Clinical and Experimental Studies on Oxidized Fragrance Terpenes as Contact Allergens

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Cover: Detail of lavender in bloom. Printed with permission from Mary (Moosey) Ruston, Mooseys Country Garden, New Zealand, www.mooseyscountrygarden.com.

I know a bank where the wild thyme blows,
Where oxlips and the nodding violet grows,
Quite over-canopied with luscious woodbine,
With sweet musk-roses and with eglantine…

From A Midsummer Night´s Dream by William Shakespeare

Abstract

The work presented in this thesis aims to improve the possibilities for diagnosing contact allergy to oxidized limonene and linalool and to, in patients, study contact allergy from structurally closely related hydroperoxides with regard to their specificity, potency and cross-reactivity. The fragrance terpenes limonene (from citrus oil) and linalool (from lavender oil) are widely used in household and industrial products. For both limonene and linalool, exposure to air at normal handling results in autoxidation and thus formation of allergenic oxidation products among which the primary oxidation products, the hydroperoxides, are the most potent allergens. Concomitant positive patch test reactions to oxidation mixtures where the main allergens are hydroperoxides are recorded in specific patch test studies. However today, oxidized terpenes are not included in routine patch testing for contact allergy.

An irritation study was performed for non-oxidized and oxidized limonene and linalool, showing that air oxidation increased irritation of both limonene and linalool and that oxidized limonene was more irritating than oxidized linalool at similar concentrations. Based on these results, a dose-response study was conducted in 3418 consecutive dermatitis patients investigating four patch test concentrations of oxidized linalool 2.0-11.0% in petrolatum (pet.). 5-7% of the patients showed positive patch test reactions to oxidized linalool. Oxidized linalool at 6.0% pet. concentration is suggested for future screening.

Concomitant positive patch test reactions and cross-reactivity of hydroperoxides were investigated in clinical studies and experimental procedures. No evidence for general cross-reactivity or formation of non-specific antigens upon administration of the investigated hydroperoxides was found. Cross-reactivity was only seen between hydroperoxides with great similarity in structure. Furthermore, three limonene hydroperoxide analogues were investigated as to allergenic potential in a modified murine local lymph node assay (LLNA) calculating on lymph nodes from individual mice. A statistically significant difference between sensitizing capacities of the investigated limonene hydroperoxides was established. Two of the limonene hydroperoxide analogues were assessed in individuals allergic to oxidized limonene. The analogue with the highest sensitizing capacity in the modified LLNA (limonene-1-hydroperoxide) gave more reactions in the tested individuals.

In conclusion, this thesis shows that it is important to study the contact allergenic potential of chemicals to which we are exposed. Seemingly harmless products may cause or worsen ACD. Such risks can only be detected by testing of relevant allergenic compounds in appropriate concentrations. The frequency of positive patch test reactions to autoxidized linalool observed among the dermatitis

patients, places this material among the most common contact allergens tested today. In addition, a new main allergen in the oxidation mixture of limonene, limonene-1-hydroperoxide, is proposed. The thesis confirms the effect of air oxidation on the allergenicity of common fragrance terpenes. Furthermore, the studies show the great specificity of the contact allergens *in vivo*. Finally, the impact on the allergenic potency by the primary oxidations products, the hydroperoxides is re-established in humans.

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. Reprints were made with permission from the publishers.

I. Air oxidation increases skin irritation from fragrance terpenes

Bråred Christensson J, Forsström P, Wennberg A-M, Karlberg A-T, Matura M.
Contact Dermatitis: 2009: 60: 32–40.

II. Oxidized linalool - a significant contact sensitizer

Bråred Christensson J, Matura M, Gruvberger B, Bruze M, Karlberg A-T.
Manuscript.

III. Hydroperoxides form specific antigens in contact allergy.

Bråred Christensson J, Matura M, Bäcktorp C, Börje A, Nilsson JLG, Karlberg A-T.
Contact Dermatitis: 2006; 55(4): 230-7.

IV. Limonene hydroperoxide analogues differ in allergenic activity.

Bråred Christensson J, Johansson S, Hagvall L, Jonsson C, Börje A, Karlberg A-T.
Contact Dermatitis: 2008; 59(6): 344-52.

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Abbreviations

ACD	Allergic contact dermatitis
APC	Antigen presenting cells
EC3	Estimated concentration to induce a stimulation index of 3
EU	European Union
FCAT	Freunds Complete Adjuvant Test
FM	Fragrance Mix,
FM II	Fragrance Mix II
GC	Gas Chromatography
GPMT	Guinea Pig Maximization Test
15-HPA	15-Hydroperoxyabietic acid
15-HPDA	15-Hydroperoxydehydroabietic acid
HPLC-UV	High Performance Liquid Chromatography with ultraviolet detector
ICD	Irritant contact dermatitis
ICDRG	International Contact Dermatitis Research Group
IL	Interleukin
LC	Langerhans cells
LDI	Laser Doppler Imaging
Lim-1-OOH	Limonene-1-hydroperoxide
Lim-2-Me-2-OOH	Limonene-2-methyl-2-hydroperoxide
Lim-2-OOH	Limonene-2-hydroperoxide
Lin-OOH	Linalool hydroperoxide
LLNA	Local lymph node assay
MHC	Major Histocompatability Complex
MP	Myroxylon Pereirae
Non-ox.	Non-oxidized
OECD	Organization for Economic Corporation and Development
Ox.	Oxidized
Pet.	Petrolatum
Ppm	Parts per million
QSAR	Quantitative structure activity relationships
ROAT	Repeated open application test
SAR	Structure activity relationships
SDS	Sodium dodecyl sulphate
SI	Stimulation Index
TNF	Tumor necrosis factor

INTRODUCTION

Fragrance materials have been utilized throughout history for religious, medicinal and aesthetic purposes. In early Egypt, resins such as myrrh and frankincense were used along with aromatic wood and bark, seeds, roots, and flowers to scent the atmosphere, often in religious ceremonies. The art of perfumery, the blending of odoriferous substances in appropriate proportions, was known to the ancient Chinese, Hindus, Egyptians, Carthaginians, Arabs, Greeks, and Romans. Initially, odorous plants or their resinous products were most probably used directly, or by extracts prepared by placing flowers, roots or leaves in fatty oils (1).

Many techniques which are still used in traditional perfume industry today, including distillation, evaporation and filtration, were developed in the 8th and 9th centuries by Arabian chemists. Following trade, new raw ingredients such as jasmine and various citrus were imported from South and East Asia. The technique of distillation spread to Europe during the Middle Ages and preparation of oils of cedarwood, rose, rosemary, sage, cinnamon and myrrh became a specialty of the European medieval pharmacies (1).

The first modern European perfume, consisting of scented oils blended in an alcohol solution, was made in 1370 and was known as Hungary Water. During the Renaissance period, perfumes were used primarily by royalty, nobility and the wealthy to mask body odours and compensate for other (un-) sanitary odours. In the 16th century, France gradually became the European center of perfume and cosmetic manufacture (1).

Still today, raw materials used in the fragrance industry include natural products of plant or animal origin. Essential oils are often obtained from plant materials by steam distillation or, in some cases, by solvent extraction by which a concrete, a solid substance with plant waxes, is obtained. Treatment of the concrete with a second agent, usually alcohol, separates the concentrated flower oil from the waxes, resulting in an "absolute". Citrus oils are extracted from fruit peels either by pressing with sponges or by mechanical maceration (1). The essential oils obtained are complex mixtures of different compounds and the composition of the oil depends on which species and what parts of the plant are used as well as on the origin of the plant, the time of harvest, and the distillation process.

Fine perfumes may contain more than 100 ingredients. A perfume is usually composed of a top note, the volatile odour first perceived; a middle note which lasts longer and provides a full, solid character; and a base note which is persistent and may last for hours on the skin. Perfumes are often classified

according to dominant odours such as floral groups (e.g. jasmine, rose or gardenia), spicy blends (e.g. carnation, clove or cinnamon), woody groups (e.g. aromatic grass, sandalwood or cedarwood) or Orientals (e.g. woody, mossy, spicy notes blended with sweet odours as vanilla or balsam, often accentuated with musk or civet) (1).

The art of perfumery has changed over the last two centuries as modern chemistry has led to industrial mass production of synthetic blends. This has vastly widened the use in the broad population and brought down prices. Since the later half of the 20th century, even fine perfumes are structured on synthetic chemicals, often in high concentrations. Industrially synthesized ingredients are often imitations of naturally occurring substances, but also production of entirely new chemicals takes place. Today, more than 4000 fragrance substances have been identified and about 2500 fragrance ingredients are in current use (2 and references therein).

Perfumes are usually alcoholic solutions and may contain about 15–30% fragrance concentrates. Eau de parfum contains 8-15% aromatic compounds while Eau de Toilette contains about 4-8% perfume concentrate. Eau de colognes usually contain about 3–5% fragrance concentrates. Recent developments include aerosol sprays and highly concentrated bath oils. Fragrances are today also found in a wide variety of domestic and industrial products. Industrial perfumes are added to products like paints, cleaning materials, rubber, plastic, paper and textiles (2 and references therein). Fragrances may be added to mask unpleasant natural odours from the products or to attract customers, e.g. a pleasant scent for toys or addition of leather odours to plastic furniture coverings (1, 2). Some fragrance materials are also used for other purposes, e.g. limonene from citrus peel oil may be used to give degreasing or cleansing properties to hand washes and polishes. Essential oils are also used in aromatherapy, herbal remedies, as antimicrobial agents, and even in locally applied medication and ointments either as a perfume ingredient or as a penetration enhancer.

Besides these beneficial and desired effects, fragrance compounds have known disadvantages as well, such as low grade toxicity, skin irritation, airway hyper-reactivity, phototoxicity and the possibility of causing contact allergy (3). Because of their widespread use, the exposure of the population to fragrance compounds is extensive. Being volatile compounds, fragrance chemicals are small and many of them are themselves contact allergens or prone to chemical processing by which they are transformed into contact allergens. In effect, with the widespread use and exposure, fragrances are common causes of contact allergy (4-7).

Contact Allergy

Contact allergy is common both in the general population and among dermatitis patients. It is estimated that 15-20% of the general population are sensitized to one or more contact allergens (4). When exposed to an allergen, a sensitized individual may develop allergic contact dermatitis (ACD). The most common causes of contact allergy are metals, especially nickel, followed by fragrances and preservatives (4, 5). The frequency and extent of exposure to allergenic chemicals are key factors for developing contact allergy, and by limiting the exposure to a certain allergenic chemical through legislation or changes in use and handling, the incidence of contact allergy is reduced over time (8). Contact allergy is considered to be life-long in the individual, although intra-individual variations have been seen in many studies when repeated tests for contact allergy have been performed (9, 10).

Both the initial induction (*sensitization*) phase and the following effector (*elicitation*) phase of contact allergy involve skin penetration, followed by binding of the hapten to skin components, and subsequent presentation of hapten-modified peptides to immunologically active T-cells.

Hapten-protein complex formation

Contact allergy is caused by haptens, which are small (<1000 Da), reactive compounds that have appropriate lipophilicity (logP≈2) to pass through the skin barrier. Haptens are too small to provoke an immune response themselves and become immunogenic after binding to macromolecules, generally considered to be proteins, in the skin (11).

The binding of the hapten to a skin protein is a critical step to make the hapten immunogenic. Most contact allergens have electrophilic properties and their functional groups are expected to react with nucleophilic groups in the side chains of the amino acids, e.g. thiol in cystein or primary amines present in lysine, thus forming covalent bonds (11). The metal ions, e. g. nickel or cobalt cations, have a different reactivity and are considered to form metal-protein chelate complexes by co-ordination bonds (11). In recent years, the possibility of antigen formation by a radical mechanism has been discussed, and evidence that protein interactions with hydroperoxides can take place by radical reactions has been given in cross-reactivity studies as well as in radical trapping experiments (11-14).

Some compounds are not reactive themselves but can be transformed into contact allergens either by skin metabolism or outside the body, the latter often by air oxidation (11). The name *prehapten* has been introduced for compounds activated outside the body, saving the name *prohapten* for compounds activated by specific enzymatic systems of the skin (15, 16). Many fragrance chemicals, e. g. limonene, linalool and linalyl acetate, have been found to be prehaptens (17-22). It has recently been

shown that the fragrance compound geraniol can be activated both by air oxidation (23) and by metabolic enzymes (24, 25) and thus is both a pre- and a prohapten.

Sensitization in contact allergy

The sensitization phase comprises the events resulting in the initial recognition of the hapten by hapten-specific T-cells and the subsequent clonal expansion of specific T-cells (Fig. 1).

At skin exposure, the hapten penetrates into the epidermis where dendritic antigen presenting cells (APC) known as Langerhans cells (LC) are found (26). The haptens associate to proteins either outside the LC before being internalized into the LC, or, in the case of lipophilic haptens, the free hapten may penetrate the LC leading to protein-binding inside the cell. The hapten-protein complex is then processed in the LC and hapten-modified peptides are presented in association with either Major Histocompatability Complex (MHC) class I or MHC class II molecules on the cell surface. The association of a hapten-modified peptide with either MHC class I or MHC class II molecules determines which group of T-cells that will be activated in a later step of the sensitization phase: antigen presentation in association with MHC class I primarily leads to CD8+ T-cells being activated while antigen presentation in association with MHC class II primarily leads to activation of CD4+ T-cells (26, 27).

Following the encounter with the allergen, the LC become activated and produce interleukin (IL)-1β, which in turn stimulates production of tumor necrosis factor (TNF)-α in the keratinocytes. As a result, the LC disentangle from the surrounding keratinocytes (27) and migrate towards the dermis. Production of matrix metalloproteinases facilitates LC penetration of the basal membrane and migration through the dermis (27). Antigen sampling also leads a maturation process by which changes in expression of adhesion molecules as well as expression of chemokine receptors occur, thus promoting LC migration to the afferent lymph vessels and towards the regional lymph node (27). Also upregulation of MHC molecules and co-stimulatory molecules take place in the process enabling the matured LC to present antigen effectively to naïve T-cells. In the paracortical area of the regional lymph node, the LC will settle as interdigitating cells with a large contact area which enhances the possibility to encounter naïve T-cells (27).

Naïve T-cells home to the paracortical areas of the lymph nodes and enter mainly by the high endothelial venules. The estimated frequency of a contact-allergen specific T-cell in a non-sensitized individual is 1 of 10^6 to 1 in 10^9 T-cells (26). At encounter, an "immunological synapse" is formed. An antigen specific signal, i.e. T-cell receptor recognition of MHC-antigen complex must be present. Secondly, interactions of costimulatory molecules on the LC and the T-cell are required. When these

two signals are present, activation and proliferation of the antigen specific naïve T-cell are induced (27).

When sensitization occurs, the naïve T-cell becomes activated resulting in clonal expansion generating antigen specific effector and memory T-cells that leave the lymph nodes via the efferent lymph vessels and pass into the blood. Their surface molecules allow them to pass into peripheral tissues and back to lymphoid organs through blood- and lymphatic vessels (26).
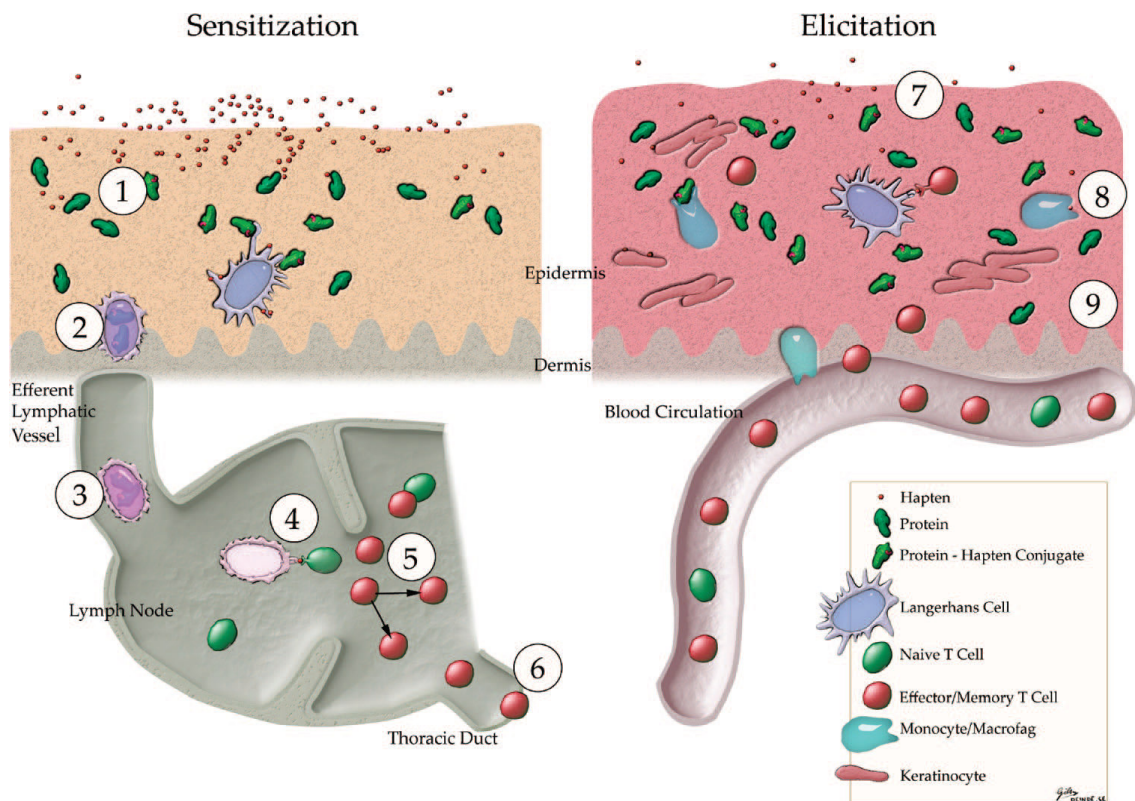

Figure 1. Sensitization and elicitation phases in contact allergy. **1** The hapten penetrates the epidermis and is internalized in the Langerhans cell (LC) either as a hapten-protein complex or a free hapten binding to proteins inside the cell. **2** Hapten-induced activation of LC and disentanglement from keratinocytes. **3** LC migrate to the draining lymph node. Hapten-protein complex is processed in the LC and presented in association with either Major Histocompatability Complex (MHC) class I or MHC class II molecules on the cell surface. **4** Presentation of antigens by LC to naïve T-cells in the paracortical areas of lymph nodes. **5** Proliferation of hapten-specific T-cells; memory and effector T-cells are formed. **6** Hapten-specific T-cells leave the lymph node and enter the circulation. **7** Re-exposure to the hapten. **8** Release of cytokines and chemokines attracting cells to the skin from the circulation, homing of hapten-specific T-cells and non-specific inflammatory cells to exposed area. **9** Inflammatory response within 2-3 days, symptoms of ACD (26). Illustration from *Karlberg et al.,* (11).

The sensitization phase usually requires at least 3 days to several weeks to complete. Normally, no clinical eczema is visible during this phase. The number of specific cells will, in the absence of further contacts with the allergen, decrease but not to the level as before the sensitization (26).

Elicitation in contact allergy

In the sensitized individual, re-exposure to the contact allergen may cause ACD. In this effector phase of contact allergy, the hapten again penetrates into the skin and binds to epidermal proteins and cells, causing release of pro-inflammatory cytokines such as IL-1β and TNF-α (26). This causes migration of activated LC towards the dermis, as well as increased expression of adhesion molecules on endothelial cells, leading to recruitment and extravasation of hapten-specific T-cells in sensitized individuals. These T-cells become activated when hapten-modified peptides are presented by APC or other cells in the skin, and the resulting release of pro-inflammatory cytokines and chemokines enhances further infiltration of inflammatory cells leading to an eczematous reaction at the site of exposure, the clinical expression of ACD. A maximum in the inflammatory reaction is usually reached within 2-3 days (26).

Patch testing

Patch testing is an established method to diagnose contact allergy, by which an eczematous reaction is obtained on intact skin in response to a localized application of a contact allergen. In this method, allergens in an appropriate vehicle are applied under occlusion in a test chamber on the skin, usually on the upper back of the patient. The standard time of application is 48 hours and the patch test readings are usually carried out on two occasions, first on day 2-3 and again on day 5-7 (28). The reactions are graded based on their morphological characteristics as – (negative), ? (doubtful), irr (irritant), + (weak or moderate positive reaction), ++ (strong positive reaction) and +++ (very strong positive reaction) according to the International Contact Dermatitis Research Group (ICDRG) (29).

The concentration of a test material used in patch testing, which is often several times higher than concentrations of the same substances found in consumer products, should be high enough to detect cases of contact allergy without provoking irritation or actively sensitizing the patient. It is important to avoid both false-positive and false-negative reactions, as this will lessen the reliability of the test. Guidelines on establishing an appropriate patch test concentration with respect to irritancy and active sensitization have been published in a review by Bruze *et al.* on behalf of the European Society of Contact Dermatitis (30). However, many test preparations have not undergone as thorough clinical studies as described by Bruze *et al.* Instead, "standard" concentrations of patch test substances have been established by traditions or following case reports, which may lead to sub-optimal diagnosing of contact allergy. Patients with a suspected ACD are usually tested with a baseline series of common

allergens, sometimes together with additional allergens specific for each individual case. The baseline patch test series is a selection of ubiquitous and frequent contact allergens, and thus important to screen with to diagnose contact allergy.

Predictive methods for skin sensitization

Predicting the allergenic activity of a chemical is of great importance and interest to avoid ACD in the population. Animal testing has been a reliable and preferred method in the past. However, a ban on *in-vivo* testing of cosmetics and toiletry ingredients for skin sensitizing properties within the European Union (EU) in 2013 (31), has intensified the search for alternative methods for prediction of contact allergenic activity.

The guinea pig has, since many decades, been used as an experimental animal in predictive testing for contact allergy and several test methods have been described. Two methods, the Buehler test and the Guinea Pig Maximization Test (GPMT), are currently recommended according to Organization for Economic Corporation and Development (OECD) guidelines (32). The Buehler test involves epidermal exposure to the test compound while the GPMT has both intradermal and epidermal exposure (33-35). Freunds Complete Adjuvant Test (FCAT), involving only intradermal induction, was specifically developed for testing of fragrance compounds, and has been validated and tested along with other guinea pig models (36, 37). This later method was chosen for the guinea pig studies in paper III. In interpreting the results of the FCAT, the number of animals with a positive reaction at skin challenge in the exposed group is compared with the number of animals reacting in a sham treated control group, i.e. induced in the same way but without the test compound. These guinea pig models thus investigate the elicitation phase rather than the sensitization phase of contact allergy. As the whole process from sensitization to elicitation is involved, these methods mirror the clinical testing situation.

The more recently developed murine local lymph node assay (LLNA) is a stable and reliable predictor of contact allergenic activity, which has been evaluated and accepted by the U.S. Food and Drug administration (FDA) and recommended by the OECD (11, 38). In this model, the test compound is repeatedly applied to the dorsum of the ears of a group of mice and the proliferation of lymphocytes in the local lymph nodes is assessed quantitatively and compared to controls (39, 40). This model includes skin penetration of the hapten and investigates the sensitization phase of contact allergy. The LLNA gives a dose-response relationship and provides a comparative measure by an EC3 (Estimated concentration to induce a stimulation index (SI) of 3) value, which may be used to classify the sensitizing capacities of different test compounds (41, 42). The disadvantages with this method are that the measurements may not differentiate between allergens and strong irritants, thus there is a possibility of false-positive responses in the LLNA from irritants at high concentrations (43).

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To assess the sensitizing capacity of various chemicals *in vitro*, cell systems using human dendritic cells have been developed. In these cell systems, dendritic cells have been studied after exposure to potential contact sensitizers as well as irritants, with regard to altered cell surface markers, gene expressions for e.g. chemokine and cytokine production and receptors, and chemokine and cytokine levels (27 and references therein). *In vitro* methods measuring the reactivity of chemicals towards peptides with nucleophilic properties, e.g. glutathione have also been developed. The data obtained is correlated to the sensitizing potency for the same chemical in the LLNA. These studies have shown good correspondence for strong and moderate sensitizers (11 and references therein).

The biological activity of a chemical compound is a function of its structural and biophysical properties. It has been shown repeatedly that the structural requirements for haptens are specific and chemical structures indicating biological activity have been identified, e. g. aldehydes and epoxides. In structure activity relationships (SARs) and quantitative structure activity relationships (QSARs), chemical structures of compounds have been correlated with chemical reactivity or biological activity, the latter based mainly on data from animal predictive methods for skin sensitization. Large data banks have been compiled and developed to allow predictions of data for homogenous series of compounds, and computer-based expert systems have been made available to forecast the sensitizing capacities of potential contact allergens (11 and references therein).

Irritation

Mechanisms of irritant contact dermatitis

Irritant contact dermatitis (ICD) is an eczematous reaction in the skin as a result of external factors but with no eliciting contact allergens. In response to exposure to irritants, changes in morphology, surface markers and cytokine production have been noted in keratinocytes, and also increase in mononuclear cells, in particular of CD4+ T-lymphocytes, has been observed. Irritants have been found to cause upregulation of different cytokines in ICD, among which IL-1 and TNF-α are considered being of great importance (44). Besides directly affecting keratinocytes, irritants can cause dermatitis in several different ways depending on the biological activity of the compound. Disrupture of the horny layer and effects on blood vessels are some examples.

A broad spectra of external factors may cause irritation. In the extreme, highly alkaline or acid compounds can cause tissue destruction after a very brief exposure, while weaker irritants, such as water, detergents, oils, heat, cold or friction generally result in a chronic cumulative ICD, visible after multiple exposures. Individuals have different thresholds for ICD, and the localization for the exposure, the age of the individual and pre-existing skin diseases all affect the onset of ICD (44).

Assessment of ICD

As the cause of ICD and the subsequential damage to the skin is different depending on the causative agent, the assessment of ICD is difficult and a specific test for ICD is not available. Several techniques and test substances have been used to assess ICD. Sodium dodecyl sulphate (SDS) has been extensively tested in experimental settings as a model substance for chemicals primarily disrupting the barrier function of the skin (45), as well as in combination with different allergens (46-49). Some other experimentally used irritants, such as nonanoic acid, croton oil or benzalkonium chloride are considered to cause ICD by different mechanisms (44 and references therein). It has been noted that increased susceptibility to one irritant is not predicitive of susceptibility to other types of irritants (50). Methods of assessment of ICD include transepidermal water loss (TEWL) which measures barrier disruption, Laser Doppler Imaging (LDI) measuring variations in blood flow, and visual scoring.

Irritation and contact allergy

Many contact allergens have irritant properties and it has been stipulated that sensitization does not come from the antigenic signal alone but together with associated irritancy, inherent in the hapten or by simultaneous exposure (49 and references therein). An increased response to an allergen is observed in earlier sensitized individuals in the presence of irritants (46-48). It has also been shown that additive effects of irritants and allergens can increase the risk of sensitization (51). Effects on skin response after exposure to different combinations of allergens and irritants have been extensively reviewed (49).

Contact allergy to fragrances

Diagnostic markers of contact allergy to fragrance materials

Fragrances are today the second most common causes of contact allergy, after nickel (52, 53). The diagnostic tools in the baseline patch testing series consist of the Fragrance Mix (FM), Myroxylon Pereirae (MP) and the more recently introduced Fragrance Mix II (FM II) (54, 55).

The FM consists of 7 defined fragrance ingredients and one natural blend. Historically, the constituents of FM have been widely used in perfumed products. However, the use of fragrances has changed over time and recently developed perfumes contain the FM ingredients to a lesser extent (56). The frequency of positive reactions to FM varies between 4% and 11% in patch-tested dermatitis patients (5-7, 52, 53), and estimates from Denmark and Norway suggest that about 1.5% of the general population are sensitized to ingredients of the FM (57, 58).

MP is a natural blend which is collected from the *Myroxylon balsamum var. pereirae-* tree which grows in El Salvador. Use of MP as a fragrance ingredient in cosmetics is not permitted in the EU, but there is still use of the extract or distillate of MP (59). MP in the baseline patch test series is mostly

regarded as a general screening agent for contact allergy to fragrances and the frequency of positive reactions varies between 2% and 8% in patch-tested dermatitis patients (2, 5, 7, 52, 53).

A recent addition to the European baseline patch test series is a group of 6 fragrance chemicals in FM II. This relatively new test mixture has shown positive patch test reactions in about 2% to 5% of tested dermatitis patients (54, 55).

Also colophonium is by some investigators considered to be a fragrance marker, and is often presented in patch test studies regarding contact allergy to fragrances (7, 60-63). The relationship of contact allergy to colophonium and fragrance markers has been debated over the years (64, 65). Although concomitant reactions to colophonium often are seen in patients reacting to fragrance markers, it is important to remember that colophonium has a large usage in many products not related to fragrances.

Contact allergy to fragrances and legislation

As high rates of contact allergy have been recorded to various chemicals, legislative measures and usage recommendations have been made to lessen the exposure to the population. Examples of such an intervention is the nickel directive, which was introduced in Denmark in 1989 and throughout the EU in 2000 (66). The use of allergenic preservatives has been regulated as well. A recent measure was taken within the EU in the Cosmetics Directive by which 26 fragrance chemicals which are potential contact allergens, must be labelled on cosmetic products as well as hygiene products and detergents when used in concentrations >10 ppm in leave-on products and >100 ppm in rinse off products (31). The monoterpenes limonene and linalool are found among the listed fragrance ingredients in the Cosmetics Directive. As data on which levels of allergens in consumer products will increase sensitization rates in the population are scarce, intervention measures usually include total ban on usage of certain compounds or theoretical estimates of "safe levels".

Terpenes and autoxidation

Terpenes constitute a large and structurally diverse family of natural products which share the common structural unit isoprene (C_5H_8). Terpenes are classified by the number of isoprene units in the molecule, the smallest being monoterpenes consisting of two isoprene units (C10). Many of the most common fragrance chemicals used today are monoterpenes, e. g. linalool, limonene, linalyl acetate and geraniol.

Mechanism of autoxidation and formation of oxidation products

When exposed to air, organic molecules with unsaturated double bonds, such as terpenes, are prone to autoxidation. In the initial step of autoxidation of unsaturated molecules, the site adjacent to a double bond is involved. When the allylic hydrogen atom is abstracted, a stabilized radical (R•) is formed which reacts with oxygen, resulting in the formation of a peroxy radical. This peroxy radical can, in

turn, abstract a hydrogen atom to form a hydroperoxide while generating a new radical R$^\bullet$, and thus propagating the oxidation chain reaction (Fig. 2) (11).
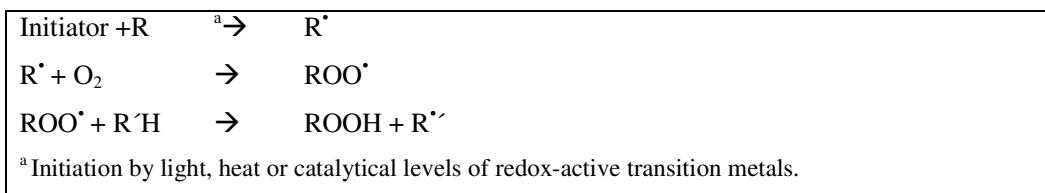
Initiator +R	$^a\rightarrow$	R$^\bullet$
R$^\bullet$ + O$_2$	$\rightarrow$	ROO$^\bullet$
ROO$^\bullet$ + R´H	$\rightarrow$	ROOH + R$^{\bullet´}$
^{a} Initiation by light, heat or catalytical levels of redox-active transition metals.		

Figure 2. General autoxidation mechanism

Many terpenes are not protein-reactive or allergenic in their basic form. However, as these molecules autoxidize, they incorporate oxygen in their structures and form primary oxidation products, hydroperoxides, which, in turn form secondary oxidation products, i. e. epoxides, aldehydes, ketones and alcohols (Fig. 3). The oxidation products can be allergenic, especially the hydroperoxides are strong sensitizers. When acting as haptens, the hydroperoxides are prone to react with proteins by a radical mechanism while the epoxides, aldehydes and ketones, if they are haptens at all, are electrophiles (11).

Effect of autoxidation on contact allergy
The discussion concerning the activation of non-allergenic compounds into potent contact allergens by air oxidation commenced in the middle of the 20th century with observations that the allergenic potential of turpentine, a mixture of monoterpenes obtained from coniferous trees, was related to the content of Δ^3-carene, and possibly a hydroperoxide of Δ^3-carene (11 and references therein). Later on, in the extensive work on colophonium, a resin obtained from coniferous trees, components and oxidation products were isolated, identified and systematically studied as to structure and allergenicity in experimental and clinical studies (67-70). A hydroperoxide, 15-hydroperoxyabietic acid (15-HPA), has been identified as the major allergen in colophonium (11, 67, 68)

Subsequently, a number of fragrance terpenes have been investigated with regard to autoxidation. Limonene, from citrus oil, was extensively studied in a manner similar to that of colophonium, and its primary and secondary oxidation products were mapped and examined in experimental and clinical studies (17-19, 60-62, 71, 72). Following this, linalool from lavender oil was studied (20, 21, 63), leading up to the works presented in this thesis. Meanwhile, also other fragrance terpenes, i. e. linalyl acetate, geraniol, caryophyllene have been investigated with regard to autoxidation (22, 23, 73).

In all fragrance terpenes listed above, the pure compounds are shown to have weak or no allergenic activity whereas the identified oxidation products have shown a range of allergenic activity from non-sensitizers to strong allergens. In all cases given above except caryophyllene, the primary oxidation

products, the hydroperoxides, have been identified as the main contact allergens with a great impact on the allergenic potential of the oxidation mixtures (18, 20-23, 60, 61). The hydroperoxides formed from fragrance terpenes so far studied, have shown a remarkably homogenous allergenic potential with an EC3 value of approximately 1%, thus classified as strong allergens according to the LLNA.

In addition, clinical studies using ox. terpenes and colophonium have repeatedly shown high frequencies of concomitant positive patch test reactions (60-63). Theoretically, concomitant reactions can be explained by concomitant sensitization or by true cross-reactions, but also by the hydroperoxides acting as oxidizing agents on skin proteins to form non-specific antigens without hapten- protein binding.

Primary oxidation products

hydroperoxides   peroxides

Secondary oxidation products

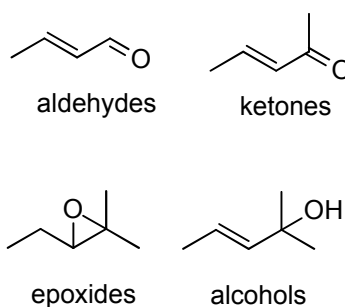aldehydes   ketones
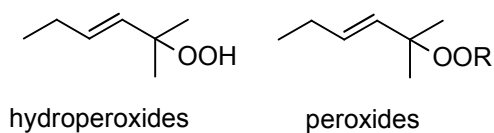
epoxides   alcohols

Figure 3: Compounds formed at autoxidation of unsaturated monoterpenes

Properties and usage of compounds and materials investigated

Colophonium

Colophonium is used in a variety of products, mainly paper size, adhesives, emulgators and soldering fluxes (74). Most colophonium used today is modified through reaction with different chemicals to improve technical properties and to lessen allergenicity. Unmodified colophonium consists of resin acids (90%) and neutral substances (10%). Autoxidation of abietic acid (Fig. 4), the main component in colophonium, results in the formation of 15-HPA, the most potent allergen in colophonium (67, 68). In the baseline patch test series, colophonium is tested at 20% in petrolatum (pet.) and renders a 2-5% frequency of positive patch test reactions in consecutive dermatitis patients (5, 7, 52, 53).
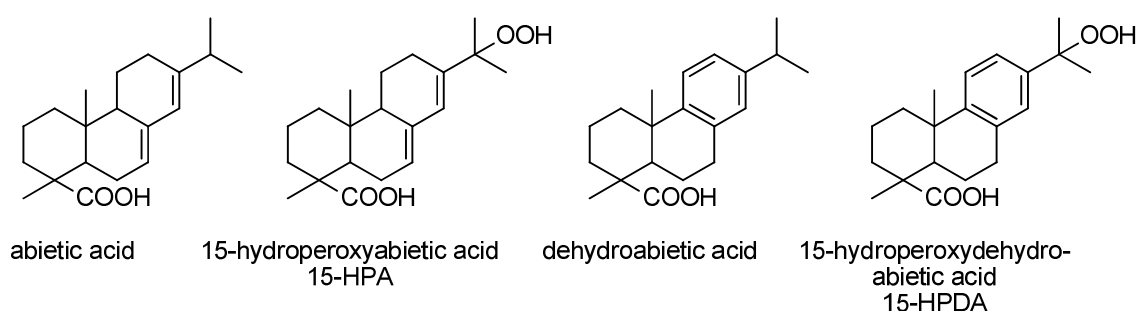

abietic acid 15-hydroperoxyabietic acid 15-HPA dehydroabietic acid 15-hydroperoxydehydro-abietic acid 15-HPDA

Figure 4. Major constituents in colophonium and their primary oxidation products (67-70).

Linalool

Linalool is a naturally occurring terpene, present in various plants, e.g. in lavender (75). It constitutes about 35-45% of natural lavender oil (75, 76). It is also synthetically produced at low costs and widely used in household and hygiene products as a fragrance chemical due to its fresh, flowery odour. According to several studies, linalool is presently among the most common fragrance ingredients used in consumer products (77-79). In cosmetics and hygiene products, the concentration of linalool is low (77, 78), but in aromatherapy and natural products, as well as in home-made soaps and fragrances, higher concentrations are possible. Pure linalool is non-allergenic or a very weak allergen and clinical studies have shown that pure linalool seldom causes positive patch test reactions in dermatitis patients (63, 80, 81). Linalool autoxidizes when exposed to air (Fig. 5). The oxidation mixture is allergenic and has been analyzed regarding main allergens (Fig. 6) (20, 21). Two hydroperoxides (Fig. 6) were found to be the main contact allergens (5:3 mixture gave EC3 value 1.6% (w/v)). One earlier clinical multicenter study has been conducted regarding ox. linalool, using a patch test concentration of 2.0% pet., showing 1.3% positive patch test reactions in the 1511 dermatitis patients tested (63).
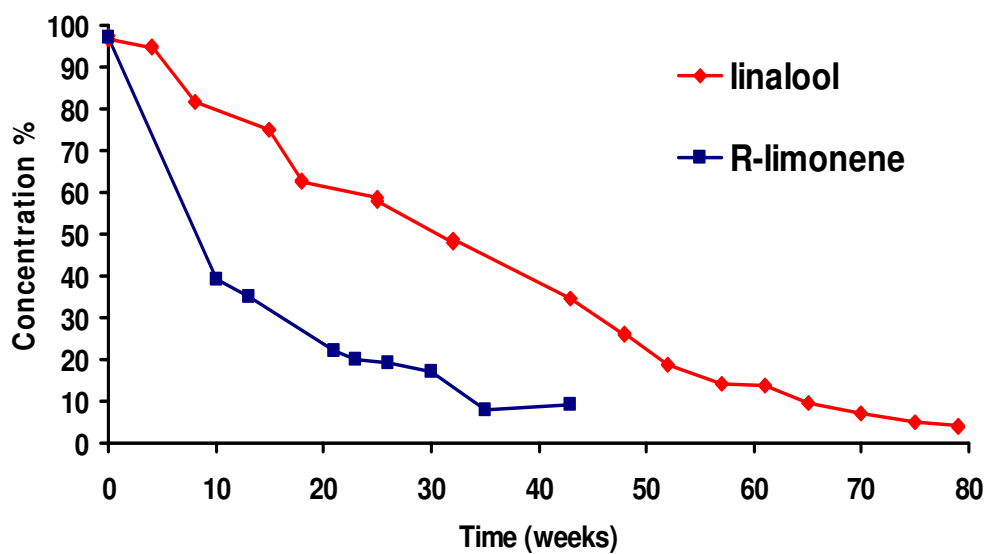
Figure 5. Decomposition rates of linalool and *R*-limonene when exposed to air. The flasks were exposed to light for 12 h per day at room temperature stirred for 1 h 4 times daily (20, 21, 60).
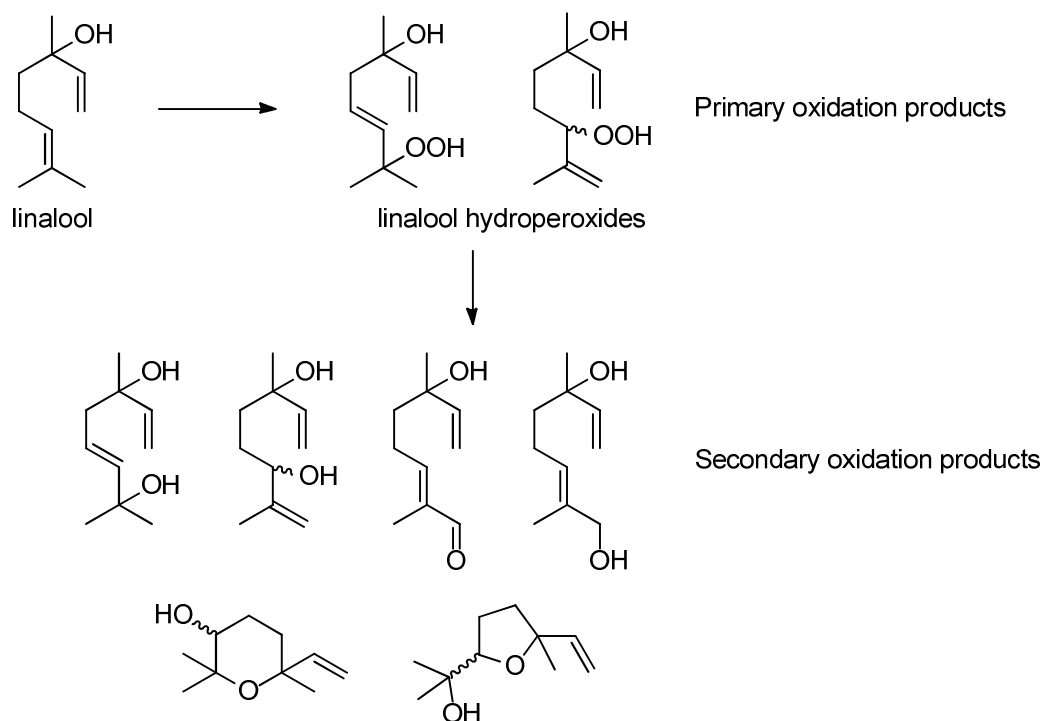

Figure 6. Identified oxidation products formed by autoxidation of linalool (20, 21).

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Limonene

R–Limonene is the main constituent (98%) of peel oil from citrus fruits. *R*-limonene and its enantiomer *S*-limonene are the mirror images of one another and are found in different ratios and amounts in different plants (82). The main source of exposure in the population is considered to be the *R*-isomer since the limonene used as an added fragrance ingredient is mainly obtained as a by-product from the citrus juice industry (personal communication with the fragrance industry). *R*-limonene or dipentene (a mixture of *R*- and *S*-limonene) are used as solvents and as industrial degreasing agents, sometimes in very high (up to 80-100%) concentrations, while industrial hand cleansers with degreasing properties may consist of 5-15% of *R*-limonene. In cosmetics and hygiene products, *R*-limonene is widely used as a fragrance chemical (77-79) in low concentrations, but, like linalool, higher concentrations are possible in aromatherapy, natural products and in home-made soaps and fragrances.

Pure *R*-limonene is a weak contact allergen in experimental studies (17, 71) and seldom causes positive patch test reactions in dermatitis patients in clinical studies (72, 80, 81). In autoxidation of *R*-limonene, the primary oxidation products, the hydroperoxides, are the most potent allergens in the oxidation mixture (limonene-1-hydroperoxide (Lim-1-OOH): EC3 value 0.33% (w/v); limonene-2-hydroperoxide (Lim-2-OOH): EC3 value 0.83% (w/v)) (14, 18, 60, 61) (Fig.7). Ox. *R*-limonene has, in earlier clinical studies, caused positive patch test reactions in a frequency of 2-3% in consecutive dermatitis patients (60-62, 72). Studies have shown similar oxidation patterns and frequencies of positive patch test reactions to *R*- and *S*-limonene in consecutively tested dermatitis patients (62).
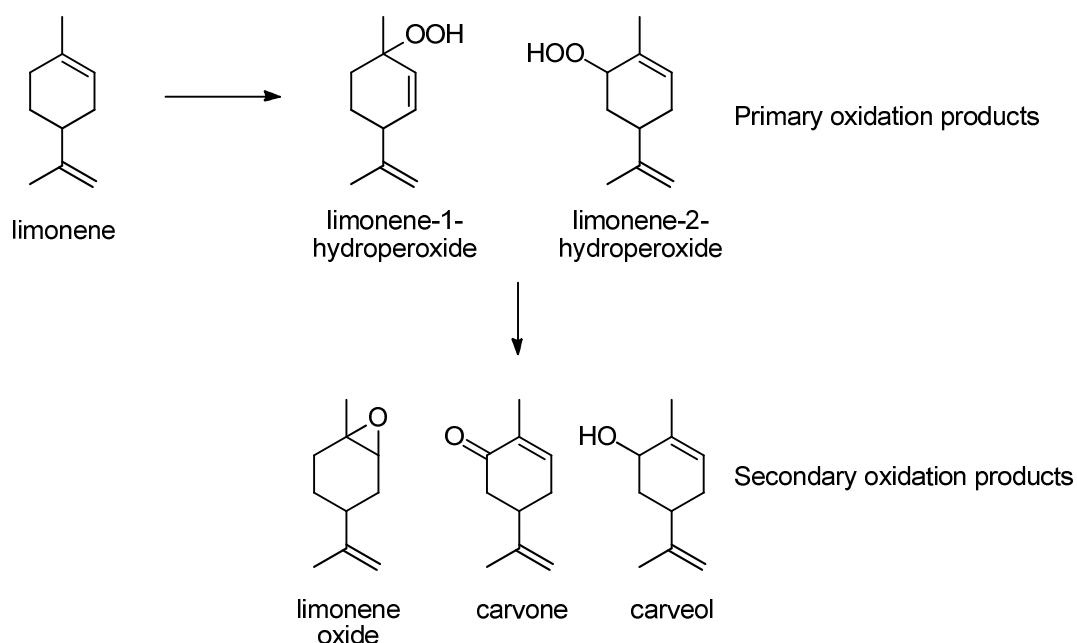

Figure 7. Identified oxidation products formed by autoxidation of *R*-limonene (14, 18, 19)

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AIMS OF THE STUDIES

The present investigations are part of a research program aiming to provide knowledge concerning the autoxidation of fragrance terpenes and the allergenic activity of their oxidation products.

The specific aims of this thesis are:

1. To improve the possibilities for diagnosing contact allergy to oxidized limonene and linalool (Papers I, II).

2. To study contact allergy in patients from structurally closely related hydroperoxides with regard to their specificity, potency and cross-reactivity (Papers III, IV).

METHODS

Chemicals

Linalool (3,7-dimethyl-1,6-octadien-3-ol), *R*-Limonene (*d*-limonene, p-mentha-1,8-diene), and cumene hydroperoxide (in mixture with cumene alcohol (4:1)) were commercially obtained. The chemicals were analyzed to confirm composition and purified by distillation before further use.

15-HPA (83), 15-hydroperoxydehydroabietic acid (15-HPDA) (Fig. 4) (84), cyclohexene hydroperoxide (Fig. 8) (13), Lim-1-OOH (14), Lim-2-OOH (Fig. 7) (14), and limonene-2-methyl-2-hydroperoxide (Lim-2-Me-2-OOH) (Fig. 8) (14) were synthesized as described in the literature. Linalool hydroperoxides (Lin-OOH) were prepared by photooxidation according to the literature (20, 21) and obtained as a 5:3 mixture of 7-hydroperoxy-3,7-dimetylocta-1,5-diene-3-ol and 6-hydroperoxy-3,7-dimetylocta-1,7-diene-3-ol (Fig. 6).
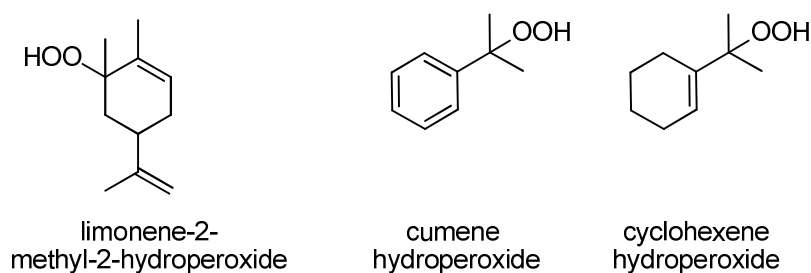

Figure 8. Additional compounds in the hydroperoxide studies (papers III and IV).

Air exposure procedure and quantifications

Both linalool and *R*-limonene were air oxidized according to a simplified experimental oxidation model. The purified terpenes were exposed to air in Erlenmeyer flasks. A daylight lamp was used to provide daylight conditions that would not be affected by seasonal variations. The flasks were exposed to light for 12 h a day at room temperature and were stirred for 1 h 4 times daily according to previous experience (17). The oxidation processes for limonene and linalool were followed with samples taken out every 1-2 weeks and stored at -20°C until analyzed. Gas Chromatography (GC) was used to analyze the remaining pure terpenes in the oxidation mixture while the High Performance Liquid Chromatography (HPLC-UV) was used to measure the hydroperoxide content as the high temperatures in the GC caused degradation of these compounds (20, 21). Pure reference compounds were used to make external calibration curves from which the concentrations of pure terpenes and hydroperoxides, respectively, were calculated.

Two batches of ox. *R*-limonene were used in the studies. A smaller batch was oxidized for 10 weeks and, at this time, contained 59% limonene and 7% Lim-2-OOH. A larger batch was oxidized for 21 weeks until the composition corresponded to the oxidation mixture previously obtained, containing 55% of limonene, 6% Lim-2-OOH and 1% Lim-1-OOH. For linalool, the oxidation mixture contained 30% linalool and 19% of linalool hydroperoxides after 45 weeks. Oxidized samples of *R*-limonene and linalool were stored under argon at -20ºC, until test preparations were made. Chemical analyses were performed on a regular basis of the stored non-ox. and ox. test materials to ensure that the compositions had not changed during storage.

Patch test materials

All patch test materials prepared at the Dermatochemistry unit, University of Gothenburg, were made using nonstabilized white petrolatum (pet.) (% w/w) according to previous experience (85). The ox. patch test preparations for the irritation studies (paper I) were kept in -20°C between the study periods whereas new test materials were made for the non-ox. test preparations at each study period. In the dose-response study (paper II), new patch test preparations were made every 2-3 months from the same batches and the test preparations of ox. linalool were kept in 5 ml syringes in the refrigerator between the tests. For the hydroperoxide studies (papers III and IV), patch test material for 15-HPA was stored at -70°C while patch test materials for limonene- and linalool hydroperoxides were stored at -20°C until used.

Standard allergens used for patch testing (papers II and III) were obtained from Chemotechnique Diagnostics AB (Vellinge, Sweden). Finn Chambers® on Scanpor® tape were used in all studies.

Laser Doppler Imaging (LDI)

The laser Doppler perfusion imager PIM 1.0 from Lisca Development AB, Linköping, Sweden was used to measure superficial skin perfusion (86). The guideline publication for laser Doppler imaging made by the standardization group of the European Society of Contact Dermatitis was followed to minimize the effect of the variables related to individuals, environment, and technique (87). A ROI (region of interest) of approximately 100 measurement sites was identified in the LDI image and the mean perfusion value was in this way calculated from each patch test area.

Clinical studies

In all the clinical patch test procedures, approximately 20 mg (88) of patch test preparations were applied in small Finn Chambers® (diameter 8 mm, inner area of 0.5 cm^2) on Scanpor tape® to the back of the patient and left under occlusion for 48 h and then removed by the patient. In papers II, III

and IV, readings for contact allergy were performed on D3-4 and D6-7 according to the International Contact Dermatitis Research Group recommendation (28).

Irritation study

In the irritation study (paper I), random patients undergoing patch testing for suspected contact dermatitis were selected at the Department of Dermatology, Sahlgrenska University Hospital. The exclusion criterium for the participating patients in this study, in addition to general excusion criteria for patch testing, was suspected fragrance allergy. 20 patients and 9 controls were included. The test preparations consisted of non-ox. linalool, ox. linalool, non-ox. *R*-limonene and ox. *R*-limonene in concentration series. On D3, irritation was assessed visually using a 10-grade scale (Table 1) (89), and by LDI.

Table 1. Visual assessment of erythema grading scale used in the irritation study (paper I) (89).

Grade	Erythematous reaction
0	No reaction
1	Marginal reaction
2	Slight perceptible erythema
3	A greater than slight reaction which is not sufficient to be classified as distinct
4	Distinct erythema
5	A greater than distinct reaction which is insufficient to be classed as well developed
6	Well developed, possibly spreading erythema
7	A greater reaction which is not sufficient to be classed as strong
8	Strong, deep erythema which may extend beyond the treatment site
9	A more intense reaction than above

Patch test studies

In the dose-response study (paper II), consecutive patients undergoing patch testing because of suspected ACD at the Department of Dermatology, Sahlgrenska University Hospital, Gothenburg, Sweden and the Department of Occupational and Environmental Dermatology, Malmö University Hospital, Malmö, Sweden, were screened with ox. linalool in addition to their regular patch testing. In total, 3418 patients were tested. Four concentrations of ox. linalool were used. In the third step of the dose-response study, concomitant reactions to other fragrance allergens and colophonium in the baseline patch test series were assessed.

For the studies on hydroperoxides (papers III and IV), individuals were selected from patch tested dermatitis patients at the Department of Dermatology, Sahlgrenska University Hospital, Göteborg. For the hydroperoxide studies in paper III, 29 individuals who had shown positive patch test reactions to colophonium in recent years were included. Patch testing was performed with colophonium and with 15-HPA, Lim-2-OOH and Lin-OOH in equimolar concentrations (61, 70). In paper IV, 7 individuals who had shown positive patch test reactions ox. *R*-limonene were included. Patch testing was

performed with non-ox. *R*-limonene, ox. *R*-limonene and with Lim-1-OOH and Lim-2-OOH (Fig. 7) in concentration series.

The clinical studies in papers I, III and IV were approved by the local ethics committee and all participants were included after informed consent.

Experimental sensitization studies

All experimental sensitization studies were approved by the local ethics committee.

Freunds complete adjuvant test (FCAT)

The protocol for the modified FCAT with closed challenge testing at elicitation is given in Fig. 9 (36, 37). In the hydroperoxide cross-reactivity study (paper III), three groups of 15 animals, one of which was a control group, were used. Induction was made with cumene hydroperoxide (Fig. 8) or Lim-2-OOH (Fig. 7) and challenge testing was performed with cumene hydroperoxide, cyclohexene hydroperoxide (Fig. 8) and Lim-2-OOH. In addition, the animals induced with cumene hydroperoxide were also challenged with 15-HPDA (Fig. 5). Visual evaluation of the skin reactions was conducted 48h and 72 h after application.
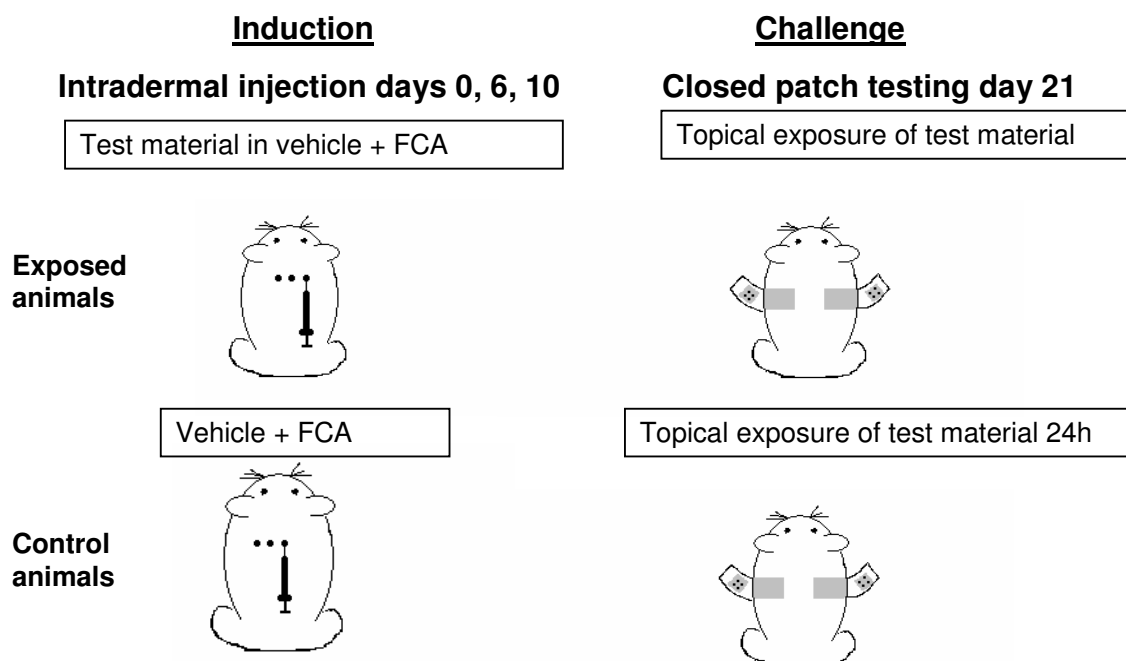

Figure 9. Protocol for Freund's Complete Adjuvant Test (FCAT) (36, 37). In this procedure, the animal is sensitized by three intradermal injections with the test compound in Freund's Complete Adjuvant (adjuvant solution containing inactivated mycobacteria). The animal is then challenged on shaved flanks with the test compound on day 21. Controls receive the same treatment but without the allergen added in the induction phase. In paper III, FCAT was used in its modified version with closed challenge testing which involves occlusion of the skin, thus enhancing skin penetration and controlling that the tested substance remains on the skin and is not removed by, e.g., the animal grooming.

Local Lymph Node Assay (LLNA)

LLNA was used in sensitization studies on non-ox. and ox. limonene and limonene hydroperoxides (paper IV). The protocol for the LLNA is given in Fig. 10. The sensitizing potencies of *R*-limonene and ox. *R*-limonene were investigated using the classical LLNA (39, 40) where each compound was tested in 3 different concentrations, using mice in groups of 4 and one control group which was sham treated. Thymidine incorporation was measured by β-scintillation counting in the pooled lymph nodes for each group. An SI giving test group/control group ratio was calculated for each concentration used. The SI values were plotted against the concentrations tested and the EC3 values were calculated by linear interpolation. Test materials which at one or more concentrations caused an SI greater than 3 were considered positive in the LLNA (41, 42). Allergens were classified according to: EC3 < 0.1% extreme contact allergen; EC3 ≥ 0.1 - < 1% strong contact allergen; EC3 ≥ 1 - < 10% moderate; and EC3 ≥ 10 - < 100% as a weak contact allergen (42).

In paper IV, Lim-1-OOH, Lim-2-OOH (Fig. 7) and Lim-2-Me-2-OOH (Fig. 8) were tested in the LLNA according to the protocol given in Fig. 10 but using only one concentration of each tested hydroperoxide (1.5%; 0.089M). Four groups of 8 mice were used, including one control group. At sacrifice, single-cell suspensions from two lymph nodes from each animal were prepared separately, and thymidine incorporation was measured for each individual animal. Individual SI values (test animal/mean value control group) were calculated.
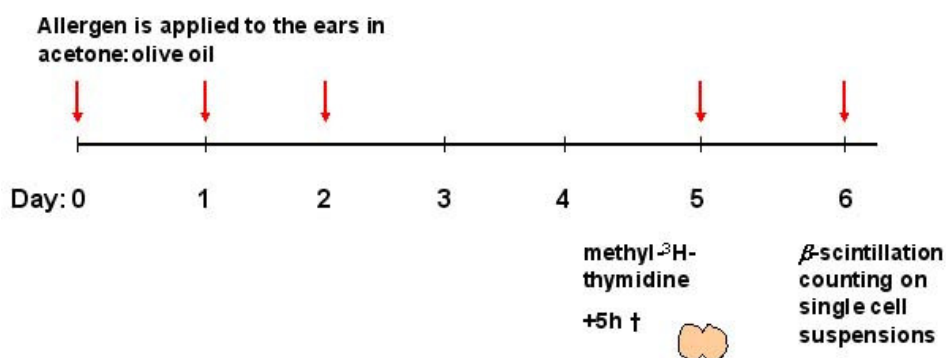

Figure 10. Protocol of the local lymph node assay (LLNA) (39, 40) The test material is applied topically to the dorsum of the ears of the mice on three consecutive days (D0, 1, and 2). On D5, all mice are injected with [methyl-3H] thymidine and sacrificed 5 h later. The draining lymph nodes are excised and single-cell suspensions of lymph node cells are prepared. In the classical LLNA, the lymph nodes are pooled for each group and thymidine incorporation is measured by β-scintillation counting. In the modified LLNA used for the statistical calculations (paper IV), two lymph nodes were taken from each animal and prepared and measured individually.

Quantum chemical calculations

In paper III, quantum chemical calculations were made using the GAUSSIAN 03 program package (90). The energies for radicals formed from cyclohexene hydroperoxide and cumene hydroperoxide (Fig. 8) were in this way calculated.

Statistics

In the irritation study (paper I), the Mann-Whitney U test compared dermatitis patients and controls, while Wilcoxon's signed rank test was used for the paired values for ox. and non-ox. terpenes as well as for paired values in increasing test concentrations. The global significance level of $P<0.05$ was corrected for multiple comparisons using Bonferroni correction and a value of $P<0.0022$ was considered statistically significant. All calculations in the dose response study (paper II) were carried out using Fisher´s exact test. In paper III, the the cross-reactivity in the FCAT guinea pig study was evaluated by Fisher´s exact test. The SI values for the three limonene hydroperoxides (paper IV) were calculated for individual mice and compared to the mean value of a group of controls using the Mann–Whitney U test. The global significance level of $P<0.05$ was corrected for multiple comparisons using Bonferroni correction and a value of $P<0.004$ was considered statistically significant.

RESULTS AND DISCUSSION

Air oxidation increases skin irritation from oxidized fragrance terpenes (Paper I)

Visual readings and LDI

By visual scoring, the oxidized patch test materials were statistically significantly more irritating compared with the non-oxidized patch test materials for linalool and limonene at each given concentration. For ox. linalool, an increase in irritancy could be statistically shown between the concentrations of 5.0% and 20% pet. while for ox. *R*-limonene, a statistically significant increase in irritation was found between 2.5% and 20%, 5.0% and 10% pet., and between 10% and 20% pet. (Fig.11).

The LDI readings for the non-oxidized and oxidized terpenes gave statistically significant differences between non-oxidized and oxidized terpenes at 20% pet., and in rising concentrations between ox. linalool 5.0% and 20% pet. and ox. *R*-limonene 2.5% and 20% pet and between 10% and 20% pet.

Conclusions from studies on irritation from ox. fragrance terpenes

The irritation study shows that air oxidation of linalool and *R*-limonene increases skin irritation. The irritation increases as the concentrations of the oxidized test preparations are raised. Ox. *R*-limonene was more irritating than ox. linalool at similar concentrations. From the extensive irritation found for ox. limonene at 20% pet., it can be concluded that hand washes and degreasers containing ox. limonene can exert considerable irritation which should be considered in labelling, usage and handling of the products. The findings that the irritation from ox. linalool was still low at 10% pet. gave rise to the question whether the patch test concentration of 2% pet., used in the previous multicenter study (63), could be raised for better detection in clinical patch testing.

Dose-response study of oxidized linalool (Paper II)

The total number of patients tested with each patch test concentration of ox. linalool and the overall number and (%) of positive reactions, doubtful reactions, and irritant reactions to each respective concentration are given in Table 2.
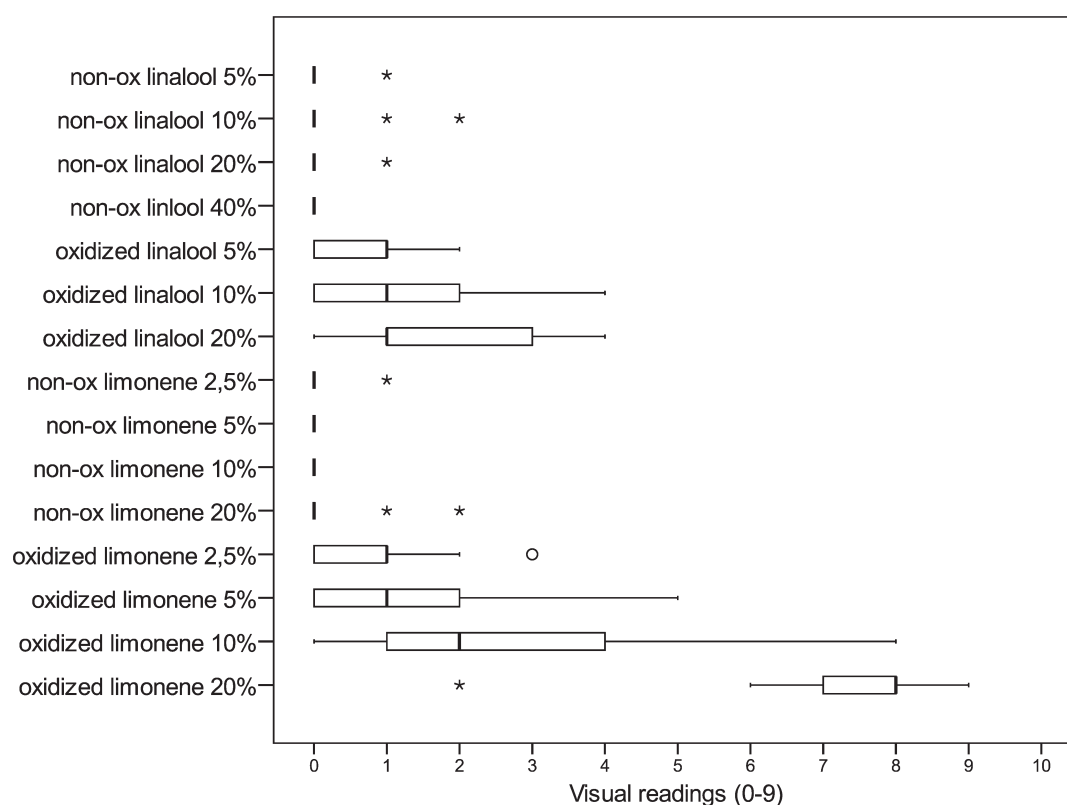
Figure 11. Boxplots of visual readings, 0-9, of non-ox. and ox. linalool and non-ox. and ox. *R*-limonene in paper I, according to erythema grading scale shown in Table 1 (89). The box represents the interquartile (IQ) range, the median is indicated by the line across the box. The whiskers indicate the range if values which are no greater than 1.5 times the IQ range. Outliers with values between 1.5 and 3 times the IQ range are indicated by circles (o). Outliers with values more than 3 times the IQ range are indicated by stars (*). Patients and controls were calculated together. (Paper I).

Table 2. Total number of patients tested with each patch test concentration of ox. linalool in the study and the number and (%) of positive reactions, doubtful reactions, and irritant reactions to each respective concentration in the dose-response study for ox. linalool [a]. (Paper II)

Ox. linalool (w/w% pet.)	Total no tested	Positive (%)		Doubtful (%)		Irritant (%)	
2.0	1693	14/1693	(0.83)	33/1693	(1.9)	0	
4.0	2075	67/2075	(3.2) [b]	106/2075	(5.1) [c]	7/2075	(0.34)
6.0	1725	91/1725	(5.3) [d]	111/1725	(6.4)	4/1725	(0.23)
11.0	1004	72/1004	(7.2) [e]	73/1004	(7.3) [f]	7/1004	(0.7)

[a] All statistics calculated with Fisher´s exact test.
[b] Increase in positive patch test reactions compared to positive reactions to ox. linalool 2% pet. $P = 1.81 \times 10^{-7}$.
[c] Increase in doubtful patch test reactions compared to doubtful reactions to ox. linalool 2% pet. $P = 9.19 \times 10^{-8}$.
[d] Increase in positive patch test reactions compared to positive reactions to ox. linalool 4% pet. $P = 0.0019$.
[e] Increase in positive patch test reactions compared to positive reactions to ox. linalool 6% pet. $P = 0.054$.
[f] Increase in doubtful patch test reactions compared to doubtful reactions to ox. linalool 4% pet. $P = 0.018$.

Statistically, a significant increase in the number of patients showing positive patch test reactions could be demonstrated for each step in rising concentrations (Table 2). Although an increase in doubtful reactions was recorded as the concentration of ox. linalool was increased, the ratios of positive reactions to doubtful reactions increased with rising concentrations. The patch test readings to the simultaneously tested concentrations were compared: only 10% of the positive patch test reactions at 4.0% pet. were positive also at 2.0% pet., 51% of the positive patch test reactions at 6.0% pet. were positive also at 4.0% pet., 74% of the positive patch test reactions at 11.0% pet. were positive also at 6.0% pet. while 39% of the positive patch test reactions at 11.0% pet. were positive also at 4.0% pet. Concomitant reactions to other fragrance markers (FM, MP, FM II) and/or colophonium were found in 39-47% of the patients showing positive patch test reactions to ox. linalool in the third part of the study, compared to 12% of the patients who were negative to ox. linalool.

Conclusions from dose-response study for ox. linalool

This study was the second multicenter patch test study performed using ox. linalool. In the earlier study on 1511 dermatitis patients, 1.3% reacted to ox. linalool tested at 2% pet. concentration (63). In this study, 5-7% of the patients tested at 6.0% pet. or 11.0% pet. had positive patch test reactions. In relation to a recent survey (53) over Swedish patch test data, positive patch test reactions to ox. linalool would be as prevalent as positive patch test reactions to the FM or MP. We interpret the high frequency of concomitant reactions to other fragrance markers in the baseline series as support for the relevancy of the positive patch test reactions. For future patch testing, we recommend the use of ox. linalool 6% pet., giving a dose per unit area of 2.4 mg/cm^2 when 20 mg of patch test preparation is applied in a small Finn chamber[®] (88), while the 11.0% pet. (4.4 mg/cm^2) patch test concentration could be used as a supplementary test in cases when a doubtful reaction is seen to the 6.0% pet. patch test concentration when it is important to verify or exclude contact allergy to ox. linalool. However, in our view, comparatively little was gained by this rise in concentration. It is also of great importance to consider the risk of active sensitization, a risk which is always present in patch testing but which should be minimized (30). Further studies should be performed to study the clinical relevancy of the positive patch test reactions by using e. g. ROAT.

Hydroperoxides form specific antigens and have a specific reactivity (Papers III and IV)

Patch test studies

In the hydroperoxide cross-reactivity study (paper III) 96.5% of the patients with previous positive reactions to colophonium reacted again at re-testing, while 46% showed positive reactions to 15-HPA. Only one of 29 individuals reacted to more than one hydroperoxide (15-HPA and Lim-2-OOH).

In the limonene hydroperoxide study, 6 of 7 patients reacted to ox. limonene at retesting. Of the 7 patients tested, all reacted to Lim-1-OOH while only 3 reacted to Lim-2-OOH.

As Lim-2-OOH was expected to be the main allergen, more concentrations of Lim-2-OOH than Lim-1-OOH were prepared in the concentration series in the first part of the study. Due to the unexpected results when the first patients all reacted to Lim-1-OOH and none to Lim-2-OOH, new analyses were performed of the test materials, and these analyses confirmed the composition of the test material. As the study progressed, more concentrations of Lim-1-OOH were added, giving a full concentration series corresponding to that of Lim-2-OOH.

Experimental studies

When the sensitizing capacity and cross-reactivity pattern for hydroperoxides was investigated in FCAT in guinea pigs, no unspecific cross-reactivity was observed (Table 3). The only statistically significant cross-reactions were reactions to cyclohexene hydroperoxide in 10 of 15 animals induced with cumene hydroperoxide.
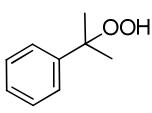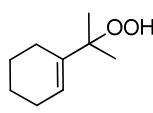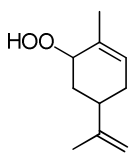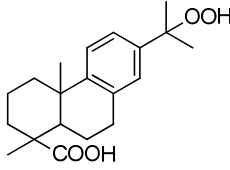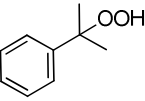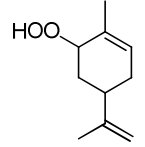
Non-ox. limonene was found to have an EC3 value of 30% (w/v) while ox. limonene gave an EC3 value of 3% (w/v) in the classical LLNA.

When three structurally related limonene hydroperoxides were tested in the extended LLNA, the SI values gave statistically significant differences between Lim-1-OOH and Lim-2-OOH (Fig. 7), between Lim-1-OOH and Lim-2-Me-2-OOH (Fig. 8), and between each of the hydroperoxides and the controls (Fig. 12).

Quantum chemical calculations

Quantum chemical calculations showed that the thermodynamic stability of three potential radicals formed from cyclohexene hydroperoxide was similar. However, the radical formed from cumene hydroperoxide, with an aromatic ring, required substantial additional energy for further intramolecular rearrangements to form epoxides (Fig. 13).

Table 3. Cross-reactivity pattern of structurally diverse hydroperoxides in sensitization tests performed in guinea pigs according to Freund's complete adjuvant test (36, 37) (paper III).

	cumene hydroperoxide	cyclohexene hydroperoxide	limonene-2-hydroperoxide Lim-2-OOH	15-hydroperoxydehydro-abietic acid 15-HPDA
cumene hydroperoxide	+	+[1]	-	-
limonene-2-hydroperoxide Lim-2-OOH	-	-	+	NT[2]

[1] Statistically significant number of positive reactions in animals induced with cumene hydroperoxide and challenge tested with cyclohexene hydroperoxide.
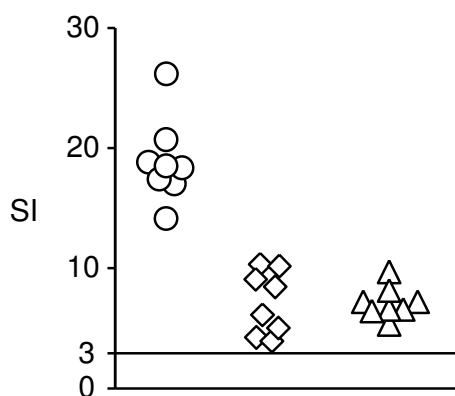[2] NT=Not tested

Figure 12. Results from the modified LLNA experiment using single-cell suspensions of the local lymph nodes from individual mice. Statistical analyses showed that when using 1.5% (0.089 M) of either hydroperoxide, Lim-1-OOH (○) was more potent to induce lymph node cell proliferation compared to Lim-2-OOH (◊) as well as to Lim-2-Me-2-OOH (Δ) giving a significant difference (*P*=0.0008) between the groups in both cases. All three hydroperoxides differed statistically significant from the controls (*P*=0.0008). No statistical difference was seen between the results obtained for Lim-2-OOH and Lim-2-Me-2-OOH. The horizontal line marks a stimulation index (SI) of 3, the cut-off limit for a compound to be considered a sensitizer (paper IV).
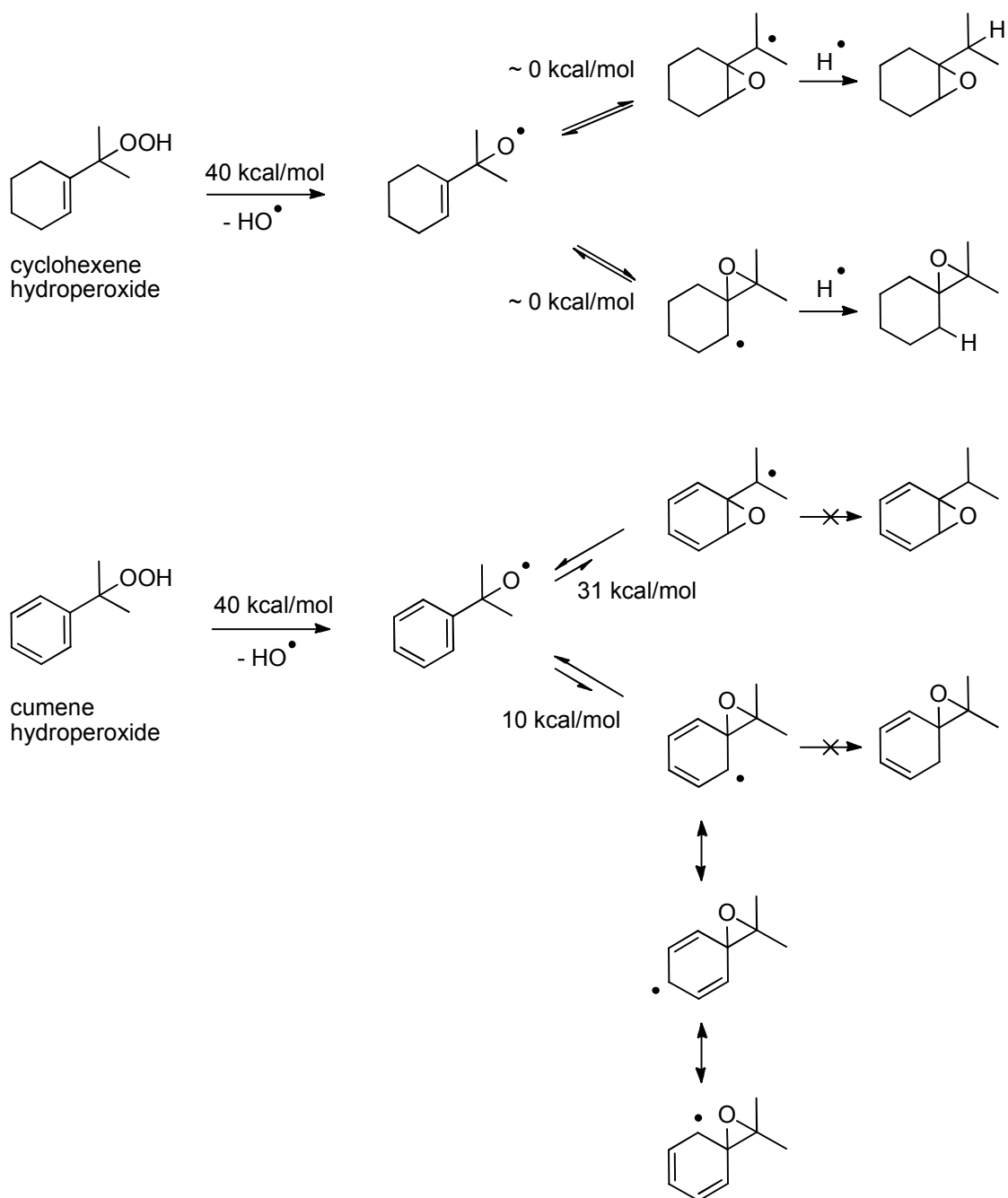
Figure 13. Reaction pathways for radicals formed from cyclohexene hydroperoxide and cumene hydroperoxide, showing reaction energies (paper III).

Conclusions from studies on hydroperoxides

Overall, specific responses were seen for the hydroperoxides investigated both in the clinical and the experimental studies. No evidence for a general cross-reactivity or formation of unspecific antigens was found. Despite structural similarities in 15-HPDA and cumene hydroperoxide, or similarities in size as cumene hydroperoxide and Lim-2-OOH, no cross-reactivities between these hydroperoxides were found. Only the structurally closely related cumene hydroperoxide and cyclohexene hydroperoxide showed significant cross-reactivity (Table 3). The finding in paper IV that closely related hydroperoxides show statistically significant differences in sensitizing potencies further demonstrates that the hydroperoxides act as specific haptens. Thus, the concomitant reactions to ox. limonene, ox. linalool and fragrance markers and/or colophonium (7, 60-63), where the main allergens are hydroperoxides, are still best explained by concomitant exposure to a wide variety of fragrance chemicals and colophonium components in products in everyday life.

The quantum chemical calculations indicate that the allylic tertiary hydroperoxide cyclohexene could react as an oxygen-centered radical or as an epoxide, in the latter case as an electrophilic hapten, while aromatic tertiary hydroperoxide cumene would most probably remain as an oxygen-centered radical when acting as a hapten. Thus, it can be concluded that the way of antigen formation giving cross-reactions between cyclohexene hydroperoxide and cumene hydroperoxide has to take place as a direct coupling between the oxygen-centered radical and skin proteins. This result agrees with the cross-reactivity previously observed between the allylic tertiary hydroperoxide 15-HPA and the aromatic tertiary hydroperoxide 15-HPDA (both Fig. 4) (91). Cross-reactivity was observed to 15-HPA in animals sensitized to 15-HPDA which cannot easily form epoxides, whereas 15-HPA also cross-reacted to epoxides of abietic acid, indicating that 15-HPA may react with skin proteins either as an radical or as an epoxide, thus generating different antigens (91).

The results from the extended LLNA on three limonene hydroperoxides confirmed the differences in the EC3 values found in the classical LLNA protocol (Lim-1-OOH: EC3 value 0.33% (w/v);Lim-2-OOH: EC3 value 0.83% (w/v); and Lim-2-Me-2-OOH: EC3 value 1.29% (w/v)) (14). By this method, one hydroperoxide, Lim-1-OOH, was shown to have a statistically significantly higher sensitizing potency than the other two tested limonene hydroperoxide analogues, while all tested limonene hydroperoxide analogues had statistically significantly higher sensitizing potencies compared to controls. The results in the clinical study supported the importance of the difference: all limonene-allergic patients reacted to Lim-1-OOH while 3 of 7 reacted to Lim-2-OOH.

In the classical LLNA, ox. limonene was shown to be a much stronger sensitizer than non-ox. limonene, which is in accordance with the results obtained in guinea pig studies (17-19, 71) and patch testing in patients (60-62, 72, 80, 81). When comparing the results from the LLNA performed on the

identified oxidation products of limonene to each other and to that of ox. limonene, it is apparent that the hydroperoxides are the strongest sensitizers and that their impact on the sensitizing capacity of ox. limonene is important (Fig. 14). This is in accordance with corresponding studies of linalool and geraniol (21, 23).

Previous clinical and experimental studies on limonene hydroperoxides have used the isolated hydroperoxide fraction of the oxidation mixture (60, 61) or synthesized Lim-2-OOH (18), which was regarded as the main allergen in the oxidation mixture of limonene. By the LLNA results and the clinical study in paper IV, a new candidate for main allergen in the oxidation mixture of limonene was identified in Lim-1-OOH. In earlier clinical studies on ox. limonene and the hydroperoxide fraction mainly containing Lim-2-OOH, 26-43% of the patients reacted to both the oxidation mixture and to the hydroperoxide fraction; 41-52% reacted only to the oxidation mixture while 16-22% reacted only to the hydroperoxide fraction (60, 61). Our results in paper IV, giving that all tested limonene-allergic patients reacted to Lim-1-OOH, is a new situation in our experience and will be further studied.
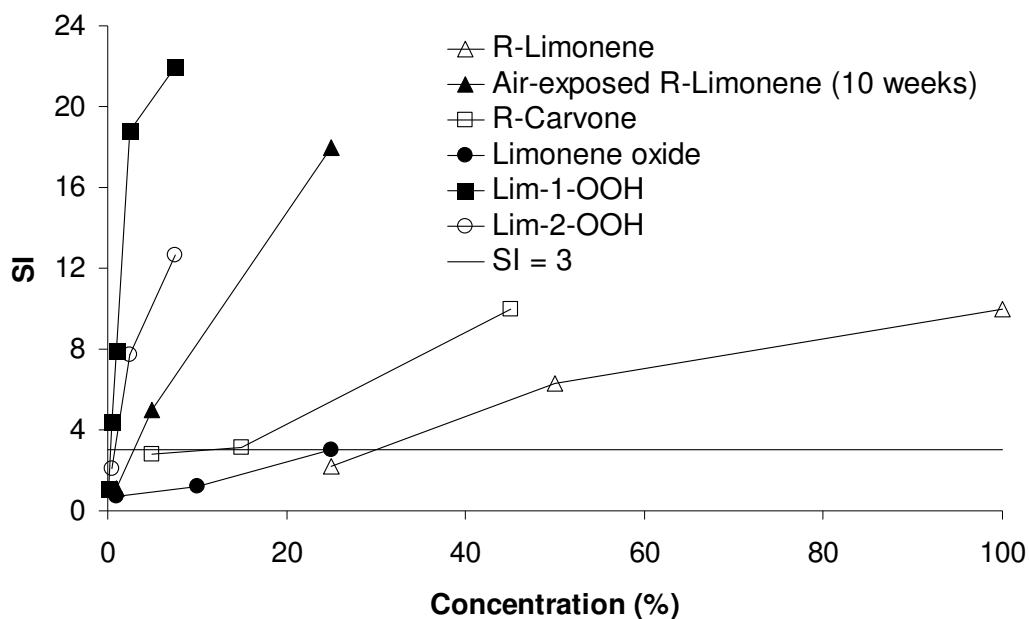

Figure 14. Dose-response curves for pure and ox. limonene and identified oxidation products when tested in the LLNA. Pure limonene (Δ), air-exposed limonene (10 weeks) (▲), carvone (□) (92), limonene oxide (●) (93), Lim-1-OOH (■) (14), Lim-2-OOH (○) (14). (Paper IV)

Methodological considerations

In the irritation study (paper I), the concentration of ox. limonene 20% caused discomfort to some of the patients and controls and the testing was discontinued. A continuous limited testing period or a shorter second test period would have been desirable in the irritation study. Using the 10 grade scale for visual assessment of the irritation reactions, differences in interpretations of readings can affect score values. Care was taken to minimize subjective differences in reading, by co-assessing the irritation reactions in initial phases. The LDI measurements gave comparatively discrete differences between the tested ox. and non-ox. terpenes and, in most cases, between the tested concentrations, perhaps due to the time that had elapsed (reading at D3). Additional assessments could have been made, e.g. 1-2-hours after removal of the patch test material as well as D3.

In the dose-response study (paper II), assessments were made on D3-4 and 6-7, and there is a possibility that the doubtful reactions showed a maximum (positive or irritant) at e.g. D2 or D 4-5 which was not graded. The clinical relevance of the positive patch test reactions, as well as the doubtful reactions, should be further studied as discussed in paper II.

In the clinical hydroperoxide cross-reactivity study (paper III), it would have been desirable to include a larger number of patients to obtain more information. However, all patients who had reacted ++/+++ to colophonium in the previous years were invited to participate. Enlarging the study to include another test centre would have been a possibility. At that time, screening with ox. limonene and ox. linalool had not been performed in Gothenburg to any extent, and patients reacting to these oxidation mixtures were not available. Likewise, in paper IV, the two limonene hydroperoxide analogues in the clinical study were tested in only 7 patients. Those were the individuals in our population who had reacted ++/+++ to ox. limonene at the time. A larger study population to ensure the reliability of the results is desirable and studies in this aspect are planned. Cross-reactivity studies in guinea pigs present some difficulties as unspecific reactions may occur, perhaps due to irritation. In the FCAT cross-reactivity study (paper III), 10/15 animals gave statistical significance from the control group, while 6/15 and 7/15 did not. In our paper, we report these findings as recorded and future studies are needed to give further evidence.

GENERAL DISCUSSION

Fragrance terpenes are ubiquitous in our environment due to their extensive use in domestic and occupational products. For most fragrance chemicals used today, little is known of air oxidation and other activation pathways with regard to contact allergy. The studies presented in this thesis give evidence that commonly utilized fragrance chemicals can cause high rates of skin sensitization. Continuous clinical research to identify relevant allergens, and development of appropriate patch test materials is essential to detect and diagnose contact allergy. Experimental research is crucial to recognize and predict allergenic activity in chemicals to which we are exposed, and to prevent exposure to new sensitizers in future products.

When diagnosing contact allergy in dermatitis patients, it is important to test with the compounds that the individuals actually come into contact with. The use of fragrance materials varies over time and new products and fashions influence exposure to the population. Although commercial perfumes may contain several hundred fragrance compounds, the main diagnostic tools, the FM and FM II together investigate contact allergy to 14 specified common sensitizers and it is important to extend and update the test panel of the baseline patch test series so that relevant allergens are included. Our dose-response study on ox. linalool shows that contact allergy can be quite common but go undetected when the compounds that the patients are exposed to are not used for screening or tested at too low concentrations. Similar systematic studies to establish optimal test concentrations are needed when new substances for patch testing are suggested as screening agents.

The high frequency of patients with positive patch test reactions to ox. linalool also shows that, although the concentrations in the products to which people are exposed are generally low, the widespread occurrence of products in daily life containing linalool presents a substantial risk for sensitization. This should bring discussion to the limits set in the EU directive (31), which cannot be considered to be safe with the high frequency of contact allergy detected in our population.

The patch test concentrations in the dose-response study (paper II) are high compared to those found in commercial consumer products. Thus, the relevancy of positive patch test reactions to these concentrations for persons normally exposed to much lower concentrations should be considered. Generally, when selecting a patch test concentration, a dose per unit area which does not give irritation or sensitization, but which gives good detection is sought (30). The location of the patch test (most often the upper back) as well as the single exposure on intact skin, requires a higher test concentration to show a positive patch test reading, compared to the concentration which can give elicitation in repeated application, or places of previous ACD where the skin may be damaged or skin memory

present, as well as in susceptible skin areas, e.g. skin folds on eye-lids or axillae. In our experience, linalool has been frequently found on the labels of products brought in by linalool-allergic patients experiencing aggravation of their eczema. However, the relevancy of the positive patch test reactions to ox. linalool has not yet been clinically assessed in a systematic way, for example by repeated open application tests with low concentrations of ox. linalool. The repeated frequent exposure to low concentrations of a compound, is typical for fragrance terpenes in consumer products and is a plausible explanation for the high rate of concomitant reactions observed to these allergens.

Our results emphasize the importance of air oxidation when patch testing with fragrance terpenes. The pure compound added to the perfume mixture is, in many cases, not the actual sensitizer. For limonene and linalool, the pure compounds have shown very few positive patch test reactions (80, 81), while the oxidation mixtures are common causes of contact allergy (60-63, 72). Also other terpenes, such as linalyl acetate and geraniol have been shown to autoxidize on air exposure, thereby forming allergenic oxidation products (22, 23). In addition, geraniol has been shown to form allergenic compounds by metabolic activation (24). Further studies are needed to investigate the chemical transformations of common fragrance compounds in everyday settings, to give a reliable assessment of their roles in contact allergy and ACD.

Not only the allergenic perspective but also irritancy must be considered when one investigates possible adverse effects of chemicals used in everyday life. In risk assessment, some account must be taken for other usage than the expected, e.g. higher doses than recommended. For fragrance terpenes, mostly low doses are used in commercial consumer products but much higher doses are possible in new modes of usage such as in aromatherapy, room-scenting aerosols or skin perfumes. Alternate or unexpected uses may also cause confusion as to when to expect exposure: a person who is avoiding fragranced products might not expect limonene to be present in a car degreaser or in an industrial handwash. On a similar note, lavender oil is currently present in medical ointments sold by pharmacies in Sweden.

This is the first time a statistically significant difference was demonstrated between the sensitizing potencies of closely related hydroperoxides using an extension of the LLNA where calculations were made on lymph nodes from individual mice, thus making statistical calculations possible. The relevancy of the findings was supported by the results in the clinical study on limonene hydroperoxides (paper IV). To the best of our knowledge, this way of using the LLNA to investigate statistical differences in sensitizing potencies of allergenic chemicals, has not been previously published. The extended LLNA provides a tool to discriminate between the sensitizing capacities of

structurally related compounds with similar EC3 values, which is of importance in SAR studies as a basis for future non-animal methods of predicting allergenic potential of chemicals.

It is generally desirable to patch test with defined compounds rather than natural products or mixtures. In earlier investigations on colophonium, ox. limonene, ox. linalool and their respective hydroperoxides or hydroperoxide fractions, patterns have emerged with 40-60% of patients reacting to both the oxidation mixture and the hydroperoxide preparation while a number react only to one of the test preparations (60, 61, 63, 70). Although the hydroperoxides are the most important allergens in the oxidation mixtures, also other sensitizers are present e. g. secondary oxidation products (aldehydes, epoxides) which may have caused ACD in certain patients. In the small clinical study on Lim-1-OOH (paper IV), all limonene-allergic patients reacted to the hydroperoxide. Using Lim-1-OOH for testing of consecutive dermatitis patients is an interesting prospect. Initially, further studies are needed to investigate if the results are applicable in larger patient groups. If confirmed, there are some practical problems using the hydroperoxide in routine testing as Lim-1-OOH is unstable and the test preparation must be handled carefully and be replaced more often than normal test preparations. Thus, in clinical practice, patch testing with a carefully controlled oxidation mixture may still be the most effective way to detect contact allergy to fragrance terpenes that undergo autoxidation.

Linalool is a ubiquitous fragrance chemical and contact allergy to linalool may have wide implications for the patient. With the widespread use of linalool in domestic and occupational products, the number of sensitized individuals can be expected to increase. Also, clinical eczema in allergic individuals may be expected. It was shown in a recent study (76), that also natural lavender oil, which consists of linalyl acetate and linalool as main components, autoxidizes in the same way as the synthetically prepared components, separately or in mixture. Thus, exposure from aromatherapy oils and so called "natural products" must also be considered. The present studies support the need for adequate risk evaluation, validation of diagnostic tools and relevant studies with regard to allergenicity for the actual chemicals to which the population is exposed.

Conclusions

In conclusion, this thesis shows that it is important to study the contact allergenic potential of chemicals to which we are exposed. Seemingly harmless products may cause or worsen ACD. Such risks can only be detected by testing of relevant allergenic compounds in appropriate concentrations.

The frequency of positive patch test reactions to autoxidized linalool observed among the dermatitis patients, places this material among the most common contact allergens tested today. In addition, a new main allergen in the oxidation mixture of limonene, Lim-1-OOH, is proposed.

The thesis confirms the effect of air oxidation on the allergenicity of common fragrance terpenes. Furthermore, the studies show the great specificity of the contact allergens *in vivo*. Finally, the impact on the allergenic potency by the primary oxidations products in fragrance terpenes, the hydroperoxides, is re-established in humans.

Future perspectives

Further studies to investigate the clinical relevancy of the high frequency of positive patch test reactions to ox. linalool in the dose-response study (paper II) should be conducted, using ox. linalool at low concentrations in use-tests such as repeated open application test (ROAT). Also, larger clinical studies to evaluate Lim-1-OOH as the main allergen in ox. limonene, should be conducted to investigate if the results from paper IV are applicable in larger groups of patients.

Investigating oxidation products from fragrance terpenes in actual consumer goods which have been stored and handled is a difficult task. Different techniques for chemical analyses of monoterpene hydroperoxides have been investigated in our group (94). Further work needs to be done to identify allergenic compounds in products present at home and at work, using appropriate analytical methods, and to investigate the clinical relevance of the detected compounds.

Studies have shown that the fragrance terpene geraniol can be metabolically activated as well as autoxidize, and thus is both a prohapten and a prehapten (23-25). Indications that linalool may be prone to metabolic transformation has been published (95). However, with regard to linalool, the epoxide formed is immediately converted to non-allergenic oxides (21). This is confirmed by the non-allergenic or very weak allergenic activity obtained from pure linalool in both LLNA (21) and clinical studies (63, 80, 81). Thus, it is important to consider individual variations in the allergenicity of fragrance terpenes, and their possibilities to act as prehaptens or prohaptens should be thoroughly investigated including clinical relevance.

Overall, the mechanisms by which some individuals are prone to develop contact allergy, as well as possible preventive measures, make interesting scopes. In guinea pigs, epidermal pre-treatment with antioxidants have been shown to reduce both rate of sensitization as well as reactions at elicitation in investigations on Lim-2-OOH (96). If this is applicable to humans, it would be an interesting strategy for prevention. Furthermore, an individual susceptibility to react to hydroperoxide antigens e.g. as a result of antioxidant deficiency, is a possibility.

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