Formation of Skin Sensitizers from Fragrance Terpenes via Oxidative Activation Routes

Chemical Analysis, Structure Elucidation, and Experimental Sensitization Studies

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LINA HAGVALL

To Jon, my wonderful husband

Abstract

The work presented in this thesis emphasizes the importance of considering oxidative activation in the toxicity assessment of fragrance chemicals. Compounds without contact allergenic properties can be activated either via autoxidation in contact with air or via cutaneous metabolism to reactive products which can cause contact allergy. It is important to prevent sensitization as the immunological memory formed in the development of contact allergy persists throughout life. The investigation of compounds susceptible to oxidative activation, thereby forming sensitizing compounds is important in the work of prevention of contact allergy. The overall aim of this thesis was to investigate mechanisms of activation via autoxidation and metabolism of single fragrance compounds and essential oils, and to study the impact of this activation on the contact allergenic activity.

The oxidative activation via autoxidation and cutaneous metabolism of the fragrance compounds geraniol and geranial was studied. It was shown that both compounds were susceptible to autoxidation, forming oxidation products with increased sensitizing capacity compared to the original compound. The oxidation products of geraniol were formed by two separate pathways, corresponding to autoxidation of each of the two double bonds in geraniol, respectively. Hydroperoxides, which previously have been identified as the most important sensitizers in the oxidation mixtures of air-exposed fragrance compounds could not be detected in air-exposed geranial. Instead, a sensitizing epoxide was detected. Geraniol and geranial were also activated metabolically. Many of the metabolites identified were also present in the autoxidation mixtures.

The autoxidation of lavender oil was studied in order to investigate if essential oils possess a natural protection against autoxidation. The results were compared to the results from the autoxidation studies of linalyl acetate and linalool, the main components of lavender oil. It was found that the autoxidation proceeded in the same way in both the pure samples and the lavender oil, and that sensitizing oxidation products were formed in both cases. The most important sensitizers formed were hydroperoxides of linalool and linalyl acetate.

This thesis adds important information on routes of autoxidation as well as on the relationship between metabolic and air induced activation of non- or weakly sensitizing compounds to sensitizers. The results presented here

indicate that other fragrance terpenes could be susceptible to oxidative acti-vation via autoxidation or cutaneous metabolism. This should be considered in the risk assessment of fragrance chemicals.

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   Fragrance Compound Geraniol Forms Contact Allergens on Air Exposure. Identification and Quantification of Oxidation Products and Effect on Skin Sensitization. Hagvall, L., Bäcktorp, C., Svensson, S., Nyman, G., Börje, A., Karlberg, A-T. *Chem. Res. Toxicol.* 2007; **20**: 807–814.

II   Autoxidation of Geranial. Hagvall, L., Börje, A., Karlberg, A-T. Manuscript.

III   Cytochrome P450 Mediated Activation of the Fragrance Compound Geraniol Forms Potent Contact Allergens. Hagvall, L., Baron, J. M., Börje, A., Weidolf, L., Merk, H., Karlberg, A-T. *Toxicol. Appl. Pharmacol.* 2008; **233**: 308–313

IV   Autoxidation of Linalyl Acetate, the Main Component of Lavender Oil, Creates Potent Contact Allergens. Sköld, M., Hagvall, L., Karlberg, A-T. *Contact Dermatitis.* 2008; **58**: 9–14.

V   Lavender Oil Lacks Natural Protection Against Autoxidation, Forming Strong Contact Allergens on Air Exposure. Hagvall, L., Sköld, M., Bråred-Christensson, J., Börje, A., Karlberg, A-T. *Contact Dermatitis.* 2008; **59**: 143–150.

Contribution Report

I   Major contribution to the formulation of the research problem; planned the LLNA experiments, performed all other experimental work; interpreted the results and wrote the manuscript.

II   Major contribution to the formulation of the research problem; planned the LLNA experiment, performed all other experimental work; interpreted the results and wrote the manuscript.

III   Formulated the research problem; performed all the *in vitro* experimental work and planned the LLNA experiments; interpreted the results and wrote the manuscript.

IV   Minor contribution to the formulation of the research problem; performed part of the synthesis of reference compounds, and participated in the planning of the LLNA experiments; contributed to the interpretation of the results and to the writing of the manuscript.

V   Major contribution to the formulation of the research problem; performed the chemical analysis work, participated in the planning of the LLNA experiments; major contribution to the interpretation of the results and to the writing of the manucript.

Abbreviations

ACD	Allergic contact dermatitis
BHT	Butylated hydroxytoluene
CYP	Cytochrome P450
EC3	Estimated concentration to produce an SI of 3
EPR	Electron paramagnetic resonance
FID	Flame ionization detector
FM	Fragrance mix
GC	Gas chromatography
HEPA	High efficiency particulate air (filter)
HPLC	High performance liquid chromatography
ICDRG	International contact dermatitis research group
LLNA	Local lymph node assay
MHC	Major histocompatibility complex
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
Pat	Patient
PBS	Phosphate buffered saline
pet	In petrolatum
rh	Recombinant human
SI	Stimulation index
SIM	Single ion monitoring
TRIS	Tris(hydroxymethyl) aminomethane
UV	Ultra violet

Contents

Chapter 1

Introduction

1.1 Fragrances, an essential part of life?

Fragrance is a word derived from the latin word for odour or smell. The use of fragrances dates back to prehistoric times, when it had religious connotations. The word perfume is derived from the latin *per fumum*, meaning through smoke and referring to the incense burned to transport prayers to the gods in heaven [1]. The great civilisations of China, India, Mesopotamia and Egypt developed the use of fragrances, which was extended into the societies of Greece, Palestine, Rome, Persia and Arabia. The Bible is full of fragrance, as the description of the life of Jesus begins with the gifts of myrrh and frankinscence at his birth and ends with the myrrh used with the binding sheets of his dead body. The following citation from Petronius, *arbiter elegantiae* (judge in taste) at the court of emperor Nero of the Roman Empire, show that fragrances were used in mundane life as well, and that the fashion of the ruling class changed quickly even then [1]:

> Wines are out of fashion, Mistresses are in
> Rose leaves are dated
> Now Cinnamon's the thing

Perfumes have also been used extensively throughout history to hide the smells of the growing cities, the smells of disease, excrements, fowl body odours and the early industries. To do the trick, these perfumes were very heavy in scent. In the 18th century, the scents became more floral and light, using rose, violet and lavender. Perfumes were now stored in exquisite glass bottles of different colours, a novel luxury of that time.

By the end of the 19th century, a system for description and structure of fragrances was developed. The fragrances were said to be vertically structured, consisting of a top note, which is the first impression of a fragrance, a middle note, which is the more lasting smell from the perfume and a bottom note, the earthy last trace of a fragrance which can remain on the skin for hours. All modern perfumes are composed according to this system. Along with the new way of composing fragrances came the use of synthetic substances and also the mass production of perfumes, making them available to the general population.

The compounds responsible for the pleasant smell of fragrances are most often monoterpenes. Monoterpenes belong to a diverse family of compounds divided into the monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}). The biosynthesis of terpenes involves condensation of isoprene (C_5) units to form the carbon skeleton, which is often further modified to contain oxygen or to form closed ring structures. Many terpenes are unsaturated molecules and are as such susceptible to autoxidation in contact with oxygen in air. This is discussed further below.

Originally, all terpenes used as fragrance ingredients were extracted from plants using methods such as steam distillation, where parts of the plant are distilled together with water vapour to extract the volatile matter, or enfleurage, where animal fat was used to slowly extract the fragrance of flowers, too delicate for distillation. These extraction methods are time consuming, especially the enfleurage. Essential oils are still being manufactured by steam distillation today, but the enfleurage has been replaced with extraction using organic solvents, such as petroleum ether, acetone, hexane, or ethyl acetate [1]. Essential oils differ in composition depending on the part of plant used, and also to some extent on the location and conditions of growth of the plants. Generally, essential oils are complex mixtures of terpenes, and a fine fragrance made from the mixing of several essential oils contains hundreds of compounds, which contribute to the complexity of the odour.

At present, the most commonly used fragrance terpenes are synthesized from terpene precursors in large scale industrial processes. The production amounts to many thousand tonnes per year and outweighs the small-scale production of essential oils by far. Fragrances are included in most hygienic, cosmetic and domestic products, as well as in products for professional use. The wide-spread use combined with the fact that many fragrance components are skin sensitizers, or can form sensitizers after activation, result in frequent allergy to fragrances. As a result of this, fragrances are one of the

most common causes of contact allergy, second only to nickel [2], and as many as 10% of the general population may be sensitized to one or more fragrance compounds [3]. It has also been shown that contact allergy to fragrances increases with increasing age and that it is more common in women than in men.

1.2 Contact allergy

It is estimated that up to 20% of the population in the western world is sensitized to one or more compounds in our environment [3]. Many of these individuals suffer from allergic contact dermatitis (ACD), the clinical manifestation of contact allergy. The immunological memory created in the development of contact allergy is life long and only symptomatic treatment of the dermatitis is available. ACD can lead to psycosocial consequences for the individual and a reduced quality of life, as the eczema often is persistent or relapsing [4]. For society, the economic consequences can be considerable, for instance due to sick leave, change of occupation or in the worst case disability pension. In view of this, prevention of contact allergy is of great importance.

Development of contact allergy is mainly due to the exposure frequency and sensitizing capacity of the sensitizing chemical, the hapten. This process is divided into the induction or sensitization phase, which results in the formation of an immunological memory, and the elicitation, which results in an inflammatory reaction, that is, ACD (Figure 1.1). In 1935, Landsteiner and Jacobs proposed that chemicals must react with, and thus modify endogenous macromolecules, in order to act as skin sensitizers [5]. Today, it is generally accepted that cutaneous proteins are the main macromolecules involved in the formation of an immunogenic complex.

1.2.1 Hapten-protein complex formation

There are several ways in which a hapten can participate in the formation of an immunogenic complex. The most common is by nucleophilic-electrophilic interactions with nucleophilic amino acid residues such as cysteine, histidine and lysine [6], where a covalent bond is formed. In these cases, the hapten-protein complex is formed by nucleophilic substitution reactions, Michael additions or nucleophilic additions [7]. The reactivity of the nucleophilic amino acid moieties is dependent of the three dimensional protein structure, as reactive groups can be shielded in hydrophobic pockets in the protein [6].

The tertiary structure of the protein can also affect the pK_a of single amino acid residues. The side chains of the free amino acids histidine, cysteine and lysine have a pK_a of 6.0, 8.3, and 10.0, respectively [8]. In epidermis, with a physiological pH of 7.4, the equilibrium is shifted so that a very low proportion of the free cysteine and lysine residues are deprotonated and reactive. In a protein structure, the pK_a of these amino acids can be different from the above mentioned values, due to interactions with neighboring residues.

Hapten-protein complexes are also thought to be formed by radical reaction mechanisms. Radicals can react with most protein residues, although aromatic amino acid residues, such as tyrosine, tryptophane and histidine are considered to be the most susceptible [9]. Many studies indicate that the antigen formation of hydroperoxides, which are strong sensitizers, occur via a radical mechanism [10–13]. This has been investigated using radical trapping experiments and EPR studies, showing that the oxygen-oxygen bond in hydroperoxides can be cleaved homolytically to form an alkoxy radical [11–13]. This radical can either react with protein directly or rearrange to form carbon-centred radicals, also capable of reacting with protein.

Compounds showing *in vivo* sensitizing capacity and at the same time lacking electrophilic or radical reactive sites are named prohaptens or prehaptens [14]. Prohaptens are non-reactive sensitizing chemicals which are activated via enzymatic conversion to sensitizing reactive metabolites in the skin. Prehaptens are non-reactive chemicals that are converted to the hapten via chemical transformations not involving enzymatic catalysis, for example by spontaneous air oxidation, also known as autoxidation. Both the cutaneous metabolism and the process of autoxidation are discussed in more detail below.

1.2.2   Sensitization

The first step in the sensitization phase is the penetration of the hapten into the skin (Figure 1.1). The penetration of chemicals into viable epidermis is governed by diffusion processes [15]. It is usually claimed that a compound should have a log P close to 2, and a molecular weight smaller than approximately 1000 Da to be able to penetrate the skin readily [16].

The immune system can not recognize small molecules, and is not triggered until the hapten has reacted with a protein, forming a hapten-protein complex [17]. These complexes are recognized by professional antigen presenting cells, the Langerhans cells, which internalize and process the hapten-protein complex. The resulting hapten-modified peptide is presented on the surface of the Langerhans cells, associated with major histocompatibility
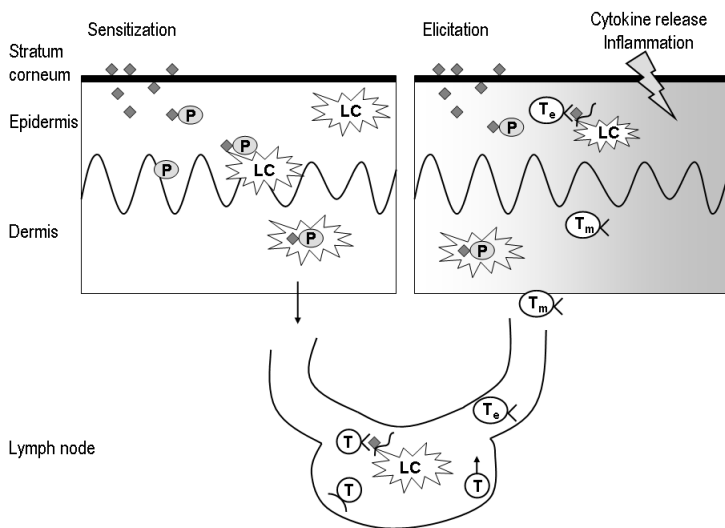
Figure 1.1: A scematic summary of the immunological mechanism of contact allergy. In the sensitization phase, the hapten penetrates into the skin and binds to protein (P) in epidermis, forming a hapten-protein complex. This is internalized and processed by Langerhans cells (LC) to the final antigen. The Langerhans cells migrate to a local lymph node, presenting the antigen to naïve T-cells (T). T-cells specific for the hapten modified peptide are activated and proliferate, forming memory T-cells (T_m) and effector T-cells (T_e) which enter the circulatory system and migrate to peripheral tissue. In the elicitation phase, the hapten-protein complex is formed and processed as above, but is now presented to specific memory T-cells present in the skin. This causes activation of the T-cells, leading to an inflammatory response.

complex (MHC) class I or MHC class II. Haptens are also thought to be able to interact directly with peptides bound to the MHC molecules [17].

The uptake of a hapten-protein complex causes the Langerhans cells to mature and migrate to the local lymph nodes. Here, hapten-modified peptides associated with MHC II are presented to naïve $CD4^+$ T-cells, whereas hapten-modified peptides associated with MHC I are presented to naïve $CD8^+$ T-cells. Antigen specific T-cells are activated, mature and proliferate into memory T-cells and effector T-cells, and thus a cellular immunological memory is formed. The sensitization process requires a few days up to several weeks for completion, whereas the subsequent elicitation phase is faster.

1.2.3   Elicitation

When an individual is sensitized, ACD is most often developed one or two days after repeated contact with the hapten [17]. Again, the hapten penetrates the skin and reacts with protein to form the hapten-protein complex (Figure 1.1). This will be internalized, processed and presented on the surface of Langerhans cells and keratinocytes. This time, memory T-cells specific for the hapten-modified peptide are already present in the circulation. When reaching the site of contact with the hapten, they recognize the hapten-modified peptide presented to them, associated with MHC class I or II by Langerhans cells, or with MHC class I by keratinocytes. Recent results suggest that also keratinocytes can present immunogenic complexes to $CD4^+$T cells [18]. The recognition of the immunogenic complex re-activates the memory T-cells into effector cells. Of these, $CD8^+$T-cells are considered to be important effector cells in contact dermatitis in mice [19]. It is not known if this is the case also in humans. Activation of the T cells causes them to release pro-inflammatory cytokines, which in turn induce the inflammatory response, resulting in ACD.

1.2.4   *In vivo* predictive test methods

The prevention of contact allergy is of great importance. Therefore, the aim of performing predictive tests on new chemicals is to prevent sensitizing chemicals from being used in sensitizing concentrations, or from reaching the market at all.

To assess the sensitizing capacity of chemicals, a number of predictive test methods has been developed. Previously, the guinea pig was the experimental animal of choice [20]. In these methods, elicitation is studied,
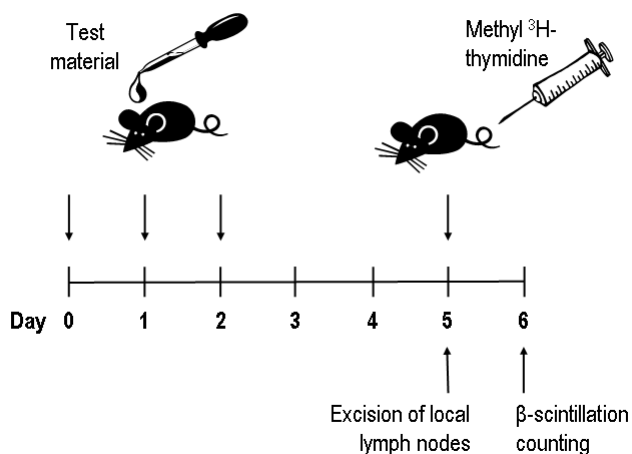
Figure 1.2: The protocol of the local lymph node assay (LLNA) [21, 22]. At days 0, 1 and 2, the test material/vehicle is applied on the dorsum of both ears. At day 5, the mice are injected with [methyl]-^{3}H-thymidine. 5 h later, the mice are sacrificed, and the thymidine incorporation is measured in the local lymph nodes using scintillation counting. A stimulation index (SI) (test group /control group ratio) of 3 is considered a positive result.

and the number of positive elicitation rections in a group of test animals in comparison with a non-exposed sham treated control group is considered a measure of the sensitizing capacity of the test compound. An advantage of these methods is that the elicitation is studied, which resembles the situation in real life. However, the guinea pig methods only give semi-quantitative information, i.e. weak sensitizer versus strong sensitizer. The use of guinea pig methods has now been restricted within the EU, due to animal welfare reasons.

Today, the murine local lymph node assay (LLNA) is a commonly used *in vivo* predictive method. In the LLNA, the hapten is applied to the dorsum of the ears, thus the penetration properties of the hapten are taken into account [21, 22] (Figure 1.2). No elicitation is performed, instead, the proliferation of lymphocytes in the local lymph nodes is measured quantitatively and compared to controls. The disadvantages of this method are that these measurements do not discriminate between proliferation of different cell types in the lymph nodes, which means that irritants can give a

false-positive response in the LLNA [23]. A major advantage of the LLNA
compared to the guinea pig methods is the quantitative assessment of the
sensitizing capacity of different haptens, which can be divided into extreme,
strong, moderate and weak, as suggested by Kimber *et al* [24].

1.2.5   Diagnostic methods in contact allergy

Patch testing (epicutaneous testing) is the standard for diagnosis of contact
allergy. It aims to provoke a miniature elicitation reaction in patients al-
ready sensitized to the test compound. The method of patch testing has
been standardized in the recommendations by the International Contact
Dermatitis Research Group (ICDRG) [25]. The test substances are diluted,
most often in white petrolatum, to an appropriate concentration. The con-
centration of the test compund is chosen so as to minimize false-positive
and false-negative reactions; usually, the highest non-irritant concentration
is used [26]. In the choice of concentration, the risk of sensitization by the
patch test must be considered, although it has been shown that active sensi-
tization by patch testing is very rare [27]. Patients are tested with a baseline
series of the most common allergens (haptens or prohaptens) [26], and some-
times also with additional compounds or materials that are suspected to be
relevant in the individual case. The test preparations are applied to the
upper back of the patient in test chambers, and are left under occlusion for
48 h [25]. Readings of the patch test reactions are performed twice, on days
2–3 and days 4–7. The reactions are interpreted and scored according to
the ICDRG guidelines as − (negative), ? (doubtful), + (weak positive), ++
(strong positive), +++ (extreme positive) or IR (irritant) [25].

1.2.6   Markers for and prevalence of contact allergy to fragrances

The diversity of compounds which provides us with refreshing, sweet or
even sensual fragrances also presents a problem when individuals are sensi-
tized to and develop ACD after contact with fragrances or perfumed prod-
ucts. As it is impossible to cover this chemical diversity in the dermatology
clinic, a fragrance mix (FM) was introduced in 1977 and modified in 1985
by Larsen [28,29] as a screening tool for the detection of fragrance sensitized
patients. The FM consists of seven compounds; eugenol, isoeugenol, geran-
iol, hydroxycitronellal, α-amylcinnamic aldehyde, cinnamic aldehyde and
cinnamic alcohol, and one natural mixture, oak moss (Figure 1.3). It has
been estimated to detect 70–80% of all cases of fragrance sensitization [29].
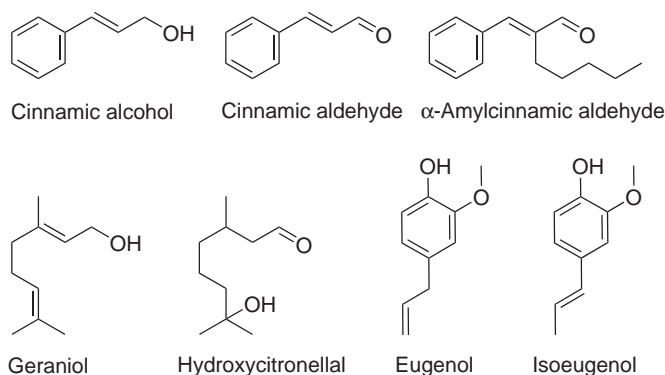
Figure 1.3: Components of the fragrance mix (FM), currently used in the baseline series for screening of contact allergy in dermatitis patients.

In recent years, a new fragrance mix (FM II) has been developed as a complement to FM [30]. FM II contains eight compounds; Lyral®(the mixture of 3-(4-hydroxy-4-methyl-pentyl) cyclohexene-1-carboxaldehyde and 4-(4-hydroxy-4-methyl-pentyl) cyclohexene-1-carboxaldehyde), citral (the mixture of geranial and neral), farnesol, citronellol, coumarin and α-hexyl-cinnamic aldehyde (Figure 1.4) [31]. It has recently been recommended that FM II is included in the baseline series for patch testing at dermatology clinics [32].

Apart from FM, the natural product balsam of Peru is used as a marker of contact allergy to fragrances in the standard series. Balsam of Peru is a natural resin obtained from the tree *Myroxylon pereirae*, used in topical medicaments for the treatment of burns and wounds, whereas extracts of the resin is frequently used in cosmetics [33]. It has been shown that concomittant reactions to FM and balsam of Peru are common and constitute a better proof of contact allergy to fragrances than a sole reaction to FM [34,35].

The prevalence of contact allergy to fragrances is high both in the general population and in patients referred to dermatology clinics. In a Danish study in 1991, the frequency of sensitization to FM was 1.1 % in an unselected population [36], whereas in a follow-up study in 1998, this frequency had increased to 2.3% [37]. Among consecutively tested dermatitis patients in Denmark, the positivity rates increased from 4.1% in 1985–1986 to 9.9% in 1997–1998 [38]. In a German multicentre study, the frequency of positive reactions to FM among consecutively tested dermatitis patients increased
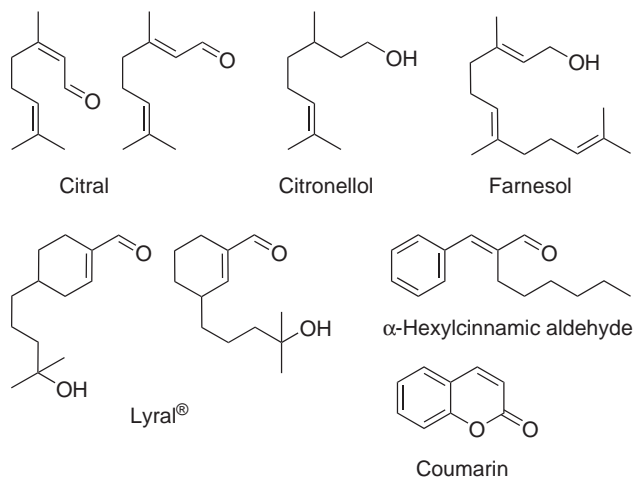
Figure 1.4: Components of fragrance mix II.

from 10.2% in 1996 to 13.1% in 1999, whereafter the proportion decreased
to 7.8% in 2002 [39]. Similar results are shown in a Belgian study, where the
reactions to FM in consecutively tested dermatitis patients increased from
7.2% in 1990 to a maximum of 13.9% in 1999, whereafter the proportion
decreased to 7.7% in 2005 [35]. These results may reflect the results of
preventive efforts such as a reduced exposure to the components of the FM,
but fragrances are still the second most common cause of ACD.

The most important limitation of the patch test method is the risk of a
false-negative diagnosis if the patient is not tested with the relevant hapten.
Many other fragrance materials apart from those included in FM and FM
II, such as essential oils, are known to cause contact allergy [40]. Ingredients
of fragrances can also undergo chemical modifications such as autoxidation,
to form new contact allergens. This type of haptens are not discovered when
testing with the pure fragrance compounds [41].

1.3   Autoxidation and contact allergy

Autoxidation is a spontaneous, air-induced oxidation of organic molecules.
It is a free radical chain reaction that results in the formation of several
products, of which the hydroperoxides are thought to be the most impor-
tant with regard to contact allergy. The oxidative deterioration of edible

Initiation   RH $\longrightarrow$ R$^\bullet$

Propagation   R$^\bullet$ + $^3O_2$ $\rightleftharpoons$ ROO$^\bullet$

                    ROO$^\bullet$ + RH $\longrightarrow$ ROOH   + R$^\bullet$

Termination   2 R$^\bullet$ $\longrightarrow$

                    R$^\bullet$ + ROO$^\bullet$ $\longrightarrow$ $\Big\}$ non-radical products

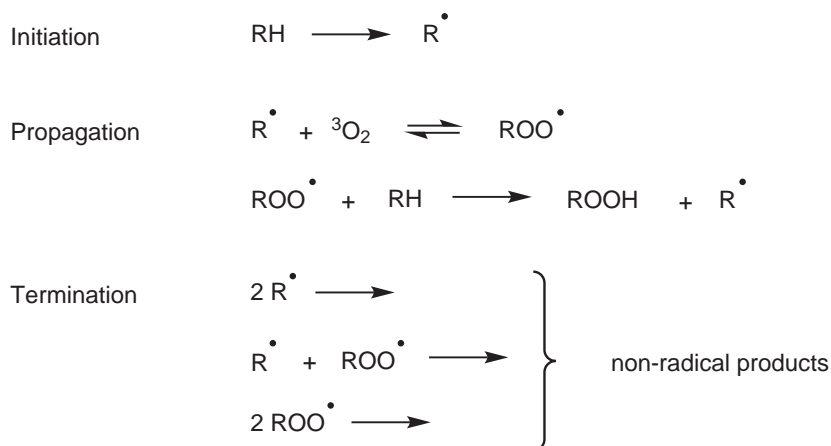   2 ROO$^\bullet$ $\longrightarrow$

Figure 1.5: General mechanism of autoxidation.

fats and oils has been the focus of research on autoxidation [42], although many terpenes also are susceptible to this, as they generally are unsaturated compounds.

1.3.1 General mechanism

Autoxidation is initiated by the formation of an alkyl radical by abstraction of a hydrogen atom by ultraviolet or visible light, heat or catalytic levels of redox-reactive transitions metals (Figure 1.5). In the propagation step, the radical reacts with oxygen to form a peroxyl radical. This step is fast, and the formed peroxyl radical then abstracts a hydrogen atom in a slower step to form a hydroperoxide, thus propagating the reaction by the creation of a new alkyl radical. The hydrogen abstraction reaction in the propagation step is selective for the most weakly bound hydrogens [43].

In the autoxidation of terpenes, radicals are preferentially created in the allylic position of a double bond, where they are stabilized by resonance, or at carbons bonded to heteroatoms such as oxygen, which also can stabilize the radical [43]. The product distribution is determined by the stabilities of the peroxyl radicals formed, which in turn are determined by the structure of the substrate. The chain reaction is terminated by the reaction of two radicals, or a radical and a peroxyl radical, or two peroxyl radicals, forming non-radical products.
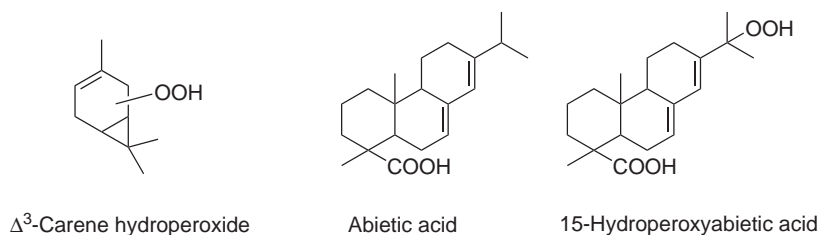
Δ^3-Carene hydroperoxide   Abietic acid   15-Hydroperoxyabietic acid

Figure 1.6: Structure of Δ^3-carene hydroperoxide, proposed as the major sensitizer in Scandinavian turpentine. Structures of abietic acid, the main component of colophony and of 15-hydroperoxyabietic acid, identified as the most important sensitizer in colophony.

1.3.2   Hydroperoxides in contact allergy

In the 1950's, it was discovered that the use of oil of turpentine caused many cases of ACD. Oil of turpentine is a volatile oil rich in monoterpenes, obtained from coniferous trees, and was at that time widely used as a solvent. A number of studies concluded that hydroperoxides formed from Δ^3-carene in the oil caused the skin reactions [44–48] (Figure 1.6). However, these hydroperoxides were never characterized. When oil of turpentine was replaced by petroleum products as solvents, and its use in other products ceased, it became an infrequent allergen [49].

Colophony, the nonvolatile fraction of exudates from coniferous trees has also been identified as a source of contact sensitization. It is still part of the baseline series for patch testing and is among the most common contact allergens in dermatitis patients. Concomittant reactions to colophony are frequent in dermatitis patients with fragrance allergy [34]. Colophony is a complex mixture of mainly diterpenes, where the main constituent is abietic acid (Figure 1.6). Abietic acid is not a sensitizer, but is easily oxidized in contact with air, forming various sensitizing oxidation products of which a hydroperoxide, 15-hydroperoxyabietic acid (Figure 1.6), has been shown to be the major sensitizing component of colophony [50, 51].

More recently, the autoxidation of fragrance terpenes has been studied. R-Limonene is a commonly used fragrance compound which is the main constituent of oil of citrus peel. It is used as a fragrance but also as a solvent in industry [52]. R-Limonene has been shown to be a non-sensitizer, but is oxidized on air exposure, forming several oxidation products [52, 53] (Figure 1.7). Among these, the hydroperoxides are the strongest sensitizers [54].
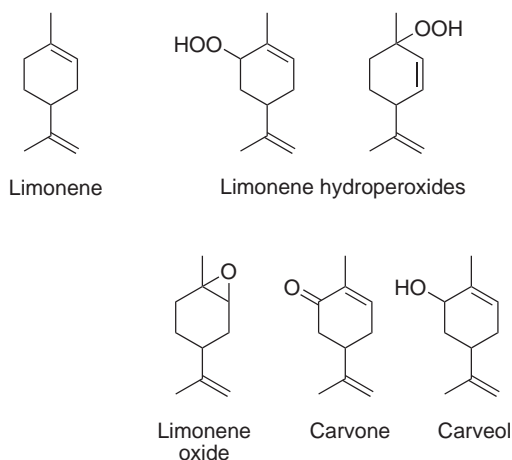
Figure 1.7: Limonene and identified oxidation products [52, 53]. The hy-droperoxides have been identified as the main sensitizers in air-exposed limonene.

Oxidized R-limonene and its hydroperoxide fraction have been identified as common causes of contact allergy when patch testing consecutively tested dermatitis patients in several clinical multicenter studies in Europe [55–57].

Linalool is one of the most commonly used fragrance compounds, origi-nating from lavender. The autoxidation of linalool has been studied, iden-tifying several oxidation products [58, 59] (Figure 1.8). As in the case of R-limonene, the hydroperoxides are the most important sensitizers. A mul-ticentre patch test study showed that oxidized linalool is a common contact allergen in dermatitis patients [41], with a frequency similar to that of oxi-dized limonene.

1.3.3   Controlling and preventing autoxidation

Autoxidation is a spontaneous process in room temperature and precence of air, therefore it is difficult to prevent. In the fragrance industry, antioxidants such as butylated hydroxytoluene (BHT) are often added to pure terpenes and essential oils (personal communication, Dr A-M Api). A study has been published, showing that the addition of antioxidants delays the start of autoxidation [60]. When the antioxidant is consumed, the autoxidation of the main compound will start. The onset of autoxidation is difficult to
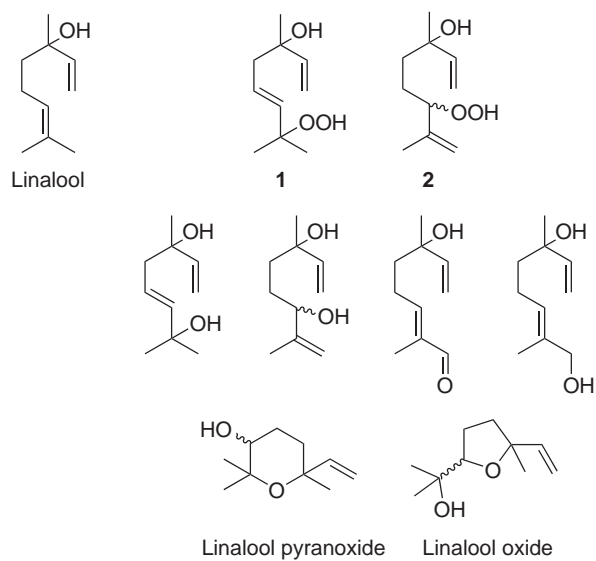
Figure 1.8: Linalool and identified oxidation products. The hydroperoxides **1** and **2** are the main sensitizers of air-exposed linalool [59].

predict, since it is dependent on the original purity of the terpenes as well as the added amount of antioxidant.

One method used to measure the degree of autoxidation of a sample is the reaction of hydroperoxides with iodide ion, forming iodine or triiodide ion [61], and subsequent titration with sodium thiosulfate which will reform ioidide ion and decolourize the solution. The amount of thiosulfate consumed in the titration is regarded as a measure of the degree of autoxidation, as well as allergenic activity of a sample. This is a rough estimate, as it does not take the formation of allergenic oxidation products other than hydroperoxides into consideration. Also, the method detects other related compounds, such as hydrogen peroxide.

1.4 Skin metabolism and contact allergy

It was assumed for a long time that the skin had no metabolic activity, acting only as an inert protective barrier to the environment [62]. It is now known that most reactions catalyzed by metabolic enzymes in the liver can also occur in the skin, catalyzed by the same or analogous enzymes.

1.4.1 Metabolic capacity of the skin

The metabolism of xenobiotics aims to render them more hydrophilic and thus more easily excreted. This is achieved in two steps, phase I and II. In phase I, hydrophilic functional groups are introduced by oxidative transformations, to form a metabolite sufficiently water soluble for rapid excretion. In most cases, phase II conjugation reactions with endogenous substrates are required for the achievement of sufficient hydrophilicity.

Both phase I and phase II metabolic enzymes have been identified in human skin [62, 63]. Examples of phase I enzymes identified, include the cytochrome P450 (CYP) superfamily, and other oxidoreductases such as alcohol dehydrogenase, aldehyde dehydrogenase, monoamine oxidases, flavincontaining monooxygenases and hydrolytic enzymes. Of these, the enzymes of the CYP family are considered to be the most important enzymes in the phase I metabolism of xenobiotics [62]. The CYP family covers a wide range of substrates, both endogenous and xenobiotic. The general reaction involves the incorporation of an oxygen atom in the structure of the substrate, such as the epoxidation of a double bond or the hydroxylation of a carbon, although other types of reactions are also catalyzed, such as the oxidation of hydroxy moieties to carbonyl compounds [64].
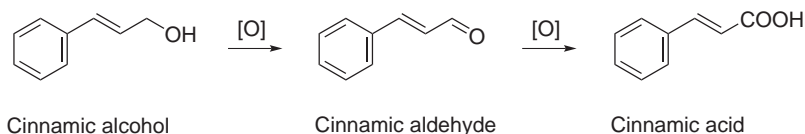
Figure 1.9: Bioactivation of cinnamic alcohol into the sensitizer cinnamic aldehyde. Further metabolic oxidation of cinnamic aldehyde yields the non-sensitizing cinnamic acid.

1.4.2   Bioactivation of prohaptens

Cinnamic alcohol is a well-known prohapten. It is a frequently used fragrance and flavour compound, with the smell and taste of cinnamon. Sensitization to cinnamic alcohol is frequent, and it is a component of FM [26]. It has been shown to be activated in human skin to cinnamic aldehyde, a known hapten [65, 66], which is also a component of FM (Figure 1.9). The aldehyde can be further oxidized in a second step to the non-toxic compound cinnamic acid. There are patients showing positive patch tests to cinnamic alcohol and negative patch tests to cinnamic aldehyde [67], which indicates that other metabolites than cinnamic aldehyde can be important in contact allergy to cinnamic alcohol. Because of this, both the alcohol and the aldehyde are used in FM.

Structure-activity relationship studies of conjugated alkenes and α,β-unsaturated oximes have revealed that these classes of compounds can be activated by CYP to highly reactive and sensitizing metabolites [68, 69]. Previously, neither of these classes of compounds have been considered to be sensitizers or prohaptens. It was shown that conjugated alkenes in or in conjunction with a six-membered ring, and α,β-unsaturated oximes were activated by CYP into strong sensitizers.

The role of bioactivation of prohaptens in the development of contact allergy is not fully investigated. A model for study of cutaneous CYP mediated bioactivation of prohaptens has recently been developed, through identification and quantification of the CYP enzymes expressed constitutively in skin [70]. The model system consists of a cocktail of these rhCYP enzymes, mixed in the same ratios as found in the skin, and provides an important tool for the investigation of skin metabolism *in vitro*.

Chapter 2

Aims of the study

The studies presented here are part of a research program with the overall goal of understanding the formation of skin sensitizers by autoxidation or by metabolism from compounds with no or low sensitizing capacity. The majority of the compounds investigated are fragrance terpenes. In this thesis the autoxidation of geraniol, geranial and linalyl acetate is studied as well as the metabolic activation of geraniol.

The specific aims of the thesis are:

1.   To investigate the autoxidation of the fragrance terpenes geraniol and geranial, and to identify the main oxidation products formed.

2.   To study sensitizing potency of autoxidized geraniol and geranial and to determine the sensitizing capacities of individual oxidation products.

3.   To study the cutaneous metabolism of geraniol, to identify the main metabolites, and to determine their sensitizing capacities. Here we wanted to compare the pattern of products formed by cutaneous metabolism and by autoxidation of geraniol.

4.   To investigate the autoxidation and sensitizing capacity of linalyl acetate, and compare the results to the corresponding results for linalool, previously investigated in the group.

5.   To investigate the products formed in autoxidized lavender oil
            and compare the product pattern with that of autoxidized lina-
            lyl acetate, linalool and β-caryophyllene. Also, the effect of au-
            toxidation on the sensitizing capacity of the lavender oil was
            investigated.

Chapter 3

Methods

3.1 Studies of autoxidation

Autoxidation studies were performed in papers I, II, III and V.

3.1.1 Air exposure procedure

Geraniol, geranial, linalyl acetate and lavender oil in samples of 50 ml were air exposed at room temperature in Erlenmeyer flasks (100 ml), covered with aluminum foil to prevent contamination. A fluorescent daylight lamp was used to provide daylight conditions that would not be affected by seasonal changes. The flasks were exposed to light 12 h a day and stirred for 1 h, 4 times a day. Minor samples were taken out every two weeks for analysis and stored at -20°C under nitrogen atmosphere.

3.1.2 Fractionation of autoxidation mixtures

Normally, samples of autoxidation mixtures were subjected to flash chromatography on silica gel columns. Repeated purifications were made from about 5 g of oxidized material. Mixtures of ethyl acetate and n-hexane were used for elution, where the proportion of ethyl acetate was gradually increased. In one case, preparative HPLC was used.

3.1.3   Identification and quantification of oxidation products

Isolated compounds were characterized using NMR spectroscopy and GC-MS. Chromatographic and spectral properties were compared with those of synthesized or commercially available reference compounds.

Quantification of terpenes and their oxidation products in oxidation mixtures was performed using HPLC-UV and GC-FID. In the HPLC-UV method, pure reference compounds were used to make external calibration curves from which the concentrations of the studied compounds could be determined. In the GC-FID method, an internal standard, 1,2,3,5-tetramethyl benzene was used. Analyses were made on pure reference compounds with added internal standard to determine the response factors. The same amount of internal standard was added to the dissolved air-exposed samples. Using the response factors, the amount of each compound in the samples could be determined.

Hydrogen peroxide was quantified in air-exposed geraniol using a derivatization method that yields a fluorescent product. Solutions of air-exposed geraniol in milli-Q water were mixed with the enzyme horse radish peroxidase and its substrate *p*-hydroxyphenylacetic acid. The amount of fluorescent product formed was measured using fluorescence detection and flow injection analysis, according to a previously published procedure [71]. External calibration curves were made using hydrogen peroxide in water. The selectivity of the derivatization reaction for hydrogen peroxide over hydroperoxides was evaluated by comparing the responses of linalool hydroperoxides **1** and **2,** and two commercially available hydroperoxides to that of hydrogen peroxide in water. It was found that the selectivity of horse radish peroxidase towards hydrogen peroxide was high (paper I).

3.2   Studies of CYP-mediated metabolism

A metabolism study was performed in paper IV. A skin-like CYP cocktail was prepared by mixing the rhCYP enzymes CYP1A1 (16.4 %), CYP1B1 (9.0 %), CYP2B6 (0.16 %), CYP2E1 (50 %), and CYP3A5 (25.5 %) to a final concentration of 44 pmol/ml, as previously described [70]. Experiments were also performed using 5 times the original concentration (220 pmol/ml), to be able to detect metabolites formed in small amounts. To determine the importance of each enzyme in the cocktail, incubations were performed using single enzymes (40 pmol/ml).

Geraniol (50 μM) was mixed with skin-like CYP cocktail or single CYPs, and MgCl$_2$ (30 mM) in TRIS buffer (300 mM, pH 7.4) and pre-incubated for 3 min, after which NADPH (1 mM) was added. The total volume was 500 μl. The samples were incubated for 60 min and the incubations were terminated by the addition of *n*-hexane/dichloromethane (1:1, 1.0 ml) containing 1,2,3,5-tetramethylbenzene as internal standard. The extracts were collected after centrifugation at 3000 rpm for 10 min and analyzed using GC/MS in SIM mode. External calibration curves were made in relation to the internal standard. The incubations were performed in duplicate and controls were run in the absence of NADPH or CYP.

3.3 Synthesis of reference compounds

All of the following synthesis work was performed using literature procedures. The yields quoted are isolated yields, obtained in our laboratory.

Some of the oxidation products identified in the autoxidation studies were available commercially. In these cases, they were purchased and purified using flash chromatography or preparative HPLC prior to use as reference compounds. The same general systems as in the purification of autoxidation mixtures were used. These compounds are generally referred to by their trivial names and these are employed also in this thesis.

3.3.1 Synthesis of hydroperoxides using photooxidation

The hydroperoxides **1-6** were synthesized according to a procedure by Bäckström et al [72] (Figure 3.1). The starting material was dissolved in a solution of the tetrabutylammonium salt of Bengal Rose in chloroform (1.5 mM) to a final concentration of approximately 0.1 M. The solution was irradiated using a Rayonet reactor and a constant flow of oxygen. The solvent was removed under reduced pressure and the crude product was purified on silica gel using mixtures of ethyl acetate and *n*-hexane as eluent.

3.3.2 Reduction of hydroperoxides to their corresponding alcohols

The geraniol alcohols **7** and **8** were prepared from hydroperoxides **3** and **4** (Figure 3.2). The hydroperoxides were dissolved in diethyl ether after which 1.1 equivalents of triphenyl phosphine were added. After the completion of the reaction, the solvent was evaporated and the two diols were separated using flash chromatography.
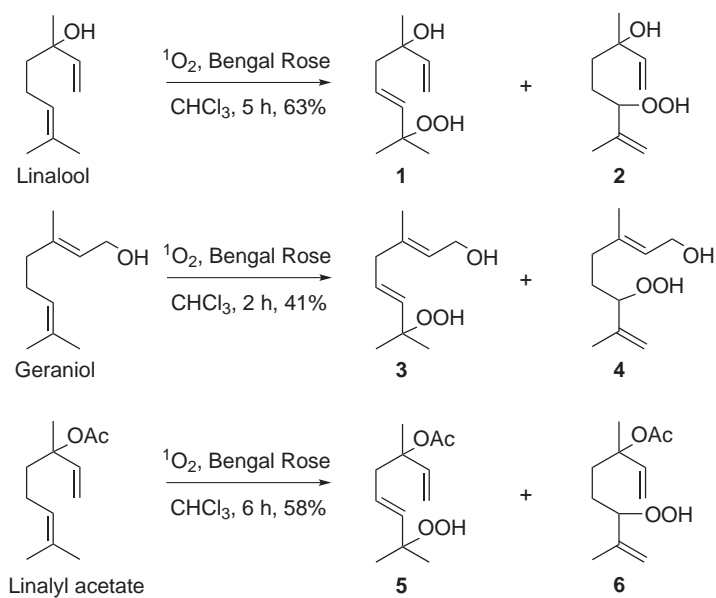
Figure 3.1: Synthesis of the hydroperoxides of geraniol, linalool and linalyl acetate.
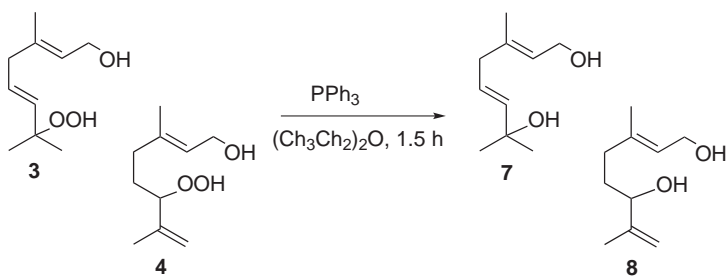
Figure 3.2: Reduction of geraniol hydroperoxides to their corresponding diols.
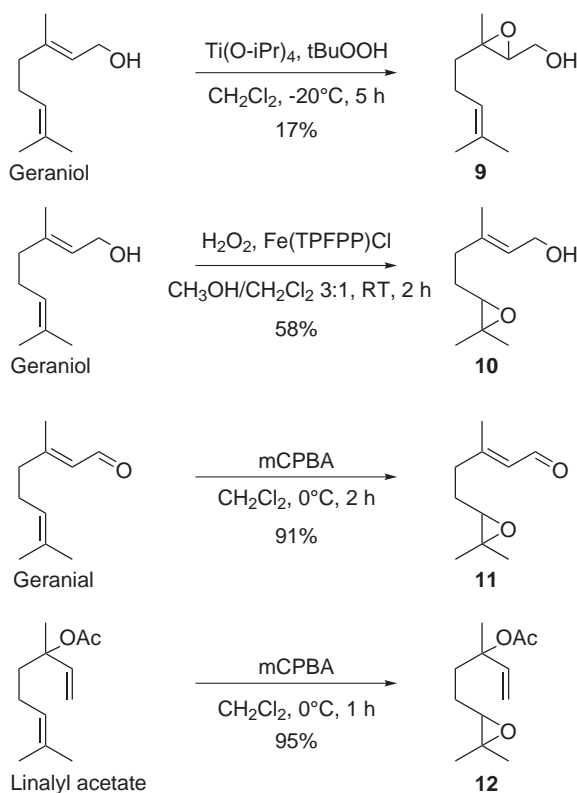
Figure 3.3: Synthesis of studied epoxides.

3.3.3   Synthesis of investigated epoxides

2,3-Epoxy-3,7-dimethyl-oct-6-en-1-ol (9) (Figure 3.3). The synthesis was performed as described in literature using the Sharpless epoxidation procedure [11].

6,7-Epoxy-3,7-dimethyl-oct-2-en-1-ol (10) (Figure 3.3). The synthesis was performed as described in literature [73], using hydrogen peroxide and a porphyrin catalyst, 5,10,15,20-tetrakis(pentafluorophenyl)21H,23H-porphine iron (III) chloride. The reaction was terminated before the second double bond was epoxidated.

6,7-Epoxy-3,7-dimethyl-oct-2-enal (11) and **6,7-epoxy-3,7-dimethyl-1-octen-3-yl acetate (12)** (Figure 3.3) were synthesized from geranial and

linalyl acetate, respectively. *m*-Chloroperbenzoic acid (1.1 eq) was added to a solution of the starting material (160 mM) in dichloromethane at 0 °C. The reaction mixture was stirred, and after the disappearance of the starting material, NaOH (2 M) was added. The organic phase was dried over $MgSO_4$ and concentrated. The crude product was purified on silica gel using mixtures of ethyl acetate and *n*-hexane as eluent.

3.4 Studies of sensitizing capacity, the LLNA

The experiments were carried out using female CBA/Ca mice, housed in cages with HEPA-filtered air flow under conventional conditions where light, humidity, and temperature were controlled. Compounds or oxidation mixtures of interest were tested in three or five concentrations, using mice in groups of four or three, respectively. The mice recieved 25 μl of a solution of the test material in the vehicle, acetone/olive oil (4:1), on the dorsum of the ears for three consecutive days (Figure 1.2). The control group was treated with equal volumes of vehicle alone. At day 5, the mice were injected intravenously through the tail vein with 20 μCi of [methyl-^{3}]H thymidine in 250 μl phosphate buffered saline (PBS). After 5 h, the mice were sacrificed, the draining lymph nodes were excised and pooled for each concentration group. Single cell suspensions of lymph node cells were prepared and the thymidine incorporation was measured using β-scintillation counting. A stimulation index (SI), that is, the increase in thymidine incorporation relative to the control group, was calculated for each concentration group. Test materials that at one or more concentrations produced an SI of 3 or greater were considered to be positive in the LLNA. The EC3 value (estimated concentration required to produce a SI of 3) used to compare relative sensitizing potencies, was calculated by linear interpolation.

In the case of hydrogen peroxide, the vehicle acetone/glycerol/water (8:1:1) was used. A pretest was performed to determine the maximum non-irritating concentration of hydrogen peroxide in the acetone/glycerol/water vehicle.

The studies were approved by the local ethics committee.

3.5 Patch testing

A patch test study was performed in paper III. Air-exposed linalool (45 weeks), linalool hydroperoxides **3** and **4** (Figure 3.1), air-exposed linalyl

acetate (45 weeks) and air-exposed lavender oil (45 weeks) were used for patch testing.

Initially, 22 patients with no personal history of fragrance sensitivity were selected among the consecutive dermatitis patients and patch tested to evaluate the irritant effect of the test preparations of oxidized lavender oil and of oxidized linalyl acetate 2.0%, 4.0%, 6.0% in petrolatum (pet.). Informed consent was obtained. No irritation was seen to the tested concentrations.

As we in parallel patch test studies found a test concentration of 4.0% pet. of air-exposed linalool to be suitable for screening, a concentration of 4% was chosen for further testing of air-exposed linalyl acetate and air-exposed lavender oil. Non-stabilized pet. was used for all patch test preparations, as the hydroperoxides present in the patch test material are more easily degraded in the presence of antioxidants [74].

In the following investigations, performed in May 2006 and June 2007, individuals with known positive patch test reactions (++ or +++) to air-exposed linalool were selected from 1985 patients with dermatitis, patch tested between 2004 and 2007. A letter was sent to 9 individuals who met the above-mentioned criteria. The response rate was 9 of 9 (100%) individuals, of whom 3 were included in the study. These patients were tested with air-exposed linalool 4.0%, 2.0%, 1.0%, and 0.5% pet., a mixture of linalool hydroperoxides 1%, 0.75%, 0.5%, 0.25%, 0.12 % and 0.06% pet., air-exposed lavender oil 4.0% pet., and air-exposed linalyl acetate 4.0% pet. The test concentrations of linalool hydroperoxides were chosen from prior experience [75]. New patch test materials were made within 2 weeks before testing. Readings were performed on days 3 and 7 according to the recommendations by the ICDRG. The study was approved by the local ethics committee.

Chapter 4

Studies of geraniol and geranial

4.1 Autoxidation of geraniol (Paper I)

The aim of this study was to investigate the autoxidation of geraniol (Figure 4.1). Geraniol occurs naturally in large amounts in many plants, such as rose. It is a widely used fragrance terpene in both cosmetics and household products [76–78]. Geraniol is considered to be a weak allergen [39], and has therefore been included in FM. However, reactions to geraniol are rare [39]. Geraniol is not an electrophile and should consequently not show any sensitizing capacity. It is therefore important to investigate if geraniol itself is a sensitizer or if the allergenic effect observed is due to the formation of sensitizing oxidation products. The two double bonds in geraniol provides six allylic positions which all could be susceptible to hydrogen atom abstraction (Figure 4.1). This indicates that the autoxidation of geraniol could proceed via two pathways, that is autoxidation of the 2,3 double bond or of the 6,7 double bond. The autoxidation of the 6,7 double bond corresponds to the autoxidation of linalool (Figure 1.8). We wanted to study if the autoxidaiton of geraniol would follow the same pathway as that of linalool or if autoxidation of the allylic alcohol moiety would dominate.

Geraniol was found to autoxidize readily at air exposure, at about the same rate as the previously investigated linalool [59] (Figure 4.2). When the oxidation mixture was fractionated, a number of oxidation products could be identified (Figure 4.1). The product distribution showed that both double
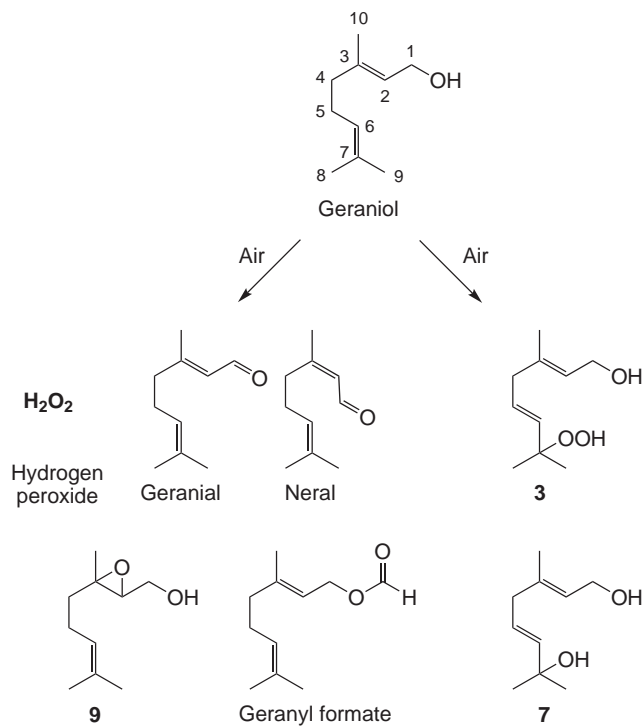
Figure 4.1: Product distribution in the oxidation mixture after autoxidation of geraniol.
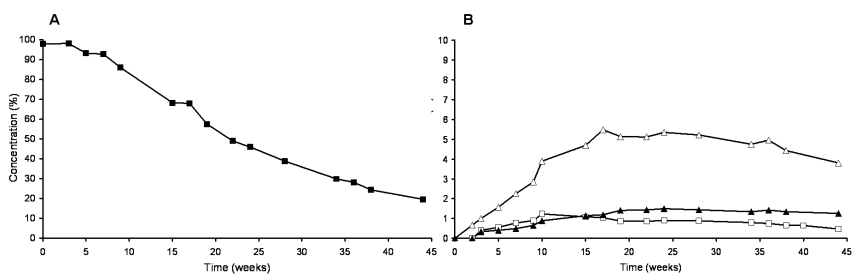

Figure 4.2: Autoxidation of geraniol (■) (A) and formation of geranial (△), neral (▲) and geraniol hydroperoxide **3** (□) (B) in the oxidation mixture.

bonds in geraniol were susceptible to autoxidation.

The hydroperoxide **3** and the diol **7** are products of autoxidation of the 6,7 double bond. This corresponds to the previously seen oxidation pathway of linalool (Figure 1.8) [59]. The aldehydes geranial and neral together with hydrogen peroxide originate from abstraction of a hydrogen atom at carbon 1. The radical thus formed, can react with oxygen to form a hydroxyhydroperoxide, which rapidly decomposes to aldehyde and hydrogen peroxide. Hydrogen peroxide was detected and quantified in the oxidation mixture and the results support the mechanism involving a hydroxyhydroperoxide (Paper I). Geranial and neral were the most abundant of the oxidation products identified, which indicates that this pathway is the most favoured (Figure 4.2).

The experimental results were confirmed using computational modeling. It was found that the most stable radical was formed by abstraction of a hydrogen atom at carbon 1. The radical formed by hydrogen abstraction at carbon 5, leading to hydroperoxide **3,** was found to have a lower stability but was still sufficiently stable to be formed.

Considering the other oxidation products formed from geraniol, geranyl formate is also believed to originate from the most favoured oxidation pathway, whereas the epoxide **9** is believed to originate from the reaction of geraniol with a hydroperoxyl radical, forming epoxide **9** and a hydroxyl radical [79].

Pure geraniol was identified as a weak sensitizer in the LLNA, which is consistent with the results from clincal studies (Table 4.1). Geraniol is a constituent of FM, used for standard screening of ACD, although it seldom gives positive test reactions [39]. The autoxidation of geraniol greatly influenced the sensitizing capacity, as the oxidation mixtures of 10 and 45 weeks of air exposure were moderate sensitizers (Table 4.1).

The increased sensitizing capacities of the air-exposed samples can be explained by the formation of the moderate sensitizers geranial and neral, and of hydroperoxide **3**, a strong sensitizer.

4.2 Autoxidation of geranial (Paper II)

The aim of this study was to investigate the autoxidation of geranial, which is the corresponding aldehyde of geraniol. As aldehydes are known to be moderate sensitizers [80], the question was raised if stronger sensitizers would be formed on air exposure or if non-sensitizing oxidation products would dominate, thus diminishing the sensitizing capacity of air-exposed geranial

Table 4.1: Sensitizing capacities of investigated compounds and oxidation mixtures. Classification according to Kimber *et al* [24].

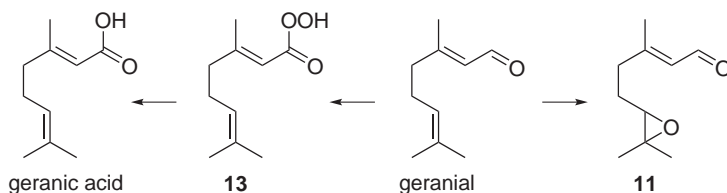| Test material	EC3 (% w/v)	EC3 (M)	Classification
Ox. geranial 5 w	1.3		strong
Ox. geraniol 10 w	4.4		moderate
Ox. geraniol 45 w	5.8		moderate
Geranial	6.8	0.45	moderate
Geraniol	22	1.45	weak
Geranyl formate	79	4.4	weak/NS
Hydrogen peroxide	—	—	NS
Neral	9.7	0.64	moderate
3,4	1.4	0.077	strong
7	—	—	NS
9	57	3.3	weak/NS
10	7.1	0.42	moderate
11	1.4	0.082	strong

Ox, air-exposed; NS, non-sensitizer.

Figure 4.3: Product distribution in the autoxidation of geranial. Peracid **13** could not be detected in the autoxidation mixture using HPLC-UV, but is thought to be the precursor of geranic acid.

compared to pure geranial. Citral, the isomeric mixture of geranial and neral, is frequently used as a fragrance and flavor substance. Citral is the major component of lemongrass (*Cymbopogon citratus*) and has been detected in 25% of domestic and occupational products [77]. A number of degradation studies of citral are published, which investigate the degradation in aqueous solution at elevated temperatures and low pH [81–83]. The products identified were the products of intramolecular reactions and were considered to be responsible for the off-odour formation in citral-containing beverages and other food products.

We found that geranial autoxidized on air exposure. The rate of autoxidation was faster than that of geraniol, more similar to the autoxidation rates of limonene and β-caryophyllene [54,84]. The oxidation mixture rapidly became viscous, indicating the formation of oligomers or polymers. After 30 weeks, the oxidation mixture was no longer suitable for analysis due to its viscosity.

The main oxidation product identified was 6,7-epoxygeranial **11** (Figure 4.3), which was formed early and accumulated with time (Figure 4.4). This is similar to the autoxidation of β-caryophyllene, where a sensitizing epoxide was formed in high amounts (Figure 4.5). Epoxide **11** was thought to originate from the reaction with hydroperoxyl radical, as previously described [79]. Geranic acid was also identified in the oxidation mixture (Figure 4.3). It can be formed by abstraction of the aldehyde hydrogen, creating an acyl radical, which can react with hydroperoxyl radical to form a peracid **13** [85,86]. The peracid can subsequently react with a molecule of geranial, forming **11** and geranic acid. The formation of the acid might affect the oxidation process as the pH of the oxidation mixture is decreased. Epoxides are known to be unstable under acidic conditions, and the formation of the acid could contribute to the degradation of **11** observed (Figure 4.4). Since

Figure 4.4: Autoxidation of geranial (■) and formation of 6,7-epoxygeranial **11** (▲).   Autoxidation rates of geraniol (△), R-limonene (□) and β-caryophyllene (○) are included for comparison.

β-Caryophyllene   Caryophyllene oxide

Figure 4.5: Autoxidation of β-caryophyllene. After 5 weeks, air exposed β-caryophyllene contained approximately 20% caryophyllene oxide [84].

acids are known to be weak or non-sensitizers [87], geranic acid was not considered to contribute to the sensitizing capacity of air-exposed geranial. Therefore, it was not quantified.

Because of the fast oxidation rate of geranial, the sensitizing capacity of 5 weeks air-exposed geranial was determined and the mixture was shown to have a strong sensitizing capacity (Table 4.1). The sensitizing capacity of **11** was determined and it was also shown to be a strong sensitizer. The strong sensitizing capacity of the 5 weeks air-exposed sample can mainly be attributed to the formation of **11**, although it is possible that other sensitizers are present in small amounts.

As the polymerization observed in air-exposed geranial was more rapid than that of the corresponding alcohol, geraniol, it is important to investigate if sensitizing oligomers are formed in the oxidation mixture of geranial. In investigations of *p-tert*-butylphenol-formaldehyde resins, a number of trimers has been identified as sensitizers [88]. This shows that oligomer oxidation products could be of importance in oxidation mixtures of compounds that are prone to polymerization.

Geranial is the major oxidation product formed in air exposed geraniol, and it is therefore most likely that **11** is formed in small amounts also in air exposed geraniol. The concentration of geranial in air exposed geraniol is decreasing with time, which might indicate further oxidation, forming **11** and geranic acid. Epoxide **11** is a strong sensitizer which could contribute to the sensitizing capacity of air exposed geraniol. The strong sensitizing capacity of air exposed geraniol is difficult to explain from the amounts of identified sensitizing oxidation products only. The further oxidation of geranial to **11** could affect the sensitizing capacity of air exposed geraniol, even if **11** is formed in amounts too low to be detected in the methods used in this study.

The general exposure to geranial in the form of citral is large in the population. Citral has previously been found to be an important sensitizer, to the extent that it has been considered to be included in the baseline series for testing in dermatitis patients. The autoxidation of geranial gave a greatly increased sensitizing capacity, as the strong sensitizer **11** is formed in high concentrations. This shows that air-exposed geranial might be an important sensitizer in the population.
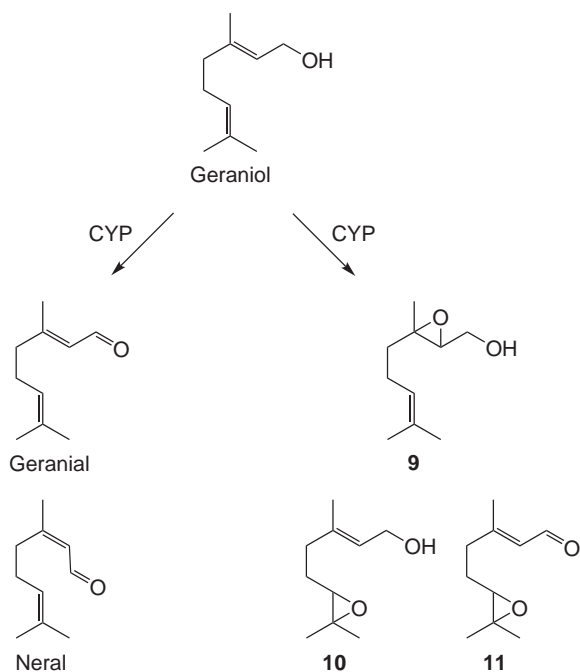
Figure 4.6: Product distribution in the metabolism experiments of geraniol.

4.3   Metabolism of geraniol (Paper III)

The aim of this study was to investigate the CYP-mediated cutaneous metabolism of geraniol. In paper I, geraniol was shown to be a weak sensitizer (Table 4.1), indicating that metabolism of geraniol in the skin to sensitizing compounds could occur. In literature, the allergenic activity of geraniol has been suggested to be caused by metabolism in the skin to geranial [9].

The cutaneous metabolism of geraniol was studied using the previously developed skin-like CYP cocktail [70]. Incubations with geraniol and the CYP cocktail showed that geranial was the main metabolite formed, followed by epoxide **10** and neral (Figures 4.6 and 4.7). Epoxide **9** was also detected in small amounts in all incubations. In the incubations using a more concentrated cocktail, the epoxide **11** could be detected. As two chemical modifications are required to form epoxide **11** from geraniol, it is not
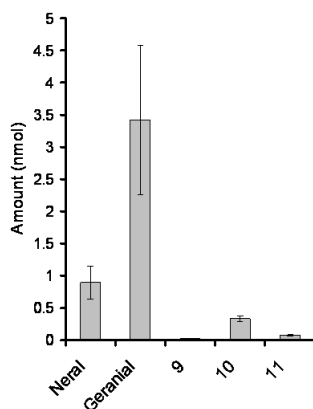
Figure 4.7: Results from the incubations with concentrated CYP cocktail.

surprising that it was detected in the incubations using the lower protein concentration.

Experiments were also carried out to determine the role of each CYP isoform present in the skin-like CYP cocktail. CYP2B6 showed the highest activity, catalyzing only oxidation to geranial and neral (Figure 4.8). This is in accordance with another studie which also has shown geraniol to be a good substrate for CYP2B6 [89]. We found that CYP1A1 and CYP3A5 showed lower activities compared to that of CYP2B6 and catalyzed not only oxidation of the alcohol to geranial and neral but also epoxidations, giving epoxides **9** and **10**. CYP1B1 and CYP2E1 showed low activities, with epoxide **9** as the only product. Epoxide **11** was not detected in any of the incubations using single CYP isoforms. It could be formed from the actions of 1A1 or 3A5 alone, although the concentration most likely would be too low to be detected in the performed experiment.

Among the different metabolites detected in this investigation, several sensitizers could be identified (Figure 6.1, Table 4.1). Most of them are discussed above, as they are also formed in the autoxidation of geraniol. Epoxide **10** was the only metabolite detected which was not also detected in the autoxidation of geraniol. It was shown to be a moderate sensitizer (Table 4.1).

The results obtained explain the weak sensitizing capacity of pure geraniol seen in animal sensitization studies. Several sensitizers are formed metabolically, and it is likely that geranial is the major hapten responsible for the
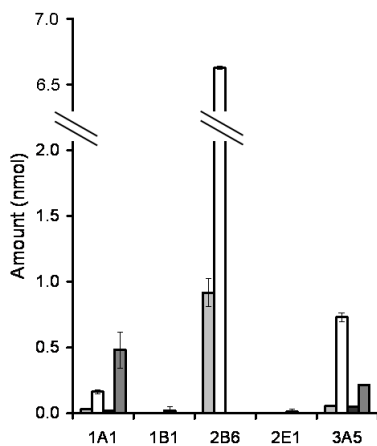
Figure 4.8: Activities of the single CYP enzymes. Concentrations detected of neral (light grey), geranial (white), epoxide **9** (black) and epoxide **10** (dark grey).

sensitizing capacity of geraniol. Epoxides **10** and **11** are probably also of importance, although they are formed in smaller amounts.

Chapter 5

Autoxidation of an essential oil

The aim of this study was to compare the autoxidation of the major constituents in an essential oil to the autoxidation of the same compounds in pure form. Lavender oil was chosen, since two of the main components, linalool and β-caryophyllene, have been studied previously [58, 59, 84]. Investigations of the autoxidation of the major component linalyl acetate were performed to complete the autoxidation studies of the pure terpenes in lavender oil.

5.1   Autoxidation of linalyl acetate (Paper IV)

Linalyl acetate is one of the most frequent fragrance ingredients in cosmetics, toiletries and household products [76, 77, 90]. The toxicological and dermatological properties of linalyl acetate have been extensively reviewed [90], but no autoxidation studies have been performed previously to the best of our knowledge. Therefore, the autoxidation of linalyl acetate was studied.

The autoxidation of linalyl acetate was expected to follow the same pathway as that of linalool, since the two molecules are very similar in structure. This was shown to be true, as the autoxidation rate of linalyl acetate was approximately the same as for linalool [59] (Figure 5.1). Also, hydroperoxides **5** and **6**, corresponding to the linalool hydroperoxides **1** and **2,** were identified in the autoxidation mixture. The ester bond in linalyl acetate (compared to the free hydroxyl group in linalool) prevented the formation
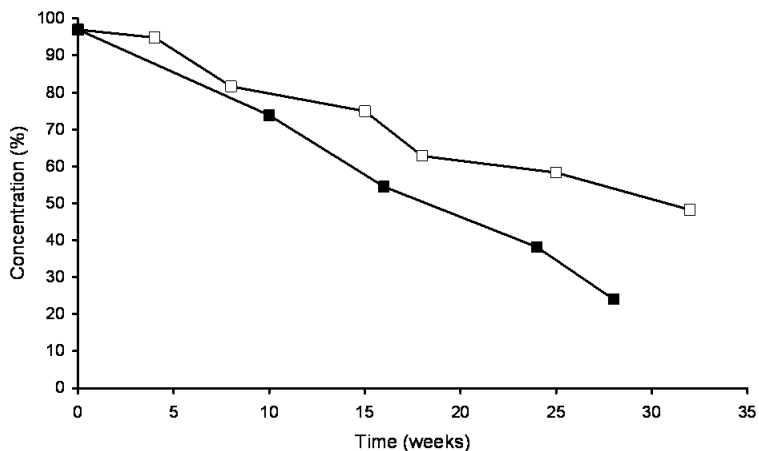
Figure 5.1: Autoxidation of linalyl acetate (■) and linalool (□) [59].

of the pyran and furan oxides previously identified in autoxidized linalool (Figure 1.8) and instead an epoxide was identified (Figure 5.2). An alcohol **14**, originating from hydroperoxide **5**, was also identified.

Linalyl acetate showed a weak sensitizing capacity in the LLNA, probably due to the electrophilic properties of the ester moiety (Table 5.1). This is in accordance with literature data on the sensitizing capacity of aliphatic esters [91]. The 10 weeks air-exposed linalyl acetate was shown to be a moderate sensitizer, as were the linalyl acetate hydroperoxides **5** and **6**. The presence of these hydroperoxides is most likely the main cause of the sensitizing capacity of air-exposed linalyl acetate.

Epoxide **12** was shown to be a non-sensitizer and although epoxides can be strong sensitizers [68], the lack of sensitizing capacity of **12** can be explained by the hydrolysis of the ester bond by esterases in the skin, forming a linalool epoxide. This epoxide is known to react intramolecularly to form linalool oxide, which has previously been shown to be a non-sensitizer [59]. The sensitizing capacity of alcohol **14** was not investigated since a linalool diol analogous to **14** (Figure 1.8) previously has been shown to be a non-sensitizer [59].

The linalyl acetate hydroperoxides seem to be slightly weaker sensitizers than the corresponding hydroperoxides of linalool (**1,2**) and geraniol (**3,4**). The EC3 value for the linalool hydroperoxides **1,2** was determined
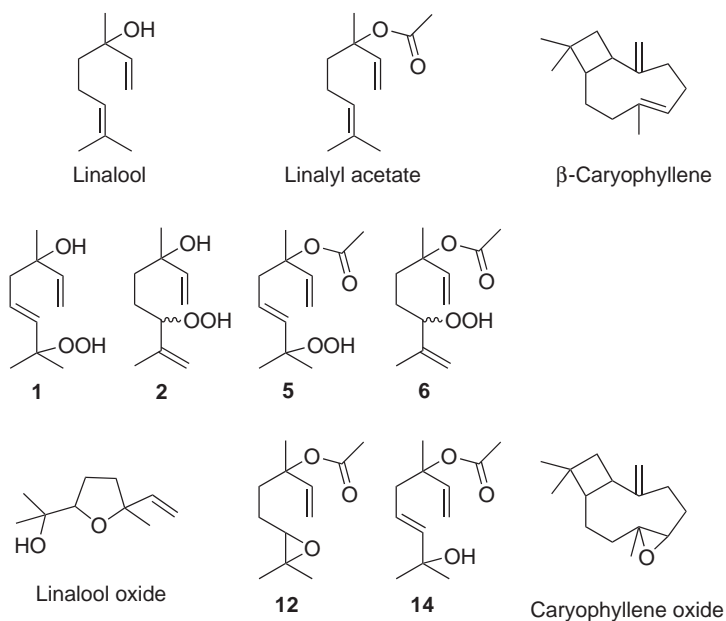
Figure 5.2: Main components of lavender oil and identified oxidation products.

Table 5.1: Sensitizing capacities of pure and air exposed linalyl acetate, lavender oil, and oxidation products of air exposed linalyl acetate.

Test material	EC3 (% w/v)	Classification
Ox. lavender oil 10 w	11	moderate/weak
Ox. linalyl acetate 10 w	3.6	moderate
Ox. synthetic mix 10 w	14	weak
Lavender oil	36	weak
Linalyl acetate	25	weak
5,6	3.6	moderate
12	—	NS

Ox, air exposed; NS, non-sensitizer.

to 0.086 M [59] and that of the geraniol hydroperoxides **3,4** to 0.077 M (Table 4.1). From the experimental data available, it is not possible to determine if this difference is due to experimental deviations or if there is a statistically significant difference in the sensitizing capacities of the linalyl acetate hydroperoxides compared to the previously tested hydroperoxides. The structures of **5** and **6** do not give any clues as to why the sensitizing capacity should be lower than those of **1**−**4**. Once again, the hydrolysis of the ester bond should be considered, thereby forming linalool hydroperoxides. In this way, the concentrations of **5** and **6** are lowered in the skin, and although the strong sensitizers **1** and **2** are formed, the concentrations of the different hydroperoxides might not be high enough to induce an equally large response as **1** and **2** alone. It is also possible that the acetylation affects penetration or irritant properties, thereby decreasing the amount of substance that penetrates into the skin.

As linalyl acetate is also a commonly used fragrance material, the impact of these results could approach the same importance as has been shown for oxidized linalool, which in a multicenter study was found to be one of the most common allergens in consecutively tested dermatitis patients [41]. As the exposure to linalyl acetate is extensive, hydroperoxides **5** and **6** could be important sensitizers in the population, although they might have a weaker sensitizing capacity compared to **1** and **2**. There are as of yet, no clinical data on the prevalence of contact allergy to oxidized linalyl acetate.

5.2 Autoxidation of lavender oil (Paper V)

Lavender oil is the essential oil obtained from steam distillation of lavender (*Lavendula angustifolia* or *Lavendula officinalis*), widely used as a fragrance ingredient and in aroma therapy [92,93]. It has also been used for centuries in traditional herbal medicine [93]. There are several case reports in the literature on contact allergy to lavender oil [94–100], although the haptens responsible for the ACD have not been identified.

The aim of this study was to investigate if the same pattern of autoxidation of the main constituents linalyl acetate, linalool and β-caryophyllene would be observed in the essential oil as was seen in previous studies of pure compounds. Two samples of lavender oil were air-exposed and the results were compared to the results from the autoxidation studies of linalyl acetate (Paper IV), linalool [59] and β-caryophyllene [84]. Also, a synthetic lavender oil, referred to as the synthetic mix, was prepared by mixing linalyl acetate, linalool and β-caryophyllene in the same relative ratios as they occur in
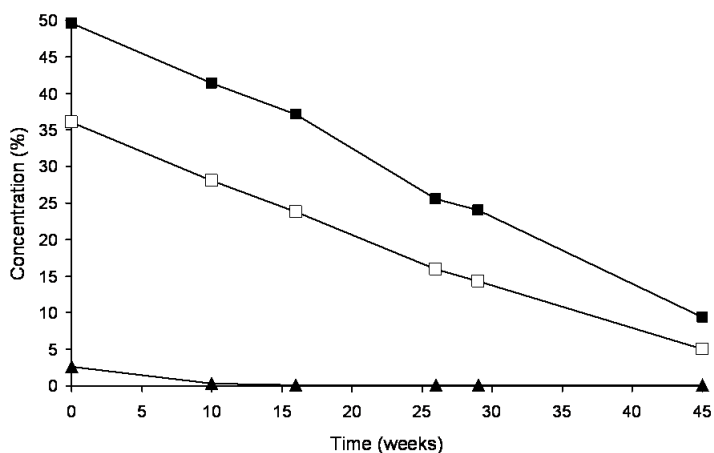
Figure 5.3: Autoxidation of linalyl acetate (■), linalool (□) and β-caryophyllene (▲) in lavender oil.

lavender oil. The synthetic mix was used to study autoxidation in a mixture of known ingredients.

Linalyl acetate, linalool and β-caryophyllene followed the same pattern of oxidation in the lavender oil as when oxidized separately (Figure 5.3). The synthetic mix was air exposed for 10 weeks and showed a decrease in the amount of linalyl acetate from 56% to 45%, in that of linalool from 40% to 32% and in that of β-caryophyllene from 3.3% to 0.37%. The rates of autoxidation of lavender oil and the synthetic mix were similar to the rates of autoxidation of the pure compounds. Thus, the oil did not contain compounds with antioxidant properties in high enough concentrations, able to prevent or delay autoxidation.

It was found that the same oxidation products were formed in air-exposed lavender oil as in the air-exposed pure samples, with the hydroperoxides **1,2,5** and **6** as the main oxidation products (Figure 5.2). This indicates that the autoxidation follows the same mechanism in the essential oil as for the pure terpenes. The amounts of hydroperoxides **1,2,5** and **6** were determined and they accumulated to the same extent as in air-exposed linalool (Figure 5.4), indicating that the stability of the hydroperoxides was not affected by the presence of antioxidant or radical scavenging compounds in the oil. Oxidation products formed in small amounts on air exposure of pure
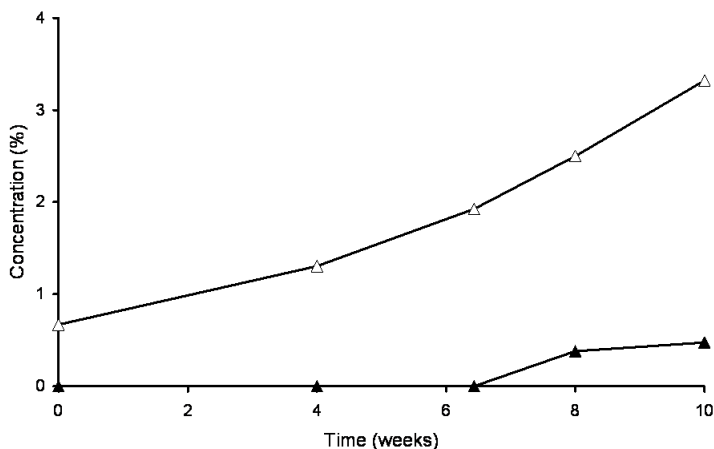
Figure 5.4: Quantification of linalool hydroperoxides **1** and **2** (▲), and linalyl acetate hydroperoxides **5** and **6** (△) in air-exposed lavender oil.

terpenes, such as the linalool pyranoxide (Figure 1.8) could not be detected. These were probably formed in concentrations too low to be detected by the methods used.

The sensitizing capacity of lavender oil as obtained, not air exposed by us, and lavender oil air exposed using the air exposure procedure was determined in the LLNA (Table 5.1). Lavender oil tested as obtained was shown to be a weak sensitizer. Its weak sensitizing capacity can be explained by the high content of linalyl acetate and also to some extent by the fact that it contained the linalyl acetate hydroperoxides **5** and **6** at the start of autoxidation (Figure 5.4). Air exposure increased the sensitizing capacity of lavender oil. After 10 weeks, the air-exposed sample showed a moderate to weak sensitizing capacity. This is in accordance with the results from the previous studies of linalool [59] and linalyl acetate (Paper IV). The autoxidation of β-caryophyllene is not considered to affect the sensitizing capacity of air-exposed lavender oil to a large extent as β-caryophyllene is present in low concentrations in the oil and caryophyllene oxide, the main oxidation product of β-caryophyllene detected, is a moderate sensitizer [84].

The synthetic mix, air exposed for 10 weeks, was also found to be a moderate to weak sensitizer with a sensitizing capacity similar to that of the 10 weeks air-exposed lavender oil. Thus, the oxidation products of the three

Table 5.2: Patch test results in four patients tested with oxidized lavender oil, linalool and linalyl acetate.

Test material	Pat. 1	Pat. 2	Pat. 3	Pat. 4
Ox. lavender oil 45 w, 4%	+	++	+++	+
Ox. linalool 45 w, 2%	+	++	+++	+
Ox. linalyl acetate 45 w, 4%	+	−	?	+
Hydroperoxides **1**,**2**, 1%	NT	−	++	++

+++, very strong positive reaction; ++, strong positive reaction; +, weak positive reaction; ?, doubtful reaction; −, negative reaction; NT, not tested. The results from testing with ox. linalool 4%, 1%, 0.5% and linalool hydroperoxides 0.75%, 0.5%, 0.25%, 0.12%, 0.06% are omitted for clarity. For the complete table, see Paper V.

compounds studied, are responsible for the sensitizing capacity of lavender oil, with those of linalyl acetate and linalool as the most important ones. The sensitizing capacity of the air-exposed lavender oil is not as high as that of the oxidation mixtures of linalool and linalyl acetate, which is explained by the lower concentration of each sensitizer in the air-exposed lavender oil compared to the oxidation mixtures of the pure samples.

A number of other compounds was identified in lavender oil, some of which probably are susceptible to autoxidation (Paper V). The results from the LLNA suggest that they are present in concentrations too low to affect the sensitizing potency of the lavender oil investigated, as that these other constituents did not make a difference in the sensitizing capacity of air-exposed lavender oil compared to the that of air-exposed synthetic mix.

A small patch test study was performed in patients with contact allergy to air exposed linalool. Four patients were tested with air-exposed linalool, air-exposed linalyl acetate and air-exposed lavender oil. Also, the linalool hydroperoxides **1** and **2** were tested (Table 5.2). All patients reacted to air-exposed lavender oil. This shows that sensitizing oxidation products formed in air-exposed lavender oil can provoke an elicitation reaction of ACD (Figure 5.2). Two patients reacted also to air-exposed linalyl acetate, indicating that linalyl acetate hydroperoxides **5** and **6** could be important sensitizers. Only two of the patients reacted to hydroperoxides **1** and **2**, which is in accordance with a previous clinical study, showing that patients reacting to oxidized linalool not always reacted to hydroperoxides **1** and **2** [41]. This indicates that other sensitizers than the hydroperoxides also

might be of importance.

Although linalyl acetate is the main constituent of lavender oil, and hydroperoxides **5** and **6** are the main primary oxidation products formed (Figure 5.4), it is not evident that hydroperoxides **5** and **6** are the most important sensitizers in air-exposed lavender oil. If their sensitizing capacity is weaker than that of **1** and **2**, as the results from paper IV indicate, **1** and **2** could be equally or more important sensitizers in lavender oil, even though they are formed in smaller amounts.

Chapter 6

General discussion

The work presented in this thesis emphasizes the importance of considering oxidative activation in the toxicity assessment of fragrance chemicals. It also adds important information on routes of autoxidation as well as the relationship between metabolic and air induced activation of non- or weakly sensitizing compounds to sensitizers.

Autoxidation is a phenomenon that has been known for over 50 years. The importance of autoxidation in the formation of sensitizing oxidation products in fragrance chemicals has generally been neglected until about 20 years ago. The skin was previously considered to be an inert barrier, protecting the body from the environment, but it is now known that human skin is able to metabolize various compounds through both phase I and phase II reactions. Today, the work of toxicity assessment of chemicals with regard to contact allergy is focused on the development of *in vitro* methods for studies of allergenic activity. In these methods, neither pre- or prohaptens are detected. There is an ambition to develop *in vitro* methods for the detection of prohaptens, but prehaptens are not considered to be as important, as autoxidation is thought to be controlled using antioxidants. This is however not the case presently, as several air exposed fragrance compounds have been shown to be important sensitizers in dermatitis patients [41, 55–57]. The autoxidation is considered as a separate pathway of activation, forming different sensitizers than those formed in the metabolic activation. The concepts prehapten and prohapten were introduced in the scientific discussion regarding compounds that need activation to become sensitizers in order to separate compounds activated by different pathways and to facilitate the development of alternative methods for the identifica-

tion of sensitizers [14]. Prohaptens are non-reactive sensitizing compounds which are activated by a specific enzymatic system, whereas prehaptens are non-reactive sensitizing compounds which are activated by simple chemical transformations. In many cases, these are helpful concepts, as the most commonly known pro- or prehaptens only fall into one of the two categories. In previous studies of pro- and prehaptens, generally only one pathway of activation was studied. Studies within the group have revealed limonene and linalool to be prehaptens, forming sensitizing hydroperoxides on air exposure [52, 53, 58, 59]. In these investigations, metabolic activation was not considered to contribute to the sensitizing capacities of these compounds, since linalool and R-limonene are non-sensitizers. Studies of the metabolism of these compounds confirmed this, since only non-sensitizing metabolites were identified [68, 101].

6.1   Contact allergy to geraniol

In this thesis, the compound geraniol was found to be activated via both autoxidation and metabolism. The studies of geraniol show that the same sensitizers were formed via both pathways. This gives a link between the two routes of activation, which has not been observed before. When comparing the two activation pathways of geraniol (Figure 6.1), the patterns of products formed in the two cases are very similar. Geranial is the main product, but also neral is formed in both cases. The main difference with regard to contact allergy is the formation of hydroperoxide **3** by autoxidation and the formation of epoxides **10** and **11** by metabolism. Thus, both pathways generate strong sensitizers in low concentrations as well as moderate sensitizers in higher concentrations.

When examining the curve of pure geraniol from the LLNA experiment, it can be seen that at high concentrations, the slope of the curve is increased (Figure 6.2). This indicates that at higher concentrations of geraniol, the concentration of sensitizing metabolites formed is high enough to give an SI greater than 3 in the mice. A question not raised in Paper III is the importance of induction of the previously identified cutaneous CYP isoforms in the development of contact allergy to geraniol. The expression of most CYP isoforms can be induced by a number of different substances, both substrates and other compounds [102, 103, and references therein]. It is not known if geraniol or other terpenes can act as inducers of cutaneous CYP isoforms, but it has recently been shown that an oxime can induce its own bioactivation pathway by inducing the expression of the CYP isoform
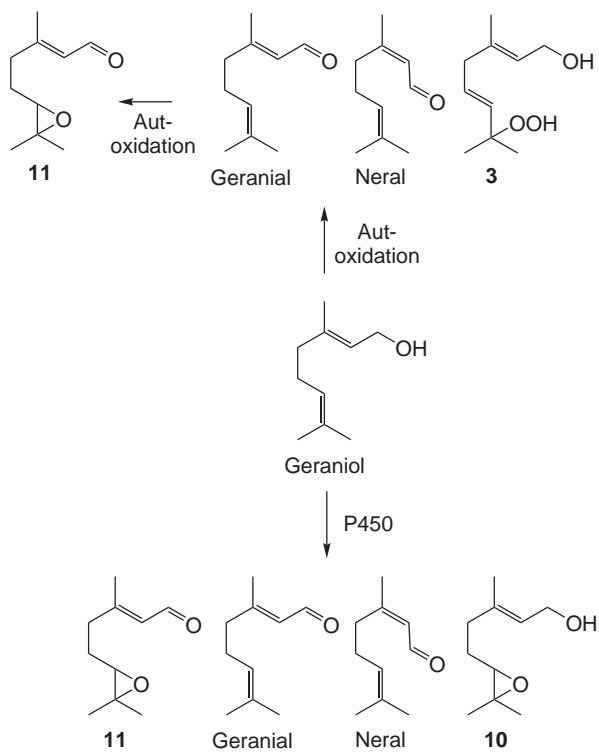
Figure 6.1: Sensitizers formed in the activation of geraniol through autoxidation and metabolism. Many of the sensitizers detected are formed in both activation pathways.
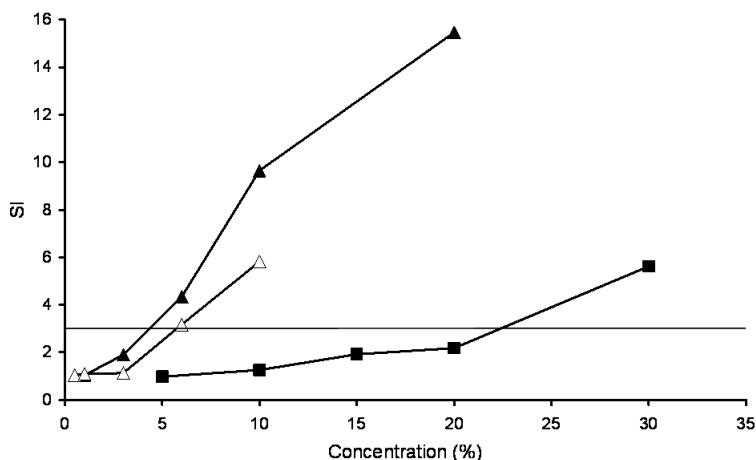
Figure 6.2: Dose response curves of 10 weeks air-exposed geraniol (▲), 45 weeks air-exposed geraniol (△) and pure geraniol (■).

mainly responsible for its metabolism [104]. The metabolic activation could increase in importance compared to the autoxidation if simultaneous exposure to geraniol and inducers of the CYPs would occur over an extended period of time, thereby increasing the amount of CYPs in the skin to a level where sensitizing metabolites are produced at a concentration sufficient for sensitization.

It is likely that the autoxidation pathway is the most important in the activation of geraniol, as the concentrations of haptens formed are higher. The higher dose of haptens in air-exposed geraniol could constitute a higher risk of sensitization to one or more of the oxidation products than the risk of being sensitized through metabolism in the skin. As the oxidation products are already present, the dose of sensitizers recieved at exposure could be higher than the concentrations built up in the metabolism. In spite of this, the metabolism is not to be overlooked, as it can further increase concentrations of products formed in both processes. Furthermore, the metabolites do not need to penetrate the skin before the reaction with protein, and most likely sensitization can occur at lower concentrations than would have been required if penetration had to be considered. It is probable that an individual sensitized to a product in the air-exposed material, which is formed in both processes, would react to the pure compound, as the amounts of

metabolites formed could be sufficient for an elicitation reaction.

In a recent multicenter study by Schnuch *et al* [67], concomitant reactions to geraniol and citral were observed in patients with contact allergy to fragrances. It is not possible to determine if the concomittant reactions observed are due to exposure to air-exposed geraniol, to metabolism of geraniol in the skin or to exposure to citral. The positive patch test reactions to geraniol can be reactions to oxidized patch test material, already containing geranial and neral, or to geranial and neral, formed metabolically in the skin. The results of the multicentre study show that the oxidative activation of geraniol via either pathway is of great importance in contact allergy to geraniol.

Geranial is one of the compounds formed in autoxidation and metabolism of geraniol and many of the results observed for the activation of geraniol apply also to the activation of geranial. The autoxidation of geranial yields the epoxyaldehyde **11**, a strong sensitizer, and the results from paper III indicate that **11** also can be formed in the cutaneous metabolism of geranial. Similarly to the activation of geraniol, the same sensitizer is formed in both activation pathways. This shows that geranial itself is a hapten as well as a pro- and a prehapten. The main difference from the previous results from the studies of limonene and linalool is that the major secondary oxidation product, the epoxyaldehyde **11**, is the most important sensitizer. In the studies of limonene and linalool, the primary oxidation products, i.e. the hydroperoxides were the dominant sensitizers in air exposed samples. To our knowledge, not many pre- or prohaptens have been studied with regard to activation via both autoxidation and metabolism. It is possible that other compounds identified as either pre- or prohaptens fall into both categories as well, forming also the same sensitizers via both pathways. Thus, in studies of activation of fragrance compounds into sensitizers, both autoxidation and metabolism should be considered.

The results presented here suggest a change in the strategy for prevention of contact allergy to geraniol and geranial, as patients sensitized to air-exposed material could develop ACD to metabolites after contact with the pure compounds through metabolic activation. An important conclusion that can be drawn from these data is that it is likely that geranial is the major hapten behind contact allergy to geraniol. What is the importance of hydroperoxide **3** and epoxide **11** relative to the aldehydes geranial and neral in contact allergy to geraniol? Only clinical studies can determine which sensitizers are the real culprits in contact allergy to geraniol.

6.2   Contact allergy to essential oils

It has been known for many decades that distillates of natural products such
as essential oils are susceptible to autoxidation, although this was not imme-
diately connected to the observation of contact dermatitis associated with
these materials [105, 106]. It was later found that sensitizing hydroperox-
ides were formed on air exposure, and that these were responsible for many
skin reactions to essential oils. There is still a belief that products origi-
nating from natural sources are protected against this type of deterioration.
Thus, an autoxidation study of one of the most commonly used essential oils,
lavender oil, was performed. It was shown that the studied constituents in
the oil autoxidized independently of each other, following the same rate of
autoxidation as in the studies of the pure compounds. Studies of the sensi-
tizing capacity of lavender oil as obtained and air-exposed lavender oil was
also performed, showing a sensitizing capacity comparable to that of au-
toxidized linalyl acetate and autoxidized linalool, the main constituents of
lavender oil. This study shows that the sensitizing capacities of essential
oils containing large amounts of linalool or linalyl acetate can be due to the
formation of sensitizing hydroperoxides during storage and handling. It is
reasonable to assume that autoxidation and formation of sensitizers could
apply to other essential oils as well, as the production of different essential
oils is performed using the same process and they generally are composed
mainly of different terpenoids which share many chemical properties such
as the susceptibility to autoxidation.

Patch testing with essential oils is reported in the literature, and many
reactions are detected, mainly to ylang ylang and lemongrass [40]. The main
constituents of lemongrass oil are the known sensitizers geranial and neral,
which occur frequently also in other essential oils. Ylang ylang oil does
not contain high concentrations of aldehydes. Instead, the main constituent
of ylang ylang is linalool [107]. It is possible that linalool hydroperoxides
formed on air exposure of the ylang ylang samples are responsible for the
positive reactions observed. The importance of oxidation products as sen-
sitizers in essential oils compared to the original components has not been
studied apart from the small clinical study presented in this thesis. No
chemical analysis of the compositions were performed in the clinical stud-
ies [40]. Thus, it is not possible to draw any conclusions from these studies
whether oxidation products have caused sensitization or not. It is impor-
tant to determine which compounds are responsible for patch test reactions
to essential oils. Therefore, further studies of essential oils should focus
on identification of potential prehaptens among the main constituents and

autoxidation studies of these compounds.

The use of synthetic fragrances is preferable, when we try to prevent ACD, as the fragrance content can be fully monitored. Essential oils of the same plant can also differ in composition depending on geographical conditions and the extraction process [108, 109]. On the other hand is it often claimed that the popular smells of essential oils can not be copied by the mixing of synthetic terpenes, even though a lot of work has been done to determine the contents of essential oils used in perfumery. Today, the composition of the most commonly used essential oils has been thoroughly investigated. Also, the production of essential oils is a handicraft and a cultural heritage, dating back to ancient times. Many essential oils are derived from plants growing in tropical regions, where the essential oils can constitute an important source of income as an export. In the light of this, how can one decide what to use? If the risk of skin sensitization is the same when using synthetic fragrances as when using essential oils, the choice is no longer based on risk assessments of skin sensitization but on commercial considerations. To be on the safe side, one can always choose the fragrance-free alternative.

6.3   Fragrances in consumer products

As the prevalence of contact allergy to fragrances increased within EU, new legislation was passed. The directive of cosmetics lists 24 fragrance compounds that must be declared on the label of the product, when used in cosmetics and hygiene products [110]. The list includes the compounds of FM, compounds for which the use has been restricted by the industry, and compounds known to give reactions in dermatology clinics. Among the compounds listed are limonene, linalool, geraniol and citral which now all have been proven to autoxidize to form sensitizing oxidation products. A GC-MS method for the simultaneous detection of the 24 fragrance compounds has been proposed [111]. This method does not include any oxidation products, such as the sensitizing hydroperoxides of limonene, although these have earned limonene the R43 label, that is, may cause skin sensitization, in the labelling of chemicals within the EU. Hydroperoxides can in general not be detected using GC, as they are thermolabile. Although it can be assumed that the hydroperoxides are formed from the parent compounds in cosmetic products, this has never been proven directly. Research in this area should focus on the identification of the actual haptens, that is, mainly the hydroperoxides in cosmetic products. This would confirm the results obtained

in the clinical studies of contact allergy to air-exposed fragrance materials.

6.4   Conclusions

It has previously been shown that autoxidation of fragrance terpenes forms hydroperoxides, which were identified as the most important sensitizers in air-exposed materials. The present work indicates that also other oxidation products, such as aldehydes and epoxides are major sensitizers formed in autoxidation. These results widen the view on activation through autoxidation. Sensitizing oxidation products identified in this work was shown to be as strong sensitizers as the hydroperoxides, which indicates that they also can be important sensitizers in the population. It is likely that also other fragrance compounds are susceptible to activation via autoxidation and metabolism, forming strong sensitizers via both activation routes. It is important to continue the investigations of activation of fragrance compounds to increase the knowledge of pro- and prehaptens in fragrances.

An important conclusion that can be drawn from the work presented here is that it is difficult to determine which test materials are relevant to use in the diagnosis of contact allergy to fragrances. The choice of test materials can lead to false negative diagnosis if the relevant hapten is not included. Contact allergy to air-exposed linalool and limonene is common in dermatitis patients [41, 55, 56], whereas reactions to the pure materials are very rare [112, 113]. Although it would be more relevant to test dermatitis patients with the air-exposed materials, this presents a problem since there is no method developed for commercial production of these test materials. It is probable that many cases of contact allergy to oxidation products of fragrance materials are not diagnosed as the relevant haptens are not available for testing.

The results presented in this thesis could be used to reduce the risk of contact sensitization to fragrances and could contribute to a scientific basis for political decisions regarding the regulation of the use of fragrances in consumer products.

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