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Permanent hair dyes
Permanent hair dyes

Exposure, diagnostics, and prevention of contact allergy

Anarita Antelmi

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Lilla Aulan, Medicinskt Forskningcentrum,
Jan Waldenströms gata 5, Skåne University Hospital, Malmö
Friday 10 March 2017 at 9.00 am.

Faculty opponent
Professor Giorgio Assennato
Emeritus of Environmental and Occupational Medicine,
Department of Biology, University of Bari, Bari, Italy
Permanent hair dyes are widely used for hair coloring and the substances used are often well known sensitizers, causing contact allergy and dermatitis in hairdressers and consumers. The aromatic amines p-phenylenediamine (PPD) and toluene-2,5-diamine (TDA) are both important components of permanent hair dyes and extremely strong sensitizers. The prevalence of contact allergy to PPD in the general population in Europe was reported to be 0.8%. Allergic contact dermatitis may especially in those colouring their hair have very dramatic clinical appearance, sometimes giving rise also to systemic reactions. Due to the daily exposure to skin irritants and contact allergens as permanent hair dyes ingredients, hairdressers have an increased risk of developing occupational hand eczema, which often can be a reason to leave the profession.

Exposure: one of the aims of this thesis was to investigate the exposure to known sensitizers in hairdyes in products bought in different countries and on the internet. We performed chemical analysis of the most important allergens (PPD and 2,5-TDA) in 52 samples of permanent hair dyes. The findings of the chemical analysis were compared with the labelling of the products. The products purchased in Europe contained concentrations of PPD and 2,5-TDA within the limits stated by the European Union (EU) regulations, whereas the concentration of PPD in products purchased outside Europe were higher and in two cases exceeded up to four times the limits of the EU regulations.

Diagnostics: the aim of study IV was to improve patch testing, trying to find the best patch test preparations (also with regard to chemical form and concentration) for the hair dye allergens PPD and 2,5-TDA. We tested 2,477 consecutive dermatitis patients at the Department of Occupational and Environmental Dermatology (DOED) in Malmö with two different groups of hair dye ingredients. As has been found previously PPD was the best hair dye marker. The patch test concentration of 1.0% in petrolatum (pet.) should remain as patch test preparation in the baseline series. The free forms, PPD and 2,5-TDA, trace more contact allergy than the respective salt. The patch testing with 2,5-TDA can be optimised and should be considered within the hairdressers series and when the suspicion of hair dye allergy is raised especially in countries where exposure to 2,5-TDA or 2,5-TDA sulphate (2,5-TDA-S) is more common than PPD.

Prevention of contact allergy: in studies I and III, we investigated in vivo the performance of protective gloves used by hairdressers, mimicking their exposure to chemicals during the hair dye procedure with oxidative dyes. In study I we observed poor protective performance by many of the glove materials commonly used by hairdressers (natural rubber latex, polyvinyl chloride, polyethylene), when tested with a permanent hair dyes containing PPD in PPD-sensitised individuals. Nitrile gloves showed an excellent performance even at the longest exposure time of 60 minutes.

In study III, the same in vivo provocation test system was used to test 3 different professional hair dyes (containing PPD, 2,5-TDA-S, and 2-methoxymethyl-PPD, respectively) and an hair dye intended for home-use (containing 2,5-TDA-S) with different glove materials in volunteers allergic to PPD and 2,5-TDA. We found that nitrile gloves protect efficiently against all the hair dyes tested. Nitrile gloves can thus be recommended to hairdressers and customers to protect the skin during hair dyeing tasks. The polyethylene gloves provided in the package of the hair dye for home-use protected the skin against the recommended hair dye in the sold kit.

Key words: permanent hair dye, contact allergy, p-phenylenediamine, toluene-2,5-diamine, hairdressers, nitrile gloves, high performance liquid chromatography, polyvinyl chloride gloves, in vivo test.

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Signature __________________________ Date ____________
Permanent hair dyes

Exposure, diagnostics, and prevention of contact allergy

Annarita Antelmi

Department of Occupational and Environmental Dermatology
Lund University
Skåne University Hospital, Malmö, Sweden

Malmö 2017
To my beloved parents
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<th>Main findings and conclusions</th>
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<td>I. Are gloves sufficiently protective when hairdressers are exposed to permanent hair dyes? An in vivo study</td>
<td>To investigate, with an in vivo method, the protective capacity of different gloves used by hairdressers against a hair dye containing p-phenylenediamine.</td>
<td>4 different glove materials (polyvinyl chloride, natural rubber latex, nitrile, and polyethylene) were tested with a hair dye containing p-phenylenediamine using an in vivo method, in 8 volunteers who were already sensitized to p-phenylenediamine.</td>
<td>Allergens formed in a p-phenylenediamine-containing hair dye can permeate through many of the gloves commonly used by hairdressers. When these gloves are used in hair dyeing the user—if sensitized—will run the risk of developing allergic contact dermatitis. Nitrile gloves gave excellent protection even with at the longest exposure time (60 minutes).</td>
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<td>II. Evaluation of concordance between labelling and content of 52 hair dye products: overview of the market of oxidative hair dye. Labelling and content of hair dye products</td>
<td>To compare the labelling with the finding of allergens by chemical analysis in hair dyes sold in different countries and on the internet.</td>
<td>Chemical analysis of the products was performed with high performance liquid chromatography to detect the content of p-phenylenediamine, toluene-2,5-diamine and 3 oxidation products of p-phenylenediamine. The labelling of the products was compared with the results of the chemical analysis.</td>
<td>Only a small group of hair dyes sold in Europe were mislabelled. Toluene-2,5-diamine or toluene-2,5-diamine sulphate are prevalent in products sold in northern Europe whereas p-phenylenediamine in southern Europe and outside Europe. 52 products purchased outside Europe contained concentrations of p-phenylenediamine that were up to four times than the limit for such concentrations stipulated in European Union legislation.</td>
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<td>III. In vivo evaluation of the protective capacity of different gloves against hair dyes</td>
<td>To further investigate the protective capacity of nitrile and polyvinyl chloride gloves against three professional hair dyes containing p-phenylenediamine, toluene-2,5-diamine sulphate and 2-methoxymethyl-p-phenylenediamine respectively. The gloves provided in home-use hair dye kits were also tested.</td>
<td>Nitrile, polyvinyl chloride and polyethylene gloves were tested in 8 subjects already sensitized to p-phenylenediamine and toluene-2,5-diamine, using 4 permanent hair dyes and the in vivo open chamber system.</td>
<td>Nitrile gloves protect the skin for up to 45 minutes against permanent hair dyes containing different hair dye allergens. The disposable polyethylene gloves are safe when used with dye for home use.</td>
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<td>IV. Is it possible to optimise patch testing with hair dye ingredients? Patch testing of 2,477 consecutive dermatitis patients in Malmö, Sweden</td>
<td>To evaluate the optimal test substance for detection of permanent hair dye contact allergy. To study the reactivity patterns of individuals who were allergic to one or more of the two main hair dye colouring substances, when these were tested as base and salt (toluene-2,5-diamine sulphate, p-phenylenediamine dihydrochloride), and to some oxidation products of p-phenylenediamine (4,4’-azodianiline, 4-nitroaniline).</td>
<td>2,477 consecutive dermatitis patients from the Department of Occupational and Environmental Dermatology in Malmö between 2013 and 2016 were patch tested with different hair dye allergens: 1.0% p-phenylenediamine and 1.0% toluene-2,5-diamine sulphate, in the first time period (July 2013 to November 2015) and an extended series of hair dye ingredients in the second time period of the study (November 2015 to September 2016).</td>
<td>When testing with p-phenylenediamine and/or toluene-2,5-diamine the salts should not be used because they trace less contact allergy to hair dyes than the respective free forms. Toluene-2,5-diamine tested as free base is, however not a better tracer of contact allergy to hair dyes than p-phenylenediamine base. When testing patients with a baseline series containing concentrations of p-phenylenediamine less than 1.0% there is a risk of underestimating the actual frequency of contact allergy and there is a clear risk of missing relevant contact allergic reactions.</td>
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</table>
List of Publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Are gloves sufficiently protective when hairdressers are exposed to permanent hair dyes? An in vivo study
   Antelmi A, Young E, Svedman C, Zimerson E, Engfeldt M, Foti C, Bruze M.
   Contact Dermatitis. 2015;72:229-36

II. Evaluation of concordance between labelling and content of 52 hair dye products: overview of the market of oxidative hair dye. Labelling and content of hair dye products
   Antelmi A, Bruze M, Zimerson E, Engfeldt M, Young E, Persson L, Foti C, Sörensen Ö, Svedman C.

III. In vivo evaluation of the protective capacity of different gloves against hair dyes
    Antelmi A, Bruze M, Zimerson E, Engfeldt E, Foti C, Svedman C
    In manuscript

IV. Is it possible to optimise patch testing with hair dye ingredients? Patch testing of 2,477 consecutive dermatitis patients in Malmö, Sweden.
    In manuscript

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# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACD</td>
<td>allergic contact dermatitis</td>
</tr>
<tr>
<td>CA</td>
<td>contact allergy</td>
</tr>
<tr>
<td>CD</td>
<td>contact dermatitis</td>
</tr>
<tr>
<td>D</td>
<td>day</td>
</tr>
<tr>
<td>DOED</td>
<td>Department of Occupational and Environmental Dermatology</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>HDhu</td>
<td>hair dye intended for home use</td>
</tr>
<tr>
<td>HDhu-TDA-S</td>
<td>hair dye for home use containing 2,5 TDA sulphate</td>
</tr>
<tr>
<td>HDp</td>
<td>hair dye for professional use</td>
</tr>
<tr>
<td>HDp-ME-PPD</td>
<td>hair dye for professional use containing ME-PPD</td>
</tr>
<tr>
<td>HDp-PPD</td>
<td>hair dye for professional use containing PPD</td>
</tr>
<tr>
<td>HDp-TDA-S</td>
<td>hair dye for professional use containing 2,5 TDA sulphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>ICD</td>
<td>irritant contact dermatitis</td>
</tr>
<tr>
<td>ICDRG</td>
<td>International Contact Dermatitis Research Group</td>
</tr>
<tr>
<td>KC</td>
<td>keratinocytes</td>
</tr>
<tr>
<td>LCs</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>4,4´-MDA</td>
<td>4,4´-diaminodiphenylmethane</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NI</td>
<td>nitrile</td>
</tr>
<tr>
<td>NRL</td>
<td>natural rubber latex</td>
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<tr>
<td>PE</td>
<td>polyethylene</td>
</tr>
<tr>
<td>pet</td>
<td>petrolatum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PPD</td>
<td>p-phenylenediamine</td>
</tr>
<tr>
<td>PPD-DHC</td>
<td>p-phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>PVC</td>
<td>polyvinyl chloride</td>
</tr>
<tr>
<td>2,5-TDA</td>
<td>toluene-2,5-diamine</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight/weight</td>
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</table>
1 Introduction

1.1 Contact allergy and allergic contact dermatitis

Contact allergy (CA) is an altered immune status of an individual induced by a particular sensitizing substance, a contact allergen (1).

Contact dermatitis (CD) is an inflammatory skin reaction caused by direct contact with allergic or irritant agents in the environment. The pathological mechanism may involve immunological hypersensitivity (giving rise to allergic contact dermatitis) no immunological hypersensitivity (resulting in irritant contact dermatitis), or it may be mixed. The group of contact dermatitis include also immediate skin reactions like non-immunological contact urticaria, immunological contact urticaria and contact urticaria syndrome that usually start within 30–60 min following the skin exposure to an eliciting substance, and clear completely within 24 h, although delayed-onset reactions may appear within 4–6 h (2).

Irritant contact dermatitis (ICD) is thus provoked by skin exposure to chemical(s) or/and physical factors that damage the skin barrier, with subsequent activation of unspecific innate immune responses (3).

Allergic contact dermatitis (ACD) is the clinical expression of CA, and consists on an inflammatory skin reaction resulting from exposure to a contact allergen. The CA immune-pathological mechanism involves allergen-specific T-cells, which are mediators of cell-mediated immunity, causing a delayed hypersensitivity as classified by Gell and Coombs (type-IV hypersensitivity) (4). The immunological events that lead to ACD are characterised by two phases: the induction phase and the elicitation phase. First, the induction phase (also known as the sensitisation phase) is when small molecules penetrate the skin barrier and conjugate with endogenous epidermal and dermal molecules. The substances that induce CA are reactive chemicals, usually with a molecular weight of < 500 Da but sometimes in the range of 500–1,000 Da. They are not antigenic by themselves and are therefore referred to as haptens. Whether or not a chemical can cause an allergic contact dermatitis depends on other factors also: the capacity of the molecule to penetrate the horny layer of the skin, its lipophilicity, and its chemical reactivity (1, 3). The hapten readily associates with major histocompatibility complex (MHC) class I
and class II molecules, which are present on epidermal Langerhans cells (LCs). After processing of the haptens, MHC I/II molecules (depending on the nature of the hapten) trigger the migration of epidermal LCs, which—through the afferent lymphatic vessels—reach the lymph nodes where they encounter naïve T-cells. The T-cells recognize the allergen-MHC complex and hapten, and specific T-cells can then expand, generating effector and memory cells, which are released into the circulation via the efferent lymphatics (5). The sensitisation phase usually goes unnoticed and requires from 4 days to several weeks. The individual is then immunologically sensitised, i.e. when encountering the allergen again in sufficient dose and for sufficient time, he/she will respond with a clinical manifestation, an ACD. This is the elicitation phase. It leads to specific T-cell activation with clinically visible development of ACD, and usually reaches its peak after 18–72 h after the exposure to the hapten. For some substances, the elicitation phase can be longer than 1–4 day—and sometimes more than 2–3 weeks (6-10). Not only the re-exposure to the initial hapten cause elicitation; cross-reacting substances will also make the clinical evaluation of ACD more difficult.

The typical appearance of ACD is erythema, infiltration, papules, oedema, and possibly vesicles. If the exposure to the allergen continues, the dermatitis may become chronic and present with scaling, fissures, and lichenification. The margins of the lesions are most often ill-defined, extending beyond the site of application of the allergen(s). Some areas have particular morphological variants: acute ACD of the scalp is erythematous with scaling; oedema is typical in regions where the skin is loose and thin —on the eyelids, the scrotum, the penis, and on the lower lobe of the ear. Allergic contact stomatitis and vulvitis usually present with erythema, or sometimes oedema. Pruritus is almost always present, and is very often severe (11).

Some variant of classical eczematous clinical manifestations of ACD are observed less frequently such as lichenoid, lymphomatoid, granulomatous, pigmented, purpuric, and erythema multiforme-like lesions. Lymphomatoid contact dermatitis is a chronic, persistent form of non-eczematous ACD, which may resemble parapsoriasis and early-stage mycosis fungoides both clinically and histopathologically. This reaction has been reported in the literature with different haptens such as p-phenylenediamine (PPD), diaminodiphenylmethane, and textile dyes (12-15). Purpuric ACD is mainly observed on the lower legs and/or feet and is caused by extravasation of erythrocytes into the dermal tissue and the epidermis, triggered by a variety of allergens (drugs, rubber, textile dyes, or plants). Lichenoid ACD mimics lichen planus and has been described associated with PPD in hair dyes (16), Primula obconica (17), nickel (18), epoxy resins (19) and drugs (20). Oral lichenoid ACD presents clinically as oral lichen planus, but usually close to the material causing the reaction—and it has been shown to be caused by metals in dental restorations (21, 22).
The location of eczematous lesions is usually limited to the site of contact with the culprit allergen, but even so, dissemination of the dermatitis with distant lesions may occur. ACD can sometimes be difficult to diagnose, for example “ectopic” ACD and airborne ACD. In ectopic ACD, the allergen can auto-transferred (for example, by the fingers) to other locations such as the face and particularly the eyelids—or to another person, as in the case of so-called connubial ACD (11). In airborne ACD, allergens are transported by air as dust particles, vapours, or gas. In most cases, the clinical manifestation involves the face, the neck, and/or décolleté. The main sources of airborne ACD are occupational allergens, cosmetics, and plants (23, 24). One case has been attributed to PPD in a temporary henna tattoo (25).

1.2 Diagnosis of contact allergy

In 1929, Bloch described in detail the technique that had previously been developed by Jadahsson—epicutaneous testing (patch testing)—in order to diagnose CA. Patch testing is still the most accurate method of establishing CA (26-28), even though in vitro techniques such as the lymphocyte transformation test give satisfying results at the group level when investigating metals, for example (29, 30). There are many advantages to the patch testing technique; thousands of allergens are available for patch testing, and it is also often possible to test the patient’s own material by patch test. There are also possible drawbacks with the technique: the patient has to come once for testing and twice for reading of the patch test. Today, the protocol for reading of patch test has been standardised (28-31) but it is well known that the readings can often be interpreted rather than registered, since the reading is subjective (27).

1.2.1 The patch testing procedure

When performing patch testing, the patient with suspected allergic contact dermatitis is re-exposed to a suspected allergen on intact skin under controlled conditions. Over the decades, the patch test technique has undergone standardizations and developments regarding the substances, concentrations, doses, vehicles, scoring, and so on (1, 32, 33). The substance with which we patch test and how we patch test with it —i.e. that the allergen is defined, the vehicle is correct, and the dose has been standardised—is the first step in accurately diagnosing CA. The baseline series must be updated regularly (34).

It is recommended that the reactions be scored according to the International Contact Dermatitis Research Group (ICDRG) criteria: –, negative reaction; ?+, doubtful reaction; +, weak positive reaction; ++, strong positive reaction; ++++, extremely positive reaction; and IR, irritant reaction (1, 35).
Doubtful reactions are reactions that do not fulfil the criteria of the ICDRG, i.e. there is only erythema and not infiltration covering the whole area, or there is only infiltration and not erythema covering the whole patch test area. By definition, a doubtful reaction is not an allergic reaction; however, it may be an allergic reaction that does not fulfil the criteria—for example, if the dose is not adequate at patch testing—i.e. the reaction may be false negative and may prove to be with retesting at a higher concentration. The easiest way to simplify this is when examining a dilution series in an allergic individual. As the dose is reduced, so will the reactivity be, and finally a doubtful reaction may be seen in a patient where it has been established that the individual is de facto allergic.

False-positive reactions are defined as positive reactions caused by irritation, with a morphology indistinguishable from a contact allergic reaction. The general principle is to patch test with the highest concentration of the allergen that does not provoke active sensitisation or irritation. Testing with serial dilutions of the test preparation and/or patch testing of controls may exclude the possibility that a reaction is false-positive. If the reaction is truly allergic, it is usually possible to decrease the concentration 100 times, giving a moderate patch test reaction without losing the possibility of eliciting a positive reaction (36).

False-negative reactions are defined as failure to elicit a positive patch test reaction even though the individual being tested has a contact allergy. An insufficient dose, a concentration that is too low, a substance that is unstable, systemic treatment with corticosteroids during patch testing, an improper vehicle or test chamber, and final reading that is too early, may all result in false-negative reactions (37-39).

Late patch test reactions are positive reactions that appear at the site of a previously negative patch test, later than day (D)7. Some allergens are known to cause late reactions. Well-known examples are when patch testing with gold or corticosteroids. A low degree of reactivity in the patient, a low concentration of the hapten, and/or slow penetration of the allergen are possible causes of late reactions. Regarding corticosteroids, the explanation is the anti-inflammatory effect of the substance. A late patch test reaction may indicate an active sensitisation caused by the patch test (40-42).

Active sensitisation is an adverse effect of patch testing. A negative patch test reaction is followed by a flare-up reaction after 10–20 days. In case of re-testing, a positive reaction appear on D3 to D7. Patch testing with serial dilutions of the allergen in question should be performed when active sensitisation is suspected (1, 35, 39, 41).
1.3 Hair dyes

1.3.1 The history of hair dyes

Throughout history, men and women have changed the natural colour of their hair or have restored the colour when, with age, it has become grey. Before the advent of synthetic organic chemistry, the use of natural products fulfilled this need (43).

Ancient Egyptians, Greeks and Romans used plant and animal extracts on a regular basis to colour the hair. Some 2,700 years ago, ancient Assyrians dyed their hair and beards black with unknown substances, or red with henna, and dusted their heads and beards with gold dust. It well known that the ancient Egyptians used henna (2-hydroxy-1,4-naphthoquinone) derived from the plant Lawsonia inermis to produce an orange tone in their hair.

In ancient Rome, one of the most popular ways for people to ornament themselves was through the use of hair dyes. The most popular hair colour was blond, which was associated with the exotic and foreign appearance of people from Gaul and Germany. The emperor Commodus, who ruled from 180 to 192 a.d., was especially famous for powdering his snow-white hair with gold. The Romans used a variety of methods and ingredients for dyeing their hair; some used henna and others used berries, vinegar, or crushed nutshells.

Little changed until the late 18th century, when industrial discoveries led to fantastic, but more toxic advances in textile dyeing. This became the foundation of modern hair dyeing practices.

In the 19th century, the English chemist William Henry Perkin made an accidental discovery that changed hair dyeing forever. In an attempt to generate a cure for malaria, Perkins created the first synthesised dye in 1863. The colour was named Mauveine (44). In the same year, his chemistry professor August Hoffman obtained a colour-changing molecule from Mauveine called p-phenylenediamine (PPD) and observed that the colourless PPD produced colour when exposed to oxidizing agents, including air (45). Twenty years later, Monnet (46) patented a process for colouring human hair based on Hofmann's observation.

In 1907, Eugene Schueller created the first chemical dye for commercial purposes, which he called “Aureole”. This product was later known as “L'Oréal”, as would the company that he founded.

The double process for dyeing hair blonde soon followed, and in 1932 hair dye was refined by chemist Lawrence Gelb who created a hair dye that actually penetrated the shaft of the hair.
His company was called “Clairol”. Later, in 1950, he introduced the first one-step hair dye product that actually lightened the hair without bleaching it.

1.3.2 Hair dyeing processes

Hair dyes are products used to colour hair and they can be roughly divided into direct and oxidative hair dyes. The oxidative hair dyes are by far the most frequently used group, representing almost 80% of the total hair dye market. The oxidative hair dyes (i.e. permanent hair dyes) basically consist of so-called precursors and cuplers, and the color formation is based on a series of oxidation and coupling reactions. The precursors are aromatic compounds derived from benzene, substituted by amino- and/or hydroxy- groups in para or ortho positions such as the aromatic amines PPD and toluene-2,5-diamine (2,5-TDA). The cuplers, are aromatic compounds derived from benzene and substituted by amino- and/or hydroxy- groups in meta position, such as resorcinol and m-aminophenol (47). The addition of alkalizing compounds is necessary for the process of hair dyeing to promote the proper pH value for the beginning of the oxidation reaction. The alkaline medium promotes the opening of the cuticles that allows the penetration of the dyes’ molecules into the cortex. The oxidizing agent permits the beginning of the reaction that occurs in the cortex and results in a colorful complex with high molar mass, which avoids the exit of molecules formed in the hair (48). The hydrogen peroxide has a dual function of oxidizing and decolourizing melanin in hair which lightens the underlying hair colour, and oxidizing the dye precursors to form synthetic colour.

The oxidative hair dye products on the market may contain various combinations of more than 100 different precursors and cuplers (49). The global market for hair dye is constantly growing, and was estimated to be 7 billion dollars in 2015. This is expected to grow by 8–10% a year over the next five years (50). According to the International Agency for Research on Cancer (IARC), 50–80% of all women in the Europe, in the USA, and in Japan have used hair dye (51).

1.3.2.1 The structure of hair

The hair is an epidermis-derived structure comprising the hair follicle in the skin and the hair shaft, which is visible on the surface of the body (52, 53).

The follicle is the essential growth structure of hair. The outside layer of each hair follicle starts from a germinating layer of the epidermis that grows down into the dermis. The dermis then grows upwards into the base of the follicle to form the dermal papilla. This allows capillaries (blood vessels) to enter the papilla and provide nutrients for the hair shaft to grow. The outer root sheath, i.e. the inner wall of the follicle, has been identified as a reservoir of multipotent keratinocyte
and melanocyte stem cells and contains keratinocytes that surrounds the dermal papilla. The outer root sheath forms a distinct bulge area between the insertion of the arrector pili muscle and the duct of the sebaceous gland. The bottom part of the follicle enlarges into an area of actively growing cells. This is called the hair bulb (54). The inner root sheath consists of three layers: Henle’s layer, Huxley’s layer, and cuticle layer. The inner root sheath cuticle layer adjoins the cuticle of the hair shaft, anchoring the hair shaft to the follicle (53).

The shaft of a hair has three layers: the cuticle (outer layer), the cortex (middle layer), and the medulla (inner layer). The cortex is the main bulk of a fully keratinized hair shaft. It gives flexibility and tensile (stretching), strength to hair and contains melanin granules, thus contributing most to the colour and the mechanical properties of the hair (55). The cortex consists of tiny fibers of keratin, running parallel to each other along the longitudinal axis of the hair shaft, and an amorphous matrix of proteins with high sulphur content (56).

The cuticle is made from 6 to 8 layers of overlapping semi-transparent keratin scales with their free edges directed upward to the tip of the hair shaft. The normal cuticle has a smooth appearance, allowing reflection of light and limiting friction between the hair shafts. It is responsible for the texture of the hair.

The medulla may be continuous, may occur intermittently along the hair shaft, or may be absent. It is a honeycomb keratin structure with air spaces inside. The primitive insulating function of the medulla is now redundant, and this layer plays no role in the process of hair cosmetics (55).

Hair colour is determined by the melanocytes found only in the matrix area of the follicle at the base of the cortex, directly above the follicular papilla. The melanin pigment is found in the cortex of the hair (57). The proportions of eumelanin and pheomelanin and the total amount of melanin determine the final natural colour of the hair.

1.3.3 Physico-chemistry of hair dyeing

Hair dyes are classified as follows according to their origin: synthetic, vegetable, and metallic.

1.3.3.1 Synthetic hair dyes

Synthetic hair dyes fall into five main categories based on the duration of their colouring effect: permanent, semi-permanent, demi-permanent, temporary (58-60) and hair bleach (61).

Permanent hair dyes give permanent hair colouring through a chemical process whereby small precursor molecules penetrate into the hair matrix; high-molecular-
weight dyes are formed by reaction of the hair dye precursors with hydrogen peroxide and they become permanently fixed inside the hair fibre.

The development of colour in the oxidative hair colouring process requires three groups of chemical reactants (48, 52, 62-67).

The first class, “primary intermediates”, comprises ortho- and para-substituted amino aromatics, aminophenols, and phenylenediamines. The second class, “couplers”, consists of meta-substituted phenols such as resorcinols and derivatives of aniline—among others, m-aminophenols and m-phenylenediamines. The third one is the oxidising agent, usually hydrogen peroxide. The process involves an oxidation of the primary intermediates to form a reactive mono- or di-imine, which then reacts with a coupler to give a colourless diphenylamine. The process of dyeing requires alkaline conditions (pH 9.0–10.0), usually provided by the use of ammonium hydroxide in the dye lotion. The oxidation process of p-phenylenediamine results in molecules much larger than the precursor, which causes the dye to bond to the hair (62). The effect of hydrogen peroxide causes the original hair colour to be lightened, which provides a blank canvas for the dye. Ammonium hydroxide is the best alkalising agent for enhancement of penetration of the dye precursors into the cortex of the hair fiber so that the dye can actually bond with the hair, it is also the most effective at promoting bleaching of melanin by hydrogen peroxide. Various combinations of primary intermediates and couplers provide a spectrum of shades of hair colour.

Temporary hair colouring are available in various forms, including rinses, shampoos, gels, sprays, and foams. Temporary dyes consist of acid water-soluble molecules of high molecular weight that do not penetrate the cuticle and that are deposited into the surface layers. After their application, the colour of the hair lasts for up to a week depending on the number of washing procedures used (68).

The semi-permanent dyes may be oxidative or non-oxidative (61). Non-oxidative semi-permanent hair dyes are used to enhance colour and to modify grey hair, but they cannot lighten the hair colour because no bleaching agents are involved (69). They withstand 5 or 6 shampooings and generally consist of different kinds of low-molecular weight organic dyes (70). Semi-permanent hair dyes have smaller molecules than temporary dyes, and they are therefore able not only to penetrate into the hair cuticle but also, in part, to diffuse throughout the cortex. For this reason, the colour will survive repeated washing, typically 4–5 shampooings or a few weeks. The common semi-permanent colourants are generally classified as follows: nitroaniline, nitrophenylendiamines, nitroaminophenols, azoic compounds, and anthraquinone compounds. These colouring agents allow achievement of a wide range of shades, which are strongly dependent on the nature of the moiety bound to the aromatic ring and on the pH of application. These kinds of dyes are also known as “dispersed dyes” and are often used in the
textile industry. Their low water solubility is increased by adding to the dye bath an organic solvent that is partially soluble in water, which improves the solubility of the dye and increases the uptake by the hair by simplifying its diffusion through the hair cuticle (by inducing swelling) (71-77).

Oxidative demi-permanent synthetic dyes are permanent hair colours that contain lower amounts of hydrogen peroxide (2%) and low levels of alkalising agents (generally monoethanolamine rather than ammonia), so hair penetration is more efficient than with non-oxidative semi-permanent dyes, but less so than with permanent dyes (78). These dyes are used to enhance the natural colour, brighten it up, or cover up to 50% of grey hair, but they have little hair-lightening potential (52,78) since the alkaline agents used employed in demi-permanent colours are less effective in removing the natural hair pigment than ammonia.

Bleaching is a chemical process for removal of some or all the natural or synthetic colour from the hair. Hydrogen peroxide and ammonium hydroxide are common bleaching agents that oxidise existing melanin (69, 77). Any colouring treatment to transform the original colour to a lighter one requires bleaching. After that, a light-coloured permanent or semi-permanent dye or toner may be applied.

1.3.3.2 Vegetable hair dyes

Vegetable hair dyes (based on plants, e.g. Lawsonia inermis, Matricaria chamomilla, and Cinchona officinalis) are temporary and usually wash out within 8-10 shampooings. The vegetable hair dyes are not used for drastic hair colour transition. Thus, the result of the hair colouring is not widely different from the original hair colour. They are considered to be less harmful to the hair and safer than the other hair dyes.

1.3.3.3 Mineral or metallic hair dyes

Metallic dyes get their colour from “metallic salts” e.g. silver nitrate or lead salts. The metallic salt reacts with the sulphur in the protein chains of the hair to create a natural looking colour in the hair over a period of repeated exposure. Requiring daily use, they darken or lighten hair gradually and can may last for weeks or months.

1.3.4 Risk of exposure—permanent hair dyes

Hair coloring involves the use of chemicals capable of removing, replacing, and/or covering up pigments naturally found inside the hair shaft. Use of these chemicals can result in a range of adverse effects, including temporary skin irritation and IgE-mediated allergic reactions. In addition, there is an ongoing debate regarding more serious health consequences of the use of hair colour, including lead.
poisoning. Due to daily exposure to several chemicals, skin irritants, and allergens, hairdressers have a high risk of developing occupational diseases such as hand eczema, asthma, rhinitis, and contact urticaria (79). The common ingredients of oxidative hair dyes, PPD and 2,5-TDA (80), are recognized as extreme skin sensitisers (49) and are a well-known cause of delayed CA in both hairdressers and consumers. The prevalence of PPD sensitization in the general European population is between 0.0% and 2.5% (81, 82). The prevalence of PPD allergy among dermatitis patients was found to be 4.3% in Asia, 4% in Europe, 6.2% in North America, and 2% in Australia (81, 83).

Clinically, hair dye contact dermatitis can present as both irritant dermatitis and allergic contact dermatitis (ACD), the latter with severe oedema of the scalp, face, eyelids, ears, and beard skin—which are the sites commonly affected by hair dye use, whereas the hands are often involved in occupational exposure of hairdressers and barbers. Moreover, cases of contact urticaria, immediate-type hypersensitivity (84, 85), and anaphylactic reactions due to PPD have been reported in the past (85-88). Cases of systemic reaction to PPD have also been reported in users of hair dyes containing PPD—in the form of erythema multiforme, which is a mucocutaneous condition of uncertain etiopathogenesis generally triggered by factors such as herpes simplex virus infection or drugs. Erythema multiforme induced by contact dermatitis is rare (89), and has been described following allergic contact dermatitis to PPD in a hair dye (90). Another infrequent clinical form of ACD is the lichenoid eruptions reported in 4 cases of users of hair dyes containing PPD (91). A case of cutaneous pseudolymphoma, (a reactive polyclonal benign lymphoproliferative process that simulates a lymphoma), described as PPD-induced, has been reported in the moustache area in a man who had been dyeing his moustache once a month for over two months (92).

A case of systemic dermatitis from a hair dye has been observed at the Department of Occupational and Environmental Dermatology (DOED) in Malmö, in a woman who had been dyeing her hair for a long time (93).

In Asian and African countries, PPD as a constituent of hair dyes has become an emerging cause of intentional self-poisoning to commit suicide through ingestion, due to the availability of the substance on the low cost market (94, 95). The acute systemic PPD poisoning presents with characteristic angioedema, upper airway obstruction, rhabdomyolysis, methemoglobinemia, myoglobinuria, and acute renal failure. It has been discussed in the scientific community to create awareness about the adverse effects of hair dye ingestion—considering also the lack of specific diagnostic tests and specific antidotes for PPD poisoning (94, 95).

Occupational risk of bladder cancer in hairdressers and in users of permanent hair dyes products has been examined in many epidemiological studies. The IARC has concluded that hairdressers and barbers are “probably” at greater risk of bladder
cancer because of their exposure to hair dyes (96). A meta-analysis conduced by Harling et al. and including 42 studies has shown good evidence of an increased risk of bladder cancer in hairdressers, particularly with hairdressers in jobs that have been held for ≥10 years (97). However, according to the IARC hair dye products have not yet been classified as carcinogenic when used for personal use (51). It has been estimated that in 1995 (98), over one-third of women over the age of 18 and more than 10% of men over the age of 40 in Europe and North America used some kind of hair dye. Thus, there has also been concern about a possibly increased risk of bladder cancer in users of hair dyes. The study by Gago-Dominguez et al. (99) suggested that there was an increased risk of bladder cancer from use of permanent hair dye, which was more pronounced in women who had used hair dyes more than 12 times per year for more than 15 years. On the other hand, further studies and expanded meta-analyses of personal hair dye exposure and bladder cancer, also focusing on the biological plausibility of a systemic hazard to human health from exposure to the key chemical in hair dyes (PPD), have not supported an association between personal hair dye use and bladder cancer risk (100, 101). The personal use of hair dye does not appear to be associated with a specific cancer risk (102-104).

1.4 Occupational skin disease

1.4.1 Occupational disease in hairdressers

1.4.1.1 Hairdressers and hand eczema

Hand dermatitis is common and can be caused by endogenous, exogenous and mixed aetiology. There are certain occupations that naturally have a higher risk of hand dermatitis (105), i.e. professions with a lot of wet work, manual work, and skin contact with chemicals. Among these is hairdressing. Previous studies on the occurrence of hand eczema in hairdressers give a cumulative prevalence of 17-42% (106, 107). Among European hairdressers, PPD sensitization is quite frequent, because it can be as high as 20% (108, 109).

Regarding hairdressers, several studies has been performed indicating that exposure to both irritants and haptens causing contact allergy contributes to this (110-113). Atopic individuals who are hairdressers have a worse prognosis. Much effort has been made to investigate what can be done to improve the working conditions for hairdressers. It has been shown that educative programmes regarding prevention do improve the skin condition, and the correct use of gloves has been emphasised. With regard to gloves the focus has been on their correct use (114-117).
For many working in risk occupations, gloves are the best means of protection—even though the use of gloves is in itself a risk factor (118-120). In the healthcare setting, gloves are a way of protecting both the patient and the healthcare professional—and this is the main driving factor in the development of new kinds of gloves. In the occupational field, the type of glove used will otherwise depend on the nature of the chemicals involved. In all environments where gloves are used, the chemical resistance of each glove material is also influenced by the exposure time and the conditions under which the work is performed. Standard *in vitro* test methods have been developed in the USA and Europe to assess the protective efficacy of gloves against different chemicals (121, 122), and these are performed by the manufacturers before marketing. Previously performed *in vitro* studies have assessed the resistance to permeation by permanent hair dye chemicals through protective gloves. Lind et al. (123) observed a considerable degree of protection by the gloves tested: natural rubber latex (NRL), polyvinylchloride (PVC), nitrile (NI), and polyethylene (PE) against PPD, toluene-2,5-diamine sulphate (2,5-TDA-S), and resorcinol without adding hydrogen peroxide. The gloves were tested for up to 4 hours and all the materials withstood permeation well, giving protection for ≥ 30 min. Lee and Lin (124) performed an in vitro investigation of the permeation behaviour of p-aminophenol, m-aminophenol, and PPD in single and mixed challenge solutions (with ethanol or hydrogen peroxide) using disposable NRL, PVC, and neoprene gloves. Their results showed that hydrogen peroxide did not accelerate the permeation of hair dye through the gloves. NRL and PVC were not recommended for repeated handling of permanent hair dyes, and good protection by neoprene gloves was found for at least 8 hours.

*In vitro* methods cannot be expected to represent all conditions found under normal working circumstances. The protective efficacy of gloves can be influenced by factors such as occlusion, sweating, stretching, and skin temperature.

1.4.1.2 Hairdressers and prevention

Some of the chemicals used in hairdressing—such as persulphate salts in hair bleaching—are airborne allergens that are responsible for occupational asthma and occupational rhinitis (125, 126).

The general advice given of a study performed by Gube et al. was related to the constant use of suitable gloves for hairdressers and the performance of tasks in which the gloves cannot easily be worn, such as cutting before dyeing. The biomonitoring of the hairdressers’ exposure to permanent hair dyes showed the usefulness of protecting oneself by wearing adequate gloves (127). An interventional study (128) has evaluated the biomonitoring of aromatic amines before and after introduction of the regular use of gloves during the hair dyeing
procedures over a period of 2 weeks. A significant protective effect on the PPD and 2,5-TDA dermal loading and biomarkers concentrations was observed, though without any specific indications about the most suitable glove material (128).
2 Aims

2.1 Studies I and III

We wanted to study the efficacy of protective gloves used by hairdressers. An in vivo method for evaluating the exposure of allergens through glove material and the risk of ACD was used with usual hair dyes tested as is, and different gloves.

In study I, gloves recommended for hairdressers in different European countries were evaluated using a hair dye containing PPD.

In study III, based on the findings from study I, NI gloves and some gloves that are mainly used on the Swedish market were evaluated using hair dyes containing PPD and 2,5-TDA, and also a recently developed hair dye containing 2-methoxymethyl-p-phenylenediamine (ME-PPD).

2.2 Study II

The objective was to investigate the content of some hair dye ingredients (primarily PPD and 2,5-TDA) in hair dye products sold in different countries of the world and on the internet, their accordance with the labelling and with the content limits required by the European legislation in order to get an estimate of the possible exposure of workers and customers.

2.3 Study IV

This study was performed to optimise the diagnostics of contact allergy to permanent hair dye. Two thousand four hundred and seventy-seven consecutive dermatitis patients from DOED in Malmö were patch tested with several hair dye allergens during the period July 2013 to September 2016.
3 Materials and Methods

3.1 Subjects

3.1.1 Studies I and III

Eight subjects (7 females and 1 male, aged 20–61 years, median 37 years) and seven subjects (6 females and 1 male, aged 19–60 years, median 27 years) were enrolled as volunteers in study I and in study III respectively. One of volunteers participated in both the studies. They had been patch tested previously at the DOED in Malmö, and had shown ++ or +++ reactions, according to ICDRG criteria (1, 35), only to 1% PPD in petrolatum (pet) in those enrolled in study I and to 1% 2,5-TDA-S and 1% PPD both in pet, in study III. They had been sensitised either through occupational use or as consumers using hair dyes or so-called black “temporary henna” tattoos. All of them took measures against exposure to PPD since found allergic and they had not had any recent reactions to products used for skin or hair care. Exclusion criteria were sensitisation to fragrances and rubber derivatives.

3.1.2 Study IV

3.1.2.1 Time period I

One thousand seven hundred and fifty-nine consecutive dermatitis patients (1,167 females and 592 males) who had been patch tested at the DOED in Malmö were patch tested with two hair dye sensitisers included in the baseline series (1% PPD in pet and 1% 2,5-TDA-S 1.0% in pet).

3.1.2.2 Time period II

Seven hundred and eighteen consecutive dermatitis patients (500 females and 218 males) who had been patch tested at the DOED in Malmö, were patch tested with PPD as included in the baseline series, and with an extended series of hair dye ingredients containing p-phenylenediamine dihydrochloride (PPD-DHC), 2,5-TDA, 2,5-TDA-S, and two newly detected oxidation products of PPD, 4-nitroaniline and 4,4’-azodianiline (129).
3.2 Gloves

3.2.1 Study I

Six different gloves were chosen from twenty-four glove samples acquired in specific hairdresser shops or from hairdressers in three European countries (Sweden, Italy, Germany). The gloves were made of 4 different materials: PVC, NRL, NI, and PE (Table 1). The aim was to test the glove materials commonly used by hairdressers, so the choice of gloves was based on workplace visits in Malmö and Bari and on recommendations of local Consumer Union regarding protective gloves.

The 4H\textsuperscript{®}-glove (made of layers of polyethylene and ethylene-vinyl-alcohol laminate) (North Safety Products by Honeywell, Smithfield, RI, USA) was used as a negative control, being highly resistant to a wide range of hazardous chemicals.

\textbf{Table 1.}

Gloves tested in study I. The thickness on palm/back of gloves was measured at the laboratory in Malmö.

<table>
<thead>
<tr>
<th>Glove</th>
<th>Gloves (commercial name)</th>
<th>Manufacturer/Suppliers</th>
<th>Thickness (mm)*</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Medical Exam gloves\textsuperscript{®}</td>
<td>Abena A/S, Aabenraa Denmark</td>
<td>0.034</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>B</td>
<td>Guanto Monouso\textsuperscript{®}</td>
<td>M.A.RE.B, Milano, Italy</td>
<td>0.029</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>C</td>
<td>Guanti in lattice\textsuperscript{®}</td>
<td>Ro.ial., Prato, Italy</td>
<td>0.038</td>
<td>Natural Rubber Latex</td>
</tr>
<tr>
<td>D</td>
<td>Walking innovative\textsuperscript{®}</td>
<td>Brenta, Venezia, Italy</td>
<td>0.035</td>
<td>Nitrile</td>
</tr>
<tr>
<td>E</td>
<td>Alfatex 30 Nitril blau\textsuperscript{®}</td>
<td>Sänger GmbH, Schrozberg, Germany</td>
<td>0.040</td>
<td>Nitrile</td>
</tr>
<tr>
<td>F</td>
<td>Vinyl 300\textsuperscript{®}</td>
<td>Latexha W. Nentwich, Diepolsau, Switzerland</td>
<td>0.037</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>H (Negative control)</td>
<td>Silver Shield 4H\textsuperscript{®}</td>
<td>North Safety Products by Honeywell, Smithfield, USA</td>
<td>0.04</td>
<td>Polyethylene-ethylene-vinyl-alcohol</td>
</tr>
</tbody>
</table>

*mean value of 3 measurements
3.2.2 Study III

This study was based on findings from study I and the choice of gloves were therefore: 2 kinds of NI gloves, 1 kind of PVC glove, and one kind of disposable glove provided in the package of a permanent hair dye intended for home-use and labelled as plastic (Table 2). As a negative control, the 4H®-glove was used.

Table 2.
Gloves tested in the study III. Data according to the manufacturers/suppliers except for the thickness on palm/back of gloves which was measured in our laboratory. Also the material of the gloves provided with the home-use hair dye was analysed.

<table>
<thead>
<tr>
<th>Gloves and Manufacturer</th>
<th>Material</th>
<th>Average Thickness (mm)*</th>
<th>Source of selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semperguard® Semperit</td>
<td>Nitrile</td>
<td>0,08</td>
<td>Used by hairdressers in Malmö</td>
</tr>
<tr>
<td>Papyrus® supplies vinyl powdered Papyrus Supplies</td>
<td>Polyvinyl chloride</td>
<td>0,10</td>
<td>Used by hairdressers in Malmö</td>
</tr>
<tr>
<td>Papyrus® supplies Nitrile indigo fine Papyrus Supplies</td>
<td>Nitrile</td>
<td>0,13</td>
<td>Used by hairdressers in Malmö</td>
</tr>
<tr>
<td>Plastic gloves contained in Schwarzkopf hair dye (Natural &amp; Easy 590)®</td>
<td>Polyethylene**</td>
<td>0,03</td>
<td>Contained in Schwarzkopf hair dye (Natural &amp; Easy 590)</td>
</tr>
<tr>
<td>4H® silver shield North by Honeywell (negative control)</td>
<td>EVOH/PE laminate</td>
<td>0,08</td>
<td>Used in the chemical industry</td>
</tr>
</tbody>
</table>

*mean value of 3 measurements; **according to the analysis performed

3.3 Hair dyes

3.3.1 Study I

A permanent hair dye with a black shade, sold in Sweden by professional shops for hairdressers, was used to perform the in vivo tests. The main colouring ingredient was PPD (1.8%) (Table 3). The colouring cream was mixed with the developer cream containing 3% hydrogen peroxide. Thus, after mixing with developer at a ratio of 1:1 (w/w) the PPD concentration in the final testing product was 0.9%.
Table 3.
Ingredients labelled on the hair dye n1.0 Black Infiniti Affinage (International Hair Cosmetics Ltd, Romsey, UK) used on study I.

<table>
<thead>
<tr>
<th>Chemicals labelled on the colouring cream</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-amino-2-hydroxytoluene, 2-amino-3-hydroxypiridine, N,N-bis(2-Hydroxyethyl)-p-phenylenediamine sulfate, ammonia, resorcinol, p-methyaminophenol sulfate, p-phenylenediamine, p-aminophenol, 2-amino-4-hydroxyethylaminoanisolesulfate, 4-chlororesorcinol, 2-methylresorcinol, 4-amino-n-cresol, 6-amino-n-cresol, HC red no. 3, HC yellow no.2, HC yellow no.4, HC blue no.2, p-aminophenol, laureth-3, dissodium EDTA, Parfum, sodium hydrosulfite, sodium sulfite, DATEMTM, ceteareth-25, cocamidopropyl Betaine, propylene glycol, cocamide MEA, glycol stearate, myristil alcohol, aqua.</td>
</tr>
</tbody>
</table>

3.3.2 Study II

Fifty-two permanent hair dye products of 27 different brands were collected during one year (2011-2012) (Table 4). The hair dyes were purchased in 11 countries (United Arab Emirates, Australia, Brazil, Germany, Greece, Israel, Italy, Kenya, Singapore, Sweden, and USA). They were bought from ordinary stores and/or in hairdresser shops. All the products could also be bought on the internet. Darker shades were chosen, since the concentration of hair dye ingredients (PPD and 2,5-TDA) in these is higher. High-performance liquid chromatography (HPLC) was used for the analysis of PPD, 2,5-TDA, and three oxidation products of PPD.
Table 4.
List of commercial name, shade, country of purchase, and labelling of products collected in study II. Main hair colouring ingredient: p-phenylenediamine (PPD), toluene-2,5-diamine (2,5-TDA) or 2,5-TDA-sulphate (2,5-TDA-S).

<table>
<thead>
<tr>
<th>Code</th>
<th>Product name</th>
<th>Shades</th>
<th>Country of purchase</th>
<th>Labelling</th>
<th>PPD</th>
<th>2,5-TDA/2,5-TDA-S</th>
<th>Product name</th>
<th>Shades</th>
<th>Country of purchase</th>
<th>Labelling</th>
<th>PPD</th>
<th>2,5-TDA/2,5-TDA-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beauty Color professional Lorvenn Paris 1BB</td>
<td>Black Blue</td>
<td>Greece</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Recital Preference L'oréal 4</td>
<td>Brown</td>
<td>Arab Emirates</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>L’Oréal Majirel Ionène G 1</td>
<td>Black</td>
<td>Greece</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Indola Professional 0.44 Natural &amp; Essentials</td>
<td>Creator Intense Copper</td>
<td>Australia</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Dikson Color Italy 10</td>
<td>Extra light Blond</td>
<td>Italy</td>
<td>Y*</td>
<td>N</td>
<td></td>
<td>Kitoco 1.0 Black A.S.P.</td>
<td>Black</td>
<td>Australia</td>
<td>Y</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Dikson CD 6.0 Natur Color</td>
<td>Dark Blond</td>
<td>Italy</td>
<td>Y*</td>
<td>Y*</td>
<td></td>
<td>Mellor &amp; Russ 12.0 Natural Dark Brown</td>
<td>Australia</td>
<td>Y</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Freelimix 5.22 3VE Maestri Italy</td>
<td>Light Intense Violet Brown</td>
<td>Italy</td>
<td>Y*</td>
<td>Y*</td>
<td></td>
<td>Garnier Nutrisse 52 Brown</td>
<td>Brazil</td>
<td>Y</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Freelimix 5.6 3VE Maestri Italy</td>
<td>Light Auburn Brown</td>
<td>Italy</td>
<td>Y*</td>
<td>Y*</td>
<td></td>
<td>Evolution of the color Alfaparf Milano</td>
<td>Black</td>
<td>Israel</td>
<td>Y</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Lakmé Cosmetics collage 5/50</td>
<td>Brown</td>
<td>Italy</td>
<td>Y</td>
<td>N</td>
<td></td>
<td>Indola Prof 5.0 Natural &amp; Essentials Light brown Natural</td>
<td>Israel</td>
<td>N</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Selective Professional Fantasia 1999</td>
<td>Blond Plum Red</td>
<td>Italy</td>
<td>Y</td>
<td>N</td>
<td></td>
<td>Bigen 59 Oriental Black</td>
<td>Kenya</td>
<td>Y</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Selective Professional Oligomineral Cream</td>
<td>Dark Mahogany</td>
<td>Italy</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Bigen Speedy Hoyu n.881 Natural Black</td>
<td>Kenya</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Garnier Mova 50 C</td>
<td>Blackcurrant Black</td>
<td>Germany</td>
<td>N</td>
<td>Y</td>
<td></td>
<td>Easyblack hairglo Black</td>
<td>Kenya</td>
<td>Y</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Garnier Mova 55</td>
<td>Black</td>
<td>Germany</td>
<td>N</td>
<td>Y</td>
<td></td>
<td>Inecto powder haircolor Rapidolv Natural Black</td>
<td>Kenya</td>
<td>Y</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Polypalette 909 Schwarzkopf &amp; Henkel</td>
<td>Blue Black</td>
<td>Germany</td>
<td>N</td>
<td>Y</td>
<td></td>
<td>Eagle's Ram Gopal &amp; Sons Black</td>
<td>Singapore</td>
<td>Y</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Color</td>
<td>Country</td>
<td>Asian Availability</td>
<td>Product Name</td>
<td>Color</td>
<td>Country</td>
<td>Asian Availability</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
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<td></td>
</tr>
<tr>
<td>13</td>
<td>Polypalette 900 Schwarzkopf &amp; Henkel</td>
<td>Black</td>
<td>Germany</td>
<td>N</td>
<td>Brown</td>
<td>Singapore</td>
<td>Y</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Indola Profession 4.0 Natural &amp; Essentials</td>
<td>Medium Brown Natural</td>
<td>Sweden</td>
<td>N</td>
<td>Excellence creme, China 1</td>
<td>Natural Black</td>
<td>Singapore</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Indola Prof 3.0 Natural &amp; Essentials</td>
<td>Dark Brown natural</td>
<td>Sweden</td>
<td>N</td>
<td>Goreeypur Mehendy 39.8</td>
<td>Brown</td>
<td>Singapore</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Infiniti Affinage 1.0 B</td>
<td>Black</td>
<td>Sweden</td>
<td>Y</td>
<td>Herbal henna Om General Stores</td>
<td>Burgundy</td>
<td>Singapore</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Koleston Perfect Wella 4.0</td>
<td>Medium Brown</td>
<td>Sweden</td>
<td>N</td>
<td>Just for Men</td>
<td>Natural Real Black</td>
<td>Singapore</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Koleston Perfect Wella 3.0</td>
<td>Dark Brown</td>
<td>Sweden</td>
<td>N</td>
<td>Liese</td>
<td>Natural Black</td>
<td>Singapore</td>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Koleston Perfect Wella 2.0</td>
<td>Black</td>
<td>Sweden</td>
<td>N</td>
<td>Max colour Gervas</td>
<td>Dark brown</td>
<td>Singapore</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>L’Oréal Diacolor richesse</td>
<td>Light Brown</td>
<td>Sweden</td>
<td>N</td>
<td>Nutrisse cream Gamier 44</td>
<td>Mahogany Brown</td>
<td>Singapore</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Natural &amp; Easy 590 Schwarzkopf</td>
<td>Ebony Black</td>
<td>Sweden</td>
<td>N</td>
<td>Top Speed Revlon I</td>
<td>Black</td>
<td>Singapore</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Mood Scandinavian Care Hardford 26</td>
<td>Black</td>
<td>Sweden</td>
<td>Y</td>
<td>Balsam Color 612RB Clariol</td>
<td>Medium reddish Brown</td>
<td>USA</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Solocolor beautymatrix 7BC</td>
<td>Medium Blonde Brown copper</td>
<td>Sweden</td>
<td>Y</td>
<td>Clariol 113 A</td>
<td>Natural dark brown</td>
<td>USA</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Excellence creme 301 L’oréal</td>
<td>Iced Dark Brown</td>
<td>Arab Emirates</td>
<td>Y</td>
<td>Nice’n Easy Clairol 124</td>
<td>Black</td>
<td>USA</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Garnier Color 1</td>
<td>Black</td>
<td>Arab Emirates</td>
<td>Y</td>
<td>Perfect 10 Clariol 2</td>
<td>Black</td>
<td>USA</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Garnier Color 4</td>
<td>Brown</td>
<td>Arab Emirates</td>
<td>Y</td>
<td>Superior Preference 2B L’oréal</td>
<td>Purest Black</td>
<td>USA</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Y: yes; N: no; *Disclosure of contents states “may contain”
3.3.3 Study III

Three permanent hair dyes (Table 5) for professional use (HDp), 1 containing PPD (HDp-PPD), 1 containing 2,5 TDA sulphate (HDp-TDA-S), 1 containing ME-PPD (HDp-ME-PPD), and 1 hair dye intended for home use (HDhu), containing 2,5-TDA sulphate (HDhu-TDA-S), were used to perform the in vivo provocation test with gloves. The hair dyes were prepared to simulate professional or home use by mixing the colouring cream with the corresponding developer containing 3% (10 vol.) or 6% (20 vol.) hydrogen peroxide with a mixing ratio of 1:1 (w/w). The concentrations in the final testing products were 0.9% PPD in HDp-PPD, 0.86% and 0.68% 2,5-TDA in HDp-TDA-S and HDhu-TDA-S, respectively.

Table 5.
List of commercial name, shade and ingredients according to the labelling of the hair dye products tested in study III.

<table>
<thead>
<tr>
<th>Hair dyes: commercial name, manufacturer, name used in the study</th>
<th>Shade</th>
<th>Chemicals declared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koleston Perfect Wella® ▼HDp-TDA-S</td>
<td>Black 2/0</td>
<td>aqua, cetaryl alcohol, glyceryl stearate SE, toluene-2,5-diamine sulfate, ammonium hydroxide, sodium laureth sulfate, lanolin alcohol, sodium lauryl sulfate, resorcinol, 2,4-diaminophenoxyethanol HCl, m-aminophenol, glycol distearate, sodium cocoyl isethionate, sodium sulfite, ascorbic acid, parfum, disodium EDTA, 1-hydroxyethyl 4,5-diamino pyrazole sulfate, 2-methyresorcinol, polyquaternium-22, geraniol, linalool, tocopherol</td>
</tr>
<tr>
<td>Infiniti Affinage® ▼HDp-PPD</td>
<td>1.0 Black</td>
<td>Ammonia, cocamide MEA, cocamidopropylbetaine, resorcinol, p-methyaminophenol sulfate, 4-chlororesorcinol, 2-methyresorcinol, N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate, 2-amino-4-hydroxyethylaminoanisole sulfate, p-phenylenediamine, p-aminophenol, 4-amino-2-aryloxytoluene; 2-amino-3-hydroxyprididine</td>
</tr>
<tr>
<td>Koleston Perfekt Innosense Wella® ▼HDp-ME-PPD</td>
<td>2.0 Black</td>
<td>aqua, cetaryl alcohol, glyceryl stearate SE, sodium laureth sulfate, ammonium hydroxide, lanolin alcohol, 2-methoxymethyl-p-phenylenediamine, sodium lauryl sulfate, 2,4-diaminophenoxyethanol HCl, hydroxyethyl-3,4-methylenedioxyaniline HCl, resorcinol, glycol distearate, sodium cocoyl isethionate, sodium sulfite, ascorbic acid, parfum, disodium EDTA, 1-hydroxyethyl 4,5-diamino pyrazole sulfate, 4-amino-2-hydroxytoluene, tocopherol</td>
</tr>
<tr>
<td>Natur &amp; Easy Schwarzkopf® ▼HDhu-TDA-S</td>
<td>590 Black</td>
<td>toluene 2.5 diamine-sulfate, aqua, cetaryl alcohol, ammonium hydroxide, sodium-laureth-6-carboxylate, coconut alcohol, sodium myeth sulfate, potassium hydroxide, resorcinol, acrylamidopropyltrimonium chloride/acrylates copolymer, cocom-glucoside, glyceryl oleate, ceteareth-12, ceteareth 20; sodium sulfite, sodium silicate, etidronic acid, ammonium sulfate, ascorbic acid, 2-4-diaminophenoxyethanol HCl, m-Aminophenol, parfum, linalool, citronellol</td>
</tr>
</tbody>
</table>

▼HDp-TDA-S: hair dye for professional use containing toluene-2,5-diamine sulphate (2,5-TDA-S); HDp-PPD: hair dye for professional use containing p-phenylenediamine (PPD); HDp-ME-PPD: hair dye for professional use containing 2-methoxymethyl-p-phenylenediamine (ME-PPD).
▼▼HDhu-TDA-S: hair dye for home use containing 2,5-TDA-S
3.4 Patch test preparations

3.4.1 Study I

PPD (>99%; Sigma-Aldrich, St. Louis, MO, USA) and acetone (≥ 99.5%; Scharlau Chemie SA, Sentmenat, Spain, and 99.9%; VWR, Fontenay-sous-Bois, France).

3.4.2 Study III

The following chemicals were used: PPD (> 99%; Sigma-Aldrich), 2,5-TDA-S (97%, Acros Organics, Geel, Belgium), ethanol (>99.5%; CCS Healthcare, Borlänge, Sweden), petrolatum (pet, Vaselinum album, Snow-White Quality; Apoteket Produktion & Laboratorier, Göteborg, Sweden).

3.4.3 Study IV

The following chemicals were used: PPD (>99%, Sigma-Aldrich), PPD-DHC (> 99%; Fisher Scientific, Bridgewater, NJ, USA), 2,5-TDA-S (97%; Acros Organics), 2,5-TDA (98%; Combi-Blocks Inc, San Diego, CA, USA), 4-nitroaniline (≥ 99%; Sigma-Aldrich, Steinheim, Germany), and 4,4´-azodianiline (95%; Acros Organics), petrolatum (pet, Vaselinum album, as above).
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Synonyms</th>
<th>CAS Number</th>
<th>MW</th>
<th>log Po/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-phenylenediamine</td>
<td>1,4-phenylenediamine, 1,4-diaminobenzene, PPD</td>
<td>106-50-3</td>
<td>108</td>
<td>0.43</td>
</tr>
<tr>
<td>toluene-2,5-diamine</td>
<td>p-toluenediamine, 2,5-diaminotoluene</td>
<td>95-70-5</td>
<td>122</td>
<td>0.92</td>
</tr>
<tr>
<td>4-nitroaniline</td>
<td>p-nitroaniline</td>
<td>100-01-6</td>
<td>138</td>
<td>1.01</td>
</tr>
<tr>
<td>4,4′-azodianiline</td>
<td>p-diaminoazobenzene</td>
<td>538-41-0</td>
<td>212</td>
<td>1.49</td>
</tr>
<tr>
<td>Bandrowski’s base</td>
<td></td>
<td>20048-27-5</td>
<td>318</td>
<td>-0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Calculated log P: 0.97</td>
</tr>
<tr>
<td>2,5-toluenediamine sulphate</td>
<td>2,5-TDA sulphate</td>
<td>615-50-9</td>
<td>n x 220</td>
<td>0.74</td>
</tr>
<tr>
<td>p-phenylenediamine dihydrochloride</td>
<td>1,4-Benzenediamine dihydrochloride</td>
<td>624-18-0</td>
<td>181</td>
<td>not known but very water soluble</td>
</tr>
</tbody>
</table>

Figure 1.
Chemical structures, Chemical Abstracts Service (CAS) numbers, and physical properties of chemicals studied and tested in study I, II, III, IV of this thesis: p-phenylenediamine, toluene-2,5-diamine, p-nitroaniline, 4,4′-azodianiline, and Bandrowski’s base, p-phenylenediamine dihydrochloride, 2,5-toluenediamine sulphate.
3.5 Patch testing

3.5.1 Study I and III

To establish the current reactivity of each volunteer, a dilution series of PPD in acetone (1.0%, 0.1%, 0.01%, 0.001%, and 0.0001%) in study I, two dilution series one of PPD in ethanol (w/v) and one of 2,5-TDA-S in pet (w/w) (1.0%, 0.1%, 0.01%, 0.001%, 0.0001%) were patch tested in study III. Finn Chambers® (Ø 8 mm) (SmartPractice, Phoenix, AZ, USA) on Scanpor® tape (Norgesplaster A/S, Vennesla, Norway) were used. Fifteen μl of test solutions applied with micropipette, for the PPD dilution series or 20 mg of pet for the 2,5-TDA-S dilution series was applied in each test chamber. For volunteers with a previous +++ reaction to PPD and/or 2,5-TDA-S, the highest concentration tested was fixed at 10 times less than the threshold of reactivity previously observed in each subject.

Patch tested were applied on the backs of subjects at the same time as the in vivo test and left for 2 days (48 h). The reading was performed on D4 and D7. The reactions were scored according to ICDRG guidelines (1, 35).

3.5.2 Study IV

3.5.2.1 Time period I

PPD (1%), 2,5-TDA-S (1%), 4,4´-diaminodiphenylmethane (4,4´-MDA) (0.25%), and benzocaine (5.0%) tested with the Swedish baseline series and an additional baseline series used in Malmö, were purchased from Chemotechnique Diagnostics, Vellinge, Sweden. For all test preparations, the vehicle was petrolatum.

3.5.2.2 Time period II

PPD, PPD-DHC, 2,5-TDA-S, and 2,5-TDA were tested at equimolar concentrations in pet (1.0%, 1.7%, 2.0%, and 1.1%, wt/wt, respectively). PPD was also tested at 0.1% in pet. The two oxidation products of PPD, 4-nitroaniline and 4,4´-azodianiline, considered to be possible strong sensitisers, and the 2,5-TDA were also tested at concentration equimolar to 0.10% PPD, the lowest PPD concentration tested, 0.13% 4-nitroaniline, 0.20% 4,4´-azodianiline, and 0.11% 2,5-TDA w/w in pet. For all test preparations, the vehicle was pet.

All test preparations were manufactured at our laboratory in Malmö except 1% PPD, 1% 2,5-TDA-S, 0.25% 4,4´-MDA, and 5.0% benzocaine, which were purchased from Chemotechnique Diagnostics, Vellinge, Sweden.
3.6 Open chamber testing

3.6.1 Study I

An in vivo system to evaluate the protective effect of gloves against developing allergic contact dermatitis was used (130, 131). The system enables the investigator to evaluate the protective effect of several glove materials at the same time, and the effect of different exposure times. Circular chambers (12 mm in height; 12 mm in inner diameter) made of chemical-resistant stainless steel were fitted in a 1-mm-thick flexible acrylic support. The bottom of the chambers were covered with the glove material to be tested tightly fitted with metallic clip. The glove material acted as a membrane between the chamber and the skin. The substance to be tested was added above the glove material (Figure 2). Three different gloves were simultaneously tested for each of the three chambers’ support devices. The testing system was taped to the skin of the back with Scanpor® tape and the subject lay in the supine position (Figure 3). The chambers were filled up to half-height (~6 mm) with the hair dye/peroxide mixture prepared within 4 min of application. Three exposure times (15, 30, and 60 min) were chosen. As a negative control, the 4H® glove was used with an exposure time of 60 min. As a positive control, we used a chamber devoid of glove membrane and filled with the hair dye directly in contact with the skin. The exposure time was 60 min. In total, each individual was tested using 20 chambers. Readings of the areas were performed according to ICDRG guidelines on D2 or D4, and D7.
Figure 2
Open chamber test system used in studies I and III.

Figure 3
Study I: glove permeation test performed with 6 different gloves and one hair dye containing p-phenylenediamine at three exposure times (15, 30, and 60 minutes). Positive and negative controls were tested at 60 minutes.
3.6.2 Study III

The in vivo provocation test was carried out with an open-chamber test system (Figure 2) already described for study I. The exposure time chosen was 45 min for three professional hair dyes and 30 min for one hair dye intended for home use. The professional hair dyes were tested with three glove types (2 NI, 1 PVC), a negative control consisting of 4H®-glove, and a positive control consisting of a chamber devoid of glove membrane, filled with the hair dye directly in contact with the skin. The PE glove supplied in the package of the HDhu was tested with the same hair dye in addition to the above, and the duration of exposure was limited to 30 min.

3.7 Gloves analysis

3.7.1 Fourier-transform infrared spectroscopy

In study III, Fourier-transform infrared (FTIR) spectroscopy was performed with an Agilent Cary 630 FTIR spectrometer (Agilent Technologies, Danbury, CT, USA), to analyse the material of the gloves supplied in the package of a hair dye for home use. The method compared the spectrum of the material analysed, with a database of the spectra of a wide range of materials.

3.8 Chemical investigations of hair dyes

3.8.1 High performance liquid chromatography (HPLC)

Analytical HPLC was used to determine if specific substances were present in the hair dye preparations and also to measure their concentrations and for the investigation of the purity of patch tested substances. The main parts of the HPLC system are a pump which delivers a solvent flow, an injector to introduce a sample into the flow, a column which brings about a separation of the substances in the sample and a detector which visualises the substances. The substances are recognised by their specific retention times and by their specific absorption of visual light and UV-radiation. Samples for analyses were prepared by dissolving a specific amount of the investigated material in a specific volume of a suitable solvent. The concentrations of the different substances in the sample were determined by comparison of the detector response from known concentrations of
the reference substances with the response from the corresponding substances the 
analysed samples. From the determined concentrations in the analysed samples the 
concentrations of these substances in the hair dye products were calculated. Every 
analytical method has got a detection limit and sometimes a limit of quantification 
which is higher than the detection limit.

In all studies separation was performed with HPLC and using diode array detector. 
In studies I, II, and III straight-phase chromatography was performed whilst 
reversed phase chromatography was used in study IV. In straight-phase 
chromatography the stationary phase is polar whilst the mobile phase is nonpolar; 
whilst is true in reversed-phase chromatography, the stationary phase is nonpolar 
whilst the mobile phase is polar. This means that in straight-phase the most polar 
components eluate last while they eluate first in reversed-phase chromatography. 
Separation can be influenced by changing the gradient elution in which the mobile 
phase composition is varied during the chromatography. However, in all papers 
presented here isocratic elution were used meaning that the mobile phase 
composition was kept constant.

3.8.1.1 Study I 
The content of PPD was analysed with straight-phase HPLC in the permanent hair 
dye tested.

3.8.1.2 Study III 
Four hair dyes containing different hair dye allergens according to the labelling 
(one with PPD, two with 2,5-TDA, and one with MME-PPD), were analyzed with 
straight-phase HPLC to quantify the concentrations of PPD and 2,5-TDA in all 
four products. Investigations were not performed for MME-PPD, since the 
reference substance could not purchased and delivered from the suppliers before 
the start of the study.

3.8.1.3 Study II 
All investigated hair dyes were analysed with straight-phase HPLC with regard to 
PPD, 2,5-TDA, Bandrowski´s base, p-nitroaniline and 4,4′-azodianiline.

3.8.1.4 Study IV 
The purity of 2,5-TDA and 2,5-TDA-S and the possibility of these containing PPD 
was investigated by reversed-phase HPLC.

The chromatographic methods and the specific conditions of the analyses 
are described in detail in Papers I, II, III and IV.
3.9 Recording of data

The results of the patch test performed in studies I, III, and IV were recorded in the computer-based registration system Daluk, in which age, gender, and contact allergies are recorded (132). In study IV, information on patch test results of consecutive dermatitis patients tested at the DOED in Malmö was retrieved from Daluk.

3.10 Ethics

Studies I, III, and IV were approved by Regional Ethical Review Board in Lund, Sweden, and conducted in accordance with ethical standards specified in the Declaration of Helsinki. The participants gave written informed consent in studies I and III. Consecutive patients in DOED are always informed that their data are stored for possible future use in research. Before the start of study IV, we published in a local newspaper information on the study so that previously patch tested individuals had the possibility to decline participation. No one declined.

3.11 Statistics

3.11.1 Studies I and III

McNemar’s test (two-sided) was used to compare the protective effects of the different gloves, pairwise. A one-sided McNemar’s test was used to compare the protective effect of each kind of glove with that of the 4H® glove (negative control). Differences were considered significant at p-value of <0.05.

In study III, for the same pairwise comparisons of gloves Fisher’s test was used to compare their protective effects against the four hair dyes.

In study I, the relationship between the degree of reactivity to PPD and the number of reactions to hair dyes/gloves system was compared using Fisher’s exact test (two-sided), dichotomising the 8 subjects into two groups (one with reactors to 1.0% PPD and/or 0.1% PPD and the other with reactors to 0.01% PPD, or lower concentrations of PPD). Spearman’s rank correlation test was used to investigate the association between degree of reactivity in the glove penetration test and the number of positive reactions at the three exposure times.
3.11.2 Study II

Fisher’s exact test (two-sided) was used to compare the number of hair dyes containing PPD and 2,5-TDA in European and non-European countries, and also within Europe (distinguishing Sweden and central/southern Europe).

Hair dyes containing PPD that were purchased in Europe were compared with regarding the concentration of PPD detected with those from outside Europe—and also between black/brown hair dyes (according to the shade declared) and blond hair dyes—using the Student’s t-test (two-sided).

3.11.3 Study IV

McNemar’s test (two-sided) was used to compare pairwise the positive reactions observed to the hair dye ingredients tested in time period II, using the highest reactivity between test readings on D3/4 and D7.

Fisher’s exact test (two-sided) was used to compare the number of simultaneous reactions to 2,5-TDA-S in PPD-sensitised patients who were divided into two groups based on the degree of reactivity to PPD (lower: + and ++; higher: +++). The distribution of simultaneous allergic reactions to 2,5-TDA-S in PPD-hypersensitive individuals, tested in time period I, was also investigated with the Cochrane-Armitage test.

In all the statistical analyses performed, any p-value of < 0.05 was considered significant.
4 Results

4.1 Study I

4.1.1 Patch tests and open-chamber test

The results of the glove testing with the open-chamber and patch test with dilution series of PPD are summarised in Table 6. Considerable differences in the protective capacities of different glove materials were observed. For the 4H® glove (H) (Table 2) tested for 60 min, no skin reactions were detected in any subject. None of the 8 PPD-sensitized subjects reacted at any exposure time with the two different NI gloves (D and E), which showed the same degree of protection as the 4H® glove. In the group of other gloves tested one PVC glove (F) had a better performance than other PVC (A), PE (B), and NRL (C), based on the number of positive reactions at various exposure times (15, 30, and 60 min).

At the exposure time of 60 min, all the gloves except NI showed a significant worse protection compared with the negative control (p > 0.3 for gloves D and E; p = 0.031 for gloves A, B, and C; p = 0.016 for glove F). The pairwise comparisons between the six gloves showed significant differences between the respective NI glove and glove F (p = 0.031), but non-significant differences (p = 0.063) between the respective NI glove and gloves A, B, and C.

One of the patients with a positive reaction to 1.0% PPD did not react in the glove-permeation tests at all. The remaining 7 subjects who were enrolled showed positive reactions in the glove permeation tests and to the positive control.

Stronger and more numerous reactions in the permeation tests were seen in 5 subjects with a lower threshold of reactivity to PPD (reacting to 0.01% or lower) that in those who reacted to 1% and 0.1%. The dichotomised comparisons at exposure times of 60 and 30 min showed significant differences (p < 0.001).

Spearman’s rank correlation test showed a significant correlation between the number of positive reactions in the glove permeation test and both the increasing exposure times, and the number of positive reactions (Figures 4 and 5).
Table 6.
Study I: threshold of reactivity and test results of the open exposure test with hair dye/gloves in 8 PPD-sensitised subjects. The lowest value of the PPD dilution series giving a positive reaction has been included whereas the highest score of positive reaction at D2/D4/D7 is presented in the table.

*=D2 test reading

<table>
<thead>
<tr>
<th>Subjects</th>
<th>no.1</th>
<th>no.2</th>
<th>no.3</th>
<th>no.4</th>
<th>no.5</th>
<th>no.6</th>
<th>no.7</th>
<th>no.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold of reactivity to PPD dilution series (%)</td>
<td>0.01</td>
<td>1.0</td>
<td>1.0</td>
<td>0.1</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Gloves</td>
<td>Exposure times (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (Polyvinyl chloride)</td>
<td>15</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>B (Polyethylene)</td>
<td>15</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>++</td>
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<td>-</td>
<td>+</td>
</tr>
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<td></td>
<td>60</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>C (Natural Rubber Latex)</td>
<td>15</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>D (Nitrile)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>60</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E (Nitrile)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>60</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F (Polyvinyl chloride)</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>G (No glove-positive control)</td>
<td>60</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>H (4H® glove-negative control)</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 4.
Study I: Increasing number of positive reactions to the glove permeation test at higher exposure time.
Figure 5. Study I: Number of positive reactions to the glove permeation test with the glove materials tested, related to increasing exposure times.

4.2 Study II

4.2.1 Content according to labelling

Thirthy-five products were labelled as containing PPD, 29 of which (55.7%) were labelled as containing PPD but not 2,5-TDA or 2,5-TDA-S and six (11.5%) were labelled as containing both.

Twenty-one products were labelled as containing 2,5-TDA and 15 (28.8%) were labelled as containing 2,5-TDA but not PPD.

Two products (3.8%) were not labelled as containing either PPD or 2,5-TDA (Figure 4). Four of the products were labelled “may contain” PPD and three 2,5-TDA.

PPD was more commonly found in products from non-European countries than in those from European countries (24 of 29 as opposed to 8 of 23; p < 0.001) while
the opposite was true for 2,5-TDA (2 of 29 as opposed to 14 of 23; p < 0.001) (Figures 6-8).

All products but four (one European and three non-European) recommended that the consumer should perform a self-validated allergy test before dyeing the hair.

---

**Figure 6.**
Study II: Overall content of PPD and 2,5-TDA with regards to labelling of the products from European or non-European countries.

**Figure 7.**
Study II: Content of PPD and 2,5-TDA according to the labelling of the products purchased in Europe with prevalence of 2,5-TDA declared.

**Figure 8.**
Study II: Content of PPD and 2,5-TDA according to the labelling of the products purchased in non-European countries with prevalence of PPD declared.
4.2.2 Content according to chemical analysis

Nine (39.1%) of the 23 products purchased in Europe (44.2% of the total group of 52 hair dyes) contained PPD at a calculated on-head concentration of <2% (range: 0.025–0.98) and 14 (60.8%) contained 2,5-TDA at a calculated on-head concentration of <4% (range 0.22–2.4).

19 (65.5%) of 29 non-European products (55.8% of 52 hair dyes collected in the study) contained PPD at a calculated on-head concentration of <2% (range: 0.20–0.96), one had a calculated on-head concentration of 7.9%, and 4 products, devoid of instructions on how to mix the hair dye with the oxidation product, had a concentration of PPD of >2% (range: 2.2–14.2%). Three products collected in non-European countries contained 2,5-TDA with a calculated on-head concentration range of 0.53–1.1.

The PPD concentration was generally higher in hair dyes purchased outside Europe, but the difference was not statistically significant (p = 0.20).

Bandrowski’s base, p-nitroaniline, and 4,4-azodianiline were detected in only one hair dye, which was purchased in a non-European country (and said to contain no couplers). The hair dye was sold in the form of a powder containing an oxidising agent, and was intended to be mixed with water as described in the folder.

All results are shown in Tables 7 and 8.
Table 7
List of commercial name, country of purchase within Europe and content of PPD and 2,5-TDA analysed with high performance liquid chromatography (HPLC). ND= concentration below the quantification limit of 0.05.

<table>
<thead>
<tr>
<th>Product name/country of purchase</th>
<th>HPLC analysis (%)</th>
<th>Product name/ country of purchase</th>
<th>HPLC analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPD 2,5-TDA</td>
<td></td>
<td>PPD 2,5-TDA</td>
</tr>
<tr>
<td>Beauty Color professional Lorvenn Paris 1BB</td>
<td>Greece ND 1.13</td>
<td>Polypalette 900 Schwarzkopf &amp; Henkel</td>
<td>Germany ND 1.25</td>
</tr>
<tr>
<td>L’Oréal Majirel Ionène G 1</td>
<td>Greece ND 2.38</td>
<td>Indola Profession 4.0 Natural &amp; Essentials</td>
<td>Sweden ND 2.08</td>
</tr>
<tr>
<td>Dikson Color Italy 10</td>
<td>Italy 0.06 ND</td>
<td>Indola Prof 3.0 Natural &amp; Essentials</td>
<td>Sweden ND 1.11</td>
</tr>
<tr>
<td>Dikson CD 6.0 Natur Color</td>
<td>Italy 0.43 ND</td>
<td>Infiniti Affinage 1.0 B</td>
<td>Sweden 1.81 ND</td>
</tr>
<tr>
<td>Freelimix 5.22 3VE Maestri Italy</td>
<td>Italy 0.18 ND</td>
<td>Koleston Perfect Wella 4.0</td>
<td>Sweden ND 1.08</td>
</tr>
<tr>
<td>Freelimix 5.6 3VE Maestri Italy</td>
<td>Italy 0.56 ND</td>
<td>Koleston Perfect Wella 3.0</td>
<td>Sweden ND 1.12</td>
</tr>
<tr>
<td>Lakmè Cosmetics collage 5/50</td>
<td>Italy 0.77 ND</td>
<td>Koleston Perfect Wella 2.0</td>
<td>Sweden ND 1.73</td>
</tr>
<tr>
<td>Selective Professional Fantasia 1999</td>
<td>Italy 0.105 ND</td>
<td>L’Oréal Diacolor richesse</td>
<td>Sweden ND 1.24</td>
</tr>
<tr>
<td>Selective Professional Oligomineral Cream</td>
<td>Italy 0.48 ND</td>
<td>Natural &amp; Easy 590 Schwarzkopf</td>
<td>Sweden ND 1.36</td>
</tr>
<tr>
<td>Garnier Movida 50 C</td>
<td>Germany ND 1.68</td>
<td>Mood Scandinavian Care Hardford 26</td>
<td>Sweden 1.96 ND</td>
</tr>
<tr>
<td>Garnier Movida 55</td>
<td>Germany ND 1.95</td>
<td>Solocolor beautymatrix 7BC</td>
<td>Sweden ND 0.22</td>
</tr>
<tr>
<td>Polypalette 909 Schwarzkopf &amp; Henkel</td>
<td>Germany ND 0.75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 8.
List of commercial name, non-European country of purchase and content of PPD and 2,5-TDA analysed with high performance liquid chromatography (HPLC). ND = concentration below the quantification limit of 0.05.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Country of purchase</th>
<th>HPLC analysis (%)</th>
<th>Product name</th>
<th>Country of purchase</th>
<th>HPLC analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PPD</td>
<td>2,5-TDA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PPD &amp; 2,5-TDA</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>PPD &amp; 2,5-TDA</strong></td>
<td></td>
</tr>
<tr>
<td>Excellence creme 301 L’oréal</td>
<td>Arab Emirates</td>
<td>0.66</td>
<td>ND</td>
<td>Eagle’s Ram Gopal &amp; Sons</td>
<td>Singapore</td>
</tr>
<tr>
<td>Gamier Color 1</td>
<td>Arab Emirates</td>
<td>2.03</td>
<td>ND</td>
<td>Excellence creme, China 1 Gopal &amp; Sons</td>
<td>Singapore</td>
</tr>
<tr>
<td>Gamier Color 4</td>
<td>Arab Emirates</td>
<td>0.70</td>
<td>ND</td>
<td>Goreeynupur Mehendy 39.8 Gopal &amp; Sons</td>
<td>Singapore</td>
</tr>
<tr>
<td>Recital Preference L’oréal 4</td>
<td>Arab Emirates</td>
<td>0.38</td>
<td>ND</td>
<td>Herbal henna Om General Stores</td>
<td>Singapore</td>
</tr>
<tr>
<td>Indola Professional 0.44 Natural &amp; Essentials</td>
<td>Australia</td>
<td>ND</td>
<td>ND</td>
<td>Just for Men</td>
<td>Singapore</td>
</tr>
<tr>
<td>Kitoco 1.0 Black A.S.P.</td>
<td>Australia</td>
<td>1.92</td>
<td>ND</td>
<td>Liese</td>
<td>Singapore</td>
</tr>
<tr>
<td>Mellor &amp; Russ 12.0</td>
<td>Australia</td>
<td>0.68</td>
<td>ND</td>
<td>Max colour Gervas</td>
<td>Singapore</td>
</tr>
<tr>
<td>Gamier Nutrisse 52 Evolution of the color Alfaparf Milano</td>
<td>Brazil</td>
<td>0.60</td>
<td>ND</td>
<td>Nutrisse cream Garnier 44</td>
<td>Singapore</td>
</tr>
<tr>
<td></td>
<td>Israel</td>
<td>4.93</td>
<td>ND</td>
<td>Top Speed Revlon 1</td>
<td>Singapore</td>
</tr>
<tr>
<td>Indola Prof 5.0 Natural &amp; Essentials</td>
<td>Israel</td>
<td>ND</td>
<td>0.53</td>
<td>Balsam Color 612RB Clairol</td>
<td>USA</td>
</tr>
<tr>
<td>Bigen 59</td>
<td>Kenya</td>
<td>15.2</td>
<td>ND</td>
<td>Clario 113 A</td>
<td>USA</td>
</tr>
<tr>
<td>Bigen Speedy Hoyu n.881</td>
<td>Kenya</td>
<td>0.88</td>
<td>2.16</td>
<td>Nice’n Easy Clairol 124</td>
<td>USA</td>
</tr>
<tr>
<td>Easyblack hairgl</td>
<td>Kenya</td>
<td>1.31</td>
<td>ND</td>
<td>Perfect 10 Clairol 2</td>
<td>USA</td>
</tr>
<tr>
<td>Inecto powder haircolor Rapidoli¥</td>
<td>Kenya</td>
<td>31.5</td>
<td>ND</td>
<td>Superior Preference 2B L’oréal</td>
<td>USA</td>
</tr>
<tr>
<td>Eagle’s Ram Gopal &amp; Sons</td>
<td>Singapore</td>
<td>14.2</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3 Study III

4.3.1 Patch tests and open-chamber tests

The gloves supplied in the package of HDhu was found to be PE (Table 2).

All the volunteers developed positive reactions to hair dyes when tested without the glove (positive control) at 45 min with the 3 HDp, and at 30 minutes with the HDhu, but two of the subjects did not react to the test with HDp-ME-PPD. No positive reactions to the 4H® glove (negative control) were observed; nor to the 2 NI gloves tested with the 4 hair dyes (Table 9). The 2 kinds of nitrile gloves gave better protection than the PVC glove against the HDp-TDA-S in 6 subjects, and the results were statistically significant (p = 0.031); the same comparison with the HDp-PPD and the HDhu-TDA-S was almost significant (p = 0.062). The 4H® glove gave significantly better protection than the PVC glove for all hair dyes except HDp-ME-PPD.

The PE glove protected all 7 individuals tested who were exposed for 30 min to the HDhu-TDA-S, and all 5 individuals tested who were exposed for 45 min to HDp-ME-PPD (Table 9). The 4H® glove and the PE glove gave the same degree of protection against HDhu-TDA-S.

Thus, the 4H® and NI gloves gave complete protection irrespective of which hair dye was tested. For the PE and PVC gloves, there was complete protection against the HDp-ME-PPD but not against the other three hair dyes (Table 9) (Figure 9).

![Figure 9.](image)

Study III: test reading at D4 of subject no. 2.
Table 9.
Study III: pattern of reactions of 7 patients to the permeation test with 5 different gloves and 4 hair dyes at the exposure times of 45 and/or 30 minutes. Test reading performed on day (D) 4; *=Indicates a negative reaction on D4 but positive on D7; Main hair dye ingredients: PPD=phenylenediamine, 2,5-TDA-S=2,5-toluenediamine sulphate, ME-PPD=2-methoxymethyl-p-phenylenediamine, HDp: hair dye for professional use, HDhu: hair dye for home use. NT= not tested.

<table>
<thead>
<tr>
<th>Subjects (no=number)</th>
<th>Hair dyes ingredients</th>
<th>Gloves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exposure times (min)</td>
</tr>
<tr>
<td>no.1</td>
<td>HDp-TDA</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-ME-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDhu-TDA</td>
<td>30</td>
</tr>
<tr>
<td>no.2</td>
<td>HDp-TDA</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-ME-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDhu-TDA</td>
<td>30</td>
</tr>
<tr>
<td>no.3</td>
<td>HDp-TDA</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-ME-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDhu-TDA</td>
<td>30</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>HDp-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-ME-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDhu-TDA</td>
<td>30</td>
</tr>
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<td>no.5</td>
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<tr>
<td></td>
<td>HDp-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-ME-PPD</td>
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</tr>
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<td></td>
<td>HDhu-TDA</td>
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<td>no.6</td>
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</tr>
<tr>
<td></td>
<td>HDp-PPD</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HDp-ME-PPD</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>no.7</td>
<td>HDp-TDA</td>
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</tr>
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</table>
4.4 Study IV

4.4.1 Time Period I

Sixty-six patients (42 females, 24 males) reacted to PPD, while 23 (17 females, 6 males) reacted to 2,5-TDA-S (Table 10). The comparison between positive reactions to PPD (1.0%) and 2,5-TDA-S (1.0%) was statistically significant (p < 0.001). A significantly (p<0.001) higher number of simultaneous allergic reactions to 2,5-TDA-S was noted in PPD-hypersensitive individuals with higher reactivity (+++ reactions) than in those with lower reactivity (+++) (Figure 5). PPD was not detected in 2,5-TDA-S.

Table 10.
Number of positive reactions to p-phenylenediamine (PPD) and toluene-2,5-diamine sulphate (2,5-TDA-S), on the total number of patients tested in time period I; ratio males(M)/females(F).

<table>
<thead>
<tr>
<th>Time period</th>
<th>Total number (M:F)</th>
<th>Series tested</th>
<th>Chemicals tested in pet.</th>
<th>Total no. positive reactions No. (%) (M:F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1,759 (592:1,167)</td>
<td>Baseline series</td>
<td>PPD 1.0%</td>
<td>66 (3.8) 24:42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2,5-TDA-S 1.0%</td>
<td>23 (1.3) 6:17</td>
</tr>
</tbody>
</table>

4.4.2 Time period II

The results of patch testing with PPD (1%) and the hair dye ingredients tested are given in Table 11 and Figure 10.

When comparing the number of positive reactors to PPD (1.0%) and the equimolar concentration as a salt, and to 2,5-TDA in its free form (1.1%) and its salt, 2,5-TDA-S, at 2% (i.e. in equimolar amounts), significantly more patients tested positive against PPD and to 2,5-TDA in their free form (p < 0.001 and p = 0.021, respectively).

A comparison between the number of positive reactions against 2,5-TDA at 1.1% and to an equimolar amount of PPD (1%) in pet showed a statistically significant difference (p < 0.001) with more reactions against PPD.
When we compared the results of testing with 0.1% PPD and 4,4’-azodianiline at the equimolar concentration of 0.2% (Table 11 and Figure 10), fewer patients reacted against the latter.

**Table 11.**
Number of positive reactions to p-phenylenediamine (PPD) and toluene-2,5-diamine sulphate (2,5-TDA-S), toluene-2,5-diamine (2,5-TDA), p-phenylenediamine dihydrochloride (PPD-DHC), 4-nitroaniline, 4,4’-azodianiline, % of the total number of patients tested in time period II, ratio males(M)/female(F).

<table>
<thead>
<tr>
<th>Time period</th>
<th>Series tested</th>
<th>Chemicals tested in pet. (%)</th>
<th>Total no. positive reactions No. (%) (M:F)</th>
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<tr>
<td>II</td>
<td>Baseline series</td>
<td>PPD 1.0</td>
<td>26 (3.6)</td>
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<tr>
<td>718 (218:500)</td>
<td>2,5-TDA-S 2.0</td>
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<td>7 (1.0)</td>
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<td>PPD 0.1</td>
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<td>PPD-DHC 1.7</td>
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<td>4,4’-Azodianiline 0.2</td>
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<td>2,5-TDA 0.11</td>
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<td>6 (0.8)</td>
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<td></td>
<td>4-Nitroaniline 0.13</td>
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<td>3 (0.4)</td>
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<td>2,5-TDA 1.1</td>
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<td>15 (2.1)</td>
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</table>

**Figure 10.**
The distribution of allergic reactions in 31 patients with at least one positive reactions to 8 hair dyes substances tested in 718 patients tested in time period II. Abbreviations: p-phenylenediamine
5 Discussion

5.1 Studies I and III

In these two studies, we investigated the protective gloves used by hairdressers since they are exposed to many irritant factors and contact sensitisers (46). A number of previous studies on prevention of contact allergy in hairdressers have shown the efficacy of interventional strategies by teaching them the proper use of protective gloves (133). Even so, hairdressers often have hand eczema and sometimes accept this as part of their job (114), or are forced to change their occupation. Although the lack of appropriate education or implementation of recommended preventive measures can play a major role, it was worth investigating the actual ability of gloves to protect against chemicals, which is a meaningful issue in occupational dermatology relevant to different professions. For many at-risk occupations, gloves are the best means of protection, even though the use of glove is a risk factor in itself (118, 119, 134, 135). In the occupational dermatology field, the type of gloves used must depend on the nature of the chemicals involved (120).

Some in vitro studies on the protective gloves used by hairdressers have already been performed (123, 124). In vitro methods have the limitations of not reproducing normal working conditions, where occlusion, sweating, stretching, and skin temperature can affect performance of the gloves. In order to overcome this and mimic the real working conditions of hairdressers, in two consecutive studies described in this thesis we used an in vivo method to test the protective capacity of glove materials commonly used by hairdressers while dyeing hair.

Simulating work exposure, we have shown that the choice of glove material is often not sufficiently protective against chemicals, and that skin contact with allergens that has permeated through the glove will give rise to allergic contact dermatitis when used. Wearing the wrong glove might even, due to the fact that wearer thinks him/herself protected, prolong the time of exposure to sensitisers.

In study III, we wanted to investigate the NI gloves further. These that have already been found superior with regard to PPD, with other hair dyes containing main hair dye ingredient other than PPD. A new hair dye substance, ME-PPD, was recently introduced in Europe (136-139) with the aim of reducing the risk of
sensitization and/or elicitation of allergic contact dermatitis. The hair dye containing ME-PPD was investigated in study III.

In study III, the further investigation of the protection provided by NI gloves against other HDp—and one HDhu—also involved the comparison with PVC gloves, which are still popular among hairdressers in Malmö, and a PE glove that was supplied in the package of the HDhu tested.

Fixed times were decided for the provocation in vivo and were 45 min in the case of HDp and 30 min for the HDhu according to the timing for application described in the leaflet.

The two NI gloves gave complete protection from all the hair dyes tested, which was comparable to that with the negative control (the 4H® glove) (Table 6).

The PE glove was completely protective against the HDhu (table 6).

When tested with HDp-ME-PPD, both PVC and PE showed excellent protection. In the study we could not patch test with a dilution series of the substance as such, so no indication of the reactivity pattern to ME-PPD could be shown. However, this hair dye has recently been introduced on the market, and the pattern of reactivity in patients primarily sensitized to ME-PPD is unknown. Previous studies have shown that when tested in PPD-allergic subjects under simulated conditions of hair dye use, ME-PPD cross-elicitated a lower degree of reactivity (140) and that the hair dye products containing ME-PPD were tolerated by 29 of 43 PPD/2,5-TDA allergic individuals (141). Accordingly, a larger group of individuals should be tested to find out the reason for the few reactions, and differentiate whether individuals had a low reactivity—or were only cross-reacting to the HDp-ME-PPD—or whether the gloves tested with this hair dye provided a good protection.

The studies have thus found that the NI gloves give excellent protection against the permanent hair dyes tested and therefore nitrile gloves should be the first choice of gloves for hairdressers.

However limited, the study indicates that the skin exposure to HDhu for 30 min wearing the gloves supplied in the package of this kind of hair dye, can be considered safe. This does not prevent the home dye consumer from the risk of sensitisation due to the hair dyeing itself.
5.2 Study II

Nowadays cosmetic products, as well as all the rest of consumer goods, can be bought on the internet or whilst travelling.

With regard to the European Union (EU) regulation No 1223/2009 on cosmetic products, amended in 2013, the concentration of PPD applied to the hair should not exceed 2%, calculated as free base, after mixing under oxidative conditions. The limit stated with regards to 2,5-TDA or 2,5-TDA-S is 4% (142). In this study, the results of the HPLC analysis of the content of PPD and 2,5-TDA on 52 products purchased in different countries indicated that the majority of the products bought in Europe contained 2,5-TDA. The products collected in non-European countries more frequently contained PPD (24 dyes) than 2,5-TDA (3 dyes), and generally at higher concentration, even when within the limits of EU legislation (table 4). The analyses indicated that all the products purchased in Europe contained concentrations of PPD and 2,5-TDA within the limits stipulated by the EU regulation (table 7). One hair dye purchased in Kenya contained PPD (31.5%) at a calculated on-head concentration of 7.9%, i.e. almost four times the limit stated by EU law.

The concentration of PPD analysed in two other hair dyes (one from Kenya and one from Singapore) was 14.2% and 3.9%, respectively. Since no information was given on how to mix the hair dyes with the oxidation agents, the consumer might actually when using these products be exposed to levels exceeding the allowed. (table 8).

The studying of labelling showed discrepancies with results of the chemical analysis in 6 (11.5%) of the samples that the labelled allergen could not be found in the analysis. The labelling could thus be said to be over-protective.

Legislation is intended to protect consumers from the risk of acquiring sensitisation by the use of the products, nevertheless even if the majority of the products analysed contained concentrations of PPD and 2,5-TDA within the limits stipulated by legislation, the risk of developing contact allergy and/or allergic contact dermatitis still exists (143). There may be several reasons for this, the regulative measures might not be sufficient, the products not used in a correct way, repeated exposure might increase the risk to develop contact allergy and the oxidation process necessary might give rise to new sensitisers, as has been shown.

Measures could be developed using safer hair dyes. As new products are developed it is of utmost importance that, as these are used, research is performed to investigate how to best follow contact allergy frequencies and if other preventive measurements to ensure that consumers and hairdressers are protected are needed.
5.3 Study IV

The use of hair dyes is extremely popular all over the world. Hair dyes have different chemical composition in different countries. PPD is more common in hair dyes products sold in southern Europe and in non-European countries, while 2,5-TDA or 2,5-TDA-S in those sold in northern Europe. The clinical symptoms of hair dye allergy may be difficult to suspect for both consumer and clinician. That can depends on many factors such as: lack of information, latency of the reaction after exposure, or unusual clinical localization (face, neck, systemic reactions). Due to these facts and the fact that the use of hair dyes is so common at least one marker of hair dye contact allergy be in the baseline patch test series. Since the number of substances patch tested in the baseline series has to be limited, and the exposure to hair dyes ingredients differs in different countries of the world, it would be optimal if only one substance could be patch tested as marker of hair dyes allergy.

It has been argued that 2,5-TDA should be included in the baseline series in countries where it is the most common allergen in permanent hair dyes. This has previously been addressed and studies have considered PPD being the more effective hair dye marker (143).

In this study we wanted to further investigate if the hair dye marker could possibly be optimised. Besides being in their free forms, PPD and 2,5-TDA can also appear as salts (PPD-DHC and 2,5-TDA-S, respectively), and as haptens usually PPD is tested in its free form whereas 2,5-TDA is patch tested as salt. In an alkaline environment, PPD-DHC and 2,5-TDA-S are converted into PPD and 2,5-TDA, respectively, but in neutral and acidic environments they exist in their salt forms.

Recently it has been demonstrated (144) that when patch testing PPD and its salt in PPD-hypersensitive individuals, PPD traces more contact allergy than PPD-DHC. Statistically significant differences were observed when comparing number of positive reactions to PPD and to 2,5-TDA compared to their respective salts (p < 0.001 in case of PPD, and p = 0.021 in case of 2,5-TDA) (Figure 10).

PPD and 2,5-TDA, instead of PPD-DHC and 2,5-TDA-S, respectively, should thus be used for patch testing.

2,5-TDA as compared to PPD is more common in hair dyes in northern Europe. CA to 2,5-TDA could therefore be expected to be more common at our department than CA to PPD. Nevertheless, when PPD and 2,5-TDA were tested simultaneously at equimolar concentrations, a significantly higher rate of contact allergy to PPD was noted (3.6% as opposed to 2.1%; p = 0.013) (Tables 11 and 12, figure 10).
PPD can thus be considered the best screening substance to trace hair dye allergy. However, it should be noted that by only using PPD as a screening substance for hair dye allergy, 9.7% of the patients (3 of 31) patch tested in time period II would have been missed if 2,5-TDA had not been patch tested. The patch test with 2,5-TDA 1% in pet included in the hairdressers series should be considered in cases of strongly suspected reactions to permanent hair dye or temporary tattoos even considering possible unusual clinical presentations.

A statistically significant difference was observed comparing the number of positive reactions to 1.0% PPD and to 0.10% PPD, 3.6% as opposed to 1.1%, respectively; \( p < 0.001 \) (Tables 11 and 12, figure 10). Hence, lowering the patch test concentration of PPD as has previously been suggested (145) will result in a significant number of false-negative reactions with consequences for consumers and hairdressers with contact allergy to hair dyes.

4,4´-Azoedianiline and 4-nitroaniline have been investigated in previous studies as oxidation products of PPD (129). 4,4´-Azoedianiline appeared as a possible potent sensitiser, whereas 4-nitroaniline showed no sensitising capacity in an animal model but tended to cross react to PPD (129, 146). In the second time period of this study, 718 patients were patch tested with the two substances, both tested at concentrations equimolar to 0.10% PPD, to avoid the potential risk of active sensitisation. Slightly fewer patch test reactions were obtained to the two oxidation products as compared to 0.10% PPD.

No late-appearing reactions, as possible signs of active sensitisation, to any of the preparations tested in the two periods of the study, were noted.
Table 12. Study IV, time period II: pattern of positive reactions observed at test reading day (D)3/D4 and/or D7 in 31 patients positive to the sensitisers in the series of hair dyes ingredients of 718 (218 M, 500 F) patients tested. Chemicals tested: p-phenylenediamine (PPD), 2,5-toluenediamine (2,5-TDA), p-phenylenediamine-dihydrochloride (PPD-DHC), 4,4'-azodianiline, 2,5-toluendiamine sulphate (2,5-TDA-S), 4-nitroaniline. For all the patch test preparations the vehicle was petrolatum (pet).

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<th>2,5-TDA-S 2.0%</th>
<th>2,5-TDA 1.1%</th>
<th>PPD-DHC 1.7%</th>
<th>PPD 0.10%</th>
<th>2,5-TDA 0.11%</th>
<th>4,4'-Azodianiline 0.20%</th>
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6 Summary and concluding remarks

In this thesis permanent hair dyes have been studied.

With regard to exposure:

- We have found that with regard to exposure to hair dyes, PPD is more frequently found in hair dyes from southern Europe and in non-European countries whereas 2,5-TDA or 2,5-TDA–S is more commonly found in products sold in northern Europe.
- When analysing the content the products within Europe never exceeded the allowed limits with regard to the EU regulations.
- The products were in the majority of cases found to be labelled in an accurate way however if the consumer using the products could actually see the concentration of the chemicals on the label this would further help the consumer in choosing the best product.

Still self testing is recommended by manufacturers on the products even if dermatologists strongly advice against this

With regard to diagnostics:

- PPD tested at 1.0% in pet has proved to be a better tracer than 2,5-TDA. The free forms of PPD and 2,5-TDA, trace more contact allergy than the respective salt.
- Lowering the concentration of PPD at testing means that relevant contact allergies will be missed.
- When new hair dye ingredients are introduced into the market it is of utmost importance that studies are made on how to patch test the potential allergens in an optimal way.

With regard to protection:

In *in vivo* testing NI gloves have proved to give the best protection and should be recommended for hairdressers when dyeing hair.
hårfärgningsprocessen, hur vi testar, kontaktallergi frekvensen och hur man bäst skall skydda sig för allergen om man tex arbetar som frisör.


I avhandlingen har vi dels studerat exponering för toluendiamin samt parafenylendiamin i hårfärger som säljs för eget bruk dvs ”over the counter” produkter i 11 olika länder. Proukterna analyserades med HPLC(high pressure-liquid chromatography) för att kontrollera att det som deklarerades på produkten verkligen stämde med innehållet. Studien visade att för de produkter som köpts i Europa efterföljdes regelverket medan om du som konsument inhandlat en produkt på nätet eller i annat land kan den innehålla betydligt mycket mer än de i Europa tillåtna koncentrationerna av PPD. Vi studerade också några oxidationsprodukter dvs ämnen vi funnit i hårfärg som fått oxidera, dessa produkter fann vi inte i de använda hårfärgarna under förutsättning att inte oxidationsmedel tillförts produkten. Egentestning av hårfärgämnet rekommenderas på majoriteten av produkternas förpackningar trots att dermatologer av sensibiliseringsskäl avråder från detta. Vi fann precis som tidigare studier att toluendiamin tycks vanligare i de nordeuropeiska länderna medan parafenylendiamin är vanligare i sydeuropa.

Frisörer löper ökad risk för handeksem, dels betingade av traumiterativa faktorer dels pga kontaktallergen. Frisörer under utbildning får lära sig att använda handskar för att skydda sig men det är fortfarande inte alltid så att frisörer i arbete använder handskar och framför allt användes det inte alltid korrekt. För att kunna ge adekvata råd om handskar är det viktigt att även läkaren har korrekt information. Handskar analyseras ofta avseende penetration av microber, vatten och in vitro för penetration av kemiska ämnen. Avseende hårfärger har dessa tidigare analyserats i in vitro experiment med tanke på penetration framför allt av färgsubstansen som sådan. Vid hårfärgning, dvs på det sätt frisören använder handsken så är det dock inte bara hårfärgämnet man kommer i kontakt med utan hårfärgen är blandad med parfymer, couplers och slutligen vid användning blandas

Acknowledgements

I would like to express my deepest appreciation and gratitude to the following people:

My supervisor Cecilia Svedman for encouraging my research over the years and for allowing me to grow as a research scientist. The discussion with her has always stimulated new insights in my research project.

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