case these positive reactions to disperse azo dyes could be a marker of sensitisation to other *para*-compounds (such as PPD or black rubber substances).

When establishing a clinical relevance of the positive patch test to the DDs, either patch testing with the suspected fabric or with an extract should be done and the relevant DDs in the textile must be detected (i.e. exposure to that DD) should be confirmed. However, the extraction procedure is not standardized. Sensitivity to a dye in the patch test preparation placed directly on the skin would be expected to be higher than testing with the dyed textile because a not so high dose of the dye would migrate onto the skin. So ideally, identification of the dye content in the suspected fabric should also be carried out although this is not always possible. Indeed, the latest reports on chemical analysis of suspected textiles were published in 2000 (104, 105).

Clinical aspects of allergic contact dermatitis and patch testing

In order to diagnose ACD from textile dyes a high index of suspicion is required, as its clinical presentation not always indicates the cause since sometimes the clinical presentation is atypical, and its appearance not always confined to sites of direct contact. DD-related dermatitis most commonly develops on the extremities and especially on the hands, followed by the trunk, face, genitalia, buttocks, and in the folds including the neck, armpits, and groin (71, 76, 77, 79, 83, 90, 93, 94, 96, 102, 103, 105, 113, 117). In children (especially those suffering from atopic dermatitis) the flexural areas of the limbs were involved more often (52.9%) compared to non-atopic dermatitis patients when patch test reactions to DDs were positive (77).

Clinical features of dermatitis related to DDs frequently had uncommon features such as atypical localization and unusual clinical patterns. It might present itself not only as typical chronic dermatitis, but also as persistent erythematous wheal-type or transient urticarial dermatitis (71), prurigo-like eczema (118), diffuse itching (71), erythema multiforme-like eruptions (71, 119), purpuric dermatitis (93, 102, 120), nummular dermatitis, erythroderma (103), or pseudolymphoma (121). Before establishing the diagnosis of DD-related dermatitis, patients were diagnosed with lichen simplex chronicus, parapsoriasis, mycosis fungoides, drug eruption, postinflammatory hyperpigmentation, pigmented purpura, or scabies (93).

According to Lazarov, chronic dermatitis was diagnosed in 35.4% of cases, acute dermatitis in 6.8%, and the remaining consisted of atypical forms of ACD in patients with textile dye allergy (103). Seidenari described 50 of 437 patients positive to DDs and presenting atypical ACD features – erythema and edema with or without marginal desquamation (69). It was reported that dermatitis could be so monomorphic and infiltrated that at first the diagnosis of an ACD was not obvious (70). It is also not uncommon to see pustular lesions or purpura on sites of DD - induced dermatitis (103). There are reports of peculiar presentations of DD-related dermatitis such as airborne contact dermatitis (118), as well as dermatitis on the incision scar from a hip replacement coming from a pair of black pants belonging to the patient (122).

Patch test reactions to DDs. Patch testing with DDs usually results in strong or very strong (++/+++) reactions. Sometimes they can be purpuric (123). Massone et al. described persistent test reactions to D Blue 124 and D Red 1, which were strong positive reactions already on the second day's (D) reading and remained active and itching on D14 and D22 (94).

Dawes Higgs describes a patient who developed a flare-up of the dermatitis in skin folds while being patch tested to DDs and to an extract from her textiles, to which she was positive as well as to D Blue 106 on D3 (108).

Late readings. Most centres read patch tests on D2 and D3 or D4 in the published studies on DDs dermatitis Several authors also reported late (D7) readings (84-87, 99, 101-103, 108, 109), while in four studies only some patients were read on D7 (79, 80, 88, 99). Several studies reported late positive reaction rates. Koopmans and Bruynzeel point out that 10% of the total reactions to the *para* dyes were late reactions. Among them 17% were for azo dyes, of which 84% were relevant (98). Ryberg et al. showed that 13 patients out of 62 (21%) positive to DDs were positive only on D7 (88). In another study 3 out of 35 (8.6%) patients positive to textile dye mixture (TDM 8.6%) and 4 of 34 patients (11.2%) positive to ingredients of TDM were positive only on D7 (86). Pratt and Taraska noted delayed positive patch test reactions to D Blue 124 on D7/10 in 2 of 32 tested patients (6.3%) and all of them were clinically relevant (99).

One of the most important adverse consequences of patch testing is active sensitisation, i.e. when subjects previously not allergic become sensitised to one or more of the test chemicals by the test procedure. The allergic test reaction then flares up around 10 or more days (late reaction) after the test application. Sometimes, however, late reactions are seen without active sensitisation being present, as some allergens are known to give late reactions in the absence of active sensitisation. According to the 4-year review of late reactions (D10 and later) by Aalto-Korte et al., some D Orange dyes induced late reactions in much higher percentages of patients than did PPD, and the authors concluded that these textile colours were primary active sensitisers (124). However, other authors point out that a delayed immune response to some DDs is more prevalent than active sensitisation (125, 126). Thus, late readings (D7) of the patch test reactions should be performed as they give important and clinically relevant information.

Purity of the patch test preparations. Purity of the test preparations is also an important issue. In 1986, Foussereau et al. (127) reported on problems with the purity of several dyes in a commercially available test series. A study by Ryberg et al. found that some commercial test preparations labelled to contain D Orange 3 did not contain this substance, but another orange dye (128). Differences also may occur from batch to batch as well as among different manufacturers. A study in Malmö showed that the raw material of DDs used for preparing patch tests contains 39-76% contaminants or other substances (128). These can be relevant, since almost 25% of patients allergic to commercial D Blue 106 and 124, D Yellow 3 or D Orange 1 did not react to (or not only to) the main spot but to other spots when patch testing with a thin layer chromatograms (TLCs) from the commercial dyes (129). When patch test preparations of dyes contain more than one compound, it may not be the dye molecule that causes the skin reaction. It also makes it difficult to compare the results of patch testing from different batches and different departments or test laboratories.

Sensitising potential

Although DDs are the most common sensitisers among textile dyes, not much has been performed determining their sensitising potential. The sensitising potential of a chemical could be determined using animal tests (the Beuhler test, the guinea pig maximisation test (GPMT), the mouse ear-swelling test, the local lymph node assay), human tests and in vitro assays. The GPMT is a standard method for analysing thresholds at elicitation and assessing cross-reactivity at challenge (130).

Hausen et al. tested 6 azo and anthraquinone dyes using the GPMT. D Blue 1 and D Blue 124 were reported to be the strongest sensitisers, followed by D Blue 3, D Orange 3 and D Red 1, while D Yellow 3 was the weakest allergen (131). In a modified Buehler test, D Orange 3 was shown to be a significant contact sensitiser (132). In several studies D Yellow 3 was found to be a weak sensitiser in the GPMT

and also in a modified local lymph node assay, although in humans D Yellow 3 is a frequent sensitiser (131, 133, 134).

D Blue 106 has proven to be a strong allergen in the guinea pig tests (116). It is reported that the sensitisation capacity of D Blue 106 is comparable to 2,4-dinitrochlorobenzene, one of the strongest contact allergens known (135, 136).

Based on the results from the biphasic murine local lymph node assay, Ahuja et al. grouped the DDs on the basis of their sensitising potency (137). According to them, strong sensitisers are D Blue 124 and D Blue 106, moderate – D Red 1 and D Blue 1, weak – D Orange 37 and D Blue 35 and very weak – D Yellow 3 and D Orange 3. On the other side, D Orange 3 is one of the most frequent allergenic DDs in humans as shown by patch testing (134).

Current assessment of skin sensitisation relies on animal tests, but developments of in vitro methods for the prediction of the sensitising potential of chemicals are also on going. Sonnenburg et al. examined eight disperse dyes and seven products from azo-cleavage of these dyes in the loose-fit coculture-based sensitisation assay of primary human keratinocytes and of allogenic dendritic cell-related cells (138). There D Blue 1, 4-nitroaniline and 4-aminoacetanilide showed no sensitising potential, whereas D Blue 124, D Yellow 3, D Orange 37, D Blue 106, D Red 1, 2-amino-*p*-cresol, and D Orange 3 were categorized as extreme sensitisers.

It should be noted that the above mentioned studies seem to have been performed without information on the purity of the chemicals.

Cross-reactivity

Definition

Cross-reactivity is defined when a subject initially sensitised to an allergen (so-called primary sensitiser) reacts to a second allergen with which he has not been in contact previously (139).

Upon the very first contact with the allergen it is unusual to expect a manifestation of the allergic reaction unless this allergen is cross-reacting. If the chemical structures of primary and secondary allergens are closely related, the immune system could not be able to recognize them as separate antigens. Regarding proteins, it is determined that cross-reactivity requires more than 70% sequence identity, and proteins having less than 50% sequence identity are very seldom cross-reactive (140), however, such kind of data for haptens is not available. It could be that substances cross-react because of common metabolites/degradation products formed on/in the skin. Theoretically it is possible that different haptens could bond to the protein and subsequently modify it to become an antigen with similar determinants (139). Unfortunately in humans, it is impossible to know for sure the actual primary sensitiser, because humans are exposed to so many substances which could include the one to which he or she “cross-reacts”.

In clinical practice cross-sensitivity is usually referred to as having simultaneously observed concomitant reactions to substances looking chemically similar, but this is not related to the knowledge of previous exposure, so in a strict sense it could not be called “cross-reactivity”.

The more comprehensive term “co-recognition” (including by definition “cross-reactivity”) is used in clinical immunology and could be usefully adopted to define positive patch tests reactions, where co-exposure to a number of similar haptens does not allow for the identification of the primary sensitiser (141).

In clinical practice patch testing potentially cross-reacting substances in dilution series at equimolar concentrations down to negative reactions could give a clue to the possible cross-reactivity of the substances (142). Equimolarity means that exposure to the tested substances is controlled and thus an ideal possibility in which to compare related substances in the sense of elicitation of the contact allergy, because the same amount of molecules of each substance that are compared are presented to the immune system provided that the penetration of the substances is similar. Patch test reactions to a primary sensitiser would appear to lower equimolar concentrations than to the cross-reacting substance. This could be because T-lymphocytes recognize a specific substance (e.g., substance A), but not so specifically as a similar structure of substance B. When the number of molecules of substance B decreases, then the activation of the T-lymphocytes is not strong enough to produce inflammation (i.e., positive patch test reaction). This might be illustrated by the responses of the T-lymphocytes in the birch-pollen-fruit syndrome. The majority of the major birch allergen Bet v 1-reactive T-lymphocytes, retrieved from birch-pollen-allergic patients, responded to stimulation with the major apple, Mal d 1, and celery, Api g 1, allergens, but they lost their reactivity with the food allergens after a few expansion cycles in vitro but remained highly Bet v 1-reactive (143). It shows that T lymphocytes react stronger to the primary sensitiser than to the secondary, cross-reactive substances.

Nevertheless, the full cross-reaction pattern of suspected contact allergens is only possible to study when the exposure is controlled as in the GPMT (139, 142).

Clinical and experimental studies

Para-amino compounds are known to cross-react with each other and with some DDs, belonging to the azo group. From the chemical point of view, *para*-compounds mean that a benzene molecule is substituted with other groups than hydrogen in opposite positions (144). Examples of the most common *para*-amino compounds are PPD, *p*-aminobenzoic acid (PABA), benzocaine, sulfanilamide, *p*-aminobenzene and the black rubber ingredients N-cyclohexyl-N-phenyl-4-phenylenediamine, N,N'-diphenyl-4-phenylenediamine, and N-isopropyl-N'-phenyl-4-phenylenediamine

PPD was reported to be a screening substance for textile dye-related dermatitis (145). Interestingly, self-reported textile-related skin problems were statistically significant when associating contact allergy to PPD, which is frequently found in cases with contact allergy to the azo dyes. However, there was no statistical correlation to the positive patch test results to DDs (145). Koopmans and Bruynzeel concluded that positive patch test reactions to PPD correlate well with reactions to *para* compounds like *p*-aminobenzene and *p*-toluenediamine, but not with DDs (including D Orange 3)(98). In the retrospective analysis of the clinical patch data of 544 patients tested with PPD and all seven additional para-amino compounds, concordance between reactions varied greatly. The stronger the positive test reaction to PPD, *p*-toluylenediamine or *p*-aminoazobenzene, the more frequently additional positive reactions to the other compounds were observed (146).

There are reports about a high frequency of simultaneous sensitivity to DDs and other similar substances. Goon and colleagues reported a high frequency of simultaneous sensitivity to D Orange 3 in patients with positive (2+ or 3+) patch tests reactions to PPD. They found that 80% of those positive to PPD also reacted to D Orange 3 (147). Ryberg et al. showed that there are statistically highly significant associations between contact allergy to PPD and contact allergy to D Orange 3, PPD and textile dye mix (TDM), and also between contact allergy to the TDM and to black rubber mix (BRM) (all $p<0.001$). Simultaneous contact allergy to PPD and D Orange 3 has been suggested to be due to either cross-sensitisation or to the metabolic conversion of PPD and D Orange 3 to a common allergen in the skin (73, 147). Contact allergy to PPD may indicate that the patient has been primarily sensitised by hair dye, temporary "black henna" tattoo dye or by PPD derivatives in BRM. Furthermore, some of the patients who were allergic to the TDM may initially have been sensitised to PPD and then reacted to DDs due to cross-reactivity, or they may have been sensitised by the exposure to a common metabolite, rather than DDs in textiles. Another explanation of the simultaneous contact allergy to the textile dyes, PPD and BRM could be attributed to a common impurity present in all the patch test preparations, according to Ryberg et al. (86).

Simultaneous reactions are frequently observed between D Blue 124 and 106 (69). A possible explanation of cross-reactivity, given in several studies (69, 116, 148) is their close chemical similarity. It was also shown that both dyes contained a low amount of the other, seen as additional spots on the chromatograms and as peaks in the HPLC analyses (128). Moreover, some of the patients, previously regarded as being allergic to D Blue 106 and 124, reacted to impurities in the preparations, but not to the purified dyes (129). So it seems that it is impossible to draw any firm conclusions regarding cross-reactivity between different chemicals from the clinical pattern of concomitant contact allergies to chemically related patch test preparations, unless patch testing with purified preparations and animal tests (GPMT) have been performed.

There are also reports about co-sensitivity of positive patch test reactions to DDs and reactive dyes, which could not be explained by the chemical similarity of the substances (149).

2. AIMS

The general aim of the work presented in this thesis was to investigate the clinical and chemical aspects of contact allergy to the disperse azo dyes D Orange 1 and D Yellow 3 as they were found to be the most frequently positive testing consecutive dermatitis patients in our department (79).

More specifically, the purposes of the studies were to investigate:

- the elicitation potential of commercial and purified D Orange 1 and D Yellow 3
- the significance of the impurities found in these commercial DDs regarding contact allergy
- a possible association between patch test reactivity to the commercial and purified D Orange 1 and D Yellow 3 in order to find the major sensitiser
- the elicitation capacity of potential metabolites from reductive cleavage of purified D Orange 1 and D Yellow 3, BRM ingredients and PPD and to compare it with the elicitation capacity of purified D Orange 1 and D Yellow 3
- the sensitising capacity of D Orange 1, *p*-aminodiphenylamine (PADPA) and 4-nitroaniline and the cross-reactivity among them as well as to D Yellow 3, its potential metabolites from azo reduction, 4-aminoacetanilide and 2-amino-*p*-cresol, and PPD in an animal study
- the presence of 8 disperse dyes (D Blue 35, D Blue 106, and D Blue 124, D Yellow 3, D Orange 1 and D Orange 3, D Red 1 and D Red 17) in low-price textiles, obtained from all over the world

3. MATERIALS AND METHODS

SUBJECTS

The 10 patients who participated in Studies I and II were recruited from the initial group of patients with contact allergy to D Orange 1 and/or D Yellow 3 identified in the consecutively tested dermatitis patients at the department. There were 3 women and 7 men (mean age 46.1 years, range 19 – 70 years). All 10 patients had had dermatitis. Their prior test reactivity to D Orange 1, D Yellow 3, PPD and BRM is shown in Table 8 as well as the clinical relevance. Patients that were tested consecutively served as control patients for 4-nitroaniline, PADPA, 4-aminoacetanilide and 2-amino-*p*-cresol.

Table 8. *Patch test reactivity to Disperse Orange 1, Disperse Yellow 3, p-phenylenediamine and black rubber mix, when patch testing was performed earlier, in conjunction with the patients` clinical work-up.*

Patient number and gender		**1 F**	**2 M**	**3 F**	**4 M**	**5 F**	**6 M**	**7 M**	**8 M**	**9 M**	**10 M**
Substance \ Year		1999	1999	1999	2005	2004	2007	1999	2005	2004	1999
DO1	*Tested concentration*	0.5% pet	0.5% pet	0.5% pet	0.5% pet	0.5% pet	1.0% pet	0.5% pet	0.5% pet	1.0% pet	0.5% pet
	*Reaction**	+++	+++	+	+++	++	++	+++	-	++	++
DY3	*Tested concentration*	0.5% pet	0.5% pet	0.5% pet	0.5% pet	0.5% pet	1.0% pet	0.5% pet	0.5% pet	1.0% pet	0.5% pet
	*Reaction**	+	++	+	-	-	+	-	+	++	++
PPD	*Tested concentration*	1.0% pet	0.94 % pet	0.94 % pet	0.94 % pet	0.94 % pet	0.94 % pet	1.0% pet	0.94 % pet	0.94 % pet	1.0% pet
	*Reaction**	+++	+++	+	-	-	-	-	++	+++	++
BRM [#]		-	++	-	+	-	++	+++	-	-	-
Clinical relevance		DDs	PPD	U	DDs	DDs	U	BRM	PPD	PPD	U

M, male; F, female; DO1, Disperse Orange 1; DY3, Disperse Yellow 3; PPD, *p*-phenylenediamine; BRM, black rubber mix; pet, petrolatum, DDs,Disperse Dyes; U, Uncertain; * , the results of patch testing are based on the strongest reaction either on D3/4 or D7; [#] - black rubber mix was tested at 0.6% pet concentration in all patients.

CHEMICALS AND PATCH TEST PREPARATIONS

The main chemicals and patch test preparations used in Studies I-III and V are listed in Tables 9 and 10.

Table 9. *Main chemicals with manufacturers/suppliers.*

Chemical	**Paper**	**Manufacturers/supplier**
Acetonitrile	I	Scharlau Chemie S.A., La Jota, Barcelona, Spain
Acetone	I-III, V	
Dichloromethane	I	
Chloroform	I, III	
Acetonitrile for TLC systems	I	Lab-Scan, Dublin, Ireland
Acetonitrile of fluorescence HPLC grade	III	
Distilled water	I, III	Milipore SA, Malsheim, France
Anhydrous sodium sulphate	I	Acros Organics, Geel, Belgium
Sodium lauryl sulfate	V	
Naphthalene sulphonate	I	Sigma Aldrich, Steinhem, Germany
4-Nitroaniline	II, V	
p-Aminodiphenylamine	II, V	
4-Aminoacetanilide	II, V	
2-Amino-*p*-cresol	II, V	
p-Phenylenediamine	II, V	
CPPD	II	Chemotechnique Diagnostics, Vellinge, Sweden
DPPD	II	
IPPD	II	
D Blue 35	III	
D Blue 124	III	
D Yellow 3	I, III	
D Orange 1	I, III	
D Orange 3	III	
D Blue 106	III	
D Red 1	III	
D Red 17	III	

Purified disperse dyes: D Orange 1, D Yellow 3	I, III, V	Department of Occupational and Environmental Dermatology, Malmö, Sweden
D Blue 124, D Blue 106, D Orange 3, D Red 1, D Red 17	III	
Freund's complete adjuvant	V	Pierce Rockford, IL, USA
2-methylol phenol	V	Fluka chemie AG, Buchs, Switzerland
Propylene glycol	V	VWR International S.A.S., Fontenay-sous-Bois, France
Dimethylacetamide	V	Sigma Chemical Co, st.Louis, MO, USA
Ethanol	V	Kemetyl AB, Haninge, Sweden

CPPD, N-cyclohexyl-N-phenyl-4-phenylenediamine; DPPD, N,N'-diphenyl-4-phenylenediamine; IPPD, N-isopropyl-N'-phenyl-4-phenylenediamine; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; D, Disperse.

Table 10. *Main patch test preparations with producers/suppliers.*

Patch test preparation	**Producer/supplier**	**Vehicle**	**Concentration (% w/w pet., %w/v in ac. or aq.)**	**Paper**
Purified disperse dyes: D Yellow 3 D Orange 1	Dept. of Occupational and Environmental Dermatology, Malmö, Sweden	ac	1.0 – 0.000001%	I
Commercial disperse dyes: D Yellow 3 D Orange 1	Chemotechnique Diagnostics, Sweden	ac	1.0 – 0.000001%	I
Naphthalene sulphonate	Sigma Aldrich, Germany	aq	1.0%	I
p-Phenylenediamine	Sigma Aldrich, Germany	ac	1.0 – 0.000001%	II
CPPD	Chemotechnique Diagnostics, Sweden	ac	1.0 – 0.000001%	II
DPPD	Chemotechnique Diagnostics, Sweden	ac	1.0 – 0.000001%	II
IPPD	Chemotechnique Diagnostics, Sweden	ac	1.0 – 0.000001%	II
4-Nitroaniline	Sigma Aldrich, Germany	ac	0.43-0.0043%	II
p-Aminodiphenylamine	Sigma Aldrich, Germany	ac	0.58-0.0058%	II
4-Aminoacetanilide	Sigma Aldrich, Germany	ac	0.56-0.0056%	II
2-Amino-*p*-cresol	Sigma Aldrich, Germany	ac	0.46-0.0046%	II

CPPD,N-cyclohexyl-N-phenyl-4-phenylenediamine;DPPD, N,N'-diphenyl-4-phenylenediamine; IPPD, N-isopropyl-N'-phenyl-4-phenylenediamine; ac, acetone; pet, petrolatum; aq, aqueous; D, disperse.

The concentration of each substance diluted in acetone is given in % w/v, and the concentration of a substance mixed in pet. is given in % w/w. Possible azo degradation way of D Orange 1 and D Yellow 3 and potential metabolite formation is shown in Fig. 2. The molecular structures and main properties of PPD and the 3 components in BRM are given in Fig. 3.

The general and specific purity of the substances, used in Studies II and V, are both given in Table 11.

Table 11. *General and specific purity of the substances used in Studies II and V.*

Substances, obtained from the manufacturers	Concentration indicated on the label	Results from GCMS and DIMS						
		Substances used						
		Disperse Orange 1	4-nitroaniline	*p*-aminodiphenylamine	DisperseYellow 3	2-amino-*p*-cresol	4-aminoacetanilide	*p*-phenylenediamine
Disperse Orange 1	Not stated	>99%*	nd	nd	nd	nd	nd	nd
4-nitroaniline	≥ 99%	nd	>99%	nd	nd	nd	nd	nd
-*p*-aminodiphenylamine	98%	nd	nd	98%	nd	nd	nd	nd
DisperseYellow 3	Not stated	nd	nd	nd	>99%#	nd	nd	nd
2-amino-*p*-cresol	97%	nd	nd	nd	nd	99%	nd	nd
4-aminoacetanilide	99%	nd	nd	nd	nd	nd	99%	nd
p-phenylenediamine	Not stated	nd	nd	nd	nd	nd	nd	99%

* - concentration before purification 15.2%; # -concentration before purification 40.6%; nd – not detected (detection limit <0.1%); GCMS – gas chromatography-mass spectroscopy; DIMS – direct injection mass spectroscopy.

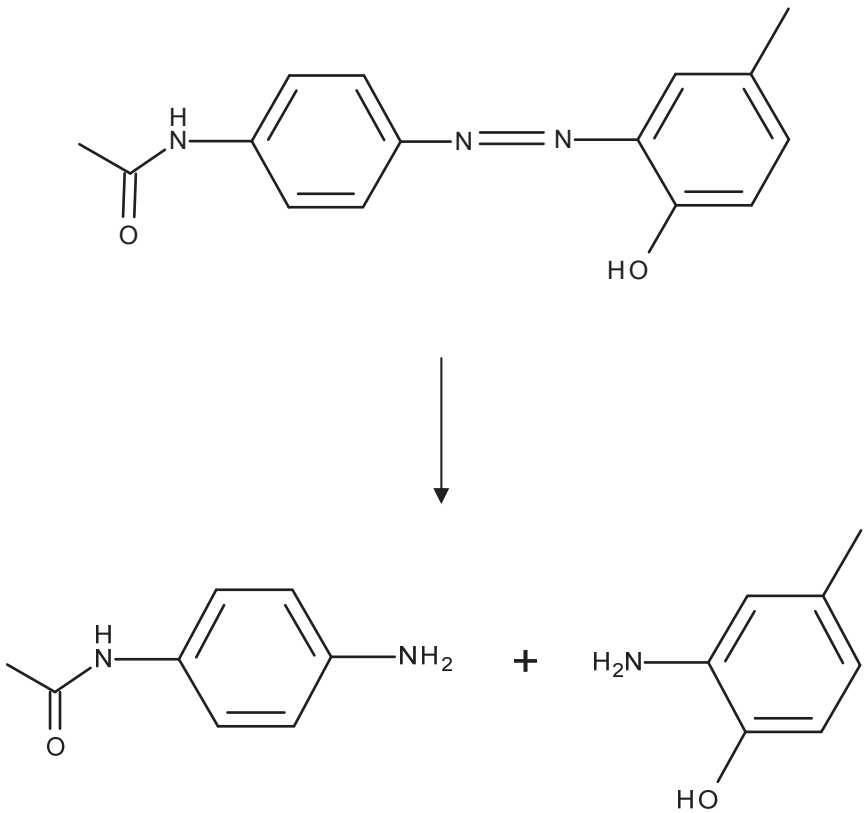
Purification of the D Orange 1 and D Yellow 3 was performed at the Department of Occupational and Environmental Dermatology, Malmö, Sweden.

Figure 2. *Possible azo degradation way of Disperse Yellow 3 (A) and Disperse Orange 1 (B) and chemical structures, Chemical Abstract Service (CAS) and Colour Index (C.I.) numbers as well as molecular weight (MW) of the investigated dyes and their metabolites.*

A. Disperse Yellow 3

CAS: 2832-40-8

MW:269



4-aminoacetanilide

CAS: 122-80-5

MW: 150

2-amino-*p*-cresol

CAS: 95-84-1

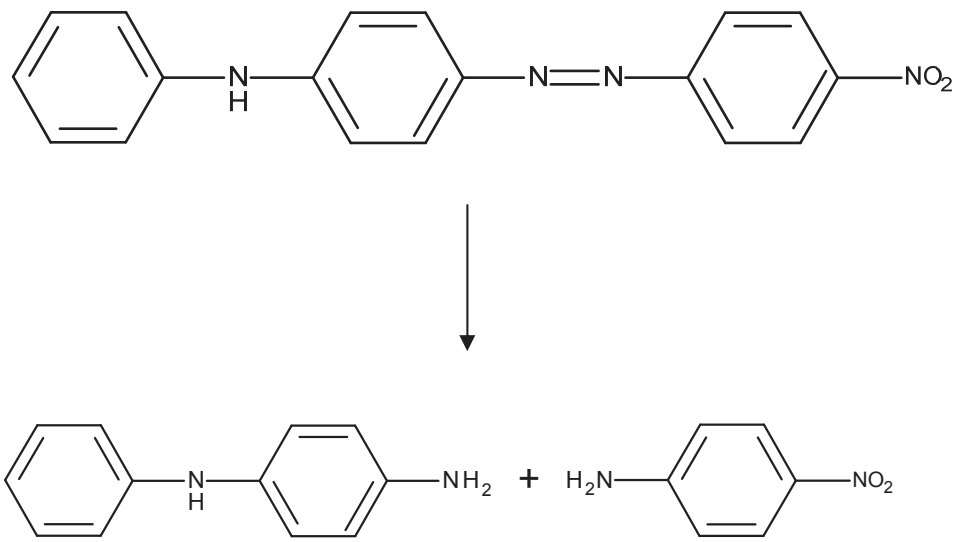
MW: 1

B. Disperse Orange 1

CAS:2581-69-3

MW: 318

C.I.: 11080



***p*-aminodiphenylamine**

CAS: 101-54-2

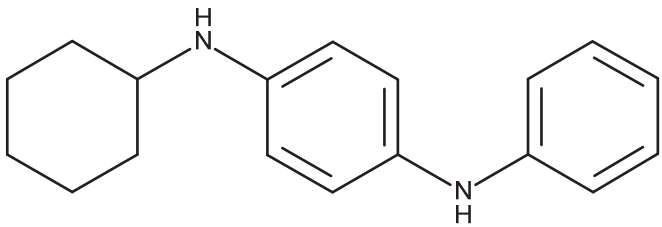
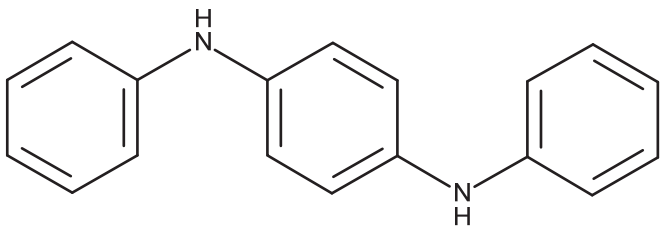
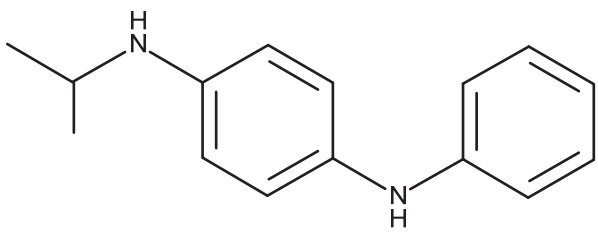
MW: 184

4-nitroaniline

CAS: 100-01-6

MW: 138

Figure 3. *Chemical structure, Chemical Abstract Service (CAS) numbers, as well as molecular weight (MW) for the investigated black rubber mix ingredients and p-phenylenediamine.*

N-cyclohexyl-N'-phenyl-4-phenylenediamine 	CAS: 101-87-1 MW: 266
N,N'-diphenyl-4-phenylenediamine 	CAS: 74-31-7 MW: 260
N-isopropyl-N'-phenyl-4-phenylenediamine 	CAS: 101-72-4 MW: 226
p-phenylenediamine 	CAS: 106-50-3 MW: 108

PATCH TESTING

In Study I all 10 patients were patch-tested with:

- one or more dilution steps of commercial and purified D Yellow 3 and D Orange 1, depending on their previous reactivity. Those who previously reacted with a + and/or ++ reaction to 1.0% were tested with dilution series starting at 1.0%; those who previously reacted with a +++ reaction to 1.0%, were patch-tested starting with 0.01% concentration and those who previously did not react, were patch-tested with the highest concentration (1.0%) only. If patients had a positive reaction on D4, they were additionally tested with a dilution series (Fig.4).
- the water-soluble residues of D Orange 1 and D Yellow 3, diluted in distilled water to a 1.0% w/v concentration. The procedure of the separation of the water-soluble and fat-soluble fractions of the dyes is described in Paper I.
- naphthalene sulphonate 1.0% aq. w/v.

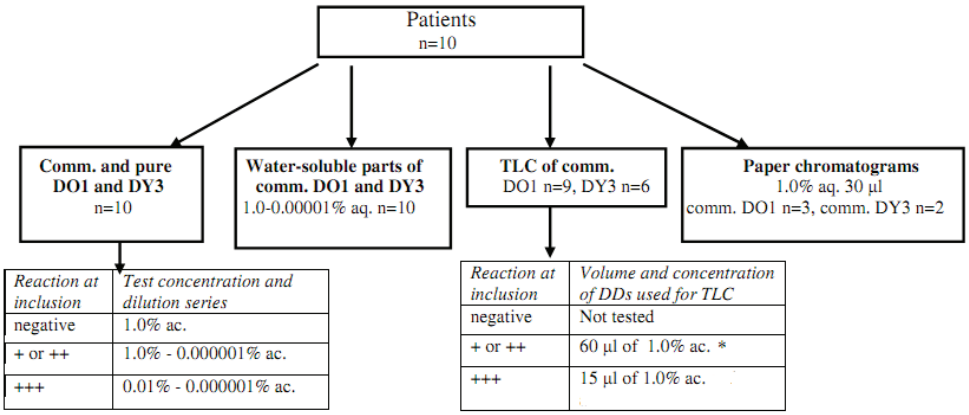
If the patients were positive to any of these preparations, they were tested with a dilution series of this preparation.

Additionally in Study II the same 10 patients were tested with:

- the series, consisting of PPD and the BRM ingredients, DPPD, CPPD and IPPD, at 1.0% w/v (all in serial dilutions in acetone starting at 1.0%).
- the presumed D Orange 1 and D Yellow 3 metabolites in serial dilutions in concentrations equimolar to the parental compound (Fig. 5). If the patients were positive on the first reading to the lowest concentration of the respective substance tested, they were additionally tested with the lower concentrations to try to find the elicitation threshold.

All patch test solutions were prepared in our department from the same batches. About 20 mg of each chemical was accurately weighed and dissolved in acetone or distilled water, yielding a 1.0% w/v preparation. From this stock solution further dilutions, from 10^{-1} to 10^{-6} % w/v, were prepared.

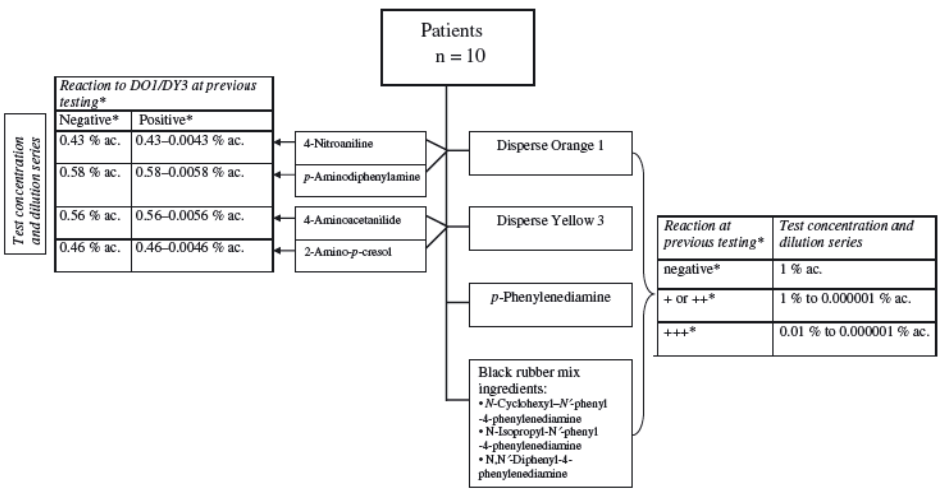
Figure 4. *The protocol for testing with Disperse Orange 1 and Disperse Yellow 3 in Study I.*



DO1, Disperse Orange 1; DY3, Disperse Yellow 3; comm., commercial; TLCs, thin-layer chromatograms; + or ++ or +++, previous positive test reactions; ac., acetone.

* 15 µl of 5% ac. concentration used if patient was negative on first reading to TLCs made from 1.0% ac. 60 µl solution.

Figure 5. *The protocol for testing with Disperse Orange 1, Disperse Yellow 3, their metabolites, black rubber mix ingredients, and p-phenylenediamine in Study II.*



+ or ++ or +++, previous positive test reactions.

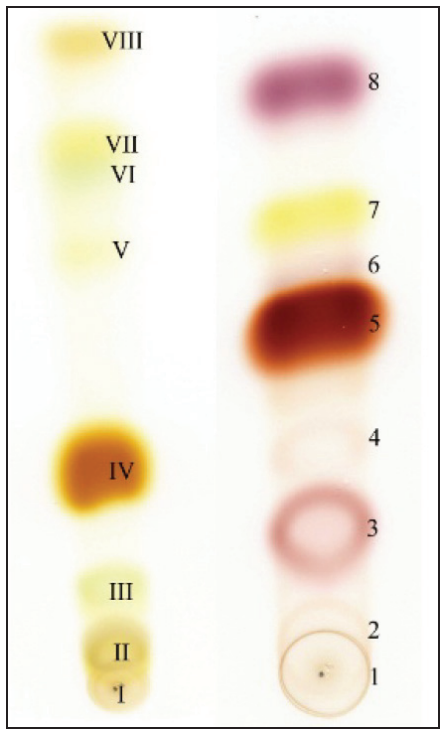
* The results of patch testing (negative, positive) are based on earlier test results prior to this study. ac, acetone.

Patch testing with thin-layer chromatograms

Thin–layer chromatography (TLC) is a method used for separating the components in a mixture of chemicals. The method is based on a stationary phase using a silica gel on a glass plate, an aluminium sheet or a plastic film, and an eluent as the mobile phase, e.g. chloroform or acetonitrile. If the sample consists of more than one chemically defined substance, the eluent will transport the different components, all having the same physico-chemical properties, different distances along the plate, giving rise to bands of separate spots.

In Study I the TLC technique was used for patch testing of individual patients with a known contact allergy to D Orange 1 and D Yellow 3 (150). To separate the ingredients in acetone solutions of D Orange 1 and D Yellow 3, TLCs were prepared on plastic films (TLC plastic roll 500×20 cm silica gel 60F254 from Merck KgaA, Darmstadt, Germany) (Fig. 6). One hundred percent chloroform was used as the mobile phase for D Orange 1 and a mixture of chloroform and acetonitrile, 86/14 (v/v) for D Yellow 3. To separate the water-soluble parts of D Orange 1 and D Yellow 3, chromatograms on filter paper (Munktell Filter AB, Grycksbo, Sweden) using distilled water as a mobile phase, were prepared. The preparation of the chromatograms is described in detail in Paper I. The TLCs prepared from the commercial dyes were compared also with TLCs prepared from the presumed metabolites (PADPA, 4-nitroaniline, 2-amino-*p*-cresol and 4-aminoacetanilide). No common spots were detected.

Figure 6. *Thin-layer chromatograms of commercial Disperse Yellow 3 (left) (86:14 mixture of chloroform and acetonitrile used as eluent) and Disperse Orange 1 (right) (100% chloroform used as eluent) patch test preparations.*



Patch test technique

In Studies I and II Finn Chambers® (Ø8 mm, Epitest Ltd, Tuusula, Finland) on Scanpor® tape (Norgesplaster A/S, Vennesla, Norway) were used for the patch testing. Fifteen µl of the test solution was applied with a micropipette to the filter paper disc in each test chamber. The TLCs and paper chromatograms with the separated spots were cut out in pieces of about 2.5×16 cm and were then applied on the upper back, to the left and/or the right of the spine of each tested patient, using Scanpor® tape to cover the chromatogram and secure them to the skin. The chambers, the TLCs, and the paper chromatograms were left on the back for 48 hrs and the readings were performed on D4 and on D7.

Evaluation of patch tests

The patch test reactions were scored according to the guidelines of the International Contact Dermatitis Research Group (151):

–	negative;
(+)/?	faint erythema;
+	erythema, infiltration, possibly papules;
++	erythema, infiltration, papules, possibly vesicles;
+++	intense erythema, infiltration and vesicles.

The minimum criterion for a positive patch test is homogeneous erythema and infiltration, i.e. +.

CHEMICAL INVESTIGATIONS

In Study III, the presence of the 8 DDs (D Red 1, D Red 17, D Blue 106, D Blue 35, D Blue 124, D Yellow 3, D Orange 1, and D Orange 3) in randomly obtained textile items was investigated at our department in Malmö. Dermatologists in different countries were contacted and were asked to send our research team cheap socks, T-shirts, underwear, scarves, tights, etc., made from 100% polyester or a poly-blend or other synthetic fibres (e.g., polyamide). They were also asked to be preferably yellow, orange or dark colours. TLCs from the extracts of the textiles were performed comparing with the 8 purified and commercial DDs, and the presence of the DD indicated by TLC had to be confirmed by HPLC.

Extracts from the textiles

The extracts were made from the textile size 20×20 cm cut into 1-2 cm pieces. Then they were put into a glass jar with a diameter of 6 cm to which 150 ml of 100 % acetone was added. Extraction was done using an ultrasonic bath for 15 min. Then the extract was vacuum-evaporated until dry (30° C) using a rotary evaporator. The obtained residues were diluted in 1-2 ml acetone and used for the application on the TLC plates.

Thin-layer chromatography of sample extracts

One µl of the extracts was repeatedly applied on the silica gel on a glass plate 20×20cm 60 F 254 with thickness of the TLC plate layer 250 µm (Merck KgaA,

Darmstadt, Germany) with a micropipette, until 5-10 µl had been deposited to 1 spot each for every 2.5 cm along a line on the lower part of the silica gel plate.

A mixture of chloroform and acetonitrile, 86/14(v/v) or 100% chloroform was used as the mobile phase. The separated components in the extracts gave a band of well defined and separated spots. The TLC plates were all inspected in visible light and in UV radiation (254 and 366 nm).

Purified and commercial solutions of D Red 1, D Red 17, D Blue 106, D Blue 124, D Yellow 3, D Orange 1, D Orange 3 and D Blue 35 (only commercial), 1% in acetone, were used as reference substances.

If there were matching spots from the textile extract and the reference (purified) dye, TLC was performed comparing extract, reference dye and the mixture of the equal parts of the extract and the reference dye.

The procedure is described in detail in Paper III.

If the matching of the spots on the TLC plate remained, HPLC of the extract and the reference dye was performed.

High performance liquid chromatography

HPLC is a method of separating chemical components, provided that they have different physic-chemical properties, which are distributed differently when carried through a stationary phase by a mobile phase. Separation of the DDs using HPLC involved a non-polar stationary phase and a polar mobile phase, containing a solvent of acetonitrile and water. The HPLC system and the linear gradient elution of the solvents used for the HPLC analysis are described in Paper III.

The identity of the substance producing a certain peak was determined by the retention time and UV spectrum. The detection limit was 0.0001 µg/cm^2.

GUINEA PIG MAXIMISATION TEST

The GPMT was performed according to the original description (152), but in order to standardize the test and objectify the evaluation of the patch test reactions, some modifications were made including statistical calculations, blind reading, and a positive control group (153, 154). The method is described in detail in Paper V.

To investigate the sensitisation capacity, the animals were induced with D Orange 1 and PADPA and challenged with the induction substance in question. To investigate the cross-reactivity among them and with 4-nitroaniline, D Yellow 3, 4-aminoacetanilide and 2-amino-*p*-cresol, and PPD, the animals were challenged with

these substances. Twenty-four test animals and twelve controls were used for each induction substance.

ETHICS

The studies described in Paper I and II were approved by the Regional Ethical Review Board in Lund, Sweden, and conducted in accordance with the ethical standards specified in the Declaration of Helsinki. All patients gave informed written consent to participate in the study.

The study described in Paper V was approved by the Lund Ethical Committee on Animal Experiments, Lund, Sweden, and conducted in accordance with ethical standards.

STATISTICAL CALCULATIONS

In Paper II the results were analysed using Fisher's exact two-sided test. Two-sided $p< 0.05$ was considered to be statistically significant.

In Paper V the number of positive animals within the test group was compared with the number of positive animals in the control group. The number of positive test animals was also compared with the number of positive animals tested with the vehicle only. Among the animals challenged with the induction substance on both the cranial and caudal patches (12 test animals and 6 control animals), only one of the patches chosen in advance was included. Statistical significance was calculated by a one-sided Fisher's exact test (comparing control and test animals) and by the McNemar test (comparing test substance with the vehicle in the same animal). When a significant value ($p<0.05$) was obtained both in comparison with the controls tested with allergen and the animals tested with vehicle alone, the compound was considered as a sensitiser.

4. RESULTS

PATCH TESTING

Patch testing with the substances present in the commercial Disperse Orange 1 and Disperse Yellow 3

All results are presented in Table 12.

The dilution series of pure D Orange 1 was positive in 8 of 10 patients and 1 patient was positive only to commercial D Orange 1. The dilution series of pure D Yellow 3 was positive in 6 of 10 patients, and 1 of 6 did not react to commercial D Yellow 3. One patient reacted to commercial and purified D Yellow 3, but did not react to the water-soluble part of commercial D Yellow 3. This patient reacted to two spots on TLCs. Another patient reacted in the same way to commercial D Orange 1. One patient was positive to the paper chromatogram from the water-soluble part of commercial D Orange 1. One patient, previously positive to D Yellow 3, did not react to D Orange 1 or D Yellow 3.

None of the 10 tested patients reacted to naphthalene sulphonate.

Patch testing with the potential metabolites from azo reduction of Disperse Orange 1 and Disperse Yellow 3

Patch testing with the potential metabolites of Disperse Orange 1: p-aminodiphenylamine and 4-nitroaniline

Of the 8 patients positive to D Orange 1 all reacted to PADPA. One of these patients, previously shown to be allergic to D Orange 1, reacted only to PADPA. All reactions to PADPA were strong (++ or +++) already on the D3 reading. Regarding the dilution series of PADPA, there were 8 positive patients of 9 tested (Table 13). The majority of the reactions was strong and disappeared abruptly from one tested concentration to the nearest lower concentration. Five of the eight patients positive to D Orange 1 reacted to 4-nitroaniline. The patch test reactions to 4-nitroaniline were weaker than to PADPA.

Table 12. Results of patch testing with the dilution series of commercial and purified Disperse Orange 1 and Disperse Yellow 3.

patient No / tested / substance	1 D4 D7	2 D4 D7	3 D4 D7	4 D4 D7	5 D4 D7	6 D4 D7	7 D4 D7	8 D4 D7	9 D4 D7	10 D4 D7
commDO1 1.0%	+++ NR	NT NT	* 	+ +	+++ NR	NT NT	+++ NR	- -	+++ NR	(+) ++
0.1%	+++ NR	NT NT	- -	(+) +	+++ NR	NT NT	++ NR	NT NT	+++ NR	(+) +
0.01%	- -	++ +++	- -	- -	+++ NR	++ +	- +	NT NT	+ NR	- -
0.001%	- -	++ ++	- -	- -	++ NR	(+) (+)	- -	NT NT	- NR	- -
0.0001%	- -	- -	- -	- -	(+) NR	- -	- -	NT NT	- NR	- -
0.00001%	- -	- -	- -	- -	++ NR	- -	- -	NT NT	- NR	- -
0.000001%	- -	- -	- -	- -	++ NR	- -	- -	NT NT	- NR	- -
pure DO1 1.0%	NT NT	NT NT	- -	(+) +	+++ NR	NT NT	NT NT	- -	+++ NR	(+) ++
0.1%	+++ tr	NT NT	- -	(+) +	+++ NR	NT NT	NT NT	NT NT	+++ NR	(+) ++
0.01%	(+) (+)	++ +++	- -	- -	+++ NR	+++ +++	+ +++	NT NT	(+) ++	(+) NR
0.001%	- -	++ +++	- -	- -	+++ NR	+ +++	(+) ++	NT NT	(+) +	- NR
0.0001%	- -	(+) (+)	- -	- -	++ NR	(+) (+)	- -	NT NT	- -	- NR
0.00001%	- -	- -	- -	- -	+ NR	- -	- -	NT NT	- -	- NR
0.000001%	- -	- -	- -	- -	+ NR	- -	- -	NT NT	- -	- NR
water soluble part of DO1 1.0%	++ +	+++ ++	- -	(+) (+)	+++ NR	+++ tr	++ +++	- -	++ +++	- (+)
0.1%	- -	+ +++	NT NT	- -	NT NT	++ NR	++ NR	NT NT	+ NR	NT NT
0.01%	- -	++ NR	NT NT	- -	NT NT	(+) NR	(+) NR	NT NT	- NR	NT NT
0.001%	- -	+ NR	NT NT	- -	NT NT	- NR	- NR	NT NT	- NR	NT NT
0.0001%	- -	- NR	NT NT	- -	NT NT	- NR	- NR	NT NT	- NR	NT NT
commDY3 1.0%	+++ tr	(+) (+)	- -	- -	++ NR	+++ NR	- -	- -	+++ +++	- (+)

	+++	(+)	NT	NT	NT	+++	NT	-	++	(+)
0.1%	tr	NR	NT	NT	NT	NR	NT	-	+++	++
	++	-	NT	NT	NT	-	NT	-	-	-
0.01%	tr	NR	NT	NT	NT	+	NT	-	-	-
	-	-	NT	NT	NT	-	NT	-	-	-
0.001%	-	NR	NT	NT	NT	-	NT	-	-	-
	+++	(+)	-	-	++	+++	-	-	+++	(+)
pure DY3 1.0%	tr	-	-	-	NR	NR	-	-	+++	+
	+++	(+)	NT	NT	NT	+	NT	-	+++	-
0.1%	tr	(+)	NT	NT	NT	NR	NT	-	+++	+
	++	++	NT	NT	NT	(+)	NT	-	++	-
0.01%	+	+	NT	NT	NT	+++	NT	-	++	-
	-	-	NT	NT	NT	-	NT	-	-	-
0.001%	NR	-	NT	NT	NT	(+)	NT	-	-	-
	-	-	NT	NT	NT	-	NT	-	-	-
0.0001%	-	-	NT	NT	NT	-	NT	-	-	-
water soluble part	++	(+)	-	-	(+)	-	-	-	-	-
of DY3 1.0%	+	-	-	-	NR	-	(+)	-	-	+++
	+	-	NT	NT	NT	NT	NT	NT	NT	NT
0.1%	NR	-	NT	NT	NT	NT	NT	NT	NT	NT
	-	-	NT	NT	NT	NT	NT	NT	NT	NT
0.01%	NR	-	NT	NT	NT	NT	NT	NT	NT	NT
naphthalene										
sulphonate 1.0%	-	-	-	-	-	-	-	-	-	-
aq.	-	-	-	-	-	-	-	-	-	-

w/v, mass concentration in acetone of the test preparation; DO1, Disperse Orange 1; DY3, Disperse Yellow 3; comm, commercial; NT , not tested; tr, reaction treated with potent topical corticosteroid; NR , not read. *case report published elsewhere (130).

Patch testing with the potential metabolites of Disperse Yellow 3: 4-aminoacetanilide and 2-amino-p-cresol

Of the 10 patients patch-tested with 4-aminoacetanilide, 3 were positive; all reacted to D Yellow 3 and 2-amino-*p*-cresol.

Of the 10 patients tested with 2-amino-*p*-cresol, 6 were positive with strong reactions on D3. All these patients were also allergic to D Yellow 3. Of the 6 patients tested with the lower than 0.56% concentration of 4-aminoacetanilide, only one was positive and reacted to 0.056%. Of the 6 patients tested with the lower than 0.46% concentration of 2-amino-*p*-cresol, 1 was positive and reacted to 0.046% (10 times diluted).

Test reactivity. In the 8 patients, who reacted positively to D Orange 1, the majority of positive D7 reactions were stronger than D3 reactions (Table 13). On the contrary, the majority of strong positive reactions to PADPA appeared on the first reading, i.e. D3/D4.

Regarding patterns of reactions to D Yellow 3 this tendency was not so strong. The majority of reactions to D Yellow 3 as well as reactions to 2-amino-*p*-cresol were seen on D3/D4.

For both D Orange 1 and D Yellow 3 the elicitation thresholds for the proposed metabolites were in most cases higher than for the parent compound. In 3 of 8 patients the thresholds for D Orange 1 and PADPA were similar.

Patch testing of the control patients. None of 118 consecutively patch-tested dermatitis patients reacted to the test solution with 4-nitroaniline 0.043%, PADPA 0.0058%, 4-aminoacetanilide 0.056% or 2-amino-p-cresol 0.046% in acetone. The concentration chosen for control testing of each substance was the lowest concentration giving a positive patch test reaction in a reasonable proportion of the 10 patch-tested patients with regard to controls needed for statistical significance. Another group of control patients were those positive to TDM, but negative to D Orange 1 or D Yellow 3. Results of their testing are presented in Table 13. Positive reactions to PADPA and 2 –amino-p-cresol were linked to positive reactions to D Orange 1 and D Yellow 3, respectively, but not to other ingredients of TDM ($p<0.05$).

Table 13. *Results of the testing of the control patients, positive to the textile dye mix but negative to Disperse Orange 1 or Disperse Yellow 3.*

	p-aminodiphenylamine 0.58%		4-nitroaniline 0.43%		4-aminoacetanilide 0.56%		2-amino-*p*-cresol 0.46%	
	Test	Control	Test	Control	Test	Control	Test	Control
Number of tested patients	8	5	8	7	6	7	6	4
Positive	8	1	5	2	3	0	6	0
p	<0.05		0.32		0.07		<0.05	

Simultaneous reactions to some *para*-amino compounds

Patch testing with the ingredients of BRM

Of 8 patients positive to D Orange 1 and PADPA, 6 reacted to IPPD. Among 8 patients positive to D Orange 1, 6 (75%) reacted to at least one BRM ingredient, and of 6 positive to D Yellow 3, 5 (83%) reacted to at least one BRM ingredient.

Patch testing with PPD

All 6 patients, who were allergic both to D Orange 1 and D Yellow 3, were positive to PPD, but 2 patients who reacted only to D Orange 1 did not react to PPD. One patient, who was previously shown to be allergic to D Yellow 3, now reacted only to PPD. Also, all 6 patients who reacted positively to D Yellow 3 and PPD reacted to 2-amino-*p*-cresol, one of the metabolites of D Yellow 3. Reactions to PPD were strongly related to positive reactions to D Yellow 3, and to one of its metabolites, 2-amino-*p*-cresol ($p<0.05$).

TESTING WITH THIN-LAYER CHROMATOGRAMS OF DISPERSE ORANGE 1 AND DISPERSE YELLOW 3

Testing with thin-layer chromatograms of commercial Disperse Orange 1

Of 9 patients, tested with commercial D Orange 1 TLCs, positive reactions were noted in 8. All reacted to the main spot (No. 5 in Fig.6), and 4 – in addition to another common spot (No. 7 in Fig.5). Of those 4, one patient was not read on D7, but the remaining 3 patients showed an allergic reaction to the additional one spot also on the D7 reading (Table 14).

Testing with thin-layer chromatograms of commercial Disperse Yellow 3

Of the 6 patients tested with commercial D Yellow 3 TLCs, 3 showed positive results. All of them reacted to the main spot (No IV in Fig.6), and 2 – to one and the same additional spot (No V in Fig.6). All 3 patients also reacted to the main spot of D Orange 1 TLCs. They also showed strong positive reactions on testing with pure and commercial D Yellow 3 (Table 11).

Paper chromatogram testing of water-soluble part of commercial Disperse Orange 1 and Disperse Yellow 3

Three patients, two of which showed a positive reaction to the water-soluble part of commercial D Orange 1, were tested with a paper chromatogram. One patient reacted to the spot on the application point. This patient reacted to the main spot on the TLCs made from commercial D Orange 1.

Two patients were tested with the paper chromatograms made from the water-soluble part of commercial D Yellow 3. None of them reacted positively.

Table 14. *The results of the patch testing with commercial Disperse Orange 1 and Disperse Yellow 3 thin-layer chromatograms.*

TLCs of D Orange 1

Patient No	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Spot No*	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7
8	- -	- -	- -	- -	- -	- -	- -	NT	- -	- -
7	(+) -	- -	- -	- +	+ NR	(+) +	- +	NT	- -	- -
6	- -	- -	- -	- -	- -	- -	- -	NT	- -	- -
5#	+++ ++	++ +++	- -	- +	+++ **NR**	++ ++	+++ +	**NT**	+++ +++	- +
4	- -	- -	- -	- -	- -	- -	- -	NT	- -	- -
3	- -	- -	- -	- -	- -	- -	- -	NT	- -	- -
2	- -	- -	- -	- -	- -	- -	- -	NT	- -	- -
1	- -	- -	- -	- -	- -	- -	- -	NT	- -	- -

TLCs of D Yellow 3

Patient No	1	2	3	4	5	6	7	8	9	10
Spot No*	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7
VIII	- -	- -	NT	NT	NT	- -	NT	- -	- -	- -
VII	- -	- -	NT	NT	NT	- -	NT	- -	- -	- -
VI	- -	- -	NT	NT	NT	- -	NT	- -	- -	- -
V	+ (+)	- -	NT	NT	NT	- -	NT	- -	++ +	- -
IV#	**++** **++**	- -	**NT**	**NT**	**NT**	+ -	**NT**	- -	(+) +++	- -
III	- -	- -	NT	NT	NT	- -	NT	- -	- (+)	- -
II	- -	- -	NT	NT	NT	- -	NT	- -	- -	- -
I	- -	- -	NT	NT	NT	- -	NT	- -	- -	- -

* Spots on each chromatogram are numbered in accordance with the registration in Fig.6, with No 1 or No I as the application spot.

Emboldened and #, main spot on chromatogram; TLCs, thin layer chromatograms; -, negative results; NT - not tested.

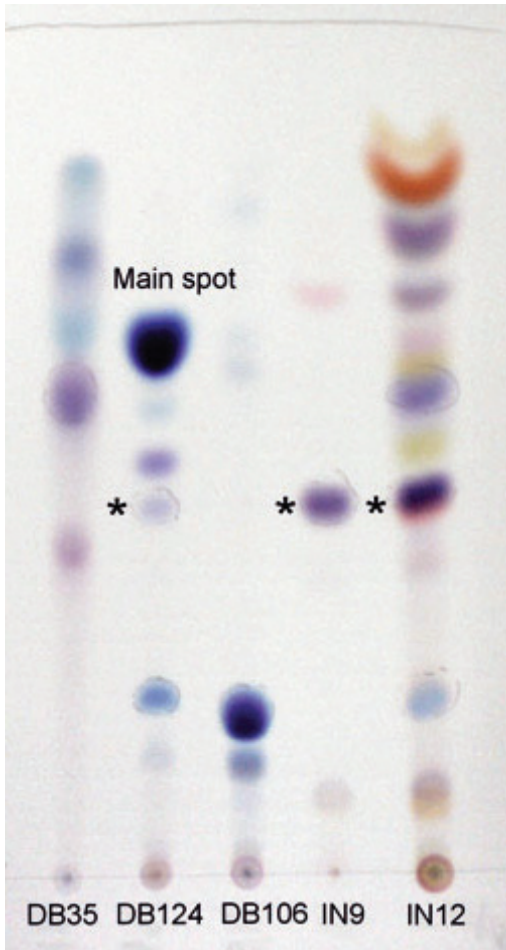
CHEMICAL INVESTIGATIONS

Thin-layer chromatography of the extracts from textiles

DDs indicated by the TLC analysis. In total 121 extracts were analyzed by TLC. Thirty-one extracts had similar spots to the main spot of the reference dyes on TLC plates. All spots on TLC plates were visible in the daylight, and no additional spots were seen in UV radiation.

The majority of the 31 extracts had matching spots with D Blue 124 (12 extracts), 10 extracts – with D Orange 1, 8 with D Red 1, 7 with D Red 17, 5 with D Orange 3 and D Blue 106, 4 with D Blue 35 and D Yellow 3. Some of the extracts had matching spots, however not to the reference dye but to impurities or to other substances present in the commercial dye preparation (Fig. 7).

Figure 7. *Similar spots when thin-layer chromatograms of the extracts (IN9, IN12) and commercial Disperse Blue 124 (DB124) and Disperse Blue 106 (DB106) dyes are compared. These spots do not represent the main spot of DB124 or DB106. Asterisks (*) indicate similar colours.*



Extracts which had similar spots to the main spot on the TLCs of the purified reference dye were also analyzed in other system. In one case the results differed and there were no matching spots (Fig. 8).

Thirty extracts were mixed with the purified dye to confirm the presence of the reference dye. Mixes were "positive" (i.e. the suspected dye spot from the extract matched the reference dye on the TLCs of the mix) in 9 cases; in two with D Yellow 3, D Red 1 and D Orange 1, and each in one case to D Blue 124, D Blue 106, and D Orange 3 (Fig. 9).

Similar colour patterns. There was a similar pattern of dyes on TLC plates among different extracts especially in the orange, blue, red, and yellow spectra. TLCs of the extracts made from different garment dyes in the same colour showed that the same colour could be composed from different dyes (Fig. 10).

Figure 8. *Thin-layer chromatograms of the extracts (L1 and L2) as compared with purified Disperse Orange 1 (DO1) and mixtures (MIX). Asterisks (*) indicate similar colours when the eluent system 84:16 chloroform/acetonitrile was used. (a) When the system was changed to 100% chloroform. (b) There were no matching colours.*

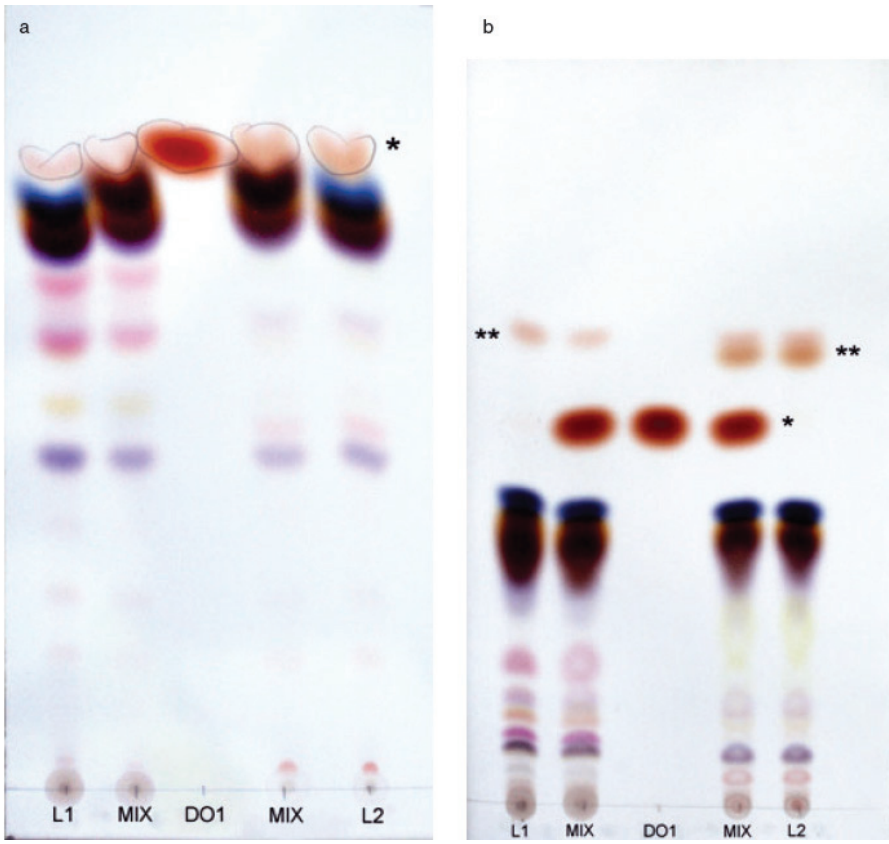


Figure 9. *Thin-layer chromatogram of the extract (I13) as compared with purified Disperse Yellow 3 (DY3), Disperse Blue 106 (DB106), Disperse Blue 124 (DB124), and mixtures (MIX). Asterisks (*) indicate similar colours.*

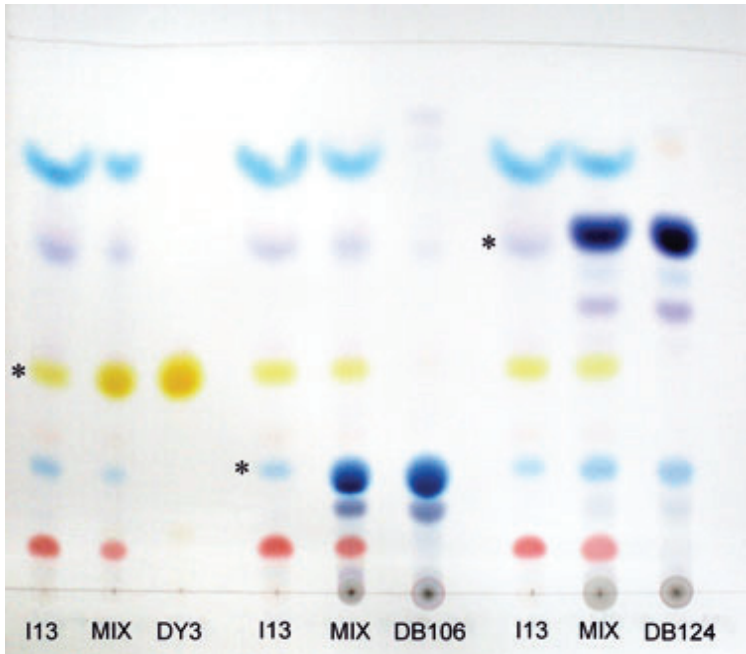
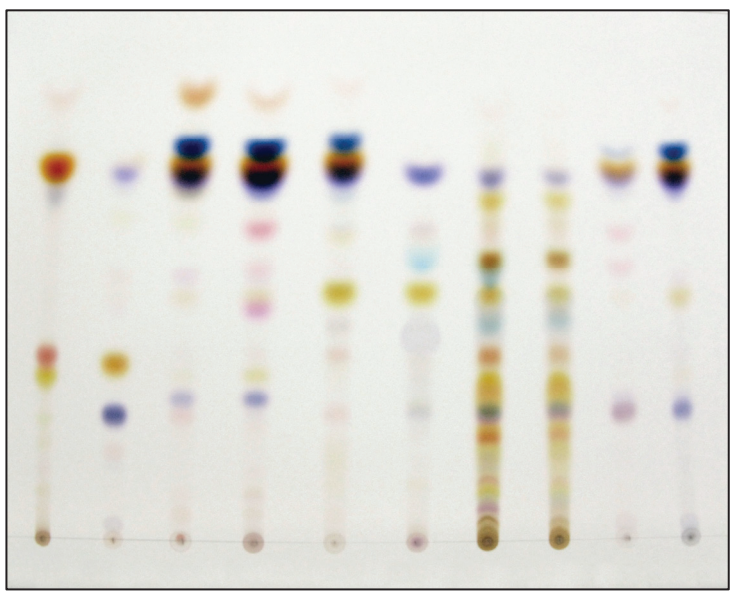


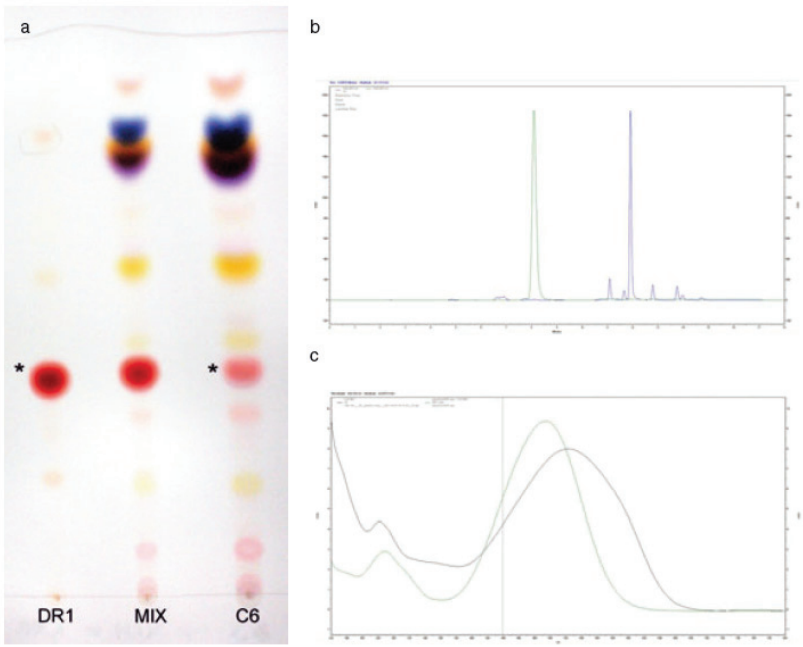
Figure 10. *Thin-layer chromatogram of the different extracts.*



High performance liquid chromatography of the extracts

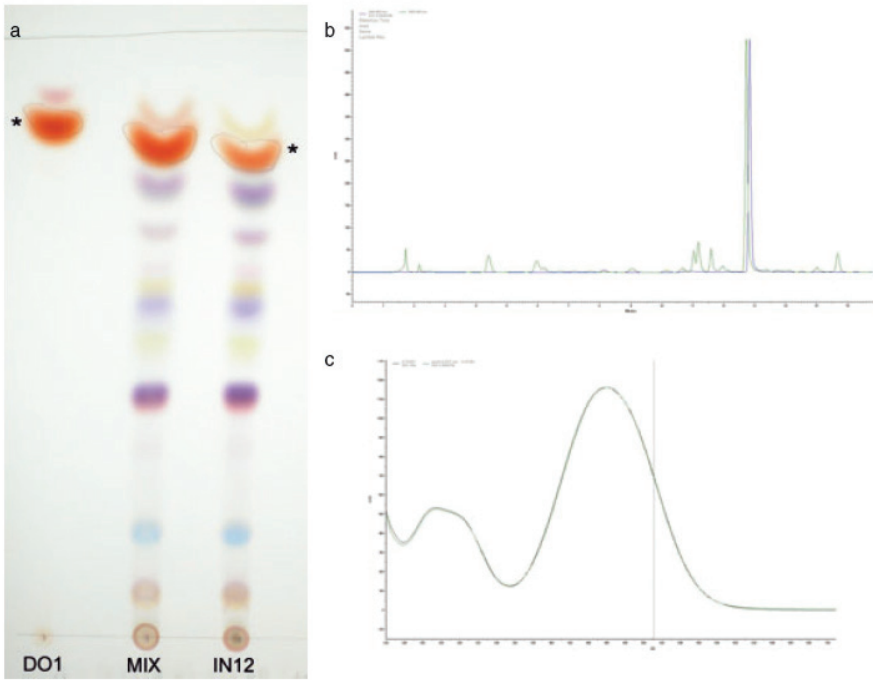
Ten samples were analyzed by HPLC. It was not possible to confirm the presence of the suspected dye from the TLC analysis (D Yellow 3, D Orange 3, D Blue 106 and D Red 1) when evaluating the retention times and UV spectra in seven samples (Fig. 11).

Figure 11. *(a) Thin-layer chromatogram of the extract from the textile (C6) as compared with the reference substance Disperse Red 1 (DR1) and a mixture of C6 and DR1 (MIX) indicates the presence of DR1 in C6, but this was not confirmed by high-performance liquid chromatography (HPLC). Asterisks (*) indicate similar colours. (b) HPLC chromatogram of purified DR1 (green line) and C6 (blue line) shows different retention times. (c) HPLC spectrogram recorded at a wavelength of 450 nm indicates that there is no DR1 (green line) in C6 (blue line); detection limit ≤0.0001 µg/cm².*



However, in a pair of brown tights from Italy (made from polyamide and elastane) D Yellow 3, D Blue 106 and D Blue 124 were confirmed by HPLC analysis. In a set of black bra and panties from India (material composition was not present on the label) – D Orange 1 was detected (Fig. 12).

Figure 12. (*a) Thin-layer chromatogram of the extract from the textile (IN12) as compared with the reference substance Disperse Orange 1 (DO1) and a mixture of IN12 and DO1 (MIX) indicates the presence of DO1 in IN12. Asterisks (*) indicate similar colours. (b) High-performance liquid chromatography (HPLC) chromatogram of purified DO1 (blue line) and IN12 (green line) shows very similar retention times. (c) HPLC spectrogram recorded at a wavelength of 450 nm indicates that DO1 (blue line) is present in IN12 (green line); detection limit ≤ 0.0001 µg/cm^2.*



GUINEA PIG MAXIMISATION TEST

As induction for every substance was performed on different occasions, results represent data from three different experiments.

Sensitising capacity

D Orange 1 and PADPA were found to be strong sensitisers in the guinea pig. Positive reactions were seen to both D Orange 1 and PADPA in 22 of 24 animals. Two control animals had reactions to D Orange 1 and two control animals reacted to PADPA (p<0.001). Only 5 of 24 animals had positive reactions to 4-nitroaniline and also two control animals to this substance (p>0.3) (Table 14).

Table 14. *Sensitising capacity of Disperse Orange 1, p-aminodiphenylamine and 4-nitroaniline.*

Induction substance	T/*n*	C/*n*	V/*n*	P/*n*
Disperse Orange 1	22/24	2/12	0/12	1/6
p-aminodiphenylamine	22/24	2/12	0/12	1/6
4-nitroaniline	5/24	2/12	2/12	4/6

T, number of the positive test reactions to the induction substance in test animals; C, number of the positive test reactions to the induction substance in control animals; V, number of the positive test reactions to the vehicle in test animals; P, number of positive test reactions to 2-methylol phenol in the positive control group. *n*, number of tested animals in the 4 groups T, C, V, and P.

Cross-reactivity

The result of the test for cross-reactivity is given in Table 4. PADPA gave a positive test reaction in 21 of 24 guinea pigs sensitised to D Orange 1 (p<0.001) (Table 15). Two animals, negative to PADPA, were positive to D Orange 1. One was negative both to PADPA and D Orange 1.

D Orange 1 was positive in 23 of 24 guinea pigs sensitised to PADPA and in 6 of 12 controls (p<0.001). Although PPD was positive in 17 of 24 guinea pigs, it was also positive in 7 of 12 controls (p>0.3).

Cross-reactivity in the animal group, in which 4-nitroaniline was the induction substance, was not assessed because sensitisation to this substance failed.

Table 15. *Cross-reactions between Disperse Orange 1, Disperse Yellow 3, their potential metabolites and p-phenylenediamine in 24 test and 12 control animals.*

Induction substance	Challenge substances						
	DO1	4-nitroaniline	PADPA	DY 3	2-APC	4-AAA	PPD
	+ *p*	+ *p*	+ *p*	+ *p*	+ *p*	+ *p*	+ *p*
DO1 *Test* *Control*	22 <0.001 2	1 >0.3 0	21 <0.001 0	0 >0.3 0	1 >0.3 0	1 >0.3 0	1 >0.3 1
PADPA *Test* *Control*	23 <0.0028 6	1 >0.3 0	22<0.001 2	2 >0.3 1	3 >0.3 1	1 >0.3 0	17 >0.3 7
4-nitroaniline *Test* *Control*	NA	5 >0.3 2	NA	NA	NA	NA	NA

PADPA, *p*-aminodiphenylamine; DO1, Disperse Orange 1; DY3, DisperseYellow 3; 2-APC, 2-amino-*p*-cresol; 4-AAA, 4-aminoacetanilide; PPD, *p*-phenylenediamine; *p*, *p*-value; +, number of positive guinea pigs; NA, Not assessed.

5. DISCUSSION

RELEVANCE OF THE SUBSTANCES PRESENT IN THE COMMERCIAL DISPERSE ORANGE 1 AND DISPERSE YELLOW 3 REGARDING CONTACT ALLERGY

One of the main aims in this thesis was to investigate the significance of the impurities found in commercial D Orange 1 and D Yellow 3 regarding contact allergy. From earlier studies we knew that commercial D Orange 1 and D Yellow 3 contained more substances than the actual dye (129).

By using TLC we could show that D Orange 1 and D Yellow 3 both contained at least six impurities each. There was one main spot of a strong colour in both TLCs, which corresponded to the main spot in the TLCs made with purified dyes. Hence, other spots could be considered impurities. From this analysis we cannot ascertain the number of impurities that are present, since one TLC spot can contain several substances. However, our TLC analysis showed a higher number of impurities in both dyes than what was previously reported (128). The TLCs of commercial dyes were checked for the presence of their potential metabolites from azo reduction: PADPA, 4-nitroaniline, 4-aminoacetanilide, and 2-amino-*p*-cresol. No metabolites could be detected when comparing TLCs of commercial D Orange 1 and D Yellow 3 with TLCs of the pure metabolites. So it is unlikely that metabolites could be the cause of the positive reactions to the other spots on TLCs.

We patch tested the dyes, in commercial and in purified forms, in serial dilutions. If the actual purified dye was the main sensitiser, we would expect a higher reactivity to the purified than to the commercial dye, since the dye content in the commercial D Orange 1 and D Yellow 3 was 15 and 41% w/w, respectively (analysis performed at our lab). Such a higher reactivity could not be shown in our study, presented in Paper I (Table 12), although there is a tendency towards lower elicitation thresholds for the purified dyes. The results are in line with the findings obtained from testing with D Blue 106 and D Blue 124 in serial dilutions (116).

All 8 patients who reacted positively when patch tested with D Orange 1 showed positive reactions to the main spot (spot No 5, containing D Orange 1) when tested with TLCs of this dye. Four of these patients also reacted to TLCs spot No 7. When tested with TLCs of D Yellow 3, three of the six patients testing positively to this dye

reacted positively to the main spot (spot No IV, containing D Yellow 3). The two patients that did not react to the TLC testing showed low patch test reactivity to D Yellow 3, and one patient was not TLC-tested. Among the 3 TLCs positive patients 2 reacted positively also to spot No V. Reactions to additional TLC spots were weaker than to the main spots for both dyes. Our results indicate the presence of at least one impurity in each dye capable of eliciting allergic reactions. These impurities have not yet been identified.

In the animal study it was also shown that D Orange 1 is a very potent sensitiser, which reflects the pattern of reactivity seen in the allergic individuals. In the serial dilution testing of patients, the elicitation thresholds varied from 0.1% to less than 0.000001% for D Orange 1 and from 0.1 – 0.01% for D Yellow 3. One patient reacted to the lowest concentration tested of D Orange 1, i.e. 0.000001% (or 0.01μg/ml), which is comparable to the lowest doses reported to give positive reactions, examples being D Blue 106, D Blue 124, and some of the main allergens in phenol formaldehyde resins (141, 160). Unfortunately this patient developed a systemic contact dermatitis with symmetrical lower leg dermatitis during the patch test procedure, and it was not possible to define her lowest elicitation threshold, which we otherwise always try to pin-point as this has considerable impact on how to judge the clinical relevance.

Testing with the water-soluble part of the commercial dyes indicated the presence of allergens also in these fractions. Of the eight patients who tested positively to D Orange 1, six reacted to the water-soluble part of commercial D Orange 1. Out of three patients tested with paper chromatograms, one showed a positive reaction to the application spot. This spot contained the least polar substances in the fraction which indicated that an allergen could be found among them. Testing of the water soluble part of commercial D Yellow 3 showed two patients with positive reactions, but testing with paper chromatograms gave no further information. No attempts have been made yet to identify the allergens in these water-soluble parts. Due to the patients' relatively high reactivity to the water-soluble parts it is unlikely that the positive reactions were caused by contamination of the extracts with the dye itself. One known additive to both the commercial dyes is naphthalene sulphonate. This substance can be excluded as a cause of the positive reactions because it was patch tested separately in all patients and none reacted positively. It was tested in a higher concentration than it could possibly have been present in any of the other test preparations in this investigation.

In patch testing, whenever one re-tests a person with an allergen that has given a positive reaction earlier, a negative test reaction on the next test occasion may occur due to variation in patch test reactivity from time to time. This is more likely to occur if the patch test reactivity to the respective allergen is low. We also saw this phenomenon in our study with negative reactions to the patch test preparations and

to the TLCs in one patient with earlier diagnosed contact allergy to D Yellow 3. On the other hand, one patient who had previously tested negatively to D Yellow 3 patch test preparations reacted to D Yellow 3 this time. It could be attributed to qualitative differences in the patch test preparations used for the previous and present patch testing (59) or to technical causes or to the individual variation of reactivity to the allergens. Patch test sensitisation on this occasion is unlikely to be the cause since the patient reacted already on D4.

PATCH TESTING WITH THE POTENTIAL METABOLITES OF DISPERSE ORANGE 1 AND DISPERSE YELLOW 3 AND *PARA*-AMINO COMPOUNDS

Reductive cleavage of the azo bond in DDs on the surface of the skin or in the skin could lead to the formation of aromatic amines which are absorbed by the skin (155). These substances could be involved in the development of the dye allergy and maybe play an essential role for the sensitisation process. If the primary sensitiser is a metabolite of the DD, the strength of the test reactions to these metabolites most likely will be stronger than to the parent dye and imply a lower elicitation threshold.

The products formed from reductive cleavage of D Orange 1 are PADPA and 4-nitroaniline. There was a good agreement in patch test results between D Orange 1 and PADPA, since all 8 patients positive to D Orange 1 reacted to PADPA. In the equimolar dilution series, D Orange 1 showed a tendency of lower elicitation thresholds than PADPA while PADPA gave stronger reactions especially on D4, indicating that PADPA reactions developed faster. 1,4-Nitroaniline gave positive test reactions in 5 of these patients. These reactions were weaker than for PADPA and D Orange 1, and its elicitation thresholds were also higher. The close association between PADPA and D Orange 1 allergies was also shown by testing control patients positive to textile dye mix (TDM) but who were also negative to D Orange 1, showing that positive reactions to PADPA are linked to positive reactions to D Orange 1, but not to other ingredients of TDM ($p < 0.05$).

PADPA has been identified as a strong sensitiser in the local lymph node assay and in the GPMT (133, 156). PADPA is also a known contact allergen for hairdressers and consumers using hair dyes (157).

In vitro experiments have shown that after 24 hours 70% of DDs still stay on the human skin, while almost 50% of 4-nitroaniline is absorbed through human skin during this time period (158, 159).

It has been shown that various aromatic amines undergo N-acetylation in keratinocytes and that N-acetylated derivatives are not capable of inducing dendritic

cell activation or a positive local lymph node assay response (133, 159, 160). It has also been demonstrated that the skin has a relatively high acetylation capacity (160). When the amount of aromatic amines exceeds the acetylation capacity in the skin, the oxidative metabolism could become more important (161), but when these amines are applied in a low enough concentration, N-acetylation may detoxify them. In the present investigation PADPA, 4-nitroaniline, 4-aminoacetanilide and 2-amino-*p*-cresol are primary amines for which acetylation could be relevant. It can be interesting to note that D Yellow 3 and 4-aminoacetanilide are acetylated aromatic amines. However, little is known about the mechanisms involved in creation of reactive haptens from the aromatic amines, but it is likely that oxidized metabolites are involved in most cases.

Testing control patients positive to TDM, but negative to D Orange 1 did not reveal a statistically significant link between positive reactions to D Orange 1 and 4-nitroaniline in equimolar concentrations. It could be caused by similar metabolite formation from D Orange 3, as some of the control patients were positive to this DD. Moreover there are data showing that during hair dyeing when PPD is mixed with the oxidizing agent, 4-nitroaniline is formed (162). As most of our control patients were also positive to PPD, they might have been exposed and sensitised to 4-nitroaniline when dyeing their hair in the past.

The products formed from reductive cleavage of D Yellow 3 are 4-aminoacetanilide and 2-amino-*p*-cresol. 4-Aminoacetanilide was positive in 3 of 6 patients who were positive to D Yellow 3 and 2-amino-*p*-cresol gave positive reactions in all 6. The positive reactions to 2-amino-*p*-cresol were strong and occurred early, but there was no tendency to appear earlier than reactions to D Yellow 3. When testing in the dilution series, the allergic reactions also ceased at a 10 or 100 times higher concentration than those of D Yellow 3. It was shown previously in an in vitro and in a local lymph node assay, that 4-aminoacetanilide is a weaker sensitiser than 2-amino-*p*-cresol, however, they are said to cross-react (133, 163). While testing control patients positive to TDM but negative to D Yellow 3, it appeared that positive reactions to 4-aminoacetanilide and 2-amino-*p*-cresol were statistically significantly linked to positive reactions to D Yellow 3.

The observed overrepresentation of the simultaneous positive reactions between D Orange 1 and PADPA as well as between D Yellow 3 and 2-amino-*p*-cresol could indicate that these substances after being formed on or in the skin have caused sensitisation, but other explanations are equally likely. These substances might be used as raw materials in the dye production and remain as impurities in the DDs. We can also suspect cross-reactivity between the DDs and these substances, as they represent exact copies of the ends of the parental dye molecule. In our study, a high purity of D Orange 1 and D Yellow 3 was shown by chemical analysis. The possibility that PADPA or 2-amino-*p*-cresol could be contaminated with D Orange 1

or D Yellow 3, respectively, is not supported by the patch test reaction pattern. It should be expected that reactions to these substances would be weaker than to the respective dye as the concentration of the DDs would be lower than in the parental dye, but this was not the case.

A standard textile garment contains around 100 μg/cm^2 of the dye, e.g., D Orange 1 (69). The majority of D Orange 1 positive patients reacted down to 0.0010% when patch tested, corresponding to 0.30 μg/cm^2 D Orange 1. If 0.3% of the dye in such a garment would be absorbed by the skin, this would be enough to cause an allergic reaction in most people sensitised to D Orange 1. It is quite reasonable to anticipate the release and subsequent uptake of this amount of dye. In our study, the lowest concentration of PADPA to which the majority of patients reacted was 0.0058%. This corresponds to 1.7 μg/cm^2 of PADPA. If the total amount of D Orange 1 at a skin exposure of 0.30 μg/cm^2 would be split by reduction of the azo bond, this would correspond to a PADPA dose of 0.17 μg/cm^2. This amount should probably be lowered to reflect what happens in the skin, since metabolic reactions seldom affect 100% of a foreign substance, but usually just a portion of it.

Several other circumstances can favour the development of an allergic reaction. New textiles can contain higher concentrations of dyes than old ones. The exposure to dyes when wearing a garment can mimic a repeated application test rather than a regular patch test. In certain skin areas there may be close contact with the incriminating garment, which in combination with increased sweating and friction leads to enhanced extraction from the fabric and better penetration of the allergens.

Reactions to PPD were strongly related to positive reactions to D Yellow 3 and to one of its metabolites, 2-amino-*p*-cresol. The two patients, who reacted to D Orange 1 but not to D Yellow 3, did not react to PPD. This is contrary to other studies, where sensitivity to PPD was not related or just weakly related to D Yellow 3 sensitivity (70, 147). We were unable to find a study indicating cross reactivity between PPD and 2-amino-*p*-cresol. In our study, we found that of nine patients, positive to PADPA, six (66.7%) were positive to PPD, while an additional patient was positive to PPD, but not to PADPA. PADPA is described to show strong cross-reactivity to IPPD (156).

Control testing of 4-nitroaniline, 4-aminoacetanilide and 2-amino-*p*-cresol showed negative results for each substance. Consecutively tested dermatitis patients served as controls. Patch test reactions in allergic patients to the dilution series of these substances and the macroscopic appearance of the positive patch test reactions consistent with an allergic reaction strongly support the allergic nature of the positive test reactions.

CHEMICAL INVESTIGATIONS

The presence of the 8 DDs included in the textile dye mix was investigated in 121 garments from different countries. The garments were solvent extracted and the resulting extracts were used for analysis. After the initial TLC, it was suspected that some of the DDs could be present in 30 of 121 (24.8%) extracts, but through further analysis by mixing extract with the reference dye and repeating the procedure, changing eluent system for TLC and performing HPLC analysis, we were able to confirm the presence of D Yellow 3, D Orange 1, D Blue 106 and D Blue 124 in 3 garments out of 121 (2.5%). As these items were obtained randomly, it is obvious that DDs most commonly used for testing dermatitis patients are not widely used for dying fabric. Our study shows that one method for identification of a DD is not enough to confirm its identity. A TLC analysis showed that a textile usually is dyed with several different dyes, although it appears to have just one colour. The textiles that most often contain several different dyes are the black or darkly-coloured ones. We found D Orange 1 in a set of black bra and panties and D Yellow 3, D Blue 106 and D Blue 124 in the brown tights. This shows that it can be difficult to predict which dyes are used in a textile just from its colour.

For a patient who needs to avoid certain textile dyes it can be very useful to have information about the material the garment is made from and which types of dyes that are used for different fibres, e.g. DDs are exclusively used for synthetic fibres and especially in polyester.

Thirty percent of the garments, some of which were also obtained within the EU, had no fibre composition or country of origin information available on labels. So it may be difficult to avoid certain types of textiles and therefore certain dyes for consumers.

When investigating the textiles, we used two forms of chromatography: thin-layer and high-performance liquid chromatography. TLC is simple to use, and it is inexpensive and quick. Conditions of the TLC can be easily modified to obtain efficient separation of different components in the mixture, but it is a qualitative and not a quantitative technique. It could be that overlapping of several components in the spot of mixture with similar retention time on the TLCs occurs. This could be revealed through changing conditions of the TLC (e.g. eluent system) or using other methods (for example, HPLC). HPLC is a much more sensitive method – the detection limit of disperse dyes in our study was 0.0001 $\mu g/cm^2$, and a standard textile garment contains around 100 $\mu g/cm^2$ of the dye (164).

Our study described in Paper III showed that the DDs in the textile dye mix are infrequently found in textiles. It could be that other disperse azo dyes are used in the textiles we are wearing today, but even if we only found these dyes in one item made in the EU, this demonstrates that customers still are at risk of getting problems from DDs. Yet it is interesting to note that we have a relatively high frequency of contact

allergy to the textile dye mix, although the use of and exposure to these dyes should be very limited nowadays. Maybe our patients were sensitised at the time during which these dyes were frequently in use, or maybe the dyes somehow have the ability to detect contact allergies to newer dyes because of chemical similarities. In any case it will be difficult to show a clinical relevance of a positive patch test to the current textile dye mix or to any of its constituents. Because of this, it should be of high priority to develop a new textile dye mix that includes the most relevant disperse dyes used nowadays.

GUINEA PIG MAXIMISATION TEST

Sensitising capacity of Disperse Orange 1, *p*-aminodiphenylamine and 4-nitroaniline

PADPA has been found to be a sensitiser in the GPMT (156), but to our knowledge D Orange 1 has not been investigated with the GPMT. We sensitised 22 of 24 (92%) guinea pigs with both substances. D Orange 1 and PADPA could be classified as strong sensitisers, when the significance levels $p<0.05$, $p<0.01$ and $p<0.001$ are used to designate sensitisers as weak, moderate or strong, respectively (154). They could be compared with strong sensitisers such as diglycidyl ether of Bisfenol F, phenyl glycidyl ether or some of the main allergens in phenol-formaldehyde resins where GPMTs were performed according to the same methodology (165-167).

In our study 4-nitroaniline in an equimolar concentration to D Orange 1 did not show a sensitizing capacity. This finding confirms results from other studies (e.g., 138) where 4-nitroaniline was found to be a non-sensitizer.

Cross-reactivity of Disperse Orange 1 and *p*-aminodiphenylamine with 4-nitroaniline, Disperse Yellow 3, its potential metabolites and *p*-phenylenediamine

The animals induced with D Orange 1 showed cross-reactivity to PADPA and the animals induced with PADPA showed cross-reactivity to D Orange 1. 4-Nitroaniline, D Yellow 3, 2-amino-*p*-cresol, 4-aminoacetanilide and PPD did not show cross-reactivity in any of the two groups. The purity of D Orange 1 was over 99% and PADPA was not detected in it (detection limit 0.1%), and D Orange 1 was not detected in PADPA (detection limit 0.1%), so the presence of PADPA in D Orange 1 or vice versa cannot explain the observed cross-reactivity.

Our results indicate that a person sensitised to D Orange 1 will react to PADPA, but not to PPD. Individuals can be exposed to PADPA using oxidative hair dyes or rubber (175). PADPA can also be used in a textile dye synthesis, so it could remain in

the final product and be transferred to textiles when dyeing items (157). Primary sensitisation to PADPA causes allergy to D Orange 1 as indicated by our study.

Pontén and colleagues hypothesised that the size of the molecule could influence cross-reactivity pattern - when sensitised with a smaller molecule, the larger but chemically similar molecule is not recognized, but when sensitised with the larger molecule, the smaller molecule is recognized (165). The hypothesis was supported in studies of phenyl glycidyl ether of bisphenol F. In our study both the larger molecule (D Orange 1) and the smaller molecule (PADPA) cross-reacted, this seems to modify the earlier findings. However, the size difference between the molecules in our study is smaller than in the studies regarding epoxy resins and phenol-formaldehyde resins. The observed simultaneous reactions could, on the other hand, be a result of metabolism and thus be an indication of the relevance of the azo reduction hypothesis. In this case, the animals would have been sensitised to PADPA both when they were induced with this substance and with D Orange 1.

Among our patients six were allergic to D Orange 1 as well as to D Yellow 3 while two patients reacted positively to D Orange 1 but not to D Yellow 3. This overrepresentation of simultaneous reactions could indicate cross- reactivity. However, the guinea pigs induced with D Orange 1 did not show cross - reactivity when tested with D Yellow 3. This argues against cross- reactivity, at least in the investigated direction. Testing D Orange 1 in guinea pigs induced with D Yellow has, however, not yet been done but could give valuable information. Some clinical reports also show that clinically relevant sensitisation to only one DD exists (69, 102, 108).

No cross-reactions were demonstrated to PPD with D Orange 1 as the primary sensitiser in our study. We have noticed in our previous study when patch testing patients that only showed contact allergy to D Orange 1 and not to other DDs, there were no positive patch test reactions to PPD (168). Interestingly, PPD did not cross - react at a statistically significant level when the primary sensitiser was PADPA, although these two molecules have chemical similarities.

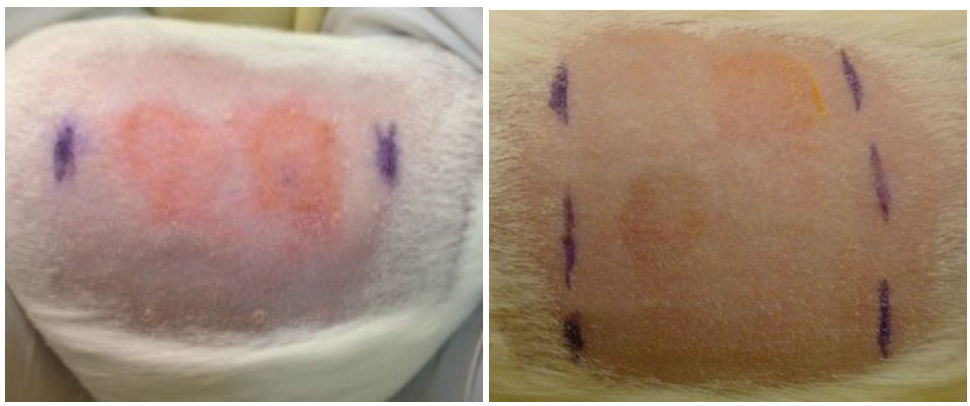
It is worth mentioning that reading of the patch test reactions of the coloured substances in guinea pigs could be complicated (Fig. 13). As the epidermis of the guinea pig contains fewer layers than in humans, a positive reaction to the sensitiser is mostly based on the erythema appearance. However, blind readings and inclusion of the control groups help to reduce possible bias and influence of e.g. coloured substances.

Whether primary sensitisation to PPD would cause cross-reaction to D Orange 1 or PADPA is not known. If PPD could have been tested at a higher concentration, we might have seen a higher degree of cross-reactivity. The GPMT study performed by Yamano and colleagues showed that when the primary sensitiser was PPD, it did not

cross-react with PADPA, but when the primary sensitiser was PADPA, cross-reactions were seen to PPD tested at a higher concentration than PADPA (156). It could be that metabolic activation plays an important role for the sensitisation capacity, and differences in skin metabolism between animals and humans should be taken into account.

Figure 13. *Positive reactions in the guinea pig when testing with coloured substances. (A) –clearly positive patch test reactions; (B) – a coloured patch test area where erythema can be masked by the colour of the substance.*

A. *B.*



THE METABOLITE HYPOTHESIS

Studies II and V were, at least partly, designed to investigate the hypothesis that D Orange 1 and D Yellow 3 are split by azo reduction and that some of the reaction products or metabolites could be the main allergen that causes the allergic reactions observed to these azo dyes. If this was the case, we would expect positive test reactions to the metabolite associated to the specific dye, that the reactions are stronger and develop faster, and that the elicitation threshold of the metabolite is lower than for the corresponding dye. Regarding the animal studies in study V we would expect to observe cross-reactivity between the dye and the metabolite which possibly then could be an indication of the metabolite having caused the reactions.

D Orange 1 showed a strong association to one of its metabolites, PADPA, and a weaker to 4-nitroaniline in patch-tested patients. Test reactions from all substances tested in equimolar concentrations had similar strength and developed with similar speed. Elicitation thresholds for D Orange 1 were lower than for PADPA. In animals, both substances cross-reacted independent of which one the animals were induced with. 4-Nitroaniline did not cross- react with D Orange 1 in the GPMT. All these

observations are consistent with cross - reactivity between D Orange 1 and PADPA. None of the observations directly support the metabolite theory, and the results regarding elicitation thresholds speak against this theory. These observations indicate that the observed simultaneous reactions to D Orange 1 and PADPA are cross - reactions.

D Yellow 3 showed a strong association to one of its metabolites, 2-amino-*p*-cresol and a weaker to 4-aminoacetanilide in patch-tested patients. Test reactions from all substances tested in equimolar concentrations had similar strength and developed with similar speed. Elicitation thresholds for D Yellow 3 were lower than for 2-amino-*p*-cresol. No animal experiments have been done yet. None of the observations directly support the metabolite theory and the results regarding elicitation thresholds speak against this theory. These observations indicate that the observed simultaneous reactions to D Yellow 3 and 2-amino-*p*-cresol are cross - reactions and with a weaker cross - reactivity to 4-aminoacetanilide in D Yellow 3 - sensitised individuals.

6. SUMMARY AND CONCLUDING REMARKS

The main aim of the work presented in this thesis was to investigate clinical and chemical aspects of contact allergy to disperse dyes. The most important findings in the thesis are as follows:

- Impurities and/or intentionally added substances in the commercial DDs can also be sensitisers, as shown when testing with TLCs as well as with the water- soluble parts of commercial D Orange 1 and D Yellow 3
- The 8 DDs (D Red 1, D Red 17, D Blue 106, D Blue 35, D Blue 124, D Yellow 3, D Orange 1, and D Orange 3), which are most frequently incriminated to be the cause of textile dye dermatitis, are very rarely used in textiles nowadays, but it is still possible to find them, not only in garments made outside the EU but also made in the EU
- When identifying DDs in the extract of the textile by TLC, it is necessary to confirm results using another system or another method
- The metabolite theory has not been supported by our results, i.e. elicitation thresholds for D Orange 1 and D Yellow 3 were lower than for PADPA and 2-amino-*p*-cresol, respectively. Test reactions from all substances tested in equimolar concentrations had similar strength and developed with similar speed. These observations indicate that the observed simultaneous reactions to D Orange 1 and PADPA on one hand, and the observed simultaneous reactions to D Yellow 3 and 2-amino-*p*-cresol on the other, are cross-reactions and with a weaker cross- reactivity to 4-aminoacetanilide in D Yellow 3- sensitised individuals
- D Orange 1 and PADPA are strong sensitisers in the GPMT. It can be assumed that individuals primarily sensitised to D Orange 1 could react to PADPA, but not to another potential metabolite from azoreduction, i.e. 4-nitroaniline, or to D Yellow 3, PPD, 4- aminoacetanilide, or 2-amino-*p*-cresol. Therefore, PPD is not suitable as a marker for the detection of patients that have been primarily sensitised to D Orange 1. It could also be stated that 4-nitroaniline cannot be the primary sensitiser in case of

sensitisation to D Orange 1. Whether D Orange 1 and PADPA cross-react cannot be stated with certainty from the results of our animal studies

On the basis of these findings the following remarks can be made:

It could be recommended to determine which DDs are actually being currently used to dye textiles and that could be of clinical importance and consequently should be included in patch test preparations used for the detection of contact allergy to textile dyes. In addition, the DDs' purity that are already used for testing patients should be checked and relevant impurities should be identified and verified whenever it is possible to define their sensitising capacity.

Furthermore, one should verify the clinical relevance of positive reactions to these dyes by patch testing suspected textiles of the patient and, if positive, try to pin- point the culprit dye. Late readings of the patch test reactions should be carried out as they give important and clinically relevant information.

One way to find a new marker of contact allergy to textile dyes would be to perform an investigation on occupational dermatitis and work-related contact allergy in workers within the textile dyeing industry. Since this kind of manufacture has more or less left Europe (for e.g. Asia), investigations would have to be performed far away from Sweden and Lithuania and this implies some difficulty.

When investigating textiles randomly obtained from all over the world, we noticed similar patterns of dyes on the TLCs among different extracts, especially in the orange, blue, red, and yellow spectra. As we found that the DDs used for patch testing are not frequently used in the textiles, it would be useful to investigate which DDs are commonly used nowadays.

It would also be useful to find out which substance(s) was/were present in the spots on the TLCs that our patients reacted to in addition to the main spot.

In recent years it was demonstrated that human skin bacteria are able to split azo dyes into the corresponding aromatic amines, some of which were sensitisers. Indeed we have shown that presumed metabolites from azo reduction of D Orange 1 and D Yellow 3 elicit positive patch test reactions in dermatitis patients positive to these dyes. As it is also known that azoreduction takes place in the skin, although it is not said to be the major metabolic pathway for azo dyes, patch testing patients with dilution series both on normal skin and disinfected skin would be helpful in order to elucidate the role of the skin bacteria.

Whether the reactions to D Orange 1 are allergic reactions to this substance per se, or due to its metabolites being the primary sensitisers could probably be elucidated testing these substances in equimolar concentrations and in serial dilutions both at induction and challenge in a GPMT.

Further insight into percutaneous absorption and metabolism in the skin of the disperse azo dyes would add more valuable knowledge with respect to the pathogenesis of textile-dye-related dermatitis.

SUMMARY IN SWEDISH

Kliniska och experimentella studier av kontaktallergi mot dispersionsfärgämnen

I Georgien har man funnit 30 000 år gamla fibrer som människor använt till att klä sig med, och det är de äldsta kända exemplen på tyg. Att fibrerna dessutom var färgade talar för att människorna redan då inte bara ville ha kläderna som skydd utan att de även var intresserade av exklusivitet! Nuförtiden tillhör dispersionsfärgämnen den klass som utgör över 20% av all färg som produceras i världen. Dispersionsfärgämnen är också de vanligaste allergiframkallande textilfärgämnena. De används för att färga syntetfibrer som polyester, acetat och polyamid. Dispersionsfärgämnen av azo-typen karakteriseras av en eller flera azogrupper (-N=N-) i den kemiska strukturen. På den yrkes- och miljödermatologiska avdelningen, Skånes Universitetssjukhus, Malmö har vi lapptestat våra hudsjuka patienter med en blandning av 8 dispersionsfärgämnen sedan 1999, och då har de flesta patienter som testats med dessa 8 färgämnen reagerat på två substanser, nämligen Disperse (D) Orange 1 och D Yellow 3, där båda två innehåller en azogrupp. Från tidigare studier visste vi också att kommersiell D Orange 1 och D Yellow 3 innehöll fler ämnen än själva färgämnet. Om det sker en reduktiv klyvning av azobindningen i dispersionsfärgämnesmolekylerna på ytan av huden eller i huden kan det leda till att aromatiska aminer bildas som kan tas upp via huden. Dessa metaboliter skulle då kunna inducera kontaktallergi. Innan detta avhandlingsarbete startade var det inte känt om de upprenade färgämnena, föroreningar i de kommersiella färgämnena eller metaboliterna är de faktiska, allergiframkallande ämnena hos en patient som reagerar positivt vid lapptestning med D Orange 1 eller D Yellow 3. Dessutom var det inte känt om de dispersionsfärgämnen som nu finns i kommersiella testserier faktiskt används i textilfärger idag.

Ett av huvudsyftena med denna avhandling var att utvärdera betydelsen av de föroreningar som finns i de kommersiella färgämnena D Orange 1 och D Yellow 3 och deras potentiella metaboliter, som kan bildas genom azoreduktion, med avseende på kontaktallergi. Ett ytterligare syfte har varit att undersöka den sensibiliserande kapaciteten hos D Orange 1 och dess metaboliter samt undersöka deras korsreaktivitet mot D Yellow 3, dess metaboliter och *p*-fenylendiamin (PPD). Ett tredje syfte var att undersöka om 8 dispersionsfärgämnen, hittills mest citerade att vara allergiframkallande, fortfarande används för att färga syntettextilier som säljs i

olika länder i världen. Dessutom gjordes en utvärdering av de många publicerade studier som handlar om kontaktallergi mot dispersionsfärgämnen som används vid färgning av textilier. Avhandlingen baserar sig på 4 vetenskapliga artiklar samt en översiktsartikel.

Det är känt sedan tidigare att vissa lapptestpreparationer av dispersionsfärgämnen innehåller föroreningar men betydelsen när det gäller kontaktallergi har inte utretts för de flesta färgämnen. I delarbete I undersöktes betydelsen av dessa föroreningar i de kommersiella färgämnena D Orange 1 och D Yellow 3. Tio patienter med känd kontaktallergi för D Orange 1 och/eller D Yellow 3 lapptestades med en spädningsserie av dessa färger, både det kommersiella färgämnet och det upprenade. Nio individer testades även med kommersiellt färgämne som hade separerats med hjälp av tunnskiktskromatografi (TLC) på en speciell plastfilm belagd med kiselgel. De testades även med papperskromatogram gjorda av de vattenlösliga delarna av respektive kommersiell färg. Remsorna sattes mot huden som en lapptest. Genom att använda TLC kunde vi visa att de kommersiella färgämnena D Orange 1 och D Yellow 3 båda innehöll minst 6 föroreningar vardera och att dessa två färger innehöll minst en förorening vardera som var sensibiliserande. Dessa föroreningar har ännu inte identifierats.

Från *in vitro*-försök är det känt att mänskliga hudbakterier kan splittra ett dispersionsfärgämne så att de korresponderande aromatiska aminerna bildas, där somliga visats vara allergiframkallande när man utfört djurstudier (local lymph node assay). Om färgmolekyler lossnar från klädesplagg och fastnar på huden skulle dessa kunna brytas ner av hudbakterier och därefter penetrera huden och inducera kontaktallergi. Vi prövade denna hypotes i delarbete II genom att lapptesta 10 patienter med känd allergi mot D Orange 1 och/eller D Yellow 3 med spädningsserier av dessa 2 upprenade färger, 4-nitroanilin och *p*- aminodifenylamin i koncentrationer ekvimolara till upprenat D Orange 1 samt även 4-aminoacetanilid och 2-amino-*p*-kresol i koncentrationer ekvimolara till upprenat D Yellow 3. Den observerade överrepresentationen av samtidiga positiva reaktioner mellan D Orange 1 och *p*-aminodifenylamin liksom mellan D Yellow 3 och 2-amino-*p*-kresol kan tyda på att dessa ämnen efter att ha bildats på eller i huden har orsakat sensibilisering. Vi kan också misstänka korsreaktivitet mellan dispersionsfärgämnena och dessa metaboliter, eftersom de utgör exakta kopior av ändarna av modersubstanserna. Testreaktionerna för D Orange 1 respektive D Yellow 3 hade en tendens till lägre tröskelvärde än vi såg för *p*-aminodifenylamin respektive 2-amino-*p*-kresol. Våra observationer stöder inte direkt metabolitteorin och resultaten avseende tröskelvärdena talar emot denna teori. Observationerna indikerar att de observerade, samtidiga reaktionerna mellan D Orange 1 och *p*-aminodifenylamin samt mellan D Yellow 3 och 2-amino-*p*-kresol är korsreaktioner med en svagare korsreaktivitet mot 4-aminoacetanilid hos D Yellow 3-sensibiliserade individer.

Vi har inte haft kunskap huruvida de dispersionsfärgämnen som används i kommersiella lapptestserier verkligen används idag för att färga syntettextiler. Därför undersöktes förekomsten av de 8 dispersionsfärgämnena, som mest beskrivits som allergena i den medicinska litteraturen, i syntettextilier inköpta på flera platser i världen (delarbete III). Textilier från 13 länder i Europa, Asien och USA analyserades. Metoden som användes var TLC. När det fanns matchande fläckar vid jämförelse mellan textilextrakten och referensfärgämnet utfördes högtrycksvätskekromatografi. Bland 121 analyserade plagg fann vi 4 av färgämnena i 3 olika plagg. Det var ett par ljusbruna tights tillverkade och sålda i Italien och som innehöll Disperse Yellow 3, Disperse Blue 124 och Disperse Blue 106, samt ett BH och trosa-set från Indien där vi fann Disperse Orange 1. Slutsatsen man kan dra är att de dispersionsfärgämnen vi idag lapptestar med inte är särskilt mycket använda i klädesplagg längre men att man fortfarande kan hitta dem i vissa plagg, till och med i sådana som är tillverkade i Europa.

I delarbete IV gjordes en utvärdering av de många publicerade studier som handlar om kontaktallergi mot dispersionsfärgämnen använda för färgning av textilier. Materialet kom från publicerade artiklar på PubMed från tidsperioden 1990-2012. Prevalensdata visas för varje studie samt för varje färgämne. Bland 54 studier beskrevs 26 dispersionsfärgämnen. Prevalenssiffror på över 1% fanns för flera färgämnen när man hade lapptestat eksempatienter. Detta gällde D Blue 106, D Blue 124 och D Orange 3. Vi fann inga data för D Blue 26 och 102, D Orange 37 och D Yellow 49, som alla listas som allergen av EU kommissionen.

I delarbete V undersöktes den allergiframkallande kapaciteten hos D Orange 1 och dess 2 metaboliter *p*-aminodifenylamin och 4-nitroanilin och korsallergin mellan dessa 3 samt även korsallergin mot D Yellow 3, dess 2 metaboliter 4-aminoacetanilid och 2-amino-*p*-kresol och en potentiellt korsreagerande substans, PPD, genom att utföra marsvinstestet guinea pig maximization test. Vi fann att D Orange 1 och *p*-aminodifenylamin var starkt allergiframkallande och att de korsreagerade med varandra. PPD, 4-nitroanilin, 4-aminoacetanilid, 2-amino-*p*-kresol eller D Yellow 3 uppvisade inte någon korsreaktivitet mot D Orange 1 eller *p*-aminodifenylamin. Den potentiella metaboliten till D Orange 1, 4-nitroanilin, var inte ett allergen i detta marsvinstest.

Med de resultat som presenterats i avhandlingen kan vi konstatera att:

- Det vore av värde att undersöka vilka dispersionsfärgämnen som används idag för att färga textilier och vilka som har klinisk relevans för att veta vilka som borde finnas i lapptestserier. När vi undersökte textilier från olika delar av världen såg vi liknande färgmönster bland många extrakt, framförallt inom det orangea, blåa, röda och gula spektrumet

- De färgämnen som används idag, deras renhet borde undersökas och relevanta föroreningar identifieras för att man ska kunna definiera den sensibiliserande kapaciteten
- Sena avläsningar av lapptestreaktioner av dessa ämnen bör utföras eftersom en del allergiska reaktioner kommer efter dag 3 eller 4
- Ett sätt att finna nya markörer för textilfärgämneskontaktallergi vore att undersöka arbetare i textilfärgsindustrin med klinisk undersökning och lapptestning av arbetsmaterial.

Det vore även av värde om man kunde identifiera de substanser som finns i de föroreningar som våra patienter reagerade allergiskt för.

Eftersom azoreduktion möjligen kan ske på eller i huden skulle man kunna lapptesta patienter med känd allergi mot D Orange 1 och/eller D Yellow 3 med dessa substanser i spädningsserie på både normal hud och desinficerad hud för att närmare kunna undersöka hudbakteriernas roll i allergiutvecklingen

För att fastställa om det är D Orange 1 i sig eller dess metaboliter som är de primära allergenen skulle man kunna testa dessa substanser ekvimolart och i spädningsserier både vid induktionen och vid eliciteringen när marsvinstest utförs.

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Paper I

STUDY

Textile Dyes Disperse Orange 1 and Yellow 3 Contain More Than One Allergen As Shown by Patch Testing with Thin-Layer Chromatograms

Laura Malinauskiene, Erik Zimerson, Magnus Bruze, Kristina Ryberg, and Marlene Isaksson

Background: **It is known that some patch-test preparations containing disperse dyes contain impurities with unknown relevance for the development or elicitation of contact allergy.**

Objective: **To evaluate the significance of the impurities found in the commercial dyes Disperse Orange 1 (DO1) and Disperse Yellow 3 (DY3) regarding contact allergy in patients with known sensitivity to them.**

Methods: **Ten patients allergic to DY3 and/or DO1 were tested with a dilution series of commercial and purified DY3 and DO1 (with water-soluble parts prepared from the commercial dyes) and with naphthalene sulfonate. Nine patients were additionally tested with thin-layer chromatograms (TLCs) made from the commercial DO1 and DY3 and with paper chromatograms made from the water-soluble part of these dyes.**

Results: **Eight of nine and three of six patients tested positively to the TLCs of DO1 and DY3, respectively. Among them, 4 of 8 and 2 of 3 patients, respectively, were positive also to another spot on the TLCs. One patient was positive to the paper chromatogram from the water-soluble part of DO1. None of the tested patients reacted to naphthalene sulfonate.**

Conclusion: **The results of our study suggest that there are more relevant allergens in the fat-soluble and water-soluble fractions of the commercial disperse dyes.**

ALLERGIC REACTIONS to textile dyes have been documented, especially in prevalence studies from southern Europe and studies from southern Sweden.[1,2] The most common dye sensitizers are grouped with the disperse dyes (DDs), which are used for coloring synthetic textile materials (polyester, polyamide, etc).

Many authors recommend Disperse Blue 106 (DB 106) and Disperse Blue 124 (DB 124) as screening allergens for textile dermatitis because they were the most prevalent dye allergens causing positive allergic patch-test reactions in most studies,[3–5] even if a Swedish study found Disperse Orange 1 (DO1) to be the most common dye allergen (with a contact allergy rate of 0.5%), followed by Disperse Yellow 3 (DY3).[2] However, DB 106 and DB 124 were patch-tested at a lower concentration in the Swedish study than in studies from southern Europe.

Even if textile colors such as DDs are chemically defined substances, it has been demonstrated that they are not pure.[6] A study from our department showed that the materials used for producing DY3 and DO1 patch-test preparations were impure.[6] For example, DO1 contains approximately 40% pure dye by weight; one of the components of the remaining mass is the water-soluble dispersing agent naphthalene sulfonate.[6] Furthermore, it was demonstrated in recent years that human skin bacteria are able to split azo dyes into the corresponding aromatic amines,[7] some of which were sensitizers in the local lymph node assay.[8,9] All these additional substances in the DDs are of unknown relevance for the induction of contact allergy or the elicitation of a contact allergic reaction. (The chemical structures of DO1 and DY3 are shown in Fig 1.)

The aims of this study were (1) to evaluate the degree of allergic patch-test reactivity to both commercial and purified DO1 and DY3 and the significance of the impurities found in these commercial DDs to contact

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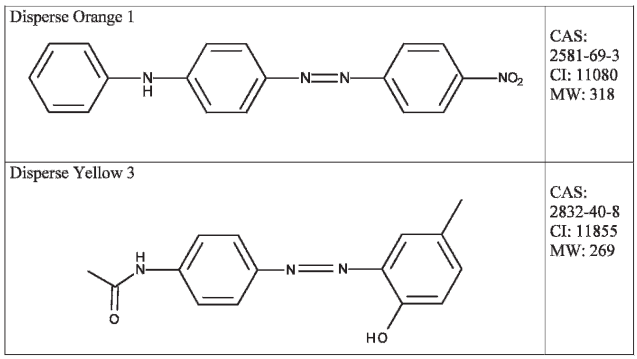

Figure 1. Chemical structure, Chemical Abstracts Service (CAS) number, color index (CI) number, and molecular weight (MW) of Disperse Orange 1 and Disperse Yellow 3.

allergy in patients with known sensitivity to them, and (2) to investigate a possible association between patch-test reactivity to commercial DO1 and DY3 and reactivity to purified DO1 and DY3 in order to find the major sensitizer.

Materials and Methods

Study Population

The study population consisted of 10 patients—3 women and 7 men (mean age, 46.1 years; range, 19–70 years)—previously tested at the Department of Occupational and Environmental Dermatology in Malmö and found to be allergic to DO1 or DY3 or both. All 10 patients had had dermatitis; three had a relevant contact allergy to DDs, two had been sensitized to p-phenylenediamine through henna tattoos, and one had been sensitized to p-phenylenediamine by dark hair dyes. One patient was primarily sensitized to black rubber, whereas in three cases of hand eczema, there was no clear association with the patient's contact allergens.

Chemicals

Chloroform, acetone, and dichloromethane of analytic grade were obtained from Scharlau Chemie S.A. (La Jota, Barcelona, Spain). Acetonitrile for the thin-layer chromatography system was obtained from Lab-Scan (Dublin, Ireland). The commercial DO1 and DY3 materials for preparing the patch-test preparations and the thin-layer chromatograms (TLCs) were bought from Chemotechnique Diagnostics (Vellinge, Sweden). Distilled water was purchased from Milipore SA (Malsheim, France), and naphthalene sulfonate was obtained from Sigma Aldrich (Steinhem, Germany). Anhydrous sodium sulfate 99% was obtained from Acrōs Organics (Geel, Belgium).

Patch-Test Preparations

The purification and identification of DO1 and DY3 were carried out in our department. The purity of the substances was greater than 99%.[6] All patch-test solutions, TLCs, and paper chromatograms for patch testing were prepared from the same batches at our department. About 20 mg of each DD, commercial and purified, were accurately weighed and dissolved in acetone, yielding a 1.0% weight per volume (w/v) preparation. From this stock solution, further dilutions from 10^{-1}% to 10^{-6}% w/v were prepared. All 10 patients were patch-tested with one or more dilution steps of commercial and purified DY3 and DO1, depending on their previous reactivity. Those who previously reacted with a + or ++ reaction to the 1.0% preparation were tested with dilution series starting at 1.0%; those who previously reacted with a +++ reaction to 1.0% were patch-tested with a 0.01% concentration to start, and those who previously did not react were patch-tested with only the highest concentration (1.0%). If patients had a positive reaction on day 4 (D4), they were additionally tested with dilution series (Fig 2).

The commercial solutions of DO1 and DY3 were separated into water-soluble and fat-soluble fractions by extraction of each dye in a solvent system consisting of water and dichloromethane. The extraction was repeated until no visible residue of the dye color could be seen in the lipid phase (Fig 3). Each test tube (1 cm in diameter), containing 3 cm of the lipid phase obtained after each washing, was

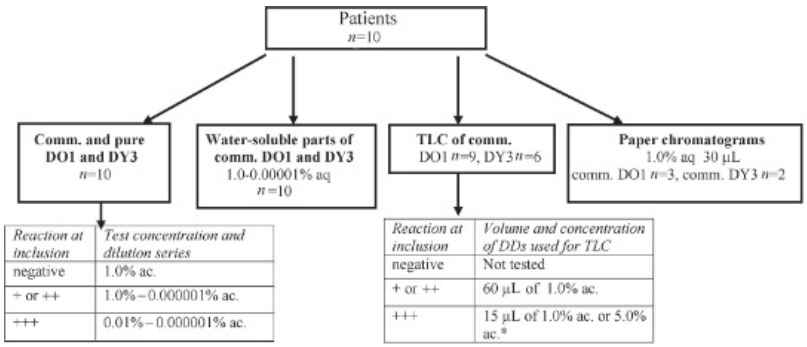

Figure 2. Protocol for testing with Disperse Orange 1 and Disperse Yellow 3. (ac. = acetone; aq = aqueous; comm. = commercial; DD = disperse dye; DO1 = Disperse Orange 1; DY3 = Disperse Yellow 3; TLC = thin-layer chromatogram.) (*Concentration used if patient had negative reaction to TLC made from 1.0% ac. 60 µL solution on first reading.)

carefully inspected for color residues across the tube length against a white background. The colored dichloromethane phases were washed with water, dehydrated by the addition of anhydrous sodium sulfate, and vacuum-evaporated with a rotary evaporator until dry (30°C). The obtained residues constituted the fat-soluble fractions of commercial DO1 and DY3. The water-soluble fractions of the dyes were evaporated in the same way, and the obtained residues constituted the water-soluble parts of the dyes. Patch-test preparations were made from these residues by diluting them in distilled water to a 1.0% w/v concentration. In addition, all patients were tested with naphthalene sulfonate 1.0% aqueous (aq) w/v. Patients who had positive reactions to any of these preparations were tested with a dilution series of the specific preparation.

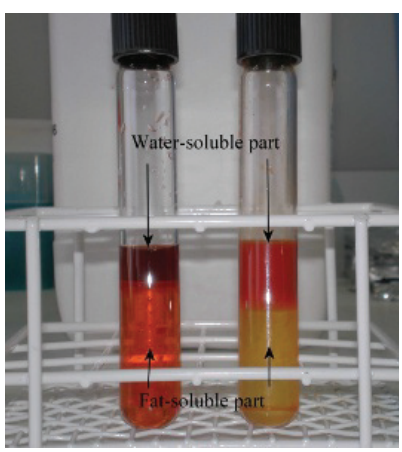

Figure 3. Water-soluble parts of commercial Disperse Orange 1 (*left*) and Disperse Yellow 3 (*right*) in process of preparation by the mixing of the commercial dyes with distilled water and dichloromethane.

Preparation of TLCs and Paper Chromatograms

The chromatograms were made according to a procedure earlier described by Bruze and colleagues.[10] TLCs From a TLC plastic roll (500 × 20 cm silica gel 60F254 [Merck KgaA, Darmstadt, Germany]), 18 × 18 cm strips were cut out. A micropipette was used to repeatedly apply 3 µL of the samples to be tested (ie, solutions of the commercial DO1 or DY3 in acetone) until 15 µL or 60 µL had been deposited on one spot each for every 2.5 cm along a line on the lower part of the silica gel plate. Double spots were applied for each sample, one to be used as a patch test and the other to be used as a reference when the test was read.

Chloroform 100% was used as the mobile phase for DO1, and a mixture of chloroform and acetonitrile (86:14 volume per volume [v/v]) was used for DY3. After separation, less substance remained on the application spot visually, and a band of well-defined and separated spots could be seen. The TLCs were all inspected under visible light and with ultraviolet (UV) radiation (254 nm and 366 nm); no additional spots were visualized in UV radiation. The plastic-roll pieces with the chromatograms were then cut into long strips to be used for patch testing (Fig 4).

Some patients were tested with TLCs made from the 1.0% solutions of commercial DY3 and DO1 but containing only 15 µL on each application spot, owing to these

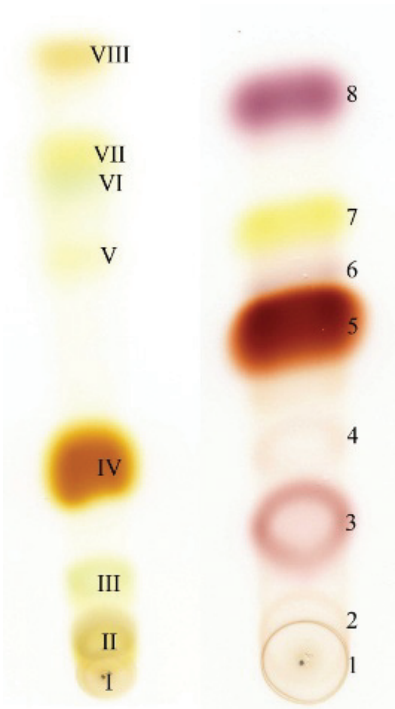

Figure 4. Thin-layer chromatograms of commercial Disperse Yellow 3 (*left*) (86:14 mixture of chloroform and acetonitrile used as eluent) and Disperse Orange 1 (*right*) (100% chloroform used as eluent) patch-test preparations.

patients' having had strong allergic reactions when previously patch-tested with these dyes; the other patients were tested with TLCs containing 60 μL of the 1.0% solution on each spot. If patients had negative results at the first reading, they were patch-tested with a new TLC containing 15 μL of the 5.0% solution on each spot made from commercial DY3 or DO1.

Paper Chromatograms We applied 30 μL of the water-soluble part of DY3 and DO1 at 1.0% aq as separate spots along a line on the lower part of the 18 × 18 cm strips of the filter paper (Munktell Filter AB, Grycksbo, Sweden).

Distilled water was used as a mobile phase. After separation, the strips were inspected in visible light and with UV radiation (254 nm and 366 nm), and detected spots were marked. The filter paper strips with the chromatograms were then cut into long strips to be used for patch testing. (In this article, these chromatograms are called "paper chromatograms.")

Patch Testing Technique

Patch testing was performed with 8 mm Finn Chambers (Epitest Ltd, Tuusula, Finland) on Scanpor tape (Norgesplaster A/S, Vennesla, Norway); 15 μL of the test solution was applied with a micropipette to the filter paper disc in each test chamber. The TLCs and paper chromatograms with the separated spots were cut out in pieces of about 2.5 × 16 cm and were then applied on the upper back of each tested patient, on either side of the spine. Scanpor tape was used to cover the chromatograms and secure them to the skin. The chambers, the TLCs, and the paper chromatograms were left on the back for 48 hours, and the readings were performed on D4 and on day 7 (D7). The reactions were scored according to the guidelines of the International Contact Dermatitis Research Group.[11]

Ethics

The study was approved by the regional ethics review board in Lund, Sweden, and was conducted in accordance with ethical standards specified in the Declaration of Helsinki. All patients gave informed written consent to participate in the study.

Results

Testing with Dilution Series of Commercial and Purified DO1 and DY3

The dilution series of pure DO1 yielded positive results in 8 of 10 patients. One patient had a positive reaction to only commercial DO1, not to the pure DO1. The dilution series of pure DY3 yielded positive results in 6 of 10 patients; 1 of 6 did not react to commercial DY3. One patient reacted to commercial and purified DY3 but did not react to the water-soluble part of commercial DY3; this patient reacted to two TLC spots. Another patient reacted in the same way to commercial DO1. One patient had a positive reaction to the paper chromatogram from the water-soluble part of commercial DO1. One patient, who previously reacted positively to DY3, did not react to DO1 or DY3. None of the 10 patients reacted to naphthalene sulfonate.

All of the above results are presented in Table 1.

Table 1. Reactions to Patch Tests with Dilution Series of Commercial and Purified Disperse Orange 1 and Disperse Yellow 3

Tested	*P1*		*P2*		*P3*		*P4*		*P5*		*P6*		*P7*		*P8*		*P9*		*P10*	
Substance	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*
Commercial DO1																				
1.0%	+++	NR	NT	NT	*	*	+	+	+++	NR	NT	NT	+++	NR	−	−	+++	NR	(+)	++
0.1%	+++	NR	NT	NT	−	−	(+)	+	+++	NR	NT	NT	++	NR	NT	NT	+++	NR	(+)	+
0.01%	−	−	++	+++	−	−	−	−	+++	NR	++	+	−	+	NT	NT	+	NR	−	−
0.001%	−	−	++	++	−	−	−	−	++	NR	(+)	(+)	−	−	NT	NT	−	NR	−	−
0.0001%	−	−	−	−	−	−	−	−	(+)	NR	−	−	−	−	NT	NT	−	NR	−	−
0.00001%	−	−	−	−	−	−	−	−	++	NR	−	−	−	−	NT	NT	−	NR	−	−
0.000001%	−	−	−	−	−	−	−	−	++	NR	−	−	−	−	NT	NT	−	NR	−	−
Pure DO1																				
1.0%	NT	NT	NT	NT	−	−	(+)	+	+++	NR	NT	NT	NT	NT	−	−	+++	NR	(+)	++
0.1%	+++	tr	NT	NT	−	−	(+)	+	+++	NR	NT	NT	NT	NT	NT	NT	+++	NR	(+)	++
0.01%	(+)	(+)	++	+++	−	−	−	−	+++	NR	+++	+++	+	+++	NT	NT	(+)	++	(+)	NR
0.001%	−	−	++	+++	−	−	−	−	+++	NR	+	+++	(+)	++	NT	NT	(+)	+	−	NR
0.0001%	−	−	(+)	(+)	−	−	−	−	++	NR	(+)	(+)	−	−	NT	NT	−	−	−	NR
0.00001%	−	−	−	−	−	−	−	−	+	NR	−	−	−	−	NT	NT	−	−	−	NR
0.000001%	−	−	−	−	−	−	−	−	+	NR	−	−	−	−	NT	NT	−	−	−	NR
DO1, water-soluble part																				
1.0%	++	+	+++	++	−	−	(+)	(+)	+++	NR	+++	tr	++	+++	−	−	++	+++	−	(+)
0.1%	−	−	+	+++	NT	NT	−	−	NT	NT	++	NR	++	NR	NT	NT	+	NR	NT	NT
0.01%	−	−	++	NR	NT	NT	−	−	NT	NT	(+)	NR	(+)	NR	NT	NT	−	NR	NT	NT
0.001%	−	−	+	NR	NT	NT	−	−	NT	NT	−	NR	−	NR	NT	NT	−	NR	NT	NT
0.0001%	−	−	−	NR	NT	NT	−	−	NT	NT	−	NR	−	NR	NT	NT	−	NR	NT	NT
Commercial DY3																				
1.0%	+++	tr	(+)	(+)	−	−	−	−	++	NR	+++	NR	−	−	−	−	+++	+++	−	(+)
0.1%	+++	tr	(+)	NR	NT	NT	NT	NT	NT	NT	+++	NR	NT	NT	−	−	++	+++	(+)	++
0.01%	++	tr	−	NR	NT	NT	NT	NT	NT	NT	+	+	NT	NT	−	−	−	−	−	−
0.001%	−	−	−	NR	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	−	−	−
Pure DY3																				
1.0%	+++	tr	(+)	−	−	−	−	−	++	NR	+++	NR	−	−	−	−	+++	+++	(+)	+
0.1%	+++	tr	(+)	(+)	NT	NT	NT	NT	NT	NT	+	NR	NT	NT	−	−	+++	+++	−	+
0.01%	++	+	++	+	NT	NT	NT	NT	NT	NT	(+)	+++	NT	NT	−	−	++	++	−	−
0.001%	−	NR	−	−	NT	NT	NT	NT	NT	NT	−	(+)	NT	NT	−	−	−	−	−	−
0.0001%	−	−	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	−	−	−

Table 1. Continued

Tested	P1		P2		P3		P4		P5		P6		P7		P8		P9		P10	
Substance	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*	*D4*	*D7*
DY3, water-soluble part																				
1.0%	++	+	(+)	–	–	–	–	–	(+)	NR	–	–	–	(+)	–	–	–	–	–	+++
0.1%	+	NR	–	–	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
0.01%	–	NR	–	–	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Naphthalene sulfonate 1% aq	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(+) = doubtful reaction; aq = aqueous; D4 = day-4 reading; D7 = day-7 reading; DO1 = Disperse Orange 1; DY3 = Disperse Yellow 3; NR = not read; NT = not tested; P = patient; tr = reaction treated with potent topical corticosteroid.

*Case report published in Malinauskiene L, Bruze M, Ryberg K, et al. Late patch test reaction to Disperse Orange 1 not related to active sensitization. Contact Dermatitis 2010;63:298–9.

TLC Testing of Commercial DO1

Of nine patients tested with commercial DO1 TLCs, positive reactions were noted in eight patients. All reacted to the main spot (see Fig 4, *spot 5*), and four reacted additionally to another common spot (see Fig 4, *spot 7*). Of those four, one patient's tests were not read on D7, but the remaining three patients had an allergic reaction to the additional one spot also on D7 (Table 2).

TLC Testing of Commercial DY3

Of the six patients tested with commercial DY3 TLCs, three showed positive results. Those three reacted to the main spot (see Fig 4, *spot IV*), and two reacted to one and the same additional spot (see Fig 4, *spot V*). All three patients also reacted to the main spot on the DO1 TLC and also showed strong positive reactions on tests with pure and commercial DY3 (see Table 1).

Paper Chromatogram Testing of the Water-Soluble Part of Commercial DO1 and DY3

Three patients, two of whom showed a positive reaction to the water-soluble part of commercial DO1, were tested with paper chromatograms. One patient reacted to the spot on the application point; this patient reacted to the main spot on the TLC made from commercial DO1.

Two patients were tested with the paper chromatograms made from the water-soluble part of commercial DY3; neither reacted positively.

Discussion

A previous study had shown that DDs used for textile dying or patch testing contain impurities.[12] Some patients were allergic to impurities and not to the actual pure dye. It is thus important to know the actual sensitizer for prevention and correct diagnosis because impurities may be present in other consumer products not connected with DDs.

In our study, we wanted to compare the elicitation potential of patch-test preparations containing commercial and purified DO1 and DY3. If only the actual purified dye was the sensitizer, a higher reactivity to this purified dye would be expected; however, this could not be shown (see Table 2). The results are in line with the findings obtained from testing with DB 106 and DB 124 in serial dilutions.[13] A study from Malmö showed that the concentration of purified DO1 and DY3 is less than 50% in the materials used to make the commercial patch-test preparations, similar to the data concerning the purity of DB 106 and

Table 2. Reactions to Patch Tests with Commercial Disperse Orange 1 and Disperse Yellow 3 Thin-Layer Chromatograms

Spot	P1		P2		P3		P4		P5		P6		P7		P8		P9		P10	
No.*	D4	D7	D4	D7	D4	D7	D4	D7	D4	D7	D4	D7	D4	D7	D4	D7	D4	D7	D4	D7
Disperse Orange 1																				
8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	−	−	−	−
7	(+)	−	−	−	−	−	−	+	+	NR	(+)	+	−	+	NT	NT	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	−	−	−	−
5†	+++	++	++	+++	−	−	−	+	+++	NR	++	++	+++	+	NT	NT	+++	+++	−	+
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	−	−	−	−
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	−	−	−	−
Disperse Yellow 3																				
VIII	−	−	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	−	−	−
VII	−	−	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	−	−	−
VI	−	−	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	−	−	−
V	+	(+)	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	++	+	−	−
IV†	++	++	−	−	NT	NT	NT	NT	NT	NT	−	+	NT	NT	−	−	(+)	+++	−	−
III	−	−	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	(+)	−	−
II	−	−	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	−	−	−
I	−	−	−	−	NT	NT	NT	NT	NT	NT	−	−	NT	NT	−	−	−	−	−	−

(+) = doubtful reaction; D4 = day-4 reading; D7 = day-7 reading; NR = not read; NT = not tested; P = patient.
*Spots on each chromatogram were numbered in accordance with the registration shown in Figure 4, with spot 1 and spot I as the application spots.
†Main spot on chromatogram.

DB 124.[6,13] Impurities in commercial DO1 and DY3 could facilitate the penetration of the actual dyes through the epidermis or act synergistically with them in eliciting an immune response.

One patient reacted to the lowest tested concentration of DO1 (0.000001%, or 0.01 μg/mL), which is comparable to the lowest doses reported to yield positive reactions to, for example, DB 106, DB 124, and some phenol formaldehyde resins.[14,15] Unfortunately, she developed systemic contact dermatitis with symmetrical dermatitis of the lower leg during patch testing, and it was not possible to define her lowest elicitation threshold.

Patch-testing with paper chromatograms was described by Mijnssen in 1969,[16] but the technique was further developed and refined at the Malmö department.[10] It has been shown to be a valuable tool for detecting individual sensitizers in compound materials.[17] The results of our study verified that patch-testing with TLCs of commercial DO1 and DY3 could yield positive reactions at the main spot as well as at other spots (impurities or intentionally added substances).

According to studies by Foussereau and Dallara in 1986, commercial textile dyes containing DY3 and DO1 seemed pure (one spot on the TLC) when analyzed with thin-layer chromatography using ethyl acetate and chloroform (3:2) as eluent.[18,19] Ryberg and colleagues recently showed that there were fewer impurities in the commercial DY3 and DO1 test preparations when compared to other DDs: TLCs made from DY3 showed two spots, and TLCs from DO1 showed three spots in their systems using glass plates.[6] They had used a mixture of chloroform and acetonitrile (86:14 v/v) as eluent. In our study, we modified the method for preparing the TLCs, looking for the best eluent system for each dye. We also managed to produce (1) seven spots from commercial DY3 by using chloroform and acetonitrile (86:14 v/v) and a plastic thin-layer chromatography plate instead of a glass plate and (2) seven spots from commercial DO1 on a plastic thin-layer chromatography plate by using chloroform 100% v/v. One main spot appeared in a strong color on both TLCs, which corresponded to the main spot on the TLC made with purified dye. Hence, other spots could be considered impurities.

Eight of 9 patients who had positive reactions to DO1, and 3 of 5 patients who reacted positively either to DY3 alone or to both DY3 and DO1, reacted to the main spot on the respective chromatogram. Besides, 4 of the 8 patients

reacting to the main spot on the DO1 TLC reacted to another but mutual spot, and 2 of 3 patients reacting positively to the main spot on the DY3 TLC also reacted to an additional but mutual spot. Reactions to the additional spots were weaker than reactions to the main spot. Cross-sensitization is one possibility, but the pattern of reactivity does not support this, since patients who have strong reactions to the main spot would be expected to also react to other spots that induced allergic reactions. However, this was not the case in 3 of 6 patients who were allergic to DO1 (see Table 2). The results thus indicate that the main sensitizing substance is the dye itself but that some impurities or intentionally added substances are allergens and may have an influence on the strength of the reaction to the commercial dye tested.

Additionally, two patients (patient 2 and patient 10) with known contact allergy to DY3 tested positively to the dilution series of the corresponding DDs but not to the TLCs. One explanation of this could be that patch-testing with the preparation on a limited area could give additive or synergistic effects between the allergens, as opposed to patch-testing with a TLC, on which the allergens are separated into different spots. Another explanation could be that the individual dose of the allergen per unit area on the TLC was too low when spread on a larger area, or that the availability of the allergen was decreased owing to adhesion to the silica on the chromatograms. This could yield false-negative reactions and should be compensated for by a larger volume or higher concentration of allergen applied to the TLC.

Testing with the water-soluble part of the commercial DDs had interesting results. It was presumed that there would be no DDs or only a minimal concentration of the corresponding DD in the water-soluble extract as the dyes are insoluble in water and the extract for testing was virtually colorless. Of the 10 patients tested, six reacted to the water-soluble part of commercial DO1 and the dilution series, but only one reacted to the water-soluble part of commercial DY3. None of the 10 patients reacted to naphthalene sulfonate, which is the water-soluble dispersing agent in commercial DO1 and DY3. Reactions to these water-soluble parts were as strong as those to the corresponding dye; one patient reacted to the water-soluble part of commercial DO1 down to a concentration of 0.0001%. If these reactions can be attributed to the residuals of the pure dye, they should not be so strong because the possible concentration of dye would be much lower than in the 1.0% patch-test solution of the commercial or purified dye.

Also, it is possible that there are other allergens besides the actual dye in the fat-soluble fraction of the commercial dyes. Patient 4 reacted to the commercial and pure DO1 and to two spots on the TLC but did not react to the water-soluble part of commercial DO1. The same applies for patient 9, who had the same pattern of reaction to commercial DY3. Hence, it is obvious that other ingredients that may be allergens and may have different physicochemical properties from those of DDs are present in both the fat- and water-soluble parts of commercial DO1 and DY3.

Negative reactions to the patch-test preparations and to the TLCs were found in one patient with earlier-diagnosed contact allergy to DY3. On the other hand, one patient who had had negative reactions to DY3 patch-test preparations reacted to the DY3 this time. This could be attributed to the qualitative differences in the patch-test preparations used for the previous and the present patch tests (as demonstrated in previous studies) or to the individual variation of reactivity to the allergens.[1] Patch-test sensitization is unlikely to be the cause on this occasion because the patient had reacted already on D4.

Conclusions

It appears that impurities in commercial DDs can also be sensitizers, as shown when testing is done with TLCs as well as with the water- and fat-soluble parts of commercial DO1 and DY3. The study also shows that patch-testing with substances in serial dilutions and patch-testing with TLCs give interesting additional information on the degree of reactivity in the individual patient and may reveal additional sensitizers in patch-test preparations, which can be chemically analyzed.[17]

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Paper II

Patch testing with the textile dyes Disperse Orange 1 and Disperse Yellow 3 and some of their potential metabolites, and simultaneous reactions to para-amino compounds

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Summary

Background. It is known that, *in vitro*, human skin bacteria are able to split disperse azo dyes into the corresponding aromatic amines, some of which are sensitizers in the local lymph node assay. We hypothesize that the molecules of disperse dyes migrate onto the skin while garments are worn, and are metabolized and degraded by commensal skin bacteria. These molecules penetrate the skin and induce sensitization.

Objectives. To evaluate the elicitation capacities of the possible azo-degradation products of the selected azo disperse dyes in patients allergic to them and to compare it with the elicitation capacities of other para-compunds.

Methods. Ten patients allergic to Disperse Yellow 3 (DY3) and/or Disperse Orange 1 (DO1) were patch tested with a dilution series of the purified dyes 4-nitroaniline and *p*-aminodiphenylamine in concentrations equimolar to those of purified DO1 in the dilution series, as well as 4-aminoacetanilide and 2-amino-*p*-cresol in concentrations equimolar to those of purified DY3 in the dilution series.

Results. Three patterns of patch test reactions could be seen. The 6 patients who were positive to DO1 and DY3 also reacted to *p*-aminodiphenylamine and 2-amino-*p*-cresol. Two patients were positive to DO1 only, and both reacted to *p*-aminodiphenylamine, but to neither 4-aminoacetanilide or 2-amino-*p*-cresol. Two patients did not react to DO1 or DY3 on this occasion.

Conclusion. We show that it is possible that the major sensitizers in contact allergy to DO1 and DY3 are their metabolites, *p*-aminodiphenylamine and 2-amino-*p*-cresol, respectively, which might be formed by the azoreductase pathway of skin bacteria.

Key words: 2-amino-*p*-cresol; 4-aminoacetanilide; 4-nitroaniline; allergic contact dermatitis; disperse dyes; *p*-aminodiphenylamine.

Disperse dyes (DDs) constitute a large dyestuff class, accounting for 22% of all dyes produced in the world (1). They are used to colour textiles, plastics, cosmetics, and food. The chemical structure of disperse azo dyes is characterized by the presence of one or more azo groups (−N = N−).

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One potential hazard to human health with azo dyes is allergic contact dermatitis, which appears in the sites where textile coloured with azo dyes comes into close contact with the skin (2).

Azo dyes can be split into aromatic amines by the liver microsome system and the intestinal microflora in mammals (3–5).

Commensal microflora are present on all body surfaces that are covered by epithelial cells and exposed to the external environment (gastrointestinal and respiratory tract, skin, etc.). The number of bacteria colonizing mucosal and skin surfaces exceeds the number of cells forming the human body. The number of skin bacteria approaches 10^{12}, and this population includes mainly gram-positive bacteria, obligate aerobes (*Micrococcus*), and facultative anaerobes (e.g. *Staphylococcus* and *Corynebacterium*) (6, 7). In recent years, it has been demonstrated *in vitro* that human skin bacteria are able to split azo dyes into the corresponding aromatic amines, some of which are sensitizers in the local lymph node assay (8). The gene encoding azoreductase from *Staphylococcus aureus* was identified and cloned, and it was shown that this azoreductase was able to cleave azo dyes to the corresponding amines (5, 9). It was also shown experimentally that skin bacteria from the genera *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Dermacoccus* and *Kocuria* were able to efficiently reduce and cleave the azo dyes Methyl Red and Orange II *in vitro* (by 74–100% in 24 hr) (10).

In vitro percutaneous absorption studies comparing human, mouse and guinea pig skin showed that human skin was the least permeable to selected azo dyes: ~30% of the applied dose was absorbed in 24 hr, and ~30% of the absorbed dose underwent azoreduction in the skin (11).

Coloured textile clothing is assumed to be the most widespread source of skin exposure to DDs. The external exposure to DDs by this route is between 1 ng/cm^2/event and 1 μg/cm^2/event. However, in the case of garments that have not been dyed according to the state-of-the-art technology, one has to assume higher release rates and exposures (12).

Thus, we can hypothesize that the DDs migrate onto the skin garments while garments are being worn, and that they are metabolized by commensal skin bacteria. The metabolites then penetrate the skin, and may induce sensitization and/or elicitation.

In a recent study, we found Disperse Orange 1 (DO1) to be the most common DD allergen among the eight DDs investigated in the mix, with a contact allergy rate of 0.5% among consecutively tested dermatitis patients, followed by Disperse Yellow 3 (DY3) (13). Combined sensitization to disperse azo dyes and other para-amino compounds [e.g. black rubber mix and *p*-phenylenediamine (PPD)], probably based on true cross-sensitization or on simultaneous positive reactions, has frequently been described (14–16). Para-amino compounds are a group of substances in which hydrogen in a benzene molecule is substituted with amino and other groups in opposite positions (17). There are experimental studies showing that a group in the para position induces a stronger immune response than the same group in other positions (e.g. ortho or meta) (18). Common or similar metabolites may also contribute to the frequently reported simultaneous reactions.

The chemical structures of DO1 and DY3, as well as their possible azo-degradation products, are shown in Fig. 1.

The aim of the present study was to evaluate the elicitation capacities of the possible azo-degradation products of DO1 and DY3, and of black rubber mix ingredients and PPD (Fig. 2), and to compare these with the elicitation capacities of purified DO1 and DY3 in patients allergic to DO1 and/or DY3.

Materials and Methods

Study population

The study population consisted of 10 patients allergic to DO1 and/or DY3. There were 3 women and 7 men (mean age 46.1 years, range 19–70 years). All 10 patients had had dermatitis. Their previous patch test reactivities to DO1, DY3, PPD and black rubber mix, and the clinically relevant contact allergies, are shown in Table 1.

Chemicals

Acetone of analytical grade was obtained from Scharlau Chemie S.A. (La Jota, Barcelona, Spain). The chemicals used for DO1 and DY3 solutions had been previously purified and identified at the Malmö department from commercial DO1 and DY3, purchased from Chemotechnique Diagnostics (Vellinge, Sweden) (19). DO1 and DY3 were purified, and their purity was >99%. These dyes were used to prepare the solutions for patch testing.

PPD and black rubber mix ingredients [*N*-cyclohexyl-N′-phenyl-4-phenylenediamine (CPPD), *N*, *N*′-diphenyl-4-phenylenediamine (DPPD), and *N*-isopropyl-*N*′-phenyl-4-phenylenediamine (IPPD)] were bought from Chemotechnique Diagnostics. 4-Nitroaniline, *p*-aminodiphenylamine, 4-aminoacetanilide and 2-amino-*p*-cresol were bought from Sigma Aldrich (Steinhem, Germany). The general purity of 4-aminoacetanilide

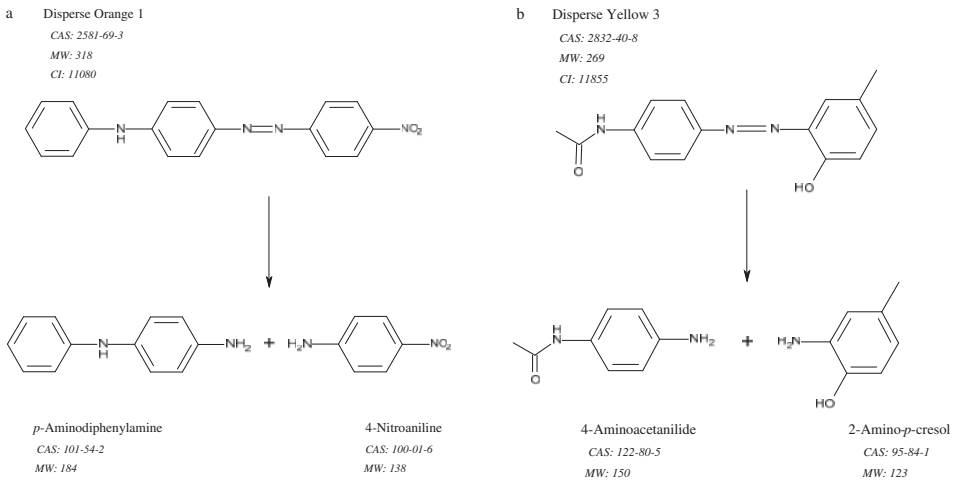

Fig. 1. Possible azo degradation pathways of Disperse Orange 1 (a) and Disperse Yellow 3 (b) and chemical structures. CAS numbers, Colour Index (CI) numbers and molecular weight (MW) of the investigated dyes and their metabolites are also shown.

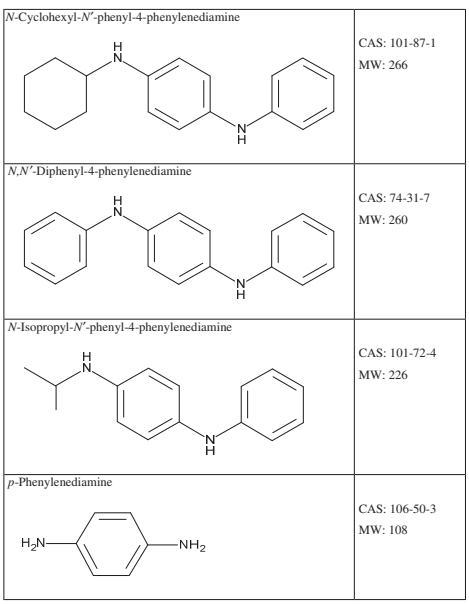

Fig. 2. Chemical structure, CAS numbers and molecular weight (MW) for the investigated black rubber mix ingredients and *p*-phenylenediamine.

was 99%, that of 2-amino-*p*-cresol was 97%, that of *p*-aminodiphenylamine was 98%, and that of 4-nitroaniline was >99%. Purified DO1 and DY3 were analysed with high-performance liquid chromatography for the presence of 4-aminoacetanilide, 2-amino-*p*-cresol, *p*-aminodiphenylamine, and 4-nitroaniline. These substances were not detected.

Patch test preparations

All patch test solutions were prepared in our department from the same batches. Approximately 20 mg each of purified DO1, purified DY3, PPD, CPPD, DPPD and IPPD was accurately weighed and dissolved in acetone, yielding a 1.0% wt/vol preparation. From this stock solution, further dilutions, from 1.0×10^{-1} to 1.0×10^{-6} % wt/vol, were prepared. All 10 patients were patch tested with one or more dilutions of the purified DY3 and DO1, depending on their previous patch test reactivity; those who previously had a +++ reaction to 1.0% were patch tested starting with a 100-fold lower concentration (0.01%), and those who previously did not react were patch-tested with the highest concentration (1.0%) only. If patients had a positive reaction on D3 or D4, they were also tested with the corresponding dilution series.

Dilution series of 4-nitroaniline and *p*-aminodiphenylamine were prepared in concentrations equimolar to the dilution series of DO1, starting at 1.0% wt/vol

Table 1. Patch test reactivity to Disperse Orange 1, Disperse Yellow 3, *p*-phenylenediamine and black rubber mix, when patch testing was performed earlier, in conjunction with the patients' clinical work-up

		Patient number, year									
Substance		1, 1999	2, 1999	3, 1999	4, 2005	5, 2004	6, 2007	7, 1999	8, 2005	9, 2004	10, 1999
DO1	Tested concentration	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.
	Reaction*	+++	+++	+	+++	++	++	+++	−	++	++
DY3	Tested concentration	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.
	Reaction*	+	++	+	−	−	+	−	+	++	++
PPD	Tested concentration	1.0% pet.	0.94% pet.	0.94% pet.	0.94% pet.	0.94% pet.	0.94% pet.	1.0% pet.	0.94% pet.	0.94% pet.	1.0% pet.
	Reaction*	+++	+++	+	−	−	−	−	++	+++	++
BRM†		−	++	−	+	−	++	+++	−	−	−
Clinically relevant contact allergy		Disperse dyes	PPD	Not found	Disperse dyes	Disperse dyes	Not found	BRM	PPD	PPD	Not found

BRM, black rubber mix; DO1, Disperse Orange 1; DY3, Disperse Yellow 3; PPD, *p*-phenylenediamine.
*The results of patch testing are based on the strongest reaction either on D3/4 or D7.
†Black rubber mix was tested at 0.6% pet. concentration in all patients.

(31 mM). 4-Aminoacetanilide and 2-amino-*p*-cresol dilution series were prepared in concentrations equimolar to the dilution series of DY3, starting at 1.0% wt/vol (37 mM). All dilution series were prepared in acetone.

Patch test technique

Finn Chambers® (Ø 8 mm; Epitest Ltd, Tuusula, Finland) on Scanpor® tape (Norgesplaster A/S, Vennesla, Norway) were used for patch testing with the dilution series of the dyes. Fifteen microlitres of the test solution was applied with a micropipette to the filter paper disc in each test chamber. The chambers were left on the back for 48 hr, and readings were performed on D3/D4 and D7 by a dermatologist. The reactions were scored according to the guidelines of the International Contact Dermatitis Research Group (20).

Patch testing

The patients were all patch tested with the same series, which included purified DO1 and DY3 (19), PPD, and the black rubber mix ingredients DPPD, CPPD, and IPPD, at 1.0% wt/vol (all in serial dilutions in acetone, starting at 1.0%). Patients were also tested with the presumed DO1 and DY3 metabolites in serial dilutions in concentrations equimolar to the parental compound (Fig. 3). If the patients were positive on the first reading to the lowest concentration of the respective substance tested, they were additionally tested with the lower concentrations.

Controls

Consecutively patch tested dermatitis patients served as controls: 16 for 4-nitroaniline, 5 for *p*-aminodiphenylamine, 118 for 4-aminoacetanilide, and 27 for 2-amino-*p*-cresol.

In order to show a relationship of the metabolites to the parental dyes, we also selected dermatitis patients positive to a textile dye mix – a mixture of eight DDs, consisting of Disperse Blue 35, DY3, DO1, Disperse Orange 3, Disperse Red 1, and Disperse Red 17 (at 0.5% wt/wt pet.), and Disperse Blue 106 and Disperse Blue 124 (at 0.1% wt/wt pet.), giving a total concentration of 3.2% – but negative to DO1 and/or DY3. We tested 4 control patients positive to textile dye mix but negative to DO1 with *p*-aminodiphenylamine 0.58%, and 7 with 4-nitroaniline 0.43%.

We tested 7 patients positive to textile dye mix but negative to DY3 with 4-aminoacetanilide 0.56% and 4 with 2-amino-*p*-cresol 0.46%. These control patients were tested with higher concentrations than the 118 controls chosen from consecutive dermatitis patients, in order to allow comparison of the results with the positive reactions to equimolar concentrations of DY3 and DO1.

Statistical analysis

Fisher's two-sided exact test was used, and we regarded $p < 0.05$ as being statistically significant when patch tests results of control patients were compared with the

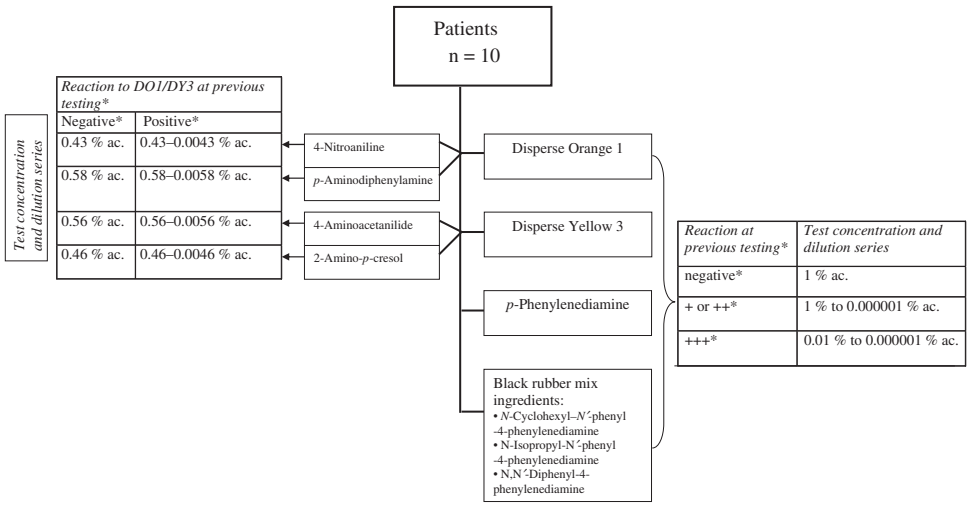

Fig. 3. The protocol for testing with Disperse Orange 1, Disperse Yellow 3, their metabolites, black rubber mixture ingredients, and *p*-phenylenediamine. + or ++ or + + +, previous positive patch test reactions. *The results of patch testing (negative, positive) are based on earlier patch test results prior to this study (see Table 1). Ac; Acetone.

results of the investigated patients. We used the test to calculate the number of controls needed to show that the concentration used was not irritant for each individual allergen ($p < 0.05$).

Ethics

The study was approved by the Regional Ethics Review Board in Lund, Sweden, and conducted in accordance with ethical standards specified in the Declaration of Helsinki. All patients gave informed written consent to participate in the study.

Results

All results are presented in Table 2.

The results indicated three different reaction patterns among patients:

(1) Six positive to DO1 and DY3– all reacted to *p*-aminodiphenylamine, PPD, and 2-amino-*p*-cresol, and 5 reacted to IPPD (Table 2). In the latter group, 1 patient had previously reacted only to DO1, but on this occasion also reacted to DY3.

(2) Two positive to DO1 and not to DY3– both reacted to *p*-aminodiphenylamine, and none reacted to CPPD, PPD, 4-aminoacetanilide, or 2-amino-*p*-cresol.

(3) Two did not react to DO1 or DY3– 1 of them had reacted previously to DY3, but on this occasion he was positive to PPD only. The other patient, previously found to be allergic to DO1, now showed a late (D14) positive reaction to *p*-aminodiphenylamine (21).

Ten patients were tested with the dilution series of DO1, and 8 of them were positive: the lowest concentrations giving a positive reaction were 0.1% in 3 patients, 0.001% in 4 patients, and 0.000001% in 1 patient.

Six of 10 patients tested with a dilution series of DY3 were positive. Four patients reacted down to 0.01%, 1–0.1%, and 1–1.0%.

The pattern of concomitant reactivity to DO1, DY3, their potential metabolites, PPD and black rubber mix ingredients is shown in Table 3.

Patch testing with the potential metabolites of DO1: *p*-aminodiphenylamine and 4-nitroaniline

Of the 8 patients positive to DO1, all reacted to *p*-aminodiphenylamine. One of these patients, previously shown to be allergic to DO1, reacted only to *p*-aminodiphenylamine. All reactions to *p*-aminodiphenylamine were already strong (++/+++) on the D3 reading.

Table 2. Results of patch testing with the dilution series of Disperse Orange 1 (DO1), Disperse Yellow 3 (DY3), their potential metabolites, black rubber mix ingredients, and *p*-phenylenediamine

	Patient number Days of testing									
	1	2	3	4	5	6	7	8	9	10
	D4	D4	D4	D4	D4	D4	D4	D4	D4	D4
Tested substance*	D7	D7	D7	D7	D7	D7	D7	D7	D7	D7
DO1										
1.0%	NT	NT	−	(+)	+++	NT	NT	−	+++	(+)
			−	+	NR			−	NR	++
0.1%	+++	NT	−	(+)	+++	NT	NT	NT	+++	(+)
	tr		−	+	NR				NR	++
0.01%	(+)	++	−	−	+++	+++	+	NT	(+)	−
	(+)	+++	−	−	NR	+++	+++		++	(+)
0.001%	−	++	−	−	+++	+	(+)	NT	(+)	−
	−	+++	−	−	NR	+++	++		+	−
0.0001%	−	(+)	−	−	++	(+)	−	NT	−	−
	−	(+)	−	−	NR	(+)	−		−	−
0.00001%	−	−	−	−	+	−	−	NT	−	−
	−	−	−	−	NR	−	−		−	−
0.000001%	−	−	−	−	+	−	−	NT	−	−
	−	−	−	−	NR	−	−		−	−
4-Nitroaniline										
0.43%	−	(+)	−	−	++	−	−	−	++	+
	−	+	−	++	NR	−	−	−	+++	++
0.043%	−	−	−	−	(+)	−	−	NT	(+)	−
	−	−	−	(+)	NR	−	−		+	−
0.0043%	−	−	−	−	−	−	−	NT	−	−
	−	−	−	−	NR	−	−		−	−
p-Aminodiphenylamine										
0.58%	+++	+++	†	++	+++	+++	+++	−	+++	++
	tr	+++		+	NR	tr	+++	−	+++	+++
0.058%	+++	+++	−	(+)	+++	+++	++	NT	+++	−
	tr	+++	−	(+)	NR	tr	+++		+++	++
0.0058%	++	+++	−	−	+++	+++	+	NT	+++	−
	+	+++	−	−	NR	tr	++		+++	−
0.00058%	−	−	NT	NT	NT	−	−	NT	−	−
	−	−				−	−		−	−
DY3										
1.0%	+++	(+)	−	−	++	+++	−	−	+++	(+)
	tr		−	−	NR	NR	−	−	+++	+
0.1%	+++	(+)	NT	NT	NT	+	NT	−	+++	−
	tr	(+)				NR		−	+++	+
0.01%	++	++	NT	NT	NT	(+)	NT	−	++	−
	+	+				+++		−	++	−
0.001%	(+)	−	NT	NT	NT	−	NT	−	−	−
	−	−				(+)		−	−	−
0.0001%	−	−	NT	NT	NT	−	NT	−	−	−
	−	−				−		−	−	−
4-Aminoacetanilide										
0.56%	++	−	−	−	−	−	−	−	++	(+)
	+	−	−	−	NR	−	−	−	+++	++
0.056%	−	−	NT	NT	NT	−	NT	−	+	−
	−	−				−		−	(+)	−
0.0056%	−	−	NT	NT	NT	−	NT	−	−	−
	−	−				−		−	−	−
2-Amino-*p*-cresol										
0.46%	+++	+++	−	−	++	+	−	−	+++	+
	tr	+++	−	−	NR	+++	−	−	+++	++
0.046%	−	−	NT	NT	NT	+	NT	−	(+)	−
	−	−				++		−	(+)	−
0.0046%	−	−	NT	NT	NT	−	NT	−	−	−
	−	−				−		−	−	−
CPPD										
1.0%	−	+++	−	−	++	NT	−	−	−	−

Table 2. Continued

	Patient number Days of testing									
Tested substance*	1 D4 D7	2 D4 D7	3 D4 D7	4 D4 D7	5 D4 D7	6 D4 D7	7 D4 D7	8 D4 D7	9 D4 D7	10 D4 D7
	−	+++	−	−	NR		(+)	−	−	−
0.1%	NT	+++	NT	−	NT	+++	−	NT	NT	NT
		+++		−		tr	−			
0.01%	NT	++	NT	−	NT	+++	−	NT	NT	NT
		++		−		tr	−			
0.001%	NT	−	NT	NT	NT	++	NT	NT	NT	NT
		−								
0.0001–0.0000001%	NT	−	NT	NT	NT	−	NT	NT	NT	NT
		−				−				
DPPD										
1.0%	−	++	−	−	+	NT	−	−	−	−
	−	+++	−	−	NR		+	−	−	−
0.1%	NT	++	NT	−	NT	+	−	NT	NT	NT
		++		−		++	−			
0.01%	NT	+++	NT	−	NT	+	−	NT	NT	NT
		(+)		−		+	−			
0.001%	NT	−	NT	NT	NT	−	NT	NT	NT	NT
		−				−				
IPPD										
1.0%	+	+++	−	(+)	+++	NT	++	−	+++	−
	(+)	+++	−	−	NR		+++	−	+++	−
0.1%	NT	+++	NT	−	NT	+++	+	NT	++	NT
		++		−		tr	+++		NR	
0.01%	NT	+	NT	−	NT	+++	(+)	NT	−	NT
		(+)		−		tr	++		NR	
0.001%	NT	++	NT	NT	NT	NT	−	NT	−	NT
		NR					−		NR	
0.0001%	NT	−	NT	NT	NT	NT	−	NT	−	NT
		NR					−		NR	
PPD										
1.0%	NT	NT	−	−	+++	+++	−	++	NT	NT
			−	−	NR	tr	−	NR		
0.1%	NT	NT	−	NT	NT	NT	NT	+	NT	NT
			−					NR		
0.01%	+++	(+)	NT	NT	NT	NT	NT	−	+++	+++
	tr	++						−	+++	+++
0.001–0.000001%	−	−	NT	NT	NT	NT	NT	−	−	−
	−	−						−	−	−

CPPD, *N*-cyclohexyl-*N*′-phenyl-4-phenylenediamine; DPPD, *N*,*N*′-diphenyl-4-phenylenediamine; IPPD, *N*-isopropyl-*N*′-phenyl-4-phenylenediamine; NR, not read; NT, not tested; PPD, *p*-phenylenediamine; tr, reaction treated with potent topical corticosteroid.
*Concentration (wt/vol) in acetone.
†Case report published elsewhere (21).

Regarding the dilution series of *p*-aminodiphenylamine, there were 8 positive patients among 9 tested (Table 2). The majority of the reactions were strong, and disappeared abruptly from one tested concentration to the nearest lower concentration.

Five of the 8 patients positive to DO1 reacted to 4-nitroaniline. The patch test reactions to 4-nitroaniline were weaker than those to *p*-aminodiphenylamine.

Patch testing with the potential metabolites of DY3: 4-aminoacetanilide and 2-amino-*p*-cresol

Of the 10 patients patch-tested with 4-aminoacetanilide, 3 were positive; all reacted to DY3 and 2-amino-*p*-cresol.

Of the 10 patients tested with 2-amino-*p*-cresol, 6 were positive, with strong reactions on D3. All of these patients were also allergic to DY3.

Table 3. Pattern of concomitant reactivity to Disperse Orange 1 (DO1), Disperse Yellow 3 (DY3), their potential metabolites, *p*-phenylenediamine (PPD) and black rubber mix (BRM) ingredients in 10 patients allergic to DO1 and/or DY3

	DO1	NA	PADPA	DY3	AAA	AC	CPPD	DPPD	IPPD	PPD
DO1	8	5	8	6	3	6	3	4	6	6
NA	5	5	5	4	2	4	2	2	3	4
PADPA	8	5	8	6	3	6	3	3	5	6
DY3	6	4	6	6	3	6	3	3	5	6
AAA	3	2	3	3	3	3	0	0	2	3
AC	6	4	6	6	3	6	3	3	5	6
CPPD	3	2	3	3	0	3	3	3	3	3
DPPD	4	2	3	3	0	3	3	4	4	3
IPPD	6	3	5	5	2	5	3	4	6	5
PPD	6	4	6	6	3	6	3	3	5	7

AAA, aminoacetanilide; AC, 2-amino-*p*-cresol; CPPD, *N*-cyclohexyl-*N*′-phenyl-4-phenylenediamine; DPPD, *N*,*N*′-diphenyl-4-phenyl enediamine; IPPD, *N*-isopropyl-*N*′-phenyl-4-phenylenediamine; NA, 4-nitroaniline; PADPA, *p*-aminodiphenylamine.

Of the 6 patients tested with the <0.56% concentration of 4-aminoacetanilide, only 1 was positive, and reacted to 0.056%.

Of the 6 patients tested with the <0.46% concentration of 2-amino-*p*-cresol, 1 was positive, and reacted to a 0.046% (diluted 10-fold).

Test reactivity

In the 8 patients who reacted positively to DO1, the majority of positive D7 reactions were stronger than D3 reactions (Table 2). In contrast, the majority of strong positive reactions to *p*-aminodiphenylamine appeared on the first reading, that is, D3/D4.

Regarding patterns of reactions to DY3, this tendency was not so strong. The majority of reactions to DY3 and to 2-amino-*p*-cresol were seen on D3/D4.

For both DO1 and DY3, the elicitation thresholds for the proposed metabolites were, in most cases, higher than those for the parent compound. In 3 of 8 patients, the thresholds for DO1 and *p*-aminodiphenylamine were similar.

Patch testing of the control patients

None of 118 consecutively patch tested dermatitis patients reacted to the test solution with 4-nitroaniline 0.043%, *p*-aminodiphenylamine 0.0058%, 4-aminoacetanilide 0.056% or 2-amino-*p*-cresol 0.046% in acetone. The concentration chosen for control testing of each substance was the lowest concentration giving a positive patch test reaction in a reasonable proportion of the patch-tested patients with respect to controls needed for statistical significance. Positive reactions to *p*-aminodiphenylamine were linked to positive reactions to DO1, but not to positive reactions to other ingredients of the textile dye mix ($p < 0.05$).

Of 5 control patients positive to textile dye mix but negative to DO1 who were tested with *p*-aminodiphenylamine 0.58%, 1 was positive ($p = 0.018$). Of 7 patients positive to textile dye mix but negative to DO1 who were tested with 4-nitroaniline 0.43%, 2 were positive ($p > 0.05$). All 7 patients positive to textile dye mix but negative to DY3 who were tested with 4-aminoacetanilide 0.56% were negative ($p < 0.05$), and all 4 who were tested with 2-amino-*p*-cresol 0.46% were negative ($p = 0.0079$).

Patch testing with the ingredients of black rubber mix

Of the 4 patients previously shown to be allergic to black rubber mix, two reacted to all ingredients (CPPD, DPPD, and IPPD), 1 to DPPD and IPPD, and 1 to black rubber mix only and not to the separate ingredients. Three patients previously not shown to be positive to black rubber mix, and now not tested with black rubber mix, but with ingredients, were positive: 3 to IPPD, 1 to DPPD, and 1 to CPPD. Of 8 patients positive to DO1 and *p*-aminodiphenylamine, 6 reacted to IPPD.

Five patients were tested with the dilution series of black rubber mix ingredients, and 4 were positive (Table 2). Two patients were positive to the dilution series of all three ingredients, and 2 were positive only to the dilution series of IPPD.

Of 8 patients positive to DO1, 6 (75%) reacted to at least one black rubber mix ingredient, and of 6 positive to DY3, 5 (83%) reacted to at least one black rubber mix ingredient.

Patch testing with PPD

Seven patients of 10 tested were positive to PPD. One patient previously shown to be positive to PPD did not react in the present testing. Of the 7 PPD-positive patients, 5 also reacted to IPPD.

All 6 patients who were allergic to both DO1 and DY3 were positive to PPD, but 2 patients who reacted only to DO1 did not react to PPD. One patient who was previously shown to be allergic to DY3 now reacted only to PPD. Also, all 6 patients who reacted positively to DY3 and PPD reacted to 2-amino-*p*-cresol, one of the metabolites of DY3. Reactions to PPD were strongly related to positive reactions to DY3 and to one of its metabolites, 2-amino-*p*-cresol ($p < 0.05$).

Five patients were tested with the dilution series of PPD; 4 of them reacted to PPD at the lowest concentration of 0.01%, and one to 0.1%.

Discussion

Reductive cleavage of the azo bond in DDs on the surface of the skin or in the skin could potentially lead to the formation of aromatic amines, which are absorbed by the skin (22).

In the present study, we wanted to compare the elicitation capacities of patch test preparations containing purified DO1 and DY3 and their potential metabolites. If the primary sensitizer is a metabolite of the DD, the strength of the reaction to these metabolites will probably be stronger, and imply a lower elicitation threshold.

There was a good agreement in patch test results between DO1 and *p*-aminodiphenylamine, as all patients positive to DO1 reacted to *p*-aminodiphenylamine, a potential metabolite of the former. A difference was observed between D4 reactions to *p*-aminodiphenylamine and those of DO1. Positive reactions to *p*-aminodiphenylamine were stronger than those to DO1 at D4 in 6 of 9 patients positive to DO1 and/or *p*-aminodiphenylamine. Reactions to the other metabolite of DO1, 4-nitroaniline, were weaker and less prevalent than the reactions to *p*-aminodiphenylamine. However, testing with the equimolar dilution series of DO1 showed a lower elicitation threshold than that for *p*-aminodiphenylamine, except in 3 of 8 patients, for whom the thresholds were similar to those of the substances. Testing of control patients positive to textile dye mix but negative to DO1 showed that positive reactions to *p*-aminodiphenylamine are linked to positive reactions to DO1 but not to positive reactions to other ingredients of the textile dye mix, suggesting that the concentration of *p*-aminodiphenylamine chosen, which was equimolar to that of the maternal compound, was not toxic or irritative.

This raises the question of whether *p*-aminodiphenylamine could be the main sensitizer in DO1 contact allergy. Actually, *p*-aminodiphenylamine has already been identified as a strong sensitizer in the local lymph node assay and the guinea pig maximization test (8, 23). Also, *p*-aminodiphenylamine is a known contact allergen for hairdressers and consumers using hair dyes (24).

One possible explanation based on experimental studies of some DDs and 4-nitroaniline could be the following. *In vitro* experiments have shown that, after 24 hr, 70% of DDs remain on human skin, whereas almost 50% of 4-nitroaniline is absorbed through human skin during this time period (11, 25). This means that the kinetics of DDs and those of at least one of the possible metabolites, 4-nitroaniline, are different. DD molecules stay on the skin for long enough to interact with azoreductases from skin bacteria. The metabolites formed penetrate the skin and initiate the immune response. When DDs are applied on the skin, they induce an immune reaction slowly, because the molecules penetrate slowly, and metabolism by skin bacteria to form immunoreactive substances also takes time. The influx of the molecules into the skin takes longer to achieve the concentration needed to induce an immune response, and the influx is constant, so positive reactions can be seen with lower concentration of the DDs. On the other hand, when already formed metabolites are applied on the skin, as in our study, they penetrate and induce an immune response more quickly, because many of the same molecules can penetrate the skin at the same time, inducing an immune response and positive patch test reactions. Because of the instant penetration of the molecules, degradation also starts instantly, and the concentration of the molecules in the skin is quickly reduced, so a lower concentration of the applied substance (fewer molecules) does not induce an immune response.

Perhaps this may also apply to *p*-aminodiphenylamine, which is a stronger sensitizer than 4-nitroaniline: testing with *p*-aminodiphenylamine provokes strong and early reactions (on D3), whereas the appearance of positive patch test reactions to DO1 requires more time.

Another explanation is that application of DO1 creates two possible allergens – *p*-aminodiphenylamine and 4-nitroaniline – that might act synergistically, inducing an allergic reaction.

In this study, we observed strong positive patch test reactions to DO1 and *p*-aminodiphenylamine that ended abruptly in the dilution series. Taking into account the similarity of the molecules of PPD and *p*-aminodiphenylamine, perhaps their metabolism or degradation in the skin is similar. It has been shown that various aromatic amines undergo N-acetylation in keratinocytes, and that N-acetylated derivatives are not capable of inducing dendritic cell activation or a positive local lymph node assay response (23, 26). Thus, the main substances responsible for the sensitization to PPD are oxidation products of PPD (27). However, it has also been shown that the skin has a very high acetylation capacity (28). Aromatic amines may be acetylated, and when the amount of substrate molecules exceeds the acetylation capacity, then oxidative metabolism could become more important in the skin (27). Therefore, when *p*-aminodiphenylamine is applied at a lower concentration, N-acetylation may detoxify it to low or non-immunogenic compounds.

Testing of control patients positive to textile dye mix but negative to DO1 did not show a statistically significant link between positive reactions to DO1 and to 4-nitroaniline at equimolar concentrations. We speculate that this could

be caused by similar metabolite formation from Disperse Orange 3, as some of the control patients were positive to this DD. Also, there are data showing that, during hair dyeing when PPD is mixed with the oxidizing agent, 4-nitroaniline is formed (29). As most of our control patients were also positive to PPD, they might have been exposed and sensitized to 4-nitroaniline when dyeing their hair in the past.

Regarding DY3, 2-amino-*p*-cresol was positive in all 6 patients who were positive to DY3. As with *p*-aminodiphenylamine, positive reactions were strong and occurred early, but there was no tendency for them to appear earlier than reactions to DY3; when the dilution series was tested, the allergic reactions also ceased at a 10-fold or 100-fold higher concentration than that of DY3. As it is also an aromatic amine, perhaps the same hypothetical mechanisms as in the *p*-aminodiphenylamine case can be assumed. It was shown previously *in vitro* and in a local lymph node assay that 4-aminoacetanilide is a weaker sensitizer that 2-amino-*p*-cresol; however, they are said to cross-react (8, 30). When control patients positive to textile dye mix but negative to DY3 were tested, it appeared that positive reactions to 4-aminoacetanilide and 2-amino-*p*-cresol were statistically significantly linked to positive reactions to DY3.

The observed overrepresentation of the simultaneous positive reactions to DO1 and *p*-aminodiphenylamine as well as to DY3 and 2-amino-*p*-cresol may also have another cause. These substances might be closely related to the raw materials used for production of the dyes, and remain as impurities in the DDs used for dyeing textiles. There could also be cross-reactivity between the DDs and these substances, as they represent exact copies of the ends of the parental molecules of the dyes. Chemical investigation for purity is therefore necessary. In our study, high purity of DO1 and DY3 was established by chemical analysis. The possibility that *p*-aminodiphenylamine or 2-amino-*p*-cresol could be contaminated with DO1 or DY3, respectively, is therefore not supported by the patch test reaction pattern. It should be expected that reactions to these substances would be weaker than to the respective dyes, as the concentration of the DDs would be lower than in the parental dye, but this was not the case.

It is possible that the levels of metabolites required for the elicitation of the positive patch reactions in our study are formed during the wearing of garments dyed with the DDs. A standard textile garment contains approximately 100 μg/cm^2 of the dye, for example DO1 (12). In our study, the lowest concentration of *p*-aminodiphenylamine to which patients reacted was 0.0058%. This corresponds to 1.7 μg/cm^2. This amount of *p*-aminodiphenylamine could be formed from 3 μg/cm^2 DO1, which corresponds to a patch test with 0.01% DO1. In our study, some patients were positive to even lower concentrations of DO1. If just a low percentage of the dye in a fabric is broken down, this could produce a relevant amount of the metabolite. Several other circumstances can favour the development of an allergic reaction. New textiles can contain higher concentrations of dyes. The exposure to dyes when wearing a garment can mimic a repeated application test rather than a regular patch test. In certain skin areas, there can be close contact that, in combination with increased sweating and friction, leads to enhanced extraction from the fabric and better penetration of the allergens.

Reactions to PPD were strongly related to positive reactions to DY3 and to one of its metabolites, 2-amino-*p*-cresol. Patients who reacted only to DO1 did not react to PPD. This is in contrast to the results of other authors, who found that PPD sensitivity is not related or is only weakly related to DY3 sensitivity (16, 31). We were unable to find a study indicating cross-reactivity between PPD and 2-amino-*p*-cresol.

p-Aminodiphenylamine, the possible metabolite of DO1, is considered to be an allergen in hair dyes, with strong cross-reactivity to PPD (27). In our study, we found that, of 9 patients positive to *p*-aminodiphenylamine, 6 (66.7%) were positive to PPD, and an additional patient was positive to PPD but not to *p*-aminodiphenylamine.

All but 1 of the patients positive to PPD were positive to IPPD, a constituent of the black rubber mix. It has previously been shown that PPD cross-reacts with IPPD, probably because of the often seen cross-reactivity between PPD and para-amino compounds (15).

Recently, cross-reactivity between IPPD and *p*-aminodiphenylamine was reported (27). In our study, 66.7% (6/9) of patients positive to DO1 and *p*-aminodiphenylamine also reacted to IPPD. On the other hand, 1 patient who was shown previously to be positive to black rubber mix did not react to the individual ingredients, but only to the black rubber mix 0.6% pet. This might be caused by a compound effect.

In this study, we consecutively tested dermatitis patients with 4-nitroaniline, 4-aminoacetanilide, and 2-amino-*p*-cresol. Patch test reactions to dilutions of these substances together with negative reactions in control patients and the macroscopic appearance of the positive patch test reactions consistent with an allergic reaction strongly support the sensitizing capacity of the chemicals.

In conclusion, the simultaneous reactions observed to DO1 and *p*-aminodiphenylamine, as well as to DY3 and

2-amino-*p*-cresol, could be attributable to cross-reactivity or to these chemicals being potential metabolites, formed by the azoreductase pathway of skin bacteria. This means that *p*-aminodiphenylamine and 2-amino-*p*-cresol would be the primary sensitizers in cases of contact allergy to DO1 and DY3, respectively. To fully establish the nature of the positive reactions to these substances, sensitization studies in the guinea pig are required.

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Paper III

Are allergenic disperse dyes used for dyeing textiles?

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Summary

Background. There are no data showing that disperse dyes, used to patch test patients, are currently being used for dyeing synthetic garments. It is unknown whether disperse dyes, which are currently routinely patch tested, are in fact present in synthetic textiles on the market.

Objectives. To determine whether eight disperse dyes, hitherto most widely cited as allergenic, are still used in textiles that are sold in various countries.

Methods. Textiles from 13 countries in Europe, Asia and the United States were analysed. The procedure used for dye identification was thin-layer chromatography. When there were matching spots from the textile extract and reference dye, high-performance liquid chromatography was performed.

Results. Of 121 analysed items, three showed positive results for some of the investigated disperse dyes. Four dyes in these items could be detected and confirmed by the use of high-performance liquid chromatography. A pair of light brown ladies' tights manufactured and purchased in Italy contained Disperse Yellow 3, Disperse Blue 124, and Disperse Blue 106, and a set of black bra and panties purchased in India contained Disperse Orange 1.

Conclusions. The eight disperse dyes that are most frequently incriminated in textile dye dermatitis are very rarely used in textiles nowadays.

Key words: allergic contact dermatitis; disperse dyes; textiles.

Disperse dyes (DDs), especially those belonging to the azo class, are the most prevalent cause of textile-related allergic contact dermatitis (1). Synthetic fabrics from fibres made entirely of or of polyester blended with polyester, acetate and nylon are dyed with DDs (2). Within the EU, azo dyes that can be metabolized to carcinogenic aromatic amines are forbidden, but up to now there is no legal prohibition on the use of allergenic azo dyes in any country (3). Approximately 50 dyes have been identified as contact allergens, and two-thirds of these are DDs, although they represent a very small fraction of the total of approximately 8000 commercially used dyes (4). According to the EU regulations for eco-labelling of textile products, the manufacturer of the garments produced in or imported to the EU must either provide a statement of non-use of certain DDs, listed as carcinogenic or allergenic, or provide a test report proving their colour fastness (5). The Oeko-Tex Association, a group of 14 textile institutes in Europe and Japan, has a list of allergenic dyes that are forbidden in clothes certified with the ecological Oeko-Tex label (6) (Table 1). In the United States, textile products that are produced in accordance with the Global Organic Textile Standard (GOTS) may be sold as organic. The GOTS prohibits the use of azo dyes

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Table 1. Disperse dyes classified as allergens and listed by the EU Commission and by Oeko-Tex

C.I. generic name	C.I. structure name	CAS number
C.I. Disperse Blue 1	C.I. 64 500	2475-45-8
C.I. Disperse Blue 3*	C.I. 61 505	2475-46-9
C.I. Disperse Blue 7	C.I. 62 500	3179-90-6
C.I. Disperse Blue 26	C.I. 63 305	–
C.I. Disperse Blue 35*†	–	12222-75-2
C.I. Disperse Blue 102	–	12222-97-8
C.I. Disperse Blue 106*†	–	12223-01-7
C.I. Disperse Blue 124*†	–	61951-51-7
C.I. Disperse Brown 1*	–	23355-64-8
C.I. Disperse Orange 1†*	C.I. 11 080	2581-69-3
C.I. Disperse Orange 3*†	C.I. 11 005	730-40-5
C.I. Disperse Orange 37	C.I. 11 132	–
C.I. Disperse Orange 76	C.I. 11 132	–
C.I. Disperse Red 1*†	C.I. 11 110	2872-52-8
C.I. Disperse Red 11	C.I. 62 015	2872-48-2
C.I. Disperse Red 17*†	C.I. 11 210	3179-89-3
C.I. Disperse Yellow 1	C.I. 10 345	119-15-3
C.I. Disperse Yellow 3*†	C.I. 11 855	2832-40-8
C.I. Disperse Yellow 9*	C.I. 10 375	6373-73-5
C.I. Disperse Yellow 39	–	–
C.I. Disperse Yellow 49	–	–

CAS, Chemical Abstract Service number, C.I., Colour Index number; – , no C.I. or CAS number for that dye.
*Present in the Textile Colours & Finish (TF-1000) series of Chemotechnique Diagnostics, Sweden.
†Investigated in the study.

that release carcinogenic arylamine compounds and DDs classified as allergenic (7, 8). In the EU, an average of 41% of clothing is imported from other countries with lower labour costs (9). There is a discrepancy between the member states in clothing import: in Sweden and Denmark, 90% of clothing products come from outside the EU, but in Portugal, almost all clothing products purchased are made in the EU (10). For the textile to be labelled 'Made in . . .' according to the EU legislation, two significant processes of manufacturing (i.e. textile spinning, weaving, finishing, or sewing) should be performed in that particular country (9).'Made in' labels are currently voluntary in the EU, and their use depends on national laws. In comparison, country of origin labelling is strictly regulated in, for example, the United States, Canada, and Japan. Nevertheless, the products imported into the EU should comply with the EU legislation. The main supplier of clothing to the EU is China, followed by Bangladesh, Pakistan, and Turkey (9). EU regulations are only applied in the EU countries, and do not have to be observed in countries outside the EU, where travellers can be exposed to garments dyed with these DDs.

There are sparse data on the presence of the dyes to which individuals are patch test-positive in the garments suspected as being the cause of their dermatitis. Chemical investigation of the textiles from the textile dye patch test-positive patients showed that dyes to which patients were patch test-positive were infrequently identified in their clothes (11). In spite of speculations that the allergenic DDs are probably no longer used, there are many reports of positive reactions to these DDs in the literature, and clinical relevance in most of the studies is considered to be high (11–14).

The primary objective of this study was to determine whether the DDs most frequently cited as allergens are used for dyeing textiles, especially in low-price garments.

Materials and Methods

Textiles

Dermatologists in different countries were contacted and asked to send us cheap socks, T-shirts, underwear, scarves, tights, etc., made from 100% polyester or blend with other synthetic fibres (e.g. polyamide), preferably with yellow, orange, or dark colours.

A total of 121 garments, mainly scarves, caps, T-shirts, socks, tights, trousers, jackets, skirts, dresses, and panties, were obtained from 13 countries. Their main characteristics are described in Table 2.

Chemicals

Chloroform and acetone of analytical grade were obtained from Scharlau Chemie S.A. (La Jota, Barcelona, Spain). Acetonitrile of fluorescence high-performance liquid chromatography (HPLC) grade was obtained from Lab-Scan (Dublin, Ireland). Distilled water was obtained from Millipore SA (Malsheim, France). Eight commercial DDs (Disperse Red 1, Disperse Red 17, Disperse Blue 106, Disperse Blue 35, Disperse Blue 124, Disperse Yellow 3, Disperse Orange 1, and Disperse Orange 3) were bought from Chemotechnique Diagnostics (Vellinge, Sweden). The reference substances for each of the DDs were isolated and identified at the Department of Occupational and Environmental Dermatology, Lund University, Skåne University Hospital, Malmö (15). The purity of the reference substances was >99%, except for Disperse Orange 3, which had a purity of >97%. We were not able to isolate and identify any reference substance for Disperse Blue 35, which is a mixture of different substances and belongs to the anthraquinone class of DDs. For comparison, we selected eight DDs that have been used for patch testing dermatitis patients in our department since 1999. Portuguese dermatologists proposed that these dyes should be included in the textile

Table 2. The main characteristics of the analysed garments

Country of purchase, number of garments	Country of origin, number of garments	Colour, number of garments	Fibre composition, number of garments
Sweden, 26	China, 29	Black, 35	100% polyester, 32
Spain, 17	Italy, 12	Orange, 24	Polyester blends*, 26
Italy, 15	Cambodia, 5	Red, 19	100% polyamide, 4
Lithuania, 5	Argentina, 5	Brown, 9	Polyamide blends†, 12
Portugal, 4	India, 5	Blue, 8	100% acrylic, 1
Bulgaria, 1	Portugal, 3	Green, 6	Elastane blends‡, 5
Argentina, 7	South Korea, 3	Yellow, 5	No identification of the textile fibres in the labels, 41
Singapore, 6	Turkey, 2	Violet, 3	
China, 10	Spain, 2	Grey, 2	
South Korea, 4	Bangladesh, 2	Multicoloured, 10	
India, 14	Hungary, 1		
South Africa, 6	Philippines, 1		
Canada, 6	No information in the labels, 51		

*Polyester blends with elastane (synonym lycra), acrylic, polyamide (synonym nylon), cotton, and viscose.
†Polyamide blends with elastane or acrylate fibres.
‡Elastane blends with cotton and viscose.

dye mix, on the basis of reports in the literature and their own experience (F Brandão, Portugal, 2011 personal communication).

All solutions and thin-layer chromatography (TLC) plates were prepared from the same batches at our department.

Extracts from the textiles

The extracts were made from 20 × 20 cm of textile, cut into 1–2 cm pieces. These were put into a glass jar with a diameter of 6 cm, to which 150 ml of 100% acetone was added. Extraction was performed with an ultrasonic bath for 15 min. The extract was then vacuum-evaporated in a rotary evaporator until dry (30°C). The obtained residues were diluted in 1–2 ml of acetone and used for application to TLC plates.

TLC of sample extracts

One millilitre of extract was repeatedly applied on the silica gel on a 20 × 20 cm glass plate (60 F 254; thickness of the TLC layer, 250 µm; Merck KgaA, Darmstadt, Germany) with a micropipette, until 5–10 µl had been deposited for each spot every 2.5 cm along a line on the lower part of the silica gel plate.

A mixture of chloroform and acetonitrile, 86:14 (vol/vol), or 100% chloroform was used as the mobile phase. The separated components in the extracts gave a band of well-defined and well-separated spots. The TLC plates were all inspected under visible and ultraviolet (UV) light (254 and 366 nm, respectively).

Purified and commercial solutions of Disperse Red 1, Disperse Red 17, Disperse Blue 106, Disperse Blue 124, Disperse Yellow 3, Disperse Orange 1, Disperse Orange 3, and Disperse Blue 35 (only commercial), 1% in acetone, were used as reference substances.

If there were matching spots from the textile extract and the reference (purified) dye, TLC was performed, comparing extract, reference dye, and the mixture of the equal parts of the extract and the reference dye. If the matching of the spots on the TLC plate remained, HPLC of the extract and the reference dye was performed.

High-performance liquid chromatography

A ThermoFinnigan system was used, consisting of a P4000 quaternary pump, a UV 6000 diode array detector, an AS3000 autoinjector, an SN4000F control module, and software controlled by ChromQuest 4.1 and monitored by Spectral Analysis for ChromQuest (ThermoFinnigan, San José, CA, USA). The injection volume was 20 µl. The column (4.6 mm internal diameter × 250 mm) was packed with Kromasil (100 Å, 5 µm; Eka Nobel, Bohus,

Table 3. The linear gradient elutions used for high-performance liquid chromatography analysis of the disperse dyes*

Time (min)	%A	%B	Flow rate (ml/min)
0	60	40	1.0
35	0	100	2.0
45	0	100	2.0

*Solvents A and B consisted of acetonitrile/water 40:60 (vol/vol) and acetonitrile, respectively.

Sweden). The detector scanned the eluate in the range 200–800 nm. HPLC was performed with a linear gradient of mobile phases, as described in Table 3. The identity of the substance producing a certain peak was determined by retention time and UV spectrum. The detection limit was 0.0001 μg/cm^2.

Results

Disperse dyes indicated by the TLC analysis

In total, 121 extracts were analysed by TLC. Three extracts were almost colourless and gave no visible spots on TLC plates.

A total of 31 extracts had similar spots to the main spots of the reference dyes on TLC plates. All spots on TLC plates were visible in daylight, and no additional spots were seen under UV light.

The majority of the 30 extracts had matching spots with Disperse Blue 124 (12 extracts), 10 with Disperse Orange 1, eight with Disperse Red 1, seven with Disperse Red 17, five with Disperse Orange 3 and Disperse Blue 106, and four with Disperse Blue 35 and Disperse Yellow 3. However, some of the extracts had matching spots not with the reference dye, but with impurities or with other substances present in the commercial dye preparation (Fig. 1).

Extracts that had similar spots to the main spot on the TLC of the purified reference dye were also analysed in another system. In one case, the results differed and there were no matching spots (Fig. 2).

Thirty extracts were mixed with the purified dye to confirm the presence of the reference dye. Mixes were 'positive' (that is, there was matching of the suspected dye spot from the extract and the reference dye on the TLC of the mix) in nine cases: in two with Disperse Yellow 3, Disperse Red 1, and Disperse Orange 1, and in one case each with Disperse Blue 124, Disperse Blue 106, and Disperse Orange 3 (Fig. 3).

Similar colour patterns

There was a similar pattern of dyes on TLC plates among different extracts, especially in the orange, blue, red and yellow spectra. TLC of the extracts made from different garments dyed in the same colour showed that the same colour could be composed of different dyes.

High-performance liquid chromatography

Ten samples were analysed by HPLC. It was not possible to confirm the presence of the suspected dye from the TLC analysis (Disperse Yellow 3, Disperse Orange 3, Disperse Blue 106, and Disperse Red 1) when evaluating the retention times and UV spectra in seven samples (Fig. 4). However, in brown tights, made and bought in Italy (made from polyamide and elastane), Disperse Yellow 3, Disperse Blue 106 and Disperse Blue 124 were confirmed by HPLC analysis. In a set of black bra and panties bought in India (material composition and country of manufacture were not present on the label), Disperse Orange 1 was detected (Fig. 5).

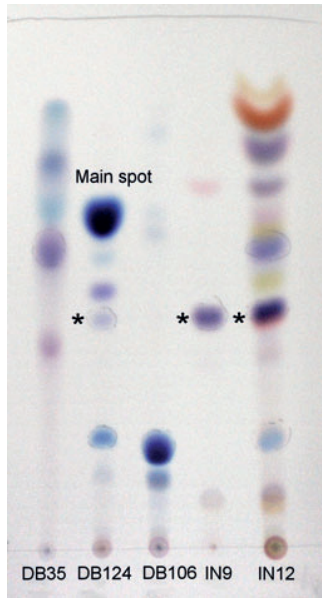

Fig. 1. Similar spots when thin-layer chromatograms of the extracts (IN9, IN12) and commercial Disperse Blue 124 (DB124) and Disperse Blue 106 (DB106) dyes are compared. These spots do not represent the main spot of DB124 or DB106. Asterisks (*) indicate similar colours.

Discussion

The prevalence of DD contact allergy varies with the population and the dyes tested. As DDs are used also in other applications, for example in toys, one study investigated commercially available toy samples (including textiles) from the market by liquid chromatography and tandem mass spectrometry for the presence of the allergenic and/or carcinogenic dyes listed by the EU, including Disperse Yellow 3, Disperse Blue 124, Disperse Orange 3, Disperse Blue 106, and Disperse Red 1.

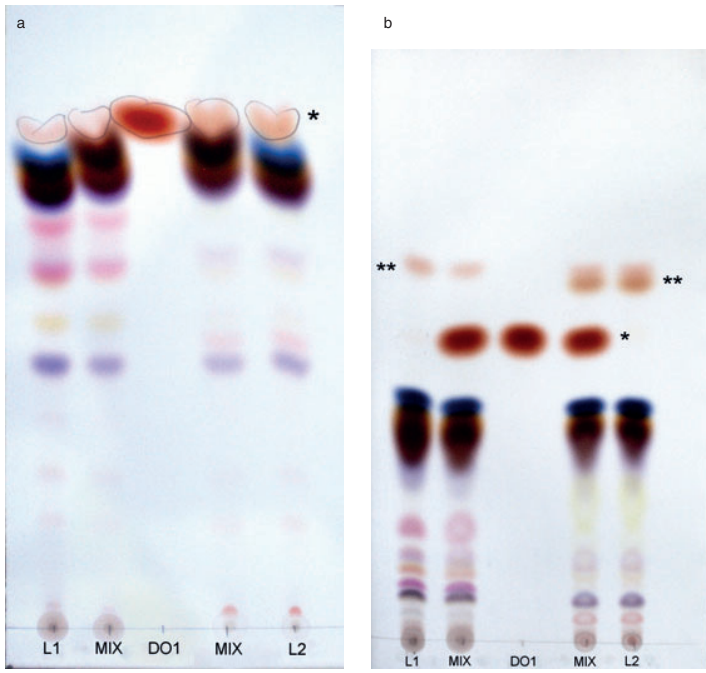

Fig. 2. Thin-layer chromatograms of the extracts (L1 and L2) as compared with purified Disperse Orange 1 (DO1) and mixtures (MIX) with it. Asterisks (*) indicate similar colours when the eluent system 84:16 chloroform/acetonitrile was used (a); when the system was changed to 100% chloroform (b), there were no matching colours.

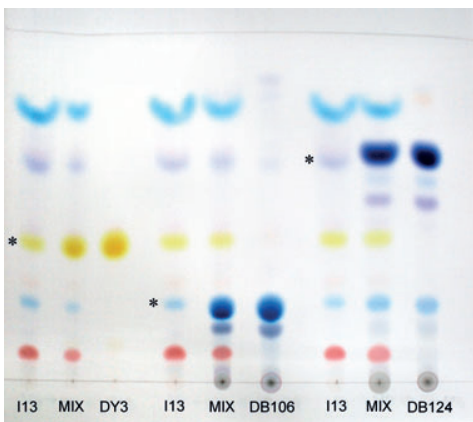

Fig. 3. Thin-layer chromatogram of the extract (I13) as compared with purified Disperse Yellow 3 (DY3), Disperse Blue 106 (DB106), Disperse Blue 124 (DB124), and mixtures (MIX) with them. Asterisks (*) indicate similar colours.

The authors were unable to find any of the investigated dyes (16). Therefore, it might be that allergenic DDs are also not used for dyeing textiles.

It was suspected that some of the eight DDs could be present in 30 of 121 (24.8%) extracts after initial TLC, but, using other methods (mixing the extract with the reference dye and repeating the procedure, changing the eluent system for TLC, and performing HPLC), we were able to confirm the presence of Disperse Yellow 3, Disperse Orange 1, Disperse Blue 106 and Disperse Blue 124 in three garments out of 121 (2.5%). As these items were obtained randomly, it is obvious that the DDs most commonly used for testing dermatitis patients are not widely used. Our study shows that one method for identification of the DD is not enough to confirm the identity of the dye. In one case, changing the eluent system for TLC from 86:14 chloroform/acetonitrile to 100% chloroform led to completely different results, and the spots did not correspond to those of the reference dye.

TLC showed that a textile is usually dyed with several different dyes, although it appears to have just one colour. The analysis also shows that the individual textile dyes usually contain impurities or that they are mixtures of

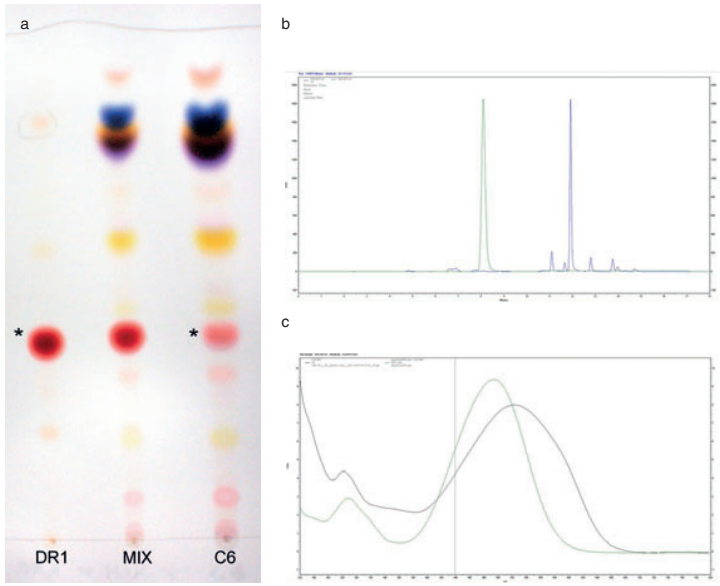

Fig. 4. (a) Thin-layer chromatogram of the extract from the textile (C6) as compared with the reference substance Disperse Red 1 (DR1) and a mixture of C6 and DR1 (MIX) indicates the presence of DR1 in C6, but this was not confirmed by high-performance liquid chromatography (HPLC). Asterisks (*) indicate similar colours. (b) HPLC chromatogram of purified DR1 (green line) and C6 (blue line) shows different retention times. (c) HPLC spectrogram recorded at a wavelength of 450 nm indicates that there is no DR1 (green line) in C6 (blue line); detection limit, ≥ 0.0001 μg/cm^2.

several dyes. The textiles that most often contain several different dyes are the black or dark ones. We found Disperse Orange 1 in the set of black bra and panties and Disperse Yellow 3, Disperse Blue 106 and Disperse Blue 124 in the brown tights. This shows that it can be difficult to predict which dyes are used in a textile just from its colour.

Thirty per cent of the garments, some of which were also obtained within the EU, had no fibre composition or country of origin information available on labels. Therefore, it may be difficult to avoid certain types of textile and thereby certain dyes for consumers.

When comparing the extracts with the commercial DDs on TLC plates, we found a significant number of the extracts with similar additional spots close to the main spot. These impurities, whether intentionally added substances or synthesis intermediates, might be relevant for the elicitation of allergic reactions. One study in Malmö showed that the raw material of DDs used for preparing patch tests contains 39–76% of contaminants or other substances (15). These can be relevant, as almost 25% of patients allergic to Disperse Blue 106 and Disperse Blue 124 did not react to the main spot but did react to the other spots on the TLCs made from the commercial forms of these dyes (17).

Our study was performed with randomly obtained items. One may suggest that investigating in a more systematic way (e.g. investigating only dark 100% polyester garments) would be more informative, but our goal was to obtain a general impression of the possibility of contact with certain disperse azo dyes in various garments.

For the investigation of textiles, we used two forms of chromatography: TLC and HPLC. TLC is simple to use, inexpensive, and quick. The TLC conditions can be easily modified to obtain efficient separation of different components in the mixture, but it is a qualitative and not a quantitative technique. It could be that overlapping of several components in the spot of the mixture with similar retention factors on the TLC plate occurs. This could be revealed by changing the TLC conditions (e.g. the eluent system) or by using other methods (e.g. HPLC). HPLC is most often a much more sensitive method – the detection limit of DDs in our study was 0.0001 μg/cm^2,

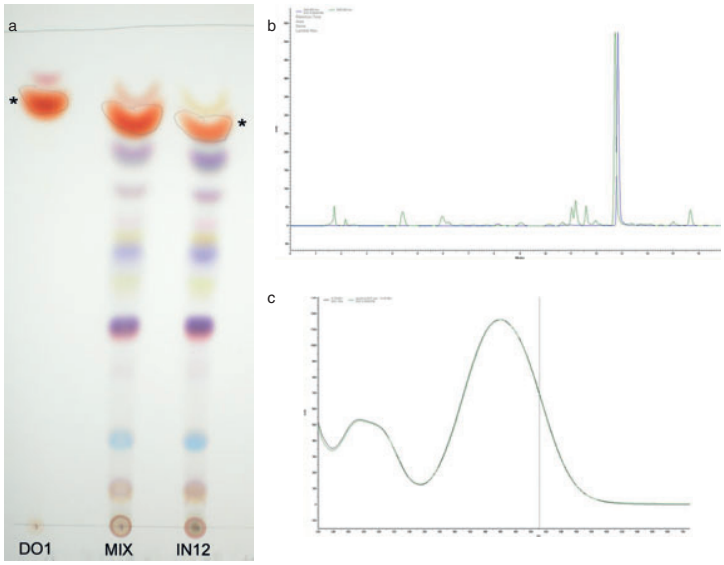

Fig. 5. (a) Thin-layer chromatogram of the extract from the textile (IN12) as compared with the reference substance Disperse Orange 1 (DO1) and a mixture of IN12 and DO1 (MIX) indicates the presence of DO1 in IN12. Asterisks (*) indicate similar colours.
(b) High-performance liquid chromatography (HPLC) chromatogram of purified DO1 (blue line) and IN12 (green line) shows very similar retention times. (c) HPLC spectrogram recorded at a wavelength of 450 nm indicates that DO1 (blue line) is present in IN12 (green line); detection limit, ≥ 0.0001 μg/cm^2.

and a standard textile garment contains approximately 100 μg/cm^2 of the dye (18).

It is very important to know whether dyes to which a patient has contact allergy are present in the textile. Indeed, in the majority of studies reporting positive reactions to DDs, clinical relevance is not stated, although it has been shown that dyes to which patients are infrequently patch test-positive are found in the suspected garment (11). Our study showed that some DDs usually implicated as allergens are infrequently found in the textiles. However, we were investigating the presence of the chemically defined dyes. Impurities that have been shown to be present in the commercial DDs, and thus in the patch test preparations, are also important in elicitation of the patch test reactions (17, 19, 20). DDs could constitute a marker of sensitization to other para-compounds [such as *p*-phenylenediamine (PPD) or black rubber substances].

For establishing the clinical relevance of positive patch test reactions to DDs, patch testing with either the suspected fabric or an extract from the fabric should be performed; that is, exposure to that DD should be confirmed. However, the extraction procedure is not standardized. In our clinic, we follow the procedure described in Materials and Methods. We use mainly acetone as a solvent for the preparation of the extracts from the textiles, because acetone can dissolve both polar and non-polar compounds, although any solvent suitable for patch testing may be used. The extraction procedure with an ultrasonic bath provides an improved technique with which to obtain more standardized extracts from the same kind of products (21).

Ideally, identification of the dye content in the suspected fabric should also be carried out, although this is not always possible. Indeed, the latest reports that we found when reviewing the literature on chemical analysis of the suspected textile were published in 2000 (22, 23).

On the other hand, the diagnosis of textile dye dermatitis can be missed even if patch testing with the commercial DD preparations is performed, because these DDs are uncommon in textiles. It has been reported that 18% of surveyed dermatitis patients suspect textiles (especially synthetic) as a cause of their skin problems, but there was no statistical correlation with positive patch test reactions to DDs (24). Interestingly, self-reported textile-related skin problems were statistically significantly

associated with contact allergy to PPD, which is frequently found in cases with contact allergy to the azo dyes (24). Therefore, it could be that other disperse azo dyes are used in the textiles that we are wearing today.

Conclusions

In this study, the majority of the investigated fabrics were found not to contain any of the eight DDs present in our baseline series. However, it is still possible to find them, not only in the garments made outside the EU, but also in those made in the EU. Our study also shows that there is a need to determine which DDs are actually being currently used to dye textiles and that could be of clinical importance, and consequently should be included in patch test preparations used for detecting contact allergy to textile dyes.

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Paper IV

Contact allergy from disperse dyes in textiles–a review

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Summary

Several disperse dyes (DDs) are still considered to be the most important allergens in textile dermatitis, but there are sparse data about their current use in textiles. The aim of this review was to evaluate published studies and reports on contact allergy to DDs published in PubMed during the last 22 years (1990–2012). Prevalence data are provided by study and by dye, as well as by the described clinical peculiarities of DD dermatitis. We reviewed 54 studies. In total, 26 DDs were tested. The average prevalence in screening studies was >1% for Disperse Blue 106, Disperse Blue 124, and Disperse Orange 3. There is a lack of data on patch testing with Disperse Blue 26, Disperse Blue 102, Disperse Orange 37, Disperse Orange 149, Disperse Yellow 23 and Disperse Yellow 49, which are listed as allergens by the EU commission. It is necessary to check the purity and identity of dyes used for patch testing, confirm the clinical relevance of positive reactions by patch testing with suspected textiles, and, if the results are positive, determine the culprit dye.

Key words: allergic contact dermatitis; disperse dyes; textiles.

Disperse dyes (DDs) are the most prevalent causes of textile-related allergic contact dermatitis (1). They are used for dyeing synthetic fabrics made from fibres composed entirely of polyester, acetate, and nylon, or a blend of these with other fibre types; they are not used to dye natural fibres (e.g. wool, cotton, and linen) (2). DDs do not chemically bond to the fibres, and their small, lipophilic molecules can therefore easily migrate onto the skin of the person who is wearing the garment, especially if the textile fastness is poor; they may be removed by rubbing and by exposure to water (3). Approximately 60% of all DDs are azo dyes, and about 25% are anthraquinone dyes, with the remainder being quinophthalone, methine, naphthalimide, naphthoquinone and nitro dyes (2). Azo dyes are currently employed to create almost the entire range of shades; they are cheap and easy to apply, so this dye class is used most often (2). Within the EU and by the International Oeko-Tex Association (a group of textile research and test institutes), some DDs (mainly azo) are classified as allergenic, and their use is restricted (4, 5) (Table 1). There are sparse data on the presence of the dyes to which individuals are patch test-positive in the garments suspected as the cause of their dermatitis. In 2003, Hatch et al. investigated textiles from textile dye patch test-positive patients, using chemical methods, and concluded that dyes to which patients were patch test-positive were infrequently identified in their clothes (6). However, some DDs are commonly patch tested, and clinical relevance is often reported.

The primary objective of this study was to review articles published in scientific journals concerning contact allergy to DDs, the frequency and prevalence of the contact allergy to the DDs present in the textiles, and the clinical peculiarities of DD dermatitis.

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Table 1. Disperse dyes classified as allergens and listed by the EU Commission and by Oeko-Tex

C.I. Generic name	C.I. number	CAS number
C.I. Disperse Blue 1	C.I. 64 500	2475-45-8
C.I. Disperse Blue 3*†	C.I. 61 505	2475-46-9
C.I. Disperse Blue 7	C.I. 62 500	3179-90-6
C.I. Disperse Blue 26	C.I. 63 305	–
C.I. Disperse Blue 35*	–	12222-75-2
C.I. Disperse Blue 102	–	12222-97-8
C.I. Disperse Blue 106*†	–	12223-01-7
C.I. Disperse Blue 124*†	–	61951-51-7
C.I. Disperse Brown 1*	–	23355-64-8
C.I. Disperse Orange 1*	C.I. 11 080	2581-69-3
C.I. Disperse Orange 3*†	C.I. 11 005	730-40-5
C.I. Disperse Orange 37	C.I. 11 132	–
C.I. Disperse Orange 76	C.I. 11 132	–
C.I. Disperse Red 1*†	C.I. 11 110	2872-52-8
C.I. Disperse Red 11†	C.I. 62 015	2872-48-2
C.I. Disperse Red 17*†	C.I. 11 210	3179-89-3
C.I. Disperse Yellow 1	C.I. 10 345	119-15-3
C.I. Disperse Yellow 3*†	C.I. 11 855	2832-40-8
C.I. Disperse Yellow 9*†	C.I. 10 375	6373-73-5
C.I. Disperse Yellow 39	–	–
C.I. Disperse Yellow 49	–	–
C.I. Disperse Orange 149	–	85136-74-9
C.I. Disperse Yellow 23	C.I. 26 070	6250-23-3

CAS, chemical abstract service; C.I., colour index number.
– indicates that there is no C.I. or CAS number for that dye.
*Present in the Textile Colours & Finish (TF-1000) series by Chemotechnique Diagnostics, Sweden, www.chemotechnique.se.
†Present in the Textile & Leather dyes series by Trolab, Germany, www.hermal.com.

Materials and Methods

Review of the literature

The National Library of Medicine (PubMed, http://www.ncbi.nlm.nih.gov.ludwig.lub.lu.se/pubmed, last accessed 5 May 2012) was searched with the MeSH terms 'disperse dyes and contact allergy', 'clothing and contact dermatitis', 'textile and contact dermatitis', and 'disperse dyes and contact dermatitis'. In addition, the journals *Contact Dermatitis* and *Dermatitis* (formerly the *American Journal of Contact Dermatitis*) were searched with the aforementioned terms. References within included articles were followed up when they were not found in the PubMed database. Only literature published in 1991–2012 and in English was included.

Methods

Three tables were created to allow comparison of prevalence data by study and by specific dye. We divided studies reporting patch testing with textile dyes into two groups, according to the patient population tested. Table 2 contains the records of those studies in which patients appeared for routine patch testing that included textile dyes (screening testing), and Table 3 contains the records of those studies in which patients were suspected to have textile dye allergic contact dermatitis (aimed testing). Additionally, Table 4 was created to record patch test results for each dye used in testing. Each record in these tables consists of dye name, number of patch test-positive patients in the study, number of patients who were patch tested, prevalence, and the reference.

Furthermore, case reports and studies containing clinical descriptions of the dermatitis caused by DDs were evaluated with regard to site and clinical features.

Results

General information

In total, we found 54 studies. Of these, eight studies could not be included because they lacked information about the tested dyes or because the study population was already included in other published study.

Twenty-four studies were conducted by patch testing eczematous patients to determine the cause of allergic contact dermatitis (Table 2). Twenty-two studies were conducted by patch testing eczematous patients who were suspected of being textile dye sensitive (Table 3).

Furthermore, 18 studies were performed in Italian clinics. The remaining studies were performed in Belgium (one study), the United States (two), Portugal (four), Israel (five), Japan (one), Austria (four), France (two), The Netherlands (two), Canada (two), Switzerland (one), China (one), North America (four), Sweden (two), the United Kingdom (three), and Sweden/Belgium (two). Three were reports from the Information Network of Departments of Dermatology, and one was a report from the European surveillance system.

Patients were adults or of mixed ages, except in five studies (15, 21, 29, 50, 51) where patients were children.

Dyes for patch testing were obtained from Fabbrica Italiana Ritrovati Medicinali Affini (FIRMA) (Firenze, Italy), Hermal/Trolab (Hamburg, Germany), or Chemotechnique Diagnostics (Vellinge, Sweden), or directly from dye manufacturers. Usually, the dyes were not checked for identity or purity [exceptions were (10, 25, 27)]. In total, 26 DDs were used for patch testing in 1% pet. Additionally, Disperse Blue 35 was tested in 0.5% pet. and 0.3% pet., and Disperse Blue 106, Disperse Blue 124, Disperse Red 1, Disperse Red 17, Disperse Orange 1, Disperse Orange 3 and Disperse Yellow 3 in 0.5%, 0.3% and 0.1% pet.

Table 2. Review of the literature on the prevalence and clinical relevance of disperse dye (DD) contact allergy in consecutively patch tested dermatitis patients (screening patch testing)

References	Study period (or publication year)	Country	Number of patients tested	Positive reactions, number (%)	Patch testing with the extract/textile	Clinical relevance according to the reference
(7)	1987–1991	Belgium	3336	28 (0.8)	Yes, 3 patients, not positive to dye allergens, were positive to textile	Not stated
(8)	1988–1990	Italy	2752	100 (3.6)	No	Not stated
(9)	1988–1990	Italy	576	19 (3.3)	No	8/19 (42%)
(10)	1990–1995	Italy	6203	236 (3.8)	No	Not stated
(11)	1991	Italy	569	2 (0.4)	No	Not stated
(12)	1992	Portugal	329	2 (0.6)	No	Not stated
(13)	1994	Portugal	78	2 (2.6)	Not stated	100%
(14)	1995–1999	Germany, Austria	1986	86 (4.3)	No	Current clinical relevance 70%
(15)	1996–2000	Italy	1098	51 (4.6)	Not stated	Past/current relevance 70%
(16)	1996–2000	Italy	6478	437 (6.7)	Not stated	371/437 (85%)
(17)	1998	Italy	1012	31 (3.1)	Yes: 10/1012 (1%) 5/10 positive (50%), but 2 were negative to the patch tests with DDs	Not stated
(18)	1999–2003	Sweden	3325	50 (1.5)	No	Not stated
(19)	2001–2002	North America	4888	146 (3.0)	Not stated	45.5% possible relevance, 5.5% past relevance, 10.3% probable relevance
(20)	2001–2002	Germany, Austria, Switzerland	3041	40 (1.3)	Not stated	Not stated
(21)	2001–2004	United States	391	8 (2.1)	Not stated	47.6%
(22)*	2002	Israel	286	15 (5.2)	Not stated	68.7% current relevance, 9.4% past relevance, 21.8% no relevance
(23)	2003–2004	North America	5136	98 (1.9)	Not stated	Not stated
(24)	2003–2005	Germany	24 980	337 (1.35)	Not stated	Not stated
(25)	2004–2005	Sweden, Belgium	1780	35 (2.0)	Not stated	Not stated
(26)	2005–2006	North America	4454	94 (2.1)	Not stated	35.5% possible relevance, 7.5% past relevance, 20.4% probable relevance, 8.6% definite
(27)	2006–2008	Sweden, Belgium	2546	65 (2.6)	Not stated	Not stated
(28)	2007–2008	Finland Italy United Kingdom	760 2938 9201	4 (0.5) 47 (1.6) 37 (0.4)	Not stated	Not stated
(29)	2008	United States	65	7 (4.6)	Not stated	Probable/possible 71.4%
(30)*	2010	China	532	Volunteers 6/205 (2.9%) Eczema patients 13 /327 (4.0%)	No	2/6 (33.0%) volunteers–past relevance 6/13 (46.0%) eczema patients – past/present relevance

*Including positive reactions to other class of dyes.

Disperse Blue 1, Disperse Blue 7, Disperse Red 11, Disperse Orange 76, Disperse Yellow 39, Disperse Yellow 54, 27 and 64 Disperse Black 2 were not tested in consecutive dermatitis patients, but were tested only in cases in which textile dye-related dermatitis was suspected.

Sex

Often, the sex distribution of patients positive for DDs was not provided, especially in earlier reports. In the vast majority of studies where the sex distribution of the positive reactions to DDs was reported, an

Table 3. Review of the literature on the prevalence and clinical relevance of disperse dye (DD) contact allergy in patients suspected or thought likely to have contact dermatitis caused by DD allergy (aimed patch testing)

References	Study period (publication date)	Country	Number of patients tested	Positive reactions, no (%)	Patch testing with the extract/textile	Clinical relevance according to the reference
(7)	1987–1991	Belgium	159	28 (17.6)	Yes, 3 patients, not positive to dye allergens, were positive to textile	Not stated
(8)	1988–1990	Italy	198	134 (67.7)	No	Not stated
(9)	1988–1990	Italy	145	23 (15.9)	No	8/19 (42%)
(31)	1988–1992	Portugal	6	6 (100%)	TLC results of the extracts from clothing were compared with those of reference dyes. In all 3 cases, DDs to which patients were patch test-positive were detected	100%
(32)	1989–1994	United States	50	12 (24.0)	Yes, positive 5/12 (41.7%)	Not stated
(33)*	1991–1997	Israel	55	22 (40.0)	Yes	Present relevance 20/22 (90.9%)
(34)	1991	Italy	2	2 (100)	No	100%
(35)	1992	Japan	1	1 (100)	Yes. Dyes were obtained from the manufacturer and analysed by TLC and mass spectrometry	100%
(36)	1993–2006	Australia	2069	114 (5.5)*	Yes (positive 12.8%)	100% in patients positive to extract/textile
(37)	1995	France	1	1 (100)	Yes	100%
(38)	1996–1999	The Netherlands	577	79 (13.7)	No	Relevant (probably) 75%
(39)	1997–1999	Canada	271	40 (14.8)	Yes, 11/271 (41%)	34/40 (85%) relevant 6/40 (15%) unknown relevance
(40)	1996–2000	Italy	130	13 (10.0) only to DDs	No	8/13 (61.5%) in patients sensitized only to DDs
(41)*	1998	Israel	103	30 (29.1)	No	100%
(42)	1998	Italy	1	1 (100)	Yes, positive. Dye detected by HPLC in the suspected garment	100%
(43)*	1999–2002	Israel	644	43 (6.7)	Yes 21/664 (3.2%) Positive 5/21 (23.8%)	Present relevance 81.4% Past relevance 6.8% No relevance 11.7%
(44)	2000	Portugal	5	5 (100)	Yes, 5/5 positive. TLC analysis revealed Disperse Blue 106	100%
(45)	2000	Switzerland	1	1 (100)	Yes: positive. Dyes obtained from manufacturer and analysed by HPLC – not detected	Not stated
(46)	2001	Canada	2	2 (100%)	Yes: negative in 1 patient, positive in another patient	100%
(47)	2004	Australia	1	1 (100)	Yes	100%
(48)	2004	Australia	1	1 (100)	Yes	Yes
(49)	2011	Italy	1	1 (100)	Yes	100%

HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.
*Including positive reactions to other classes of dyes.

overrepresentation of women was seen: from 56% to 100% (8, 9, 16, 17, 19, 33, 35, 39, 40, 43, 44, 47, 49, 52, 53). Only in one study did males slightly predominate (51%) (54).

Atopy

Among 16 studies in which atopic status (allergic rhinitis/conjunctivitis, asthma with/without atopic dermatitis) was described in patients positive for DDs, its

Table 4. Prevalence of each disperse dye by study

		Aimed testing			Screening		
Disperse dye	Concentration (wt/wt)	Number of patients positive/total tested	%	Studies (no.)	Number of patients positive/total tested	%	Studies (no.)
Disperse Blue 1	1.0% pet.	1/19	5.3	2		NR	
Disperse Blue 3	1.0% pet.	14/1441	1.0	13	3/2682	0.2	3
Disperse Blue 7	1.0% pet.	2/12	16.7	3	NR		
Disperse Blue 35	1.0% pet.	30/1779	1.7	13	11/4135	0.3	3
	0.5% pet.		NR		3/3325	0.1	1
	0.3% pet.		NR		4/2376	0.2	2
Disperse Blue 85	1.0% pet.	31/1599	2.0	9	15/2682	0.6	3
Disperse Blue 106	1.0% pet.	342/2051	16.7	16	639/35334	1.9	13
	0.3% pet.		NR		3/2049	0.2	1
	0.1% pet.		NR		5/3325	0.2	1
Disperse Blue 124	1.0% pet.	376/2363	15.5	15	517/19964	1.7	14
	0.3% pet.		NR		4/2049	0.2	1
	0.1% pet.		NR		6/3325	0.2	1
Disperse Blue 153	1.0% pet.	7/1453	0.7	5	3/2682	0.2	3
Disperse Red 1	1.0% pet.	171/2266	7.5%	17	236/30120	0.8	13
	0.5% pet.		NR		6/3325	0.2	1
Disperse Red 11	1.0% pet.	0/24	0	2	NR		
Disperse Red 17	1.0% pet.	64/1883	3.4	16	17/6511	0.3	5
	0.5% pet.		NR		5/3325	0.2	
Disperse Red 35	1.0% pet.	0/1	0	1		NR	
Disperse Orange 1	1.0% pet.	34/1498	2.3	9	52/6184	0.9	4
	0.5% pet.		NR		17/3325	0.5	1
Disperse Orange 3	1.0% pet.	244/2256	10.6	17	334/27899	1.2	12
	0.5% pet.		NR		1/3325	0.03	1
Disperse Orange 13	1.0% pet.	11/810	1.4	5	1/2355	0.04	2
Disperse Orange 76	1.0% pet.	26/282	9.2	4		NR	
Disperse Yellow 1	1.0% pet.	2/40	5.0	1		NR	
Disperse Yellow 3	1.0% pet.	157/2265	6.95	18	218/28053	0.8	12
	0.5% pet.		NR		8/3325	0.2	1
Disperse Yellow 9	1.0% pet.	26/1607	1.6	13	2/2355	0.06	2
Disperse Yellow 39	1.0% pet.	0/6	0	1		NR	
Disperse Yellow 54	1.0% pet.	6/131	4.6	2		NR	
Disperse Yellow 27	1.0% pet.	1/104	1.0	2		NR	
Disperse Yellow 64	1.0% pet.	1/5	20.0	1		NR	
Disperse Brown 1	1.0% pet.	22/1498	1.5	10	2/2355	0.1	2
Disperse Black 1	1.0% pet.	18/137	13.1	3	1/569	0.2	1
Disperse Black 2	1.0% pet.	4/6	66.7	1		NR	

NR, no reports found.

prevalence ranged from 0% to 72% among an adult or mixed population (15, 32, 33, 40, 41, 43, 46, 53, 54) (excluding case reports of 1 patient).

Textile identification

Of 18 studies in which testing with the suspected textile or with an extract made from the textile was carried out, textile composition was reported in five (17, 35, 37, 39, 46). The reports concerned 100% navy blue or black 100% polyester (35, 37), dark wool synthetic mix (17), 100% wool dyed a dark blue–green colour (46), black/blue/dark green 100% acetate or 100% polyester, 52% acetate/42% cotton, and blue cotton/polyester (39). In other articles, 'textile' was mainly referred to as 'synthetic material' or 'stockings', or not described at all.

Prevalence of contact allergy to DDs

In a few studies, testing of patients was performed with a textile series, and thus the prevalence data obtained concerned positive reactions to allergens included in the series. A majority of the positive reactions were to DDs. Nevertheless, when possible, we calculated the prevalence rate only for DDs, and if this was not possible, the prevalence of positive reactions to textile series was recorded.

In those studies in which patients appeared for routine patch testing and DDs were included (Table 2), prevalence values ranged from 0.4% (11, 27) to 6.7% (16). Prevalence values in patient populations known to be or very probably sensitized to DDs ranged from 5.5% to 100% (Table 3).

The amount of prevalence data collected for various DDs varies considerably (Table 4). Disperse Blue 124 was evaluated in 34 studies, with the highest number of tested patients, followed by Disperse Red 1 (32 studies), and Disperse Blue 106, and Disperse Yellow 3, which were evaluated in 31 studies.

There was considerable variation in the prevalence data for the aimed testing studies, but the lowest value was usually higher than the lowest prevalence value for the dye in screening studies. Average prevalence values were highest for Disperse Blue 106 (1.9%), Disperse Blue 124 (1.7%) and Disperse Orange 3 (1.2%) in screening testing, but in aimed testing Disperse Black 2 was positive in 66.7% of cases (although this is based on the data from only one study), Disperse Yellow 64 in 20%, and Disperse Blue 7 in 16.7%.

Clinical relevance and patch testing with patients' textile

Of 46 reviewed studies (Tables 2 and 3), patch testing with the suspected textile or its extract was reported in 18 (7, 17, 31–33 ,35–37, 39, 42–49). In two studies, identification of the culprit dye in the patient's textile was based on the similarity with the reference dye in a thin-layer chromatography (TLC) system (31, 44). In one case, dyes obtained from the manufacturer were investigated with mass spectrometry, and the DDs to which the patient was positive were detected (35). One study confirmed the presence of the dye by high-performance liquid chromatography (HPLC) (42). Of 28 studies in which the clinical relevance was stated, testing with the suspected garment or extract was carried out in 13. The clinical relevance of positive reactions as assessed by the authors was >70% in the majority of studies: in the aimed testing, 75–100%; and in the screening testing, 42–78%. In almost all studies, the most common DDs found to be positive on patch testing and thus clinically relevant were Disperse Blue 106 and Disperse Blue 124. Clinical relevance was mostly judged by the disappearance of dermatitis after the patient had ceased wearing dark synthetic clothing.

Monosensitization

Six studies reported rates of monosensitization to DDs (7, 8, 10, 15, 42, 48). The prevalence rate varied from 2.3% to 17%. The monosensitized patients most frequently had positive patch test reactions to Disperse Blue 124, Disperse Blue 106, Disperse Red 1, Disperse Orange 3, Disperse Yellow 3, and Disperse Red 17, but no associations with positive reactions to *p*-phenylenediamine (PPD) or clinical relevance were reported. Only one case report, by Foti et al., showed the monosensitization to Disperse Yellow 27 to be of clinical importance, by detecting the dye in the extract of the patient's clothes with HPLC (42).

Children

A recently published systematic review and meta-analysis of allergens responsible for allergic contact dermatitis among children by Bonitsis et al. (55) also covers children's sensitivity to several DDs: Disperse Blue 124, Disperse Blue 106, Disperse Orange 3, Disperse Red 1, and Disperse Yellow 3. According to this review, the prevalence of positive reactions in at least 1% of tested children was found to be statistically significant only when they were positive to Disperse Blue 124 but not to other DDs.

The North American Contact Dermatitis group compared sensitivity to Disperse Blue 106 in children and adults, and did not find a significant difference (21): the prevalence rate was 2.1% in children and 2.4% in adults (Table 5). The Portuguese Contact Dermatitis group, in a study performed in 1992, found a low prevalence of positive reactions to DDs – in 0.3% of 327 tested children (12). Studies performed in Italy (15, 50, 51) found that the most prevalent DD contact allergens in children are Disperse Blue 106 and Disperse Red 1, followed by Disperse Blue 124, Disperse Orange 3, and Disperse Yellow 3. Seidenari et al. described the sensitivities of 23 DD-positive children. In their study, the most prevalent sensitizers were Disperse Red 1 and Disperse Orange 3 (10).

Clinical picture

In 22 studies in which the clinical picture was described, DD-related dermatitis most commonly developed on the extremities (upper more frequently than lower), and especially on the hands, followed by the trunk, face, genitalia, buttocks, and the folds, including the neck, axillae, and groin (8, 14–18, 31–34, 36, 42–44, 51, 54). In children with positive patch test reactions to DDs, especially those suffering from atopic dermatitis, the flexural areas of the limbs were involved more often (52.9%) in those with atopic dermatitis than in those without it (15).

Table 5. Contact sensitization to disperse dyes in children

	Reference						
Dye tested	(15), n = 1098	(50), n = 1094	(29), n = 65	(12), n = 329	(21), n = 391	(51), n = 670	(10)*, n = 23
Disperse Yellow 3	17 (1.5%)	15 (1.4%)	ND	1 (0.3%)	ND	4 (0.6%)	4 (17.6%)
Disperse Blue 106	4 (0.4%)	44 (4.0%)	7 (8.8%)	ND	8 (2.1%)	ND	ND
Disperse Blue 124	14 (1.3%)	20 (1.8%)	ND	ND	ND	5 (0.7%)	4 (17.6%)
Disperse Orange 3	15 (1.4%)	20 (1.8%)	ND	1 (0.3%)	ND	6 (0.9%)	12 (52.2%)
Disperse Red 1	8 (0.7%)	25 (2.3%)	ND	1 (0.3%)	ND	8 (1.2%)	14 (60.1%)

ND, not done.
*Aimed testing.

Frequently, the clinical features of dermatitis related to DDs had uncommon features, an atypical localization, and unusual clinical patterns. It could present not only like typical chronic dermatitis, but also as persistent erythematous wheal-type or transient urticarial dermatitis (8), prurigo-like eczema (49), diffuse itching (8), erythema multiforme-like eruptions (8, 56), purpuric dermatitis (33, 42, 57), nummular dermatitis, erythroderma (43), or pseudolymphoma (52). Before the diagnosis of DD-related dermatitis was established, patients were diagnosed with lichen simplex chronicus, parapsoriasis, mycosis fungoides, drug eruption, post-inflammatory hyperpigmentation, pigmented purpura, or scabies (33).

According to Lazarov, chronic dermatitis was diagnosed in 35.4% of cases, and acute dermatitis in 6.8%; the remainder consisted of atypical forms of allergic contact dermatitis in patients with textile dye allergy (43). Seidenari described 50 of 437 patients positive to DDs and presenting with atypical allergic contact dermatitis features – erythema and oedema with or without marginal desquamation (16). It has been reported that dermatitis can be so monomorphic and infiltrated that, at first, the diagnosis of allergic contact dermatitis may not be obvious (7). It is also not uncommon to see sterile pustular lesions or purpura on sites of DD-induced dermatitis (43). There are reports of peculiar presentations of DD-related dermatitis, such as airborne contact dermatitis (58), as well as dermatitis on the incision scar of hip replacement caused by the black trousers of the patient (59).

Patch test reactions to DDs

Patch testing with DDs usually results in strong (++/+ + +) reactions. Sometimes, they can be purpuric (60). Massone et al. described persistent patch test reactions to Disperse Blue 124 and Disperse Red 1, which were already strongly positive on the D2 reading, and remained active and itching on D14 and D22 (34).

Dawes Higgs described a patient who developed a flare-up of the dermatitis in skin folds when patch tested with DDs and an extract from her textile, to which she was positive, as well as to Disperse Blue 106 at D3 (48). We also described a patient with systemic contact dermatitis that developed during patch testing with Disperse Orange 1 (61). The patient was tested with the dilution series of Disperse Orange 1, and was positive to the 0.000001% concentration.

Late readings

Times given for the interval between the removal of patch tests and the reading of the skin response were 2 days and 3–4 days. Not all authors reported the reading procedure.

Of the identified studies, 11 also performed late (D7) readings (23–26, 39, 41–43, 48, 49), whereas in four only some patients were read on D7 (18, 19, 27, 39). Several studies reported late positive reaction rates. Koopmans and Bruynzeel pointed out that 10% of the total reactions to the *para*-dyes were late reactions, 17% of them being for azo dyes, of which 84% were relevant (38). Ryberg et al. showed that 13 patients of 62 (21%) positive to DDs were positive only on D7 (27) in one study, and 3 of 35 (8.6%) patients positive to textile dye mixture (TDM) and 4 of 34 patients (11.2%) positive to ingredients of TDM were positive only on D7 in another study (25). Pratt and Taraska noted delayed positive patch test reactions to Disperse Blue 124 on D7/10 in 2 of 32 tested patients (6.3%), and all of them were clinically relevant (39).

One of the most important adverse consequences of patch testing is active sensitization, when subjects previously not allergic become sensitized to one or more of the test chemicals by the test procedure. The allergic test reaction then shows up at 10 or more days (late reaction) after the test application. Sometimes, however, late reactions are seen without active sensitization being present, as some allergens are known to give late reactions in the absence of active sensitization. According to the 4-year review of late reactions by Aalto-Korte et al., some Disperse Orange dyes induced late reactions in a much higher percentage of patients than PPD did, and

the authors concluded that these textile colours were primary active sensitizers (62). However, other authors have pointed out that a delayed immune response to some DDs is more prevalent than active sensitization (63, 64).

Discussion

More work has been done since the last review was published by Hatch and Maibach on the prevalence of textile dye allergic contact dermatitis: more countries are represented and more patients are tested (65). The prevalence of DD contact allergy varies with the population and the dyes tested. The available data indicate that positive patch test reaction prevalence rates for at least three dyes (Disperse Blue 106, Disperse Blue 124, and Disperse Orange 3) were >1% in screening of dermatitis patients. Therefore, according to Bruze et al., they should be included in the baseline series (66).

On the other hand, there are some DDs for which the prevalence rate in aimed testing is >10% (Disperse Blue 7, Disperse Yellow 64, Disperse Black 1, and Disperse Black 2), but they are not included in the series of the commercial patch test manufacturers (e.g. Trolab or Chemotechnique), so relevant allergies to these dyes could be missed if testing is performed only with commercial series.

There is a lack of data on patch testing with Disperse Blue 26, Disperse Blue 102, Disperse Orange 37, Disperse Orange 149, Disperse Yellow 23 and Disperse Yellow 49, which are listed by the EU Commission and by Oeko-Tex as allergens. It is also obvious that there are many more allergenic DDs than those that are classified as such by these institutions, so Oeko-Tex labelling or a label 'Made in the EU' does not mean that no allergenic dyes are used, as was also pointed out by Carozza and Nestle in their case report, where they described allergic contact dermatitis caused by 'ecological' (i.e. not listed in Oeko-Tex standard) disperse textile dyes (45).

The purity of the patch test preparations is also an important issue. In 1986, Foussereau et al. (67) reported on problems with the purity of several dyes in a commercially available patch test series. A study by Ryberg et al. found that some commercial patch test preparations labelled as containing Disperse Orange 3 did not contain that dye, but did contain another orange DD (68). Differences may also occur from batch to batch, as well as among different manufacturers. A study in Malmö showed that the raw material of DDs used for preparing patch tests contained 39–76% contaminants or other substances (68). These can be relevant, as almost 25% of patients allergic to commercial Disperse Blue 106, Disperse Blue 124, Disperse Yellow 3 or Disperse Orange 1 did not react to (or not only to) the main spot, but to other spots on the TLC plates made from the commercial dyes (61, 69). When patch test preparations of dyes contain more than one compound, it may not be the dye molecule that causes the skin reaction, and it is also difficult to compare the results of patch testing with different batches and in different clinics.

The high frequency of sensitization to DDs in women constantly reported in the studies may reflect the proportions in the study population, but it could also be caused by women's tendency to wear tighter-fitting clothes, lace underwear, or tights, which are always synthetic. Therefore, whether women are more susceptible to sensitization to DDs is not yet known.

It is very important to know whether dyes to which a patient has a contact allergy are used in textiles. Indeed, in the majority of studies reporting positive reactions to DDs, clinical relevance was not stated, although it was shown that dyes to which patients are patch test-positive are infrequently found in the suspected garment (6). As some of the DDs (e.g. Disperse Blue 106) are potent sensitizers (70), it seems that the clinical relevance of the positive patch test reactions could sometimes be overestimated. Unfortunately, not all studies provide the fibre composition of the 'culprit' textile; this could indirectly indicate the possibility of the DDs being present in the garment. DDs are not used to dye all types of synthetic fibre, and are not used for wool or cotton, where other types of dye are employed. Thus, positive patch test reactions to a disperse azo dye and to such types of textile could not be related aetiologically. On the other hand, other substances causing positive patch test reactions could be present in the textile, and positive patch test reactions to disperse azo dyes could be a marker of sensitization to other *para*-compounds (such as PPD or black rubber substances).

When establishing the clinical relevance of the positive patch test reaction to the DDs, patch testing either with the suspected fabric or with an extract from it should be performed, and the relevant DDs in the textile must be detected; that is, exposure to that DD should be confirmed. However, the extraction procedure is not standardized. Sensitivity to a dye placed directly on the skin would be expected to be higher than that seen in testing with the dyed textile, because a lower concentration of the dye would migrate onto the skin. Therefore, ideally, identification of the dye content in the suspected fabric should also be carried out, although this is not always possible. Indeed, the latest reports that we found when reviewing the literature on chemical analysis of the suspected textile were published in 2000 (44, 45).

For patients allergic to DDs, the common advice is to avoid dark, synthetic clothes. This is partially correct, as Disperse Yellow 3 and Disperse Orange 3 are found in ladies' tights and 'stockings' (not specified in the description), which are not necessarily dark, but are rather brown, or even beige (71). Also, one colour may be obtained by mixing several dyes, so even light colours may contain several allergenic DDs. It seems that the most appropriate advice would be to wear garments made from non-synthetic fibres.

A few studies reported figures for monosensitization to a particular dye, but only one of them reported the clinical relevance of this finding or an association with positive reactions to other *para*-compounds (42). In almost all of the case reports that we reviewed, sensitization to at least two DDs was reported. It seems that co-sensitization to several dyes, usually of similar structure or having the same impurities, exists, but it could be that a particular sensitization to one dye can be important. These DDs, for which monosensitization is reported, are some of the most prevalent dye allergens. Some of them have been shown to be strong allergens. This could mean that a positive reaction to one DD is related to the strength of the allergen, and shows a general predisposition of the immune system to recognize a particular pattern of the structure, rather than that this is the culprit allergen for the patient's dermatitis. The DD can be a marker for group sensitization, as is seen, for example, with the corticosteroid budesonide (72).

It is difficult to prove that the dyed textile contains the primary sensitizer. The primary sensitizer may be another substance or be a cross-sensitizer (10). It was reported that 18% of the surveyed dermatitis patients suspected textiles (especially synthetic) as a cause of their skin problems, but there was no statistical correlation with the positive patch test reactions to DDs (73). Interestingly, self-reported textile-related skin problems were statistically significantly associated with contact allergy to PPD, which is frequently found in cases with contact allergy to the azo dyes (73).

In order to diagnose allergic contact dermatitis caused by textile dyes, a high index of suspicion is required, as its clinical presentation does not always indicate the cause, because the clinical presentation is usually atypical, and its appearance is not always confined to sites of direct contact. On the other hand, a diagnosis of textile dye dermatitis could be missed even if patch testing with the commercial DD preparations is performed, because it could be that these DDs are currently uncommon in textiles. Hatch and Maibach identified the prevalence rates for 18 DDs described as allergens (65). We identified 26 allergenic DDs, but this still represents a very small fraction of the total of ~8000 commercially used dyes, in whiche DDs are one of the largest classes used. A study in Malmö showed that, in the 121 textile items analysed with different chemical methods, the most commonly named allergenic DDs were detected in three garments only (74). Therefore, it could be that other DDs are used in the textiles that we are wearing today.

Conclusions

Allergic contact dermatitis caused by DDs shows a polymorphic clinical picture, which is often atypical. Specific textile dye series contain substances that are currently employed in a limited group of garments or not used anymore, because new dyes are continuously being introduced. It is necessary to check the purity and identity of dyes used for patch testing, confirm the clinical relevance of positive reactions by patch testing suspected textiles, and, if the results are positive, determine the culprit dye. Late readings (D7) of the patch test reactions should be encouraged, as they give important and clinically relevant information.

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Paper V

Sensitizing capacity of Disperse Orange 1 and its potential metabolites from azo reduction and their cross-reactivity pattern

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Abstract

Background: Simultaneous contact allergies to Disperse □D □Orange 1, 4-nitroaniline and *p*-aminodiphenylamine □□AD□A□as well as to other disperse azo dyes and to *p*-phenylenediamine □□□D□have been reported. Cross-reactivity is one of the possible explanations to simultaneous reactions between □□D and disperse azo dyes. Some metabolites from the azo reduction of these disperse azo dyes could be sensitizers as human skin bacteria produce azo reductases.

Aim: To investigate the sensitizing capacity of D Orange 1, □AD□A and 4-nitroaniline, and the cross-reactivity between these substances and D □ellow 3, its potential metabolites from azo reduction □4-aminoacetanilide and 2-amino-*p*-cresol□and □□D.

Method: The guinea pig maximization test.

Results: It was found that both D Orange 1 and □AD□A are strong sensitizer and cross-react with each other. We were unable to sensitize guinea pigs with 4-nitroaniline tested in e□uimolar concentrations to D Orange 1.

Conclusions: The results indicate that patients sensitized primarily to D Orange 1 will react also to □AD□A, which could be found mainly in hair dyes. □□D, 4-nitroaniline, 4-aminoacetanilide, 2-amino-p-cresol, and D □ellow 3, did not show any cross-reactivity to D Orange 1 or □AD□A.

Key words: 4-nitroaniline, *p*-aminodiphenylamine, 4-aminoacetanilide, 2-amino-*p*-cresol, *p*-phenylenediamine, guinea pig maximization test, azo reduction, metabolite.

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Disperse □D□Orange 1 is a textile azo dye. It is known to be a sensitizer in humans □1□but this has never been investigated in animal studies. Simultaneous contact allergies to D Orange 1, 4-nitroaniline, and *p*-aminodiphenylamine □□AD□A□as well as to other disperse azo dyes and to *p*-phenylenediamine □□□D□have been reported □2-4□. Cross-reactivity is one of the possible explanations to simultaneous reactions between disperse azo dyes, □□D and its derivatives. Some metabolites from the azo reduction of the disperse azo dyes may be the primary sensitizers in case of contact allergy to these dyes, since it has been shown that human skin bacteria produce azo reductases and azo reduction takes place in the human skin □5, 6□. Another explanation may be common contaminants. In humans it is, however, impossible to demonstrate whether the positive patch test reactions are manifestations of cross-reactivity or a concomitant sensitization to these chemicals. The guinea pig maximization test □□□MT□is a useful tool for the investigation of the sensitizing capacity of a chemical and for the elucidation of cross-reaction patterns among structurally related sensitizers □7□.

In order to investigate the sensitizing capacity of D Orange 1 and its two metabolites from azo reduction □□AD□A and 4-nitroaniline□and the cross-reactivity to D □ellow 3, its potential metabolites from azo reduction □4-aminoacetanilide and 2-amino-*p*-cresol□and to □□D we conducted this study using the □□MT.

Materials and Methods

Substances

Acetone of analytical grade was obtained from Scharlau Chemie S. A. □La □ota, Barcelona, Spain□ D Orange 1 and D □ellow 3 had been purified and identified earlier at the Malmö department from commercial D Orange 1 and D □ellow 3 □8□, purchased from Chemotechni□ue Diagnostics □Vellinge, Sweden□.

□□D was bought from Chemotechni□ue Diagnostics. 4-□itroaniline, □AD□A, 4-aminoacetanilide and 2-amino-*p*-cresol were bought from Sigma Aldrich □Steinhem, □ermany□□Figure 1□. The general and specific purities of the substances are given in Table 1 □□.

Freund□s complete ad□uvant □FCA□ was obtained from □ierce □Rockford, IL, USA□. 2-methylol phenol □2-M□□was bought from Fluka chemie A□ □Buchs, Switzerland□ □ropylene glycol was obtained from VWR International S.A.S. □Fontenay-sous-Bois, France□, sodium

lauryl sulfate from Acros Organics (Geel, Belgium), dimethylacetamide from Sigma Chemical Co (St. Louis, MO, USA), and ethanol from Kemetyl AB (Haninge, Sweden).

Gas chromatography mass spectrometry (GCMS)

Separation of components in the samples of the substances used in the GMT for induction and challenges was performed with an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with an HP-MSI capillary column (Agilent Technologies) with a length of 30 m, an internal diameter of 0.250 mm and a film thickness of 0.25 m. Helium of alphagaz 2 quality (Air Liquide, Malmö, Sweden) was the carrier gas with a flow rate of 1.0 ml/min. The injection was split-less and the inlet was heated to 250°C. The injection volume was 1 l. The temperature program was isothermal at 70°C for 3 min, then rose with 8°C/min^{-1} to a final temperature of 300°C and isothermal at this temperature for 10 min. The gas chromatograph was connected to a Jeol JCmate II mass spectrometer (Jeol Datum Ltd., Tokyo, Japan). Electron-ionization (EI) mass spectra were recorded with m/z from 50 to 600 u, with scan duration 0.3 s and interscan delay 0.2 s. The temperature of the ion source was 250°C and the GCMS interface temperature was 250°C. The electron energy was 70 eV. The National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, U.S.A.) library of mass spectra was used for identification.

Direct inlet mass spectrometry (DIMS)

The Jeol JCmate II mass spectrometer was used with the gas chromatograph disconnected but with the same settings of the MS parameters. Around 1 µg of the substance to be analyzed was introduced into the MS and gradually heated from room temperature to 400°C during 25 min.

Ethics

The study was approved by the Lund Ethical Committee on Animal Experiments, Lund, Sweden, and conducted in accordance with ethical standards (approval No. M 28-12).

Guinea pig maximization test

The □□MT was performed according to the original description □10□. In order to standardize the test and ob□ectify the evaluation of the patch test reactions, some modifications were made including statistical calculations, blind reading, and a positive control group □□, 11, 12□. Female albino guinea pigs weighing 400 □□20□g of the Hartley□Dunkin strain □H□ Lidköpings Kaninfarm, Lidköping, Sweden□were used.

Topical irritancy. Topical irritancy was determined by applying different concentrations of each substance used for induction as a closed patch test for 2 days on both the neck and the flank of 3-4 animals. One week before testing, the animals were pre-treated with FCA. Concentrations that did not cause irritation and did not dye the skin of the guinea pig were chosen for topical induction and elicitation.

Concentrations. E□uimolar concentrations were used for all the substances used in the study. The concentrations used for induction and challenge are given in Table 2.

Induction. Twenty-four test animals were used for induction according to the following procedure:

Day (D) 0: three intradermal in□ections in a row at the site of each shoulder were given:

□i□ 0.1 ml of FCA in water 40□ w□v □FCA□water 50:50 v□v□□

□ii□ 0.1 ml of a solution of induction substance □w□v□dissolved in acetone and diluted in propylene glycol□

□iii□ 0.1 ml of mixture of 40□ w□v FCA in acetone□propylene glycol and with the same concentration of induction substance as in □ii□.

D6: pretreatment of a 2□4 cm area on the neck for topical induction with 0.2 ml sodium lauryl sulfate 10□ w□v in dimethylacetamide□acetone□ethanol □□5□ 4:3:3 v□v□v one day before topical application of the induction substance.

D7: topical induction on the neck with 0.2 ml of induction substance on a 2□4 cm piece of filter paper □130 g□m^3□Munktell1 Filter AB, □rycksbo, Sweden□placed on Durapore □3M Health Care, St. □aul, M□, USA□. The patches were covered with impermeable plastic adhesive tape □Acrylastic, Beiersdorf A□, □ermany□and held in place by adhesive bandage. The patch was left on for two days.

Controls. Twelve controls were given exactly the same treatment as described for the test animals, but with the induction substance excluded. In addition, six controls were given the known sensitizer 2-M□. These animals were used as a positive control in order to ob□ectify the evaluation of test reactions and as an indication of the accuracy of the induction procedure □□□.

Challenge.

D21, Challenge I, sensitization rate (right flank, two patches): 12 24 test animals were challenged with the induction substance on both the cranial and the caudal patch. 6 + 6 test animals were challenged with the induction substance on either the cranial patch or the caudal patch with vehicle alone on the other. Al-test Imeco AB, Södert 1e, Sweden on Durapore was used for patch testing. Thirty microlitres of the induction substance used for induction diluted in acetone was applied. Acrylastic and an outer layer of Durapore held the tests in place. The patches were removed after 1 D. Six of 12 control animals were tested with the induction substance on both patches, and 3 + 3 animals were tested with the induction substance on either the cranial or the caudal patch, with vehicle alone on the other patch 13.
Challenge II, cross-reactions (left flank, six patches): on the same occasion as challenge I: the same 24 test animals as in challenge I and 12 control animals were in addition to 4-nitroaniline and AD A challenged with D, D ellow 3, 4-aminoacetanilide and 2-amino-*p*-cresol. The positions of test substances were based on a Latin s uare table.
Evaluation. D23: the minimum criterion for an allergic positive reaction is a confluent erythema. All tests were evaluated blindly one day after the patches had been removed, i.e. two days after the application. First, the right flanks challenge I were read and thereafter, still blindly and without knowledge of the readings of the right flanks, the left flanks challenge II were read.
The procedure concerning the control group sensitized and challenged with 2-M is described elsewhere .
Induction for each of the 3 substances was performed on different occasions and freshly-made solutions of the substances were always used.

Statistical calculation

The number of positive animals within the test group was compared with the number of positive animals in the control group. The number of positive test animals was also compared with the number of positive animals tested with vehicle only. Among the animals challenged with the induction substance on both the cranial and caudal patches 12 test animals and 6 control animals, only one of the patches chosen in advance was included. Statistical significance was calculated with one-sided Fisher s exact test comparing control and test animals and with Mc emar test comparing test substance with the vehicle in the same animal. When a significant value p 0.05 was obtained both in the comparison with the test group and the controls tested with the allergen and the comparison between positively tested

animals and animals tested with the vehicle alone, the compound was considered as a sensitizer.

Results

As induction for each substance was performed on different occasions, the results represent data from three different experiments.

Purity

The investigation of the purity of the test substances showed that the general purity was 99% and higher with the exception of PADPA, where the general purity was 98%. Specific purity of all used substances was high (Table 1).

Sensitizing capacity

D Orange 1 and PADPA were found to be strong sensitizers in the guinea pig. Positive reactions were seen to both D Orange 1 and PADPA in 22/24 animals. Two control animals had reactions to D Orange 1 and two control animals reacted to PADPA ($p<0.001$). Only 5/24 animals had positive reactions to 4-nitroaniline and also two control animals to this substance ($p>0.3$) (Table 3).

Cross-reactivity

The result of the test for cross-reactivity is given in Table 4. PADPA gave a positive test reaction in 21 of 24 guinea pigs sensitized to D Orange 1 ($p<0.001$) (Table 4). Two animals, negative to PADPA, were positive to D Orange 1. One was negative both to PADPA and D Orange 1.

D Orange 1 was positive in 23 of 24 guinea pigs sensitized to PADPA and in 6/12 controls ($p<0.001$). Although PPD was positive in 17/24 guinea pigs, it was also positive in 7/12 controls ($p>0.3$).

Cross-reactivity in the animal group, in which 4-nitroaniline was the induction substance, was not assessed because sensitization to this substance failed.

Discussion

Purity

□ hen assessing contact sensitization and cross-reactivity it is very important to ascertain that experiments are performed using as pure substances as possible. □t could be that contaminants or impurities are allergens by themselves, as was shown when testing patients with the commercial D □lue 106 and 124, and D Orange 1 and D □ellow 3, respectively (14, 15). The concept general purity includes chemically undefined impurities which can have un□nown biological significance (9). □nvestigation of the specific purity means detecting the presence of certain substances (e.g. degradation products or raw materials used for the synthesis) which may be expected and thus also have an impact on the results of a sensitization study (9). □yberg et al. have showed that D □lue 124 was present in D □lue 106 and vice versa in commercial patch tests preparations, which were made from commercial dyes obtained from the various manufacturers (8).

□t is not so common to report on the purity of the substances used for animal studies (e.g., the local lymph node assay or the □euhler test) and the bias of the possible influence of other chemicals present in the substance of interest remains. Substances used in this □P□ T were confirmed to be of high general and specific purity, so the possibility that other components than the investigated substances influenced the results is virtually excluded.

Sensitization

Disperse dyes are the most common sensitizers among textile dyes (17), but not so many investigations have been performed determining their sensitizing capacity.

Although the sensitizing capacity of a chemical could be determined using animal tests, human tests and in vitro assays, the □P□ T is a standard method for analyzing sensitization capacity and assessing cross-reactivity patterns at challenge (7, 9, 17).

The most investigated disperse dyes regarding their sensitizing capacity are D □lue 124 and 106, D Orange 3 and D □ellow 3 (18, 19). □ hen discussing sensitizing capacity it is important to □now that the investigated substances do not contain other substances, but in these aforementioned studies the purity of the tested dyes were not reported. □t is □nown from chemical investigations of commercial disperse dye patch- tests preparations that the difference between the concentration stated by the manufacturer and detectable dye amount can differ up to five times (8).

D □lue 106 has proven to be a strong contact allergen in the guinea pig tests (20). □t is reported that the sensitization capacity of D □lue 106 is comparable to 2,4-dinitrochlorobenzene, one of the strongest contact allergens □nown (22, 23).

□ased on the results from the biphasic murine local lymph node assay, Ahu□a et al. grouped the disperse dyes on the basis of their sensitizing potency (24). According to them, D □ellow 3 and D Orange 3 were wea□sensitizers. D □ellow 3 was found to be a wea□sensitizer also in the □P□ T and a modified local lymph node assay (18, 20). □nterestingly, D Orange 3 and D □ellow 3 are one of the most fre□uently reported allergenic disperse dyes in humans as shown by patch testing, probably due to a high exposure (16).

Sonnenburg et al. examined several disperse dyes and products from azo-cleavage of these dyes in the loose-fit coculture-based sensitization assay of primary human □eratinocytes and of allogenic dendritic cell-related cells (25). □n this assay 4-nitroaniline and 4-aminoacetanilide showed no sensitizing potential, whereas D □ellow 3 and 2-amino-*p*-cresol were categorized as extreme sensitizers. PADPA was found to be a sensitizer in the □P□ T (26), but to our □nowledge a □P□ T was not performed previously with D Orange 1. □ e sensitized 22 of 24 (92%) guinea pigs with both substances. □ hen the significance levels $p<0.05$, $p<0.01$ and $p<0.001$ are used to designate sensitizers as wea□, moderate or strong, respectively, D Orange 1 and PADPA could be classified as strong sensitizers (9). They could be compared with strong sensitizers such as diglycidyl ether of □isfenol □, phenyl glycidyl ether or the main allergens in phenol-formaldehyde resins where □P□ Ts were performed according to the same methodology (9, 12, 27, 28).

□n our study 4-nitroaniline in an e□uimolar concentration to D Orange 1 did not show a sensitizing capacity. This finding confirms results from other studies (25, 29) where 4-nitroaniline was found to be a non-sensitizer even when a higher concentration was used for sensitization in the □P□ T (29).

Cross-reactivity

The cross-reaction pattern of suspected contact allergens is only possible to study when the exposure to them is controlled as in the □P□ T (30, 31).

PPD was reported to be a screening substance for textile dye-related dermatitis, but several clinical studies concluded that there was no statistical correlation between the positive patch test results to disperse dyes and PPD (32, 33) with the exception of D Orange 3. □n a few studies a statistically highly significant association between contact allergies to PPD and D Orange 3 was detected (34, 35). □ oreover, simultaneous reactions are fre□uently observed between D □lue 124 and 106 (36). □t is referred to as cross-reactivity in some publications, but now it is □nown that each dye may contain a low amount of the other dye. Another

explanation is that D □lue 124 can easily be converted to D □lue 106 through hydrolysis in the s□in (8).

□n the present study we demonstrated that D Orange 1 is a sensitizer in the □P□ T. □ hether the reactions to D Orange 1 are reactions to this substance *per se* or due to its metabolite PADPA cannot be stated. □f D Orange 1 is fully metabolized to PADPA during azo reduction on the s□in by s□in bacteria and/or in the s□in, then there is not a true cross-reactivity between these substances. Also, it has not been shown that D Orange 1 is azo reduced *in vivo.* □f only a part of or nothing of D Orange 1 is metabolized, then it is possible that a true cross-reactivity with PADPA occurs. The same applies to the concomitant reactions observed to D Orange 1 when guinea pigs were induced with PADPA. The purity of D Orange 1 was over 99% and PADPA was not detected in it as well as PADPA did not contain D Orange 1, so the presence of PADPA in D Orange 1 or vice versa could not explain the observed challenge reactions. To elucidate whether D Orange 1 or PADPA is the primary sensitizer, testing these 2 substances in e□uimolar concentrations and serial dilutions both at induction and at challenge would have been needed in a □P□ T. □ oreover, 4-nitroaniline is excluded from being the ma□or sensitizer in case of contact allergy to D Orange 1, as it was positive only in one guinea pig with a positive reaction to D Orange 1. Also this substance showed no sensitizing capacity when tested in an e□uimolar concentration to D Orange 1.

Our results indicate that a person sensitized to D Orange 1 will react to PADPA, but not to PPD. □umans can be exposed to PADPA using oxidative hair dyes or rubber items (37). PADPA can also be used in a textile dye synthesis, so it might remain in the final product and be transferred to textiles when dyeing them (37). A primary sensitization to PADPA causes contact allergy to D Orange 1 as indicated by our study.

This study also shows that cross-reactivity among disperse azo dyes is not universal. □uinea pigs sensitized to D Orange 1 did not react to D □ellow 3 when tested in e□uimolar concentrations. Some clinical reports also show that a clinically relevant sensitization to only one disperse dye exists (34, 38, 39).

□o cross-reactions were demonstrated to PPD with D Orange 1 as the sensitizer in our study. □ e have noticed in our previous study that when patch testing patients sensitized to D Orange 1 and not to the other disperse azo dyes, there were no concomitant positive patch test reactions to PPD (2). □nterestingly, guinea pigs induced with PADPA did not react at a statistically significant level when tested with PPD. As these two molecules have chemical similarities, cross-reactivity would be expected to occur. □ ore experiments are needed to □now whether guinea pigs sensitized to PPD would react when challenged with PADPA.

It is worth mentioning that reading of the patch test reactions of the coloured substances in guinea pigs might be complicated, although during irritancy testing we have chosen concentrations which did not dye the patch test area (Fig. 1). As the epidermis of the guinea pig contains fewer layers than that of humans, a positive reaction to the sensitizer is mostly based on the erythema appearance. Blind readings and inclusion of the positive control group help to reduce possible bias of the over-interpretation of the positive or negative results. Whether primary sensitization to PPD would cause cross-reactions to D Orange 1 is not known. The GPMT study performed by Tamano et al. (26) showed that when the guinea pigs were sensitized with PPD, they reacted on challenge to PADPA, but when they were induced with PADPA, they did not react to PPD, even when challenged with a higher concentration, which was not equimolar to PADPA (26). It is possible that metabolic activation plays an important role for the sensitization capacity, and differences in skin metabolism between animals and humans should also be taken into account.

Conclusions

D Orange 1 and PADPA are strong sensitizers in the GPMT. It can be assumed that individuals primarily sensitized to D Orange 1 could react to PADPA, but not to another potential metabolite from azo reduction, i.e. 4-nitroaniline, or to Disperse Yellow 3, PPD, 4-aminoacetanilide, or 2-amino-*p*-cresol. Therefore, PPD does not seem to be a suitable marker for the detection of patients that have been primarily sensitized to D Orange 1. Whether D Orange 1 and PADPA cross-react cannot be stated with certainty from the results of this study. .

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Figure 1. Chemical structure, Chemical Abstract Service (CAS) and Colour □ndex (C.□) numbers as well as molecular weight (□ □) for the investigated substances.

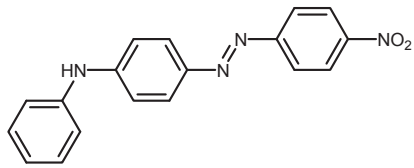
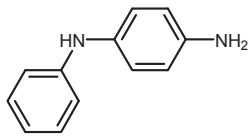
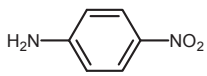
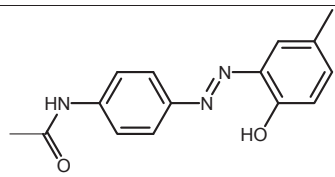
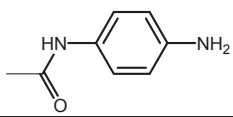
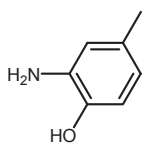
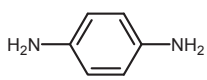
	**Disperse Orange 1** *CAS:2581-69-3* *MW: 318* *C.I.: 11080* *log $P_{o/w}$: 5*
	***p*-** *CAS: 101-54-2* *MW: 184* *log $P_{o/w}$: 2.7*
	**4-nitroaniline** *CAS: 100-01-6* *MW: 138* *log $P_{o/w}$: 1.01*
	**Disperse Yellow 3** *CAS: 2832-40-8* *MW: 269* *C.I.: 11855* *log $P_{o/w}$: 3.48*
	**4-aminoacetanilide** *CAS: 122-80-5* *log $P_{o/w}$: 0.14*
	**2-amino-*p*-cresol** *CAS: 95-84-1* *MW: 123* *log $P_{o/w}$: 1.33*
	***p*-phenylenediamine** *CAS: 106-50-3* *MW: 108* *log $P_{o/w}$: 0.43*

Table 1. **General and specific purity of the substances used in the guinea pig maximization test**

Substances, obtained from the manufacturers	Concentration indicated on the label	Results from GCMS and DIMS						
		Used substances						
		Disperse Orange 1	4-nitroaniline	*p*-aminodiphenylamine	Disperse □ellow 3	2-amino-p-cresol	4-aminoacetanilide	*p*-phenylenediamine
Disperse Orange 1	□ot stated	**>99%***	nd	nd	nd	nd	nd	nd
4-nitroaniline	≥ 99%	nd	**>99%**	nd	nd	nd	nd	nd
p-aminodiphenylamine	98%	nd	nd	**98%**	nd	nd	nd	nd
Disperse □ellow 3	□ot stated	nd	nd	nd	**>99%**#	nd	nd	nd
2-amino-*p*-cresol	97%	nd	nd	nd	nd	**99%**	nd	nd
4-aminoacetanilide	99%	nd	nd	nd	nd	nd	**99%**	nd
p-phenylenediamine	□ot stated	nd	nd	nd	nd	nd	nd	**99%**

Abbreviations□□- concentration before purification 15.2%□# -concentration before purification 40.6%□nd □not detected (detection limit <0.1%)□□C□ S □gas chromatography-mass spectroscopy□D□□ S □direct in□ection mass spectroscopy.
Purification of the D Orange 1 and D □ellow 3 was performed at the Department of Occupational and □nvironmental Dermatology, □ alm□, Sweden.

Table 2. **Concentrations (%w/v) used for induction and challenge in the guinea pig maximization test**

Substance	Intradermal sensitization	Topical sensitization	Challenge I	Challenge II
Disperse Orange 1	1.20%	2.30%	0.57%	0.57%
4-nitroaniline	0.50%	1.0%	0.25%	0.25%
p-aminodiphenylamine	0.65%	1.30%	0.33%	0.33%
Disperse □ellow 3	-	-		0.49%
2-amino-*p*-cresol	-	-		0.22%
4-aminoacetanilide	-	-		0.27%
p-phenylenediamine	-	-		0.20%

Table 3. **Sensitizing capacity of Disperse Orange 1, *p*-aminodiphenylamine and 4-nitroaniline**

□nduction substance	T/*n*	C/*n*	□/*n*	P/*n*
Disperse Orange 1	22/24	2/12	0/12	1/6
p-aminodiphenylamine	22/24	2/12	0/12	1/6
4-nitroaniline	5/24	2/12	2/12	4/6

Abbreviations□T, number of the positive test reactions to the induction substance in test animals□C, number of the positive test reactions to the induction substance in control animals□□, number of the positive test reactions to the vehicle in test animals□P, number of positive test reactions to 2-methylol phenol in the positive control group. *n*, number of tested animals in the 4 groups T, C, □, and P.

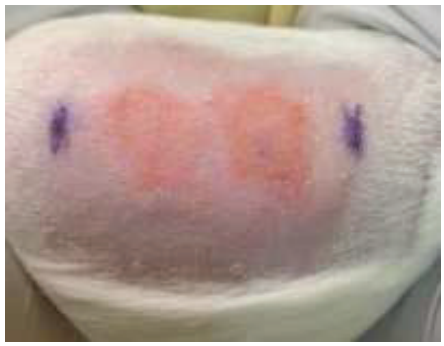
Table 4. **Cross-reactions between Disperse Orange 1, Disperse Yellow 3, their potential metabolites and *p*-phenylenediamine in 24 test and 12 control animals**

□nduction substance	Challenge substances											
	DO1		4- nitroaniline		PADPA		D□ 3		2-APC		4-AAA		PPD	
	□	*p*	□	*p*	□	*p*	□	*p*	□	*p*	□	*p*	□	*p*
DO1														
Test	22	<0.001	1	>0.3	21	<0.001	0	>0.3	1	>0.3	1	>0.3	1	>0.3
Control	2		0		0		0		0		0		1	
PADPA														
Test	23	<0.0028	1	>0.3	22	<0.001	2	>0.3	3	>0.3	1	>0.3	17	>0.3
Control	6		0		2		1		1		0		7	
4-nitroaniline														
Test	□A		5	>0.3	□A		□A		□A		□A		□A	
Control			2											

Abbreviations□PADPA, *p*-aminodiphenylamine□DO1, Disperse Orange 1□D□3, Disperse□ellow 3□2-APC, 2-amino-*p*-cresol□4-AAA, 4-aminoacetanilide□PPD, *p*-phenylenediamine□*p*, *p*-value□□, number of positive guinea pigs□□A, not assessed.

Figure 1. Positive reactions in the guinea pig when testing with coloured substances. A □a clearly positive patch test reactions□□ □a coloured patch test area when erythema can be mas□ed by the colour of the substance.

A.



□.