GÖTEBORG UNIVERSITY

Contact Allergy to Autoxidized Fragrance Terpenes

Chemical Characterization, Analysis, and Studies of Contact Allergenic Activity

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Department of Chemistry
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Dermatochemistry and Skin Allergy
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2005

AKADEMISK AVHANDLING

för avläggande av filosofie doktorsexamen i kemi som enligt beslut av ordföranden för naturvetenskapliga fakultetens lärarförslagsnämnd kommer att försvaras offentligt fredagen den 29 april 2005 kl 13.00 i sal KB, Kemigården 4, Göteborgs universitet och Chalmers tekniska högskola. Avhandlingen försvaras på svenska.

Fakultetsopponent är Professor Christer Hansson, Avdelningen för dermatologi och venereologi, Lunds universitet, Sverige.

ABSTRACT

Fragrances are ubiquitous in our environment. Not only cosmetics and toiletries contain fragrance materials but most household and occupational products are scented. Because of their widespread use fragrances are next to nickel the most common cause of contact allergy in the population and among eczema patients and thus constitute a significant clinical problem. Terpenes are because of their odorous properties frequently used in fragrances. Due to air oxidation (autoxidation) terpenes may form oxidation products with allergenic properties on air exposure.

In this thesis, the autoxidation at room temperature of the commonly used fragrance terpenes linalool, β-caryophyllene, β-myrcene, and linalyl acetate was investigated. The main focus was on the formation of hydroperoxides since they are known to be strong contact allergens. The effect of autoxidation on the contact allergenic activity of the compounds was investigated by testing the sensitizing capacity before and after air exposure. The oxidized terpenes were also used for screening in consecutive dermatitis patients.

All terpenes studied autoxidized on air exposure. The autoxidation of linalool and β-caryophyllene was carefully examined. Many oxidation products were identified in oxidized linalool, including two hydroperoxides, which were shown to be strong contact allergens. In oxidized β-caryophyllene no hydroperoxides could be detected. The only oxidation product identified was the moderately sensitizing caryophyllene oxide. Accordingly, oxidized linalool showed a relatively strong allergenic activity, while oxidized β-caryophyllene was only weakly sensitizing. Autoxidation of linalyl acetate gave a similar pattern of oxidation products as linalool and the allergenic activity was affected to the same extent. β-Myrcene polymerized rapidly when air exposed and no oxidation products were identified but an increased sensitizing capacity was observed after air exposure compared to the pure compound.

The importance of formation of stable hydroperoxides was also evident in the patch test study on consecutive dermatitis patients. A high frequency of positive reactions (1.7%) was seen to oxidized linalool and/or the hydroperoxide fraction of linalool while fewer reactions were observed to oxidized β-caryophyllene and β-myrcene. However, it is important to consider also the more frequent exposure to linalool in the population.

Essential oils are often claimed to be protected from autoxidation by occurrence of natural antioxidants. Lavender oil, an essential oil containing linalool, linalyl acetate, and β-caryophyllene, was included in these studies. The compounds were found to oxidize also in the oil, and the same oxidation products could be identified as in the oxidation mixtures of the pure compounds, including the hydroperoxides of linalool and linalyl acetate.

The results show the importance of investigating the effect of autoxidation on the allergenic activity for each compound of interest. The formation of stable hydroperoxides seems to be essential for a significant increase in sensitizing capacity which means that not only the degradation of a compound needs to be investigated but also the composition of the obtained oxidation mixture. In order to develop effective preventive strategies it is necessary to know the true allergens with which people come in contact. To be able to reduce the frequency of contact allergy to fragrances, compounds with low allergenic potential and low content of oxidation/degradation products should preferentially be used.

Keywords: autoxidation, contact allergy, essential oil, FCAT, fragrance, hydroperoxide, LLNA, patch testing, radical reaction, sensitization, structural elucidation, terpene
ISBN: 91-628-6446-7

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DOCTORAL THESIS

Submitted for partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry

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Printed by Intellecta DocuSys AB
Göteborg 2005
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LIST OF PUBLICATIONS

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

I **Studies on the autoxidation and sensitizing capacity of the fragrance chemical linalool, identifying a linalool hydroperoxide**
Sköld, M., Börje, A., Matura, M., and Karlberg, A.-T.
Contact Dermatitis **46**, 267-272 (2002).

II **Contact allergens formed on air exposure of linalool. Identification and quantification of primary and secondary oxidation products and the effect on skin sensitization**
Sköld, M., Börje, A., Harambasic, E., and Karlberg, A.-T.
Chemical Research in Toxicology **17**, 1697-1705 (2004).

III **The fragrance chemical β-caryophyllene – air oxidation and skin sensitization**
Sköld, M., Karlberg A.-T., Matura, M., and Börje, A.
Submitted.

IV **Selected oxidized fragrance terpenes are common contact allergens**
Matura, M., Sköld, M., Börje, A., Andersen, K. E., Bruze, M., Frosch, P., Goossens, A., Johansen, J. D., Svedman, C. White, I. R., and Karlberg, A.-T.
Contact Dermatitis In press.

V **Autoxidation of linalool, linalyl acetate, and β-caryophyllene in air-exposed lavender oil. Identification of oxidation products and effect on skin sensitization**
Sköld, M., Hagvall, L., Börje, A., and Karlberg, A.-T.
Manuscript

CONTENTS

ABBREVIATIONS

ACD	Allergic contact dermatitis
AOO	Acetone:olive oil 4:1
APCI	Atmospheric pressure chemical ionization
DMAPP	Dimethylallyl diphosphate
dpm	Disintegrations per minute
EC3	Estimated concentration to induce a stimulation index of 3
ESI	Electrospray ionization
FCA	Freund's complete adjuvant
FCAT	Freund's complete adjuvant test
FIA	Freund's incomplete adjuvant
FID	Flame ionization detector
FM	Fragrance mix
FPP	Farnesyl diphosphate
GC	Gas chromatography
GC/MS	Gas chromatography – mass spectrometry
GPP	Geranyl diphosphate
HPLC	High performance liquid chromatography
ICDRG	The international contact dermatitis research group
IPP	Isopentenyl diphosphate
LC	Langerhans cells
LC/MS	Liquid chromatography – mass spectrometry
LLNA	Local lymph node assay
MHC	Major histocompatibilty complex
NMR	Nuclear magnetic resonance
OECD	Organization for economic co-operation and development
PBS	Phosphate buffered saline
SI	Stimulation index
TCA	Trichloroacetic acid

INTRODUCTION

Fragrances are ubiquitous in our environment and their usage is not limited to products used primarily for their scent like perfumes, eaux de cologne and deodorants, but they are also frequently present in household and occupational products. Because of their widespread use, fragrances are next to nickel the most common cause of contact allergy and constitute a significant clinical problem. Terpenes are compounds of natural origin many of which are commonly used in fragrances. The majority of the terpenes are unsaturated hydrocarbons and therefore prone to oxidation when air-exposed. It was shown in previous studies that the fragrance terpene limonene, readily autoxidizes and forms contact allergenic oxidation products.[1] Autoxidation of fragrance terpenes can contribute to contact allergy to fragrances. In the present study, some frequently used fragrance terpenes and their ability to autoxidize and form allergenic oxidation products have been investigated.

Terpenes

Biosynthesis of terpenes

Terpenes form a large and structurally diverse family of natural products that originate from the head-to-tail condensation of a variable number of isoprene units (C_5).[2] The terpenes are divided into monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), and tetraterpenes (C_{40}). The isoprene units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) can be derived by two biochemical pathways: the mevalonate pathway and the deoxyxyluose phosphate pathway.[3] The condensation of DMAPP and IPP mediated by the enzyme prenyl transferase yields geranyl diphosphate (GPP) from which the monoterpenes are formed (Figure 1). Condensation of GPP with another molecule of IPP, leads to farnesyl diphosphate (FPP), the precursor of sesquiterpenes. Further condensations with isoprene units will give the larger terpenes. Depending on the enzyme systems present in a particular organism, different terpenes will be formed from the respective precursors. The enzymes will also control the stereochemistry of the final product.

Biological activity

Mono- and sesquiterpenes are common constituents of essential oils. Essential oils are volatile oils of rather complex compositions naturally occurring in plants. The

terpenes of essential oils play a role in plant-animal interactions as protection against insects, bacteria, and fungi and also for attraction of pollinating species.[4] We can benefit from the biological activities of terpenes and use them for their pharmacological properties e. g. antiseptic activity. More important is the usage in fragrances based on the pleasant smell from many of the terpenes, especially the volatile monoterpenes. However, being biologically active compounds, terpenes might also cause adverse effects e. g. skin sensitization and phototoxic reactions.

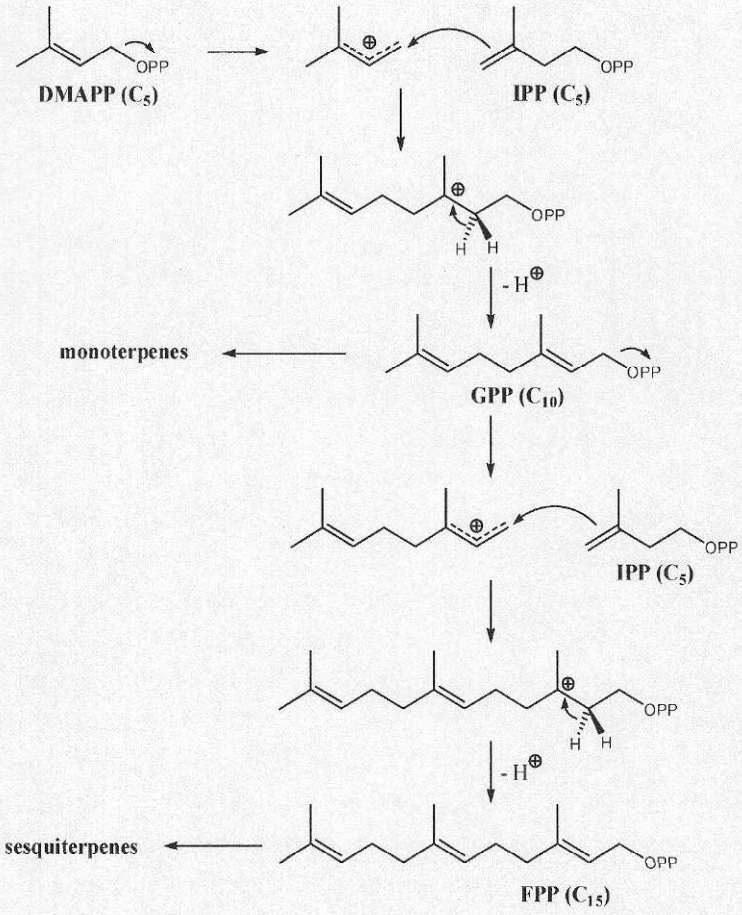


Figure 1. The biosynthetic pathway of mono- and sesquiterpenes. Enzymatic condensation of DMAPP and IPP yields GPP, the precursor of monoterpenes. Condensation with an additional IPP (C_5) unit leads to the sesquiterpene precursor, FPP.

Allergic contact dermatitis

Mechanism of allergic contact dermatitis

Allergic contact dermatitis (ACD) is the clinical manifestation of contact allergy, a delayed-type hypersensitivity (type IV immunological reaction).[5] One has to distinguish between a sensitization phase and an elicitation phase of contact allergy. In the sensitization phase an immunological memory is created which makes a person contact allergic to a specific compound (hapten). This process requires several days to weeks. In the elicitation phase, the already sensitized individual is re-exposed to the hapten, which might result in ACD within 1-2 days. Once an individual has become sensitized, the contact allergy will persist throughout life.

To cause sensitization, a hapten needs to penetrate the skin and react with a macromolecule in the skin to form an antigen. The antigen is taken up by Langerhans cells (LC) in the epidermis. The processed antigen is presented on the surface of the LC together with an MHC (major histocompatibility complex) class II molecule. The antigen-carrying LC become activated and travel via the afferent lymphatics to the local lymph nodes. In the lymph nodes, the LC present the antigen to naïve T-cells. A T-cell that recognizes the specific combination of the antigen and the MHC class II molecule will be activated and proliferate. Effector T-cells and memory T-cells with the right specificity are formed and the individual is sensitized to the hapten (Figure 2).[5]

At subsequent contacts with the hapten, the elicitation phase is initiated. The first events of this process are the same as in the sensitization phase; the hapten penetrates the skin, reacts with macromolecules, and is taken up, processed and presented by LC. In the elicitation phase the antigen can be presented also by other cells in the epidermis, e. g. keratinocytes. As the individual is sensitized to the hapten, the antigen-presenting cells can interact with the existing specific T-cells already in the skin. This results in a local cytokine and chemokine release, which in turn leads to the arrival of more T-cells and non-specific cells like macrophages and leucocytes, and an eczematous inflammatory reaction might develop in the skin (Figure 2).[5]

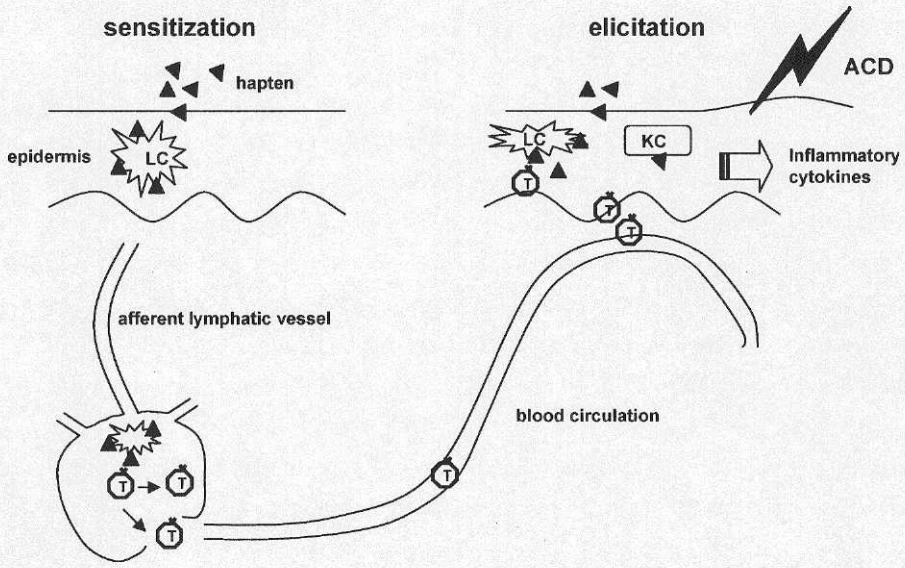


Figure 2. Schematic presentation of sensitization and elicitation in allergic contact dermatitis.[5] In the sensitization phase, the antigen (hapten-protein complex) is carried by LC to the draining lymph node where the antigen is presented to T-cells. If sensitization occurs, there will be specific memory T-cells circulating in the body. At subsequent contacts with the hapten (elicitation phase), the specific T-cells recognize the antigen on the LC and keratinocytes (KC) and become activated to release inflammatory cytokines. Leucocytes and macrophages are recruited to the site, and an inflammation is seen in the skin (ACD) 24-48 h after contact with the hapten.

Chemical properties of haptens and antigen formation

Haptens are compounds of natural or synthetic origin, present in our environment, that can cause skin sensitization. The first requirement for a compound to act as a hapten is the ability to penetrate the skin barrier. Haptens are therefore relatively lipophilic compounds (log P ~ 2)[6] of low molecular weight (<1000).[7] These molecules are too small to be immunogenic in themselves and haptens must therefore also be able to react with macromolecules (proteins) in the skin to form antigens. More than 3500 substances are known to cause contact allergy.[8]

As mentioned, a compound must have the ability to bind to a protein or other macromolecules and form a non-self antigen, to be a sensitizer. Normally this binding occurs by the formation of a covalent bond. Most haptens are electrophiles that can react with nucleophilic groups in the side chains of certain amino acids in proteins, e. g. lysine, cysteine, methionine, histidine, tryptophane and tyrosine.[9] The

nucleophilic reactions involved in skin sensitization can be divided into three groups: nucleophilic substitutions, Michael additions, and nucleophilic additions.[9]

Hydroperoxides are haptens that are believed to form antigens according a radical mechanism. Radicals can react with the aromatic side chains of the amino acids tyrosine, phenylalanine, tryptophan, and histidine.[10] The antigen formation of hydroperoxides has been studied in chemical trapping experiments, and the results support a radical mechanism.[11-14] Furthermore, organic hydroperoxides are known to be tumour promoters and it is believed that radicals are responsible for the promoting activity. The production of radicals from hydroperoxides has therefore been studied also in this field. Hydroperoxides were added to cultures of keratinocytes and resulted in the formation of radicals.[15-17]

Some compounds have a chemical structure without any electrophilic or radical reactive sites, but they still possess allergenic activities. They are so called prohaptens that need to be activated to become sensitizers. The skin is a metabolising organ and many compounds are metabolically transformed when penetrating through the skin. This can result in activation or detoxification of the original molecules.[18] Compounds may also be converted to reactive molecules before skin absorption, in contact with air, through a process called autoxidation (see below).

Animal predictive test methods for skin sensitization

There are several predictive tests for determining the allergenic activity of chemicals, involving both experimental animals and humans. The guinea pig is considered to be a suitable experimental animal in predictive testing for contact allergy and has been used for decades. Freund's complete adjuvant test (FCAT) is one of many protocols involving guinea pigs and it was developed specifically for investigating the allergenic activity of fragrance chemicals. We have used FCAT in its modified version, with closed challenge testing.[19,20] The induction procedure includes three intradermal injections of the test compound, in a Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA)/water emulsion (Figure 3). In guinea pig studies, the whole process from induction (sensitization) to elicitation is involved and the allergenic activity is measured/investigated in the elicitation phase. The number of animals with a positive reaction in the exposed group is compared with the number of animals reacting in the control group.

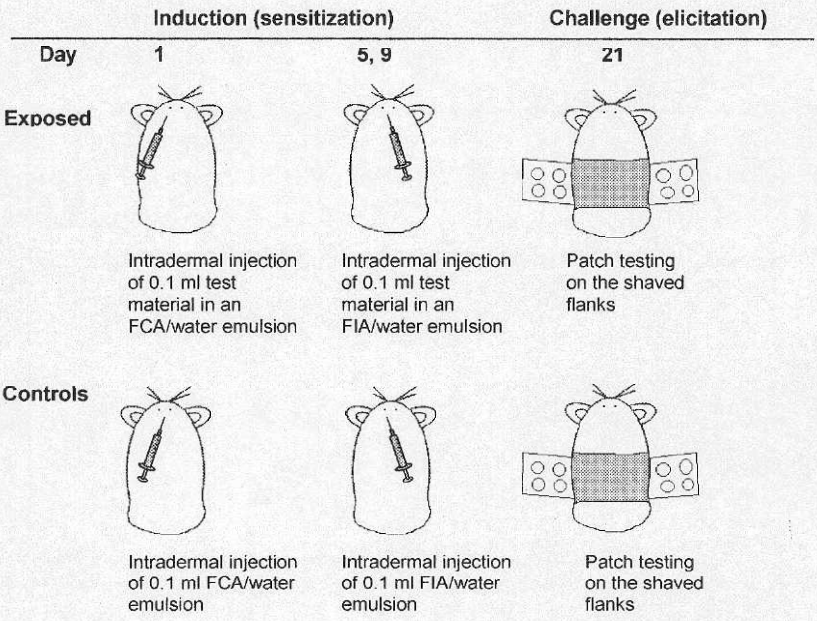


Figure 3. The Freund's complete adjuvant test (FCAT) in its modified version, with closed challenge testing.[19,20] FIA was used instead of FCA at the second and third inductions.

A predictive test method in mice, the local lymph node assay (LLNA) has recently been adopted by the OECD as an alternative method to the guinea pig tests, for identifying skin sensitizing compounds.[21] In this method the sensitization phase of contact allergy is studied and the skin sensitizing potential is measured by the increased proliferation in the draining local lymph nodes caused by the tested compound (Figure 4). The method generates quantitative dose-response data, and the skin sensitizing potency of different chemicals can be determined and compared using a calculated EC3 value. The EC3 value is the estimated concentration of a compound to induce a 3-fold increase of proliferation in the lymph nodes, compared to vehicle treated control animals.[22]

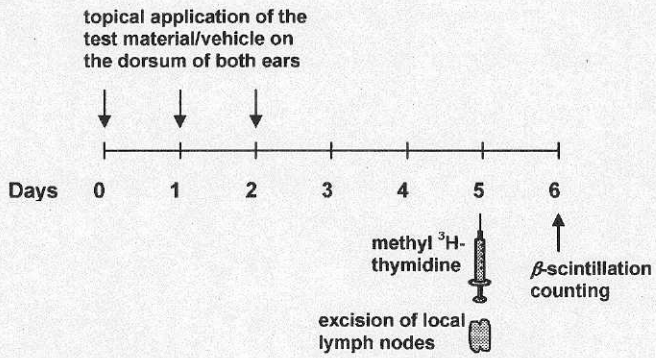


Figure 4. The protocol of the local lymph node assay (LLNA).

Patch testing

Patch testing is a well-established method of diagnosing contact allergy. It aims to reproduce an eczematous reaction, by applying allergens under occlusion on intact skin on patients suspected to have a contact allergy.

The allergen to be tested is diluted in a vehicle, most often white petrolatum, and applied on the skin in a test chamber for 48 h. The preferred site is the upper back. The general principle is to use the highest concentration of a test material that does not provoke any irritation, to avoid false-positive and false-negative reactions.[23] Patients with a suspected contact allergy are tested with a standard series of common allergens, sometimes together with additional allergens specific for each individual case. Patch test reading is carried out twice, in most dermatology clinics on day 2-3 and again on day 4-7. The reactions are scored according to their morphological characteristics as – (negative), ?+ (doubtful reaction), + (weak positive reaction), ++ (strong positive reaction), +++ (extreme positive reaction), or IR (irritant reaction), as recommended by the ICDRG.[24]

Contact allergy to fragrances

Use and properties of fragrance chemicals

There are approximately 3500 fragrance materials available from which the perfumers can create fragrances. The materials are traditionally divided into three groups: natural essential oils, synthetic chemicals, and semi-synthetic chemicals.[25] To have an odor, a compound must have a relatively low molecular weight and an appreciable vapour pressure at room temperature. Since a perfume may contain everything from a few to over 300 fragrance compounds and more than 100

fragrance compounds have been reported to be contact allergens, the risk for a perfume to contain one or several allergens is high.[26]

Fragrances are ubiquitous in our environment and not only cosmetics and toiletries contain fragrance materials but almost all household and occupational products are also scented.[27] Even unscented products are not necessarily fragrance-free, because unscented products may contain a "masking fragrance".[28] In the old days, a certain smell was accepted for chemicals used in industry and different occupations. This is no longer the case and large quantities of fragrances are used to mask the odours from products in occupational use. Thus, it can be stated that virtually everyone is in daily contact with fragrance materials.

Diagnostic markers of contact allergy to fragrances

The main tool for diagnosing contact allergy to fragrances, included in European standard patch test series, is the fragrance mix (FM). The ingredients in the FM are seven fragrance chemicals and one natural extract, oak moss absolute. The chemicals include: three cinnamic derivatives (α-amylcinnamaldehyde, cinnamaldehyde, cinnamic alcohol), two eugenol derivatives (eugenol, isoeugenol), and two linear monoterpenes (geraniol, hydroxycitronellal). Some of the constituents can be regarded as haptens, while some are prohaptens, and need to be metabolically activated.[29] Oak moss absolute is an extract of the lichen *Evernia prunastri* and is a mixture of many ingredients of which chlorotranol and atranol have been identified as the main contributors to the allergenic activity.[30] The FM is estimated to detect approximately 50-80% of fragrance allergies[31] and is regarded as a good diagnostic marker of contact allergy to fragrances. It is obvious that a mixture of only a few compounds can not detect contact allergy to all sensitizing fragrance chemicals and new markers for contact allergy to fragrances are searched for.

The natural product Myroxylon pereirae (balsam of Peru) is also used as a diagnostic marker for fragrance allergy included in the European standard series. It originates from a tree, *Myroxylon pereirae*, which grows in Central America and it has been used as a fragrance ingredient due to its odorous properties. Colophonium is another natural product obtained from pine trees that often co-reacts with fragrance allergens. Positive patch test reactions to both materials are highly associated with contact allergy to fragrances.[32]

Frequency of contact allergy to fragrances

In a Danish study, the frequency of contact allergy to FM in an unselected population was found to be 1.1% in 1991.[33] In 1998, the frequency had increased to 2.3% in a corresponding general population.[34] The frequency of contact allergy to fragrances in dermatitis patients, measured as the frequency of positive reactions to the FM, has been investigated in several studies. FM usually ranks as second after nickel among the most common contact allergens.[35] In a European multicenter study, FM caused allergic reactions at a frequency of 8.3%.[36] In Denmark, the changes of prevalence of contact allergy to common allergens among consecutively tested patients were investigated over a 12-year period. The FM was the only patch test material to which sensitivity increased significantly, from 4.1% (1985-86) to 9.9% (1997-98).[37] The rate of positive reactions to the FM in a large UK patch test population was found to be relatively constant from 1980 to 1996. The mean annual frequency of positive reactions was 8.5% in females and 6.7% in males.[38] In a German study, the frequency of contact allergy to fragrances in dermatitis patients from 1996 to 2002 was investigated. The highest frequency of positive reactions to FM was obtained in 1998 (13.1%), whereafter the proportion decreased to the lowest frequency in 2002 (7.8%).[39] This may be the result of preventive efforts i. e. a reduced exposure to the FM constituents.

Autoxidation

Autoxidation is the air-induced oxidation of organic molecules. It is a free radical reaction that results primarily in the formation of hydroperoxides. Research on autoxidation has in many cases focused on the oxidative deterioration of unsaturated fats, which has for many years been a concern of chemists working with lipids and food.[40] However, terpenes are often unsaturated molecules and are therefore also susceptible to oxidation in the presence of air.

Mechanism of autoxidation

In the initiation step of autoxidation a radical (R·) is generated (Scheme 1) which may be caused by exposure to light, heat, or catalytical levels of redox-active transition metals.[40] The reaction of R· with oxygen, in the propagation step, is fast and leads to the formation of a peroxyl radical. The peroxyl radical can abstract a hydrogen atom and form a hydroperoxide, and on the same time propagate the oxidation chain reaction. The hydrogen atom abstraction reaction is slow and selective for the most weakly bound hydrogens.[40]

Initiation: $RH \rightarrow R\cdot$

Propagation: $R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow ROOH + R\cdot$

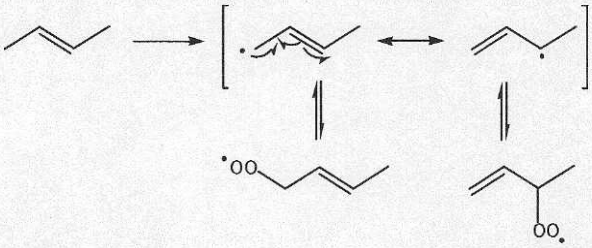
Termination: $2\ R\cdot \rightarrow$

$R\cdot + ROO\cdot \rightarrow$ } non-radical products

$2\ ROO\cdot \rightarrow$

Scheme 1. Basic autoxidation mechanism.

In unsaturated molecules, radicals are generated preferentially adjacent to a double bond, by abstraction of an allylic hydrogen atom. The resulting radical is stabilized by resonance and addition of oxygen can therefore take place in different positions (Scheme 2). What site that predominantly reacts with oxygen is determined by the relative stabilities of the formed peroxyl radicals. This, in turn, is determined by the substituents on the peroxyl bearing carbons.[41]



Scheme 2. Schematic presentation of autoxidation of an unsaturated molecule.

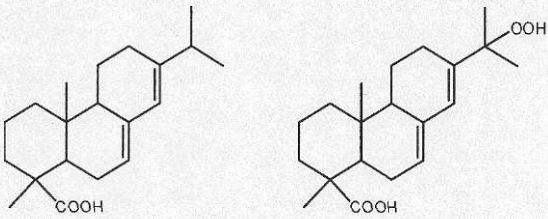
Effect of autoxidation on allergenic activity

Many terpenes are simple hydrocarbons and consequently they are not protein reactive nor allergenic. However, via autoxidation these molecules can incorporate oxygen in their structures and form primary oxidation products (hydroperoxides), and secondary oxidation products (epoxides, aldehydes, ketones, alcohols). Hydroperoxides are, as previously mentioned, haptens that react with proteins according to a radical mechanism. Epoxides, aldehydes, and ketones are electrophiles and might also be haptens, depending on the structure of the molecule.

Oil of turpentine is a volatile, monoterpene-rich oil obtained by distillation of turpentine oleoresin, and has been widely used as a solvent in e. g. paints. Since its use resulted in many cases of ACD, investigations were conducted to try to find the

cause of skin sensitization. Already in the 1950's studies on the influence of autoxidation on its allergenic activity were conducted.[42-48] The investigations showed that the allergenic activity of oil of turpentine could be related to the degree of oxidation, and that pure unoxidized oil caused no reactions in sensitized patients. It was found that hydroperoxides of one of the constituents, Δ^3-carene, were the major sensitizers in the oil. Replacement of Δ^3-carene-rich turpentine oils with those with low or negligible concentrations, in combination with the withdrawal of oil of turpentine from general use, resulted in a decline in the number of cases of contact allergy.[49]

Colophonium is a common cause of contact allergy.[50] It is a complex mixture of mainly diterpene resin acids obtained as the distillation residue of oleoresin mainly from trees of the *Pinus* species [51] Abietic acid, a main constituent of colophonium, is easily autoxidized when air exposed, forming allergenic oxidation products. A hydroperoxide, 15-hydroperoxyabietic acid (Figure 5), has been shown to be the major contact allergen in colophonium.[52]



abietic acid **15-hydroperoxyabietic acid**

Figure 5. Structures of abietic acid, one of the major resin acids in colophonium, and 15-hydroperoxyabietic acid, the main allergen in colophonium, formed by autoxidation of abietic acid.

Also the autoxidation of *R*-limonene, and the effect on its allergenic activity has been studied. *R*-Limonene is a monoterpene and the main constituent of volatile oils from several citrus fruits. It is produced mainly from citrus peel by pressing and distillation.[53] It is frequently used as a fragrance chemical, but it is also used as an alternative solvent to e. g. chlorinated hydrocarbons.[1] *R*-Limonene itself was shown to not be a skin sensitizer but air-exposed *R*-limonene was found to be allergenic. Several different oxidation products were identified (Figure 6), of which some were found to be sensitizers.[1,54] Also in this case, hydroperoxides were identified as the main allergens.[53] It has also been shown, in several patch test studies, that contact

allergy to oxidized *R*-limonene is common among dermatitis patients with suspected allergic contact dermatitis.[55-57]

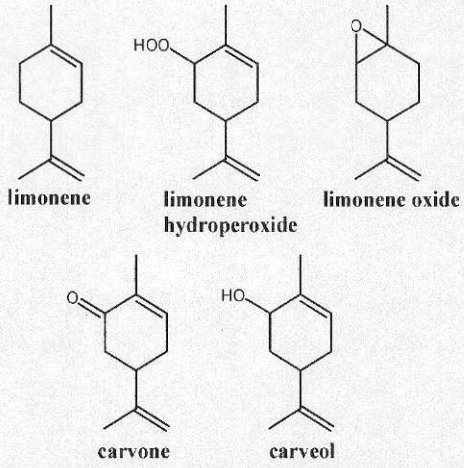


Figure 6. Structures of limonene and oxidation products identified in autoxidized limonene.

Properties and usage of the fragrance materials investigated

The fragrance terpenes investigated in this work are frequently used fragrance chemicals. They were chosen on basis of their structures (Figure 7), which lack functional groups that would make them protein reactive and thus allergenic. However, they are unsaturated compounds which in accordance with previously studied terpenes, could make them susceptible to oxidation in the presence of air and prone to create oxidation products with potentially contact allergenic properties.

Linalool

Linalool is a naturally occurring monoterpene alcohol, present in large amounts in various plants, e. g. in lavender.[58] It is frequently used as a fragrance chemical due to its fresh, flowery odor. Linalool has been in public use since before the 1900's[59] and is according to several studies still one of the most frequently used fragrance chemicals in scented products.[60-63] Despite its great usage, reports on contact allergy to linalool are rare.[64-66] Autoxidation of linalool has been described, but not investigated in detail.[67-71]

β-Caryophyllene

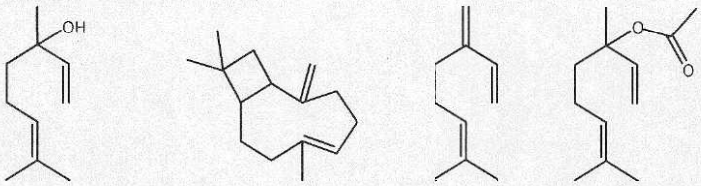
β-Caryophyllene is a sesquiterpene present in natural products such as the oil of cloves, cinnamon leaves, and copaiba balsam. The odor of β-caryophyllene is described as woody and spicy,[72] and it has been commonly used as a fragrance chemical since the 1930's.[73] Caryophyllene has been detected in a relatively large number of scented products,[63,74] but there are no reports of contact allergy to caryophyllene. One study describes autoxidation of caryophyllene performed under accelerated conditions which resulted in rapid decomposition of caryophyllene and the formation of caryophyllene oxide.[75]

β-Myrcene

β-Myrcene is a monoterpene found in e. g. bay oil, oil of hops, and verbena oil, with a spicy, balsamic scent,[76] that has been in public use since the 1950's. It is used in e. g. deodorants,[74] but is otherwise less frequently used as a fragrance chemical than linalool and caryophyllene. In an earlier study myrcene was autoxidized at elevated temperatures and some secondary oxidation products were identified.[77] Regarding the allergenic activity of myrcene, positive reactions to myrcene were found in two patients who also reacted to oxidized tea tree oil.[78]

Linalyl acetate

Linalyl acetate is a naturally occurring monoterpene, present in e. g. lavender.[58] It is structurally very similar to linalool, differing only by the acetylated hydroxyl group. Linalyl acetate is also a frequently used fragrance chemical.[61-63] There are no studies on autoxidation of linalyl acetate in the literature to the best of my knowledge. Studies of the skin sensitizing properties of linalyl acetate have been reviewed.[79] Linalyl acetate caused sensitization in a few subjects in human maximization tests and in guinea pig experiments linalyl acetate caused some reactions when tested at 20% and 10% concentrations.[79] Frosch et al.[36] reported no reactions to linalyl acetate when patch testing 100 consecutive dermatitis patients.



linalool **β-caryophyllene** **β-myrcene** **linalyl acetate**

Figure 7. Structures of the fragrance terpenes investigated in this thesis.

Lavender oil

Lavender oil is the essential oil obtained from steam distillation of the freshly cut flowering tops of lavender.[80] The oil has a sweet, floral, herbaceous odor. It has been used for centuries in various products, both as fragrance and for medicinal purposes,[81] since it is traditionally believed to have antibacterial, antifungal, smooth muscle relaxing, sedative, and antidepressive properties. Today the oil is used in aroma therapy or incorporated into cosmetics, perfumes, soaps etc. as a pleasant fragrance but also as an antimicrobial agent.[82] The main constituents of lavender oil are linalool and linalyl acetate, together with minor amounts of β-caryophyllene. There are several reports in the literature on contact allergy to lavender oil.[65,83-87] However, the true allergens in lavender oil are unknown. Already in 1954 autoxidation of lavender oil was reported.[88] The oxidation was measured as the peroxide content of the oil, but no oxidation products were identified.

AIMS OF THE STUDY

The present investigations are part of a project with the overall goal of providing knowledge concerning the autoxidation of fragrance terpenes and how this affects their allergenic activity. The knowledge obtained can be used to reduce the risk of sensitization to these materials.

The specific aims of the present thesis were:

1. to investigate the autoxidation of linalool, β-caryophyllene, β-myrcene, and linalyl acetate at room temperature, with emphasis on the formation of primary oxidation products,
2. to investigate the effect of autoxidation on the contact allergenic activity of linalool, β-caryophyllene, β-myrcene, and linalyl acetate,
3. to investigate the prevalence of contact allergy to oxidized linalool, β-caryophyllene, and β-myrcene in consecutive dermatitis patients,
4. to investigate the autoxidation of linalool, β-caryophyllene, and linalyl acetate in lavender oil, and the effect of autoxidation on the sensitizing capacity of lavender oil.

EXPERIMENTAL PROCEDURES

Air exposure procedure

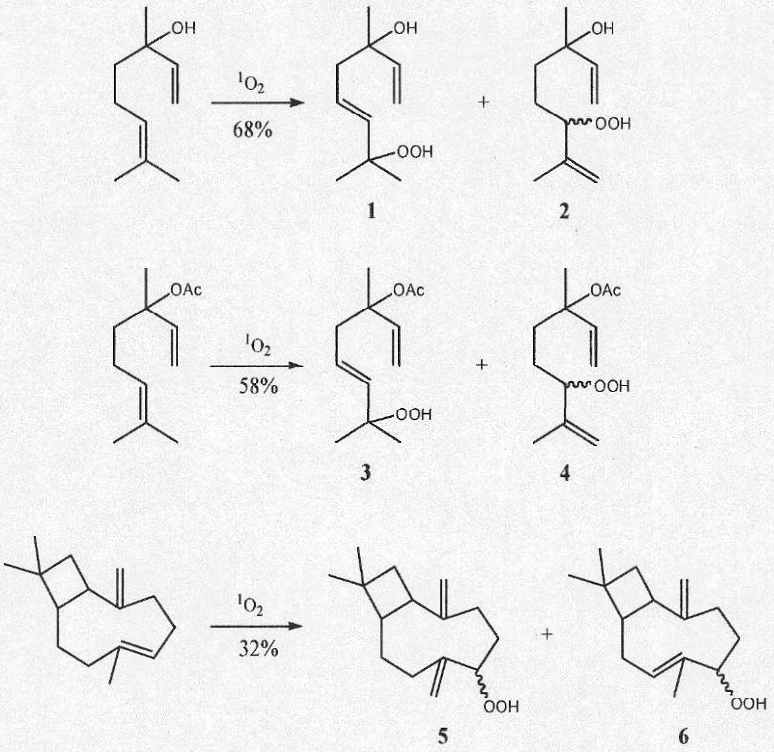
Linalool, β-caryophyllene, β-myrcene, linalyl acetate, and lavender oil were air exposed in Erlenmeyer flasks, in daylight at room temperature. The flasks were covered with aluminum foil in order to prevent contamination. The terpene oils were stirred for 1h, 4 times a day, according to previous studies.[1] The oxidation/degradation of linalool, β-caryophyllene, and β-myrcene was followed on a continuous basis, and samples were taken about every second week to be analyzed with GC, to determine the concentrations of the remaining terpenes over time. Linalyl acetate and lavender oil were air exposed mainly in order to get oxidized materials for isolation and identification of the oxidation products formed. The samples taken from the air-exposed terpenes were stored at –20 °C under an atmosphere of nitrogen.

Synthesis of potential oxidation products from linalool, β-caryophyllene, and linalyl acetate

Autoxidized terpenes contain complex mixtures of oxidation products. To facilitate the identification of these oxidation products, potential oxidation products were synthesized, and used as reference compounds. Some of the synthesized compounds were also used in the sensitization experiments.

Synthesis of primary oxidation products from linalool, β-caryophyllene, and linalyl acetate

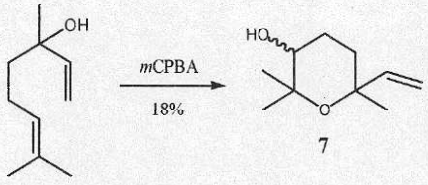
Linalool, β-caryophyllene, and linalyl acetate were photooxidized according to a procedure described by Bäckström et al.[89] (Scheme 3). The terpenes, in a concentration of approximately 0.1 M, were added to a chloroform solution of tetrabutylammonium salt of Bengal rose. The solutions were irradiated for 5-6 h using a Rayonet reactor and a constant flow of oxygen.



Scheme 3. Synthesis of hydroperoxides of linalool, linalyl acetate, and *β*-caryophyllene by photooxidation.

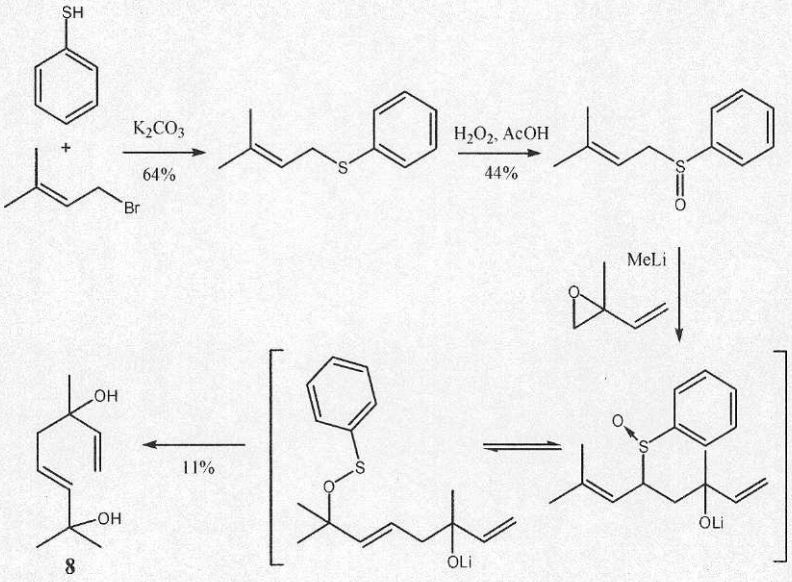
Synthesis of secondary oxidation products of linalool

2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol **7**. 2,2,6-Trimethyl-6-vinyl tetrahydro-*2H*-pyran-3-ol was synthesized by adding *m*CPBA to a CH_2Cl_2 solution of linalool according to a procedure described in the literature (Scheme 4).[13]



Scheme 4. Synthesis of 2,2,6-trimethyl-6-vinyltetrahydro-*2H*-pyran-3-ol

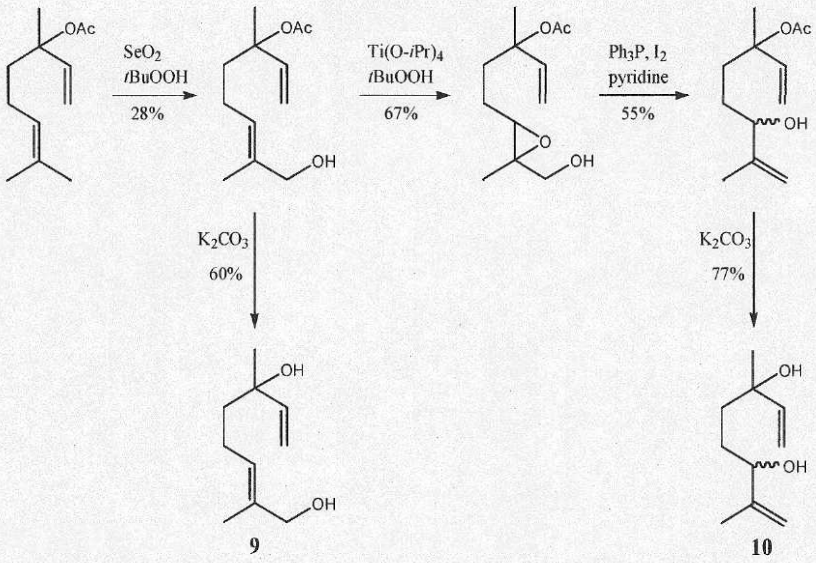
2,6-Dimethylocta-3,7-diene-2,6-diol **8**. (3-Methyl-but-2-enyl)-phenyl sulfide was synthesized from thiophenol and 1-bromo-3-methyl-2-butene, using K_2CO_3 as base, in DMF. The obtained sulfide was oxidized with AcOH and H_2O_2, as described in the literature.[90] The sulfoxide was treated with methyl lithium to get the lithio anion that subsequently was allowed to react with 2-methyl-2-vinyloxirane (Scheme 5).[91]



Scheme 5. Synthesis of 2,6-dimethylocta-3,7-diene-2,6-diol

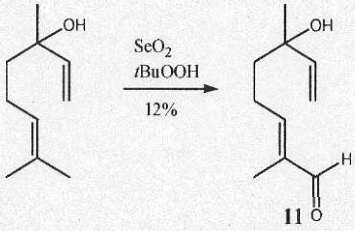
2,6-Dimethylocta-2,7-diene-1,6-diol **9**. Linalyl acetate was oxidized to the allylic alcohol using SeO_2 and *t*BuOOH.[92] The acetate group was subsequently removed with anhydrous K_2CO_3 (Scheme 6).

2,6-Dimethylocta-1,7-diene-3,6-diol **10**. The synthesis was performed using the procedure described by Liu et al.[92] Linalyl acetate was oxidized using SeO_2 and *t*BuOOH to the allylic alcohol that was epoxidized using $Ti(O\text{-}iPr)_4$ and *t*BuOOH. The epoxide was treated with Ph_3P, I_2, and pyridine to get the rearranged alcohol. The acetate group was finally removed with anhydrous K_2CO_3 (Scheme 6).



Scheme 6. Synthesis of 2,6-dimethylocta-2,7-diene-1,6-diol (**9**) and 2,6-dimethylocta-1,7-diene-3,6-diol (**10**)

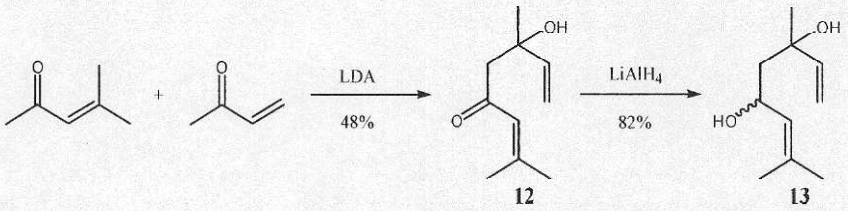
6-Hydroxy-2,6-dimethylocta-2,7-dienal **11**. Linalool was oxidized to the allylic aldehyde using SeO_2 and *t*BuOOH (Scheme 7).



Scheme 7. Synthesis of 6-hydroxy-2,6-dimethylocta-2,7-dienal

6-Hydroxy-2,6-dimethylocta-2,7-diene-4-one **12**. The synthesis was performed as described in the literature.[93] Mesityl oxide was added to a THF solution of LDA to yield the enolate. Subsequent addition of methyl vinyl ketone resulted in 6-hydroxy-2,6-dimethylocta-2,7-diene-4-one (Scheme 8).

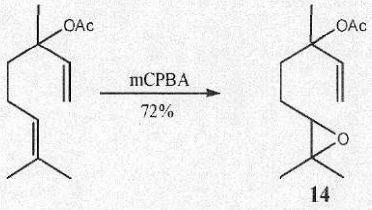
3,7-Dimethylocta-1,6-diene-3,5-diol **13**. 6-Hydroxy-2,6-dimethylocta-2,7-diene-4-one was reduced with $LiAlH_4$ in diethyl ether to get the corresponding alcohol (Scheme 8).[93]



Scheme 8. Synthesis of 6-hydroxy-2,6-dimethylocta-2,7-diene-4-one (**12**) and 3,7-dimethylocta-1,6-diene-3,5-diol (**13**)

Synthesis of a secondary oxidation product of linalyl acetate

6,7-Epoxy-3,7-dimethyl-1-octene-3-yl acetate **14**. The synthesis was performed by adding *m*CPBA to a CH_2Cl_2 solution of linalyl acetate (Scheme 9).



Scheme 9. Synthesis of 6,7-epoxy-3,7-dimethyl-1-octene-3-yl acetate

Isolation and identification of oxidation products

The oxidized materials obtained from air exposure were subjected to flash chromatography on silica gel columns, eluting with mixtures of hexane and ethyl acetate, in varying proportions. The oxidation mixtures were fractionated and further purifications were made using flash chromatography, and in some cases, preparative HPLC. A Nucleosil preparative column was used with an eluent consisting of 5% 2-propanol, 35% *tert*-butyl methyl ether and 60% *n*-hexane. Isolated compounds were characterized with ^1H and ^{13}C-NMR spectroscopy, and GC/MS. The chromatographic and spectral properties of the isolated compounds were compared with those of the synthesized or the commercially available reference compounds.

Analysis and quantification of oxidation products

The oxidation mixtures were analyzed to determine the concentrations of the remaining terpenes and to quantify some of the oxidation products found. The original terpenes as well as the secondary oxidation products linalool oxide and caryophyllene oxide, were quantified over time using an on-column GC-method with 1,2,3,5-tetramethylbenzene as internal standard and a flame ionization detector (FID).[53]

HPLC-methods suitable for the analysis of thermolabile hydroperoxides were developed using an analytical silica column and a diode array detector. The mobile phase consisted of *tert*-butyl methyl ether and *n*-hexane in different proportions depending on the oxidation mixture to be analyzed. Gradient elution was performed in the cases of oxidized linalool and oxidized lavender oil, and blank subtractions of the gradient were performed. Isocratic elution was performed in the analysis of oxidized β-caryophyllene. The major linalool hydroperoxide was quantified using an external calibration curve.

Sensitization studies

Freund's complete adjuvant test (FCAT)

FCAT was used in the sensitization studies of linalool (paper **I**) and β-caryophyllene (paper **III**). The method was performed in its modified version with closed challenge testing.[19,20] The experimental groups consisted of 14-16 animals and the control groups consisted of 14-15 animals. The experiments were performed in female Dunkin-Hartley albino guinea pigs housed in Macrolon cages, kept on a guinea pig standard diet and water *ad libitum* using the following protocol:

Induction: On days 0, 5(6), and 9(10), the animals received intradermal injections (0.1 ml) on the upper back. The test substances were dissolved in an FCA/water emulsion for the first injection and in an FIA/water emulsion for the second and third injections. The sham treated control animals were given the FCA/water emulsion and the FIA/water emulsions only.

Challenge: Closed challenge testing was performed three weeks after the first injection, using Finn chambers®. The test material in non-stabilized petrolatum was applied on the shaved flanks for 24 h and reactions were assessed at 48 and 72 h

after start of exposure. The minimum criterion for a positive reaction was a confluent erythema. The test concentrations were shown in pre-tests on FCA-treated animals to be non-irritating. The results from the FCAT experiments were statistically evaluated using the Fisher exact test. A p-value of <0.05 was considered statistically significant.

The studies were approved by the local ethics committee.

Local lymph node assay (LLNA)

LLNA[94] was used in the sensitization studies of linalool (paper **II**), β-caryophyllene (paper **III**), linalyl acetate (paper **V**), and lavender oil (paper **V**).

The experiments were carried out using female CBA/Ca mice housed in cages with hepa-filtered airflow, under conventional conditions in light-, humidity- and temperature controlled rooms. The mice, in groups of four, received 25 μl of the test material dissolved in acetone:olive oil (AOO) at one of three different concentrations, on the dorsum of both ears for three consecutive days. The control groups were treated with equal volumes of AOO alone. Five days after the initial treatment, all 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 group. Single-cell suspensions of lymph-node cells were prepared and the thymidine incorporation was measured by β-scintillation counting. The increase in thymidine incorporation relative to vehicle-treated controls was derived for each experimental group and recorded as stimulation index (SI). Test materials that at one or more concentrations caused an SI greater than 3 were considered to be positive in the LLNA. The EC3 value (the estimated concentration required to induce an SI of 3) was calculated by linear interpolation.[95]

The studies were approved by the local ethics committee.

Patch test study

Oxidized linalool, a hydroperoxide fraction of oxidized linalool, oxidized β-caryophyllene, caryophyllene oxide, and oxidized β-myrcene were used for patch testing in consecutive dermatitis patients, in a multicenter study in six European dermatology clinics (Dortmund, Gentofte, Leuven, London, Malmö, and Odense).

Patch tests were applied in small Finn Chambers® on Scanpor® tape. White, non-stabilized petrolatum was used as vehicle. The test materials were applied on the back of the patients and kept on for 48 h. The reactions were assessed on days 2 and 4(3) in two centers and on days 3 and 6(7) in the others. The reactions were scored according to the ICDRG standard recommendation.[24] The consecutive dermatitis patients were also patch tested with the fragrance mix (FM) and other allergens used as markers for fragrance allergy (e. g. Myroxylon pereirae and colophonium). A questionnaire with clinical data was filled out for every patient before testing. The patients' history of adverse reactions to fragrances was included and categorized according to the following statements: (a) certain: has reacted with an itching dermatitis to at least one fine perfume or after shave and also to other scented products; (b) probable: has reacted to one or more scented products, but no certain product has been identified as the cause of the clinical reaction; (c) questionable: has reacted to various cosmetics with or without fragrances: (d) none: has never reacted to scented materials.

Pretests in dermatitis patients without suspected contact allergy to fragrances, showed the oxidized fragrance terpene materials to be non-irritating in the concentrations used for screening.

The studies were approved by the local ethics committees and all participants were included after informed consent.

RESULTS AND DISCUSSION

Oxidative decomposition of linalool, β-caryophyllene, and β-myrcene on air exposure (Papers II and III)

Linalool, β-caryophyllene, and β-myrcene were air-exposed at room temperature and samples were taken on a regular basis for GC-analyses, to determine the remaining amounts of the original terpenes in the air-exposed samples. The analyses showed that the concentrations of the studied terpenes started to decrease immediately at the start of air exposure (Figure 8). The oxidative decompositions continued until the original compounds were almost totally consumed. The oxidation rates for β-caryophyllene and β-myrcene were high and approximately the same as the oxidation rate for the previously studied *R*-limonene.[96] After 10 weeks 75% of the original β-caryophyllene was consumed and after 48 weeks only 1% remained. Due to the fast polymerization of β-myrcene on air exposure it was not possible to follow the oxidative degradation for more than 16 weeks when approximately 70% of the β-myrcene was degraded. The oxidation of linalool was slower, after 10 weeks 80% linalool remained and after about 80 weeks 4% remained.

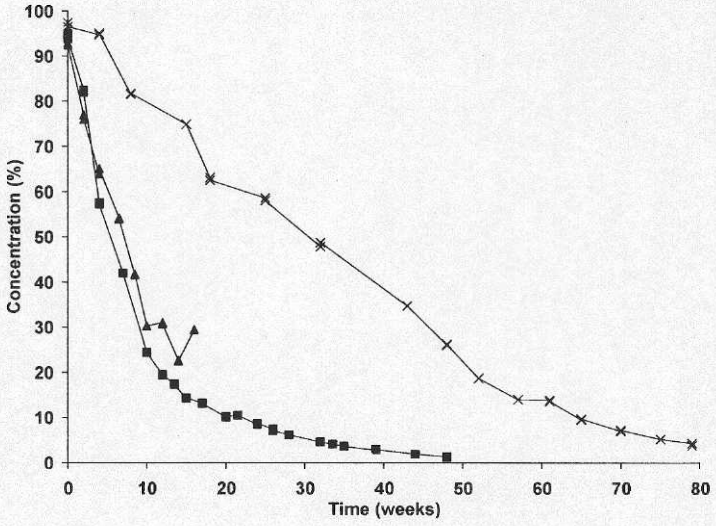


Figure 8. Comparison of oxidation rates; concentrations of linalool (×), β-myrcene (▲), and β-caryophyllene (■), in air-exposed samples, over time.

Identification and quantification of oxidation products in autoxidized linalool (Papers I and II)

Chromatograms of autoxidized linalool showed the presence of a number of different oxidation products. Based on mechanistic considerations regarding autoxidation of linalool reference compounds were synthesized in order to facilitate the identification of the oxidation products in the oxidation mixture.

Allylic hydrogen atoms can be abstracted from three positions in linalool, leading to the formation of allylic radicals **1a** (trans and cis isomers) and **1c**, and their resonance forms **1b** and **1d** (Figure 9). The radicals can react with oxygen and give the peroxyl radicals **2a - 2d**. Subsequent hydrogen atom abstraction will give the hydroperoxides **1**, **2**, **15**, **16**, and **17**. Hydroperoxides formed in the autoxidation process are known to decompose to secondary oxidation products, for example alcohols, aldehydes, ketones, and epoxides.

Hydroperoxides are highly reactive compounds and therefore difficult to synthesize. Photooxidation is one way of making hydroperoxides and in the case of linalool, the hydroperoxides **1** and **2** were synthesized using this method. A number of secondary oxidation products were also synthesized. The compounds in Figure 10 are potential oxidation products of linalool, that were synthesized, or purchased (**18**) and used as reference compounds.

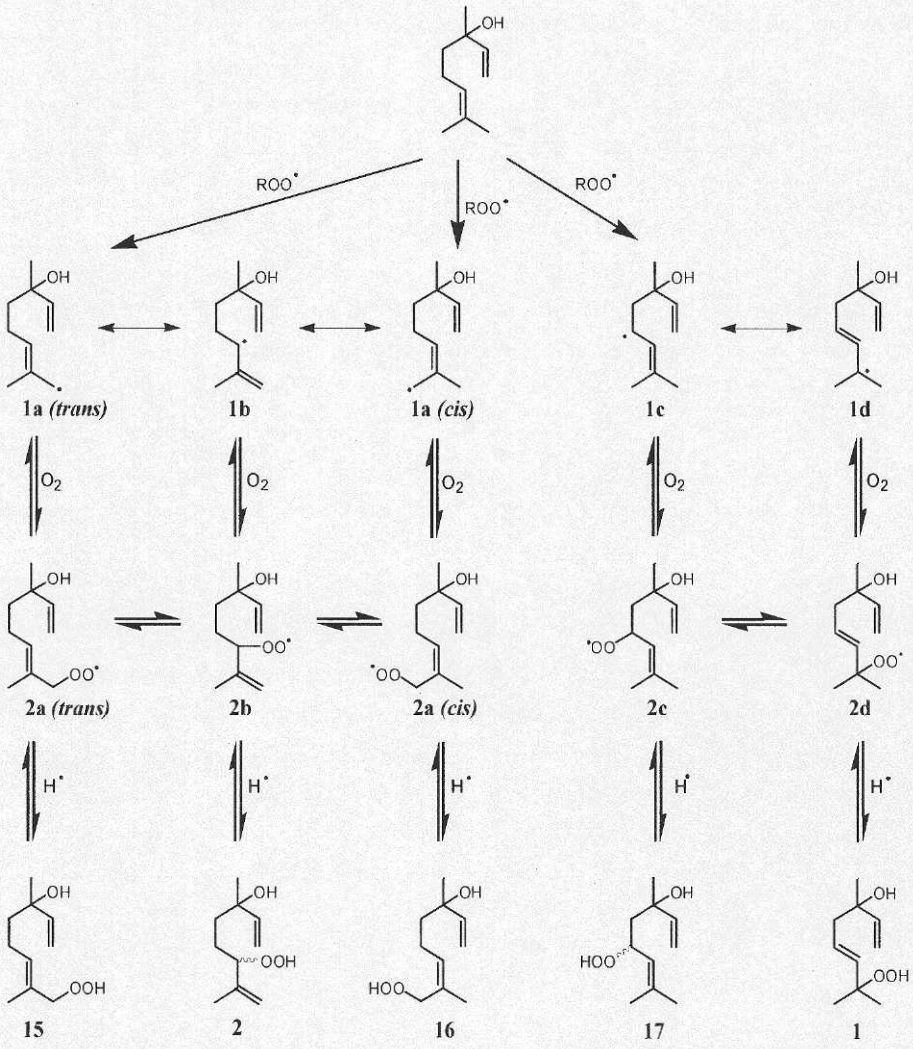


Figure 9. Autoxidation of linalool. Abstraction of allylic hydrogen atoms gives rise to the allylic radicals **1a-1d**. These radicals can react reversibly with oxygen to give the peroxyl radicals **2a-2d**. Subsequent hydrogen atom abstraction will give the hydroperoxides **1**, **2**, **15**, **16**, and **17**. The hydroperoxides will eventually decompose and form corresponding secondary oxidation products.

The chromatographic and spectral properties of the isolated oxidation products were compared to those of the reference compounds. Compounds **1**, **2**, **7-11**, and **18** (Figure 10) were found to be present in autoxidized linalool.

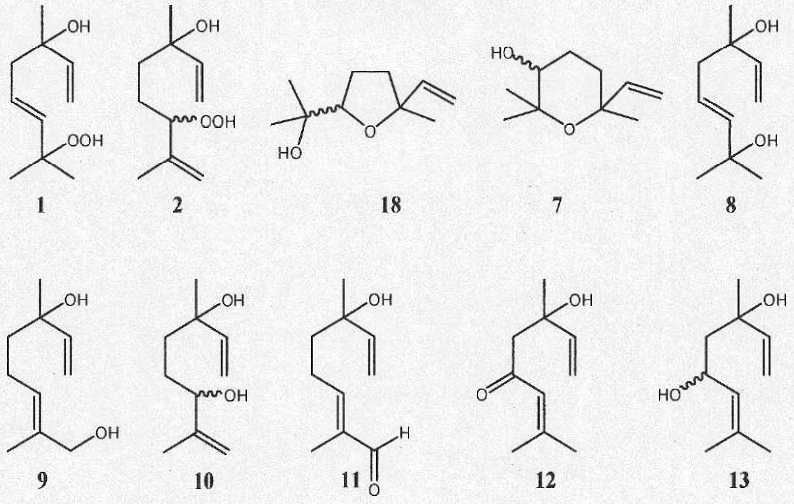


Figure 10. Structures of synthesized reference compounds **1**, **2**, and **7-13**, and oxidation products identified in air-exposed linalool **1**, **2**, **7-11**, and **18**.

The linalool oxides in the oxidation samples were quantified using GC. The furan-derivative **18** was formed in a relatively high amount, reaching a plateau at a concentration of 20% after about 50 weeks. The pyran-derivative **7** was formed to a lesser extent and reached a maximum concentration of 4% after 79 weeks (Figure 11). The hydroperoxide **1** was quantified using HPLC. It was found to be formed in high amounts and reached a maximum concentration of about 15% after 48 weeks (Figure 11). The diagram shows that, after this point, degradation is the dominating process and the concentration of the hydroperoxide declines.

Hydroperoxide **1** was the major hydroperoxide formed and the corresponding alcohol **8** was also detected in oxidized linalool. The other synthesized hydroperoxide **2**, was also found but in lower amounts. The diol **10**, which could be formed as a secondary oxidation product from this hydroperoxide, was also identified. Indirect evidence for the formation of the primary hydroperoxide **15** (Figure 9), was obtained by the detection of diol **9** and aldehyde **11** (Figure 10), which are secondary oxidation products of this hydroperoxide. The ketone **12** and the diol **13** (Figure 10), would be possible degradation products from the secondary hydroperoxide **17** (Figure 9), but neither the hydroperoxide nor the corresponding secondary oxidation products were found in the oxidation mixture.

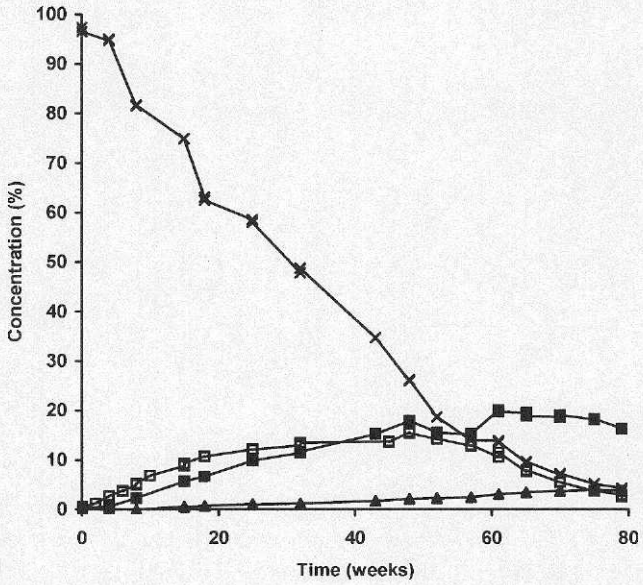


Figure 11. Concentrations of linalool (×), linalool oxide **18** (■), linalool hydroperoxide **1** (□), and linalool oxide **7** (▲), in air-exposed linalool over time.

Electron-donating groups are known to stabilize peroxyl radicals by a hyperconjugative effect.[41] The difference in alkyl substitution on the peroxyl-bearing carbons can therefore influence the equilibrium between the alkyl radicals and their corresponding peroxyl radicals, and consequently affect the proportion of the resulting hydroperoxides and secondary oxidation products in the oxidation mixture. This suggests that peroxyl radical **2b** would be favored over the primary peroxyl radical **2a**, and the tertiary peroxyl radical **2d** over the secondary peroxyl radical **2c** (Figure 9). This is consistent with the fact that the hydroperoxides **1** and **2** were the only ones found in oxidized linalool. The reason for hydroperoxide **1** being formed in higher amounts than hydroperoxide **2** is probably that the secondary hydrogen atom is more easily abstracted (leading to the allylic radical **1c** ↔ **1d**) than the primary hydrogen atom (leading to allylic radical **1a** ↔ **1b**).

A probable explanation for the formation of the furan- and pyran derivatives of linalool, is that an epoxide can be formed as a secondary oxidation product from the linalool hydroperoxide (Figure 12). This epoxide can via an intramolecular attack by the hydroxyl group in linalool, on either of the two epoxide carbons, give the cyclic ethers. Results in the literature[97,98] confirm the difficulty of forming this

epoxide without obtaining the oxides, and state that the ring formation is promoted by acidic conditions. The pH in different states of oxidized linalool was measured and it was found to decrease with longer oxidation times, which will favor the formation of the oxides.

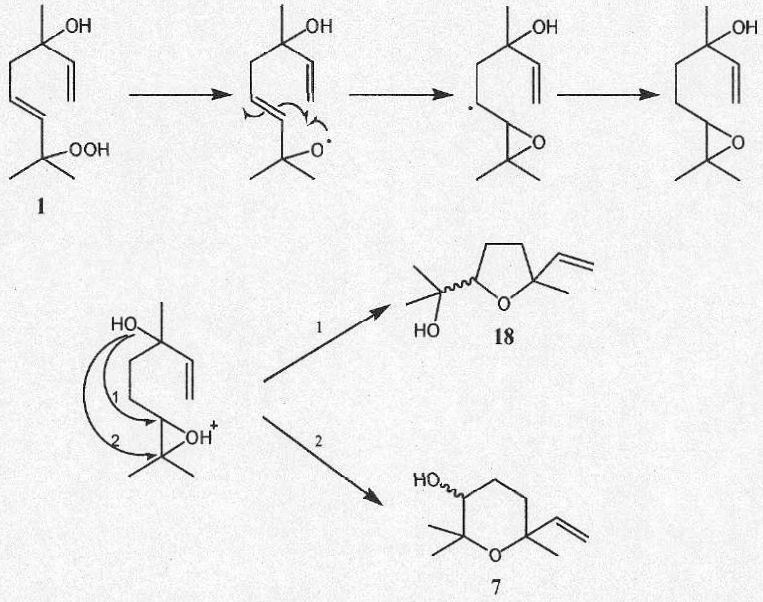


Figure 12. Proposed mechanism for the formation of the furan- and pyranoxides of linalool. A secondary oxidation product that can be formed from the tertiary hydroperoxide, an epoxide, will after attack from the hydroxyl group, give the two oxides. The ring formation is promoted by acidic conditions.

Identification and quantification of oxidation products in autoxidized β-caryophyllene (Paper III)

Autoxidation of β-caryophyllene resulted in a much less complex oxidation mixture, compared to autoxidized linalool. The only oxidation product identified, caryophyllene oxide **19** (Figure 13), was isolated using flash chromatography, with commercial caryophyllene oxide as a reference compound.

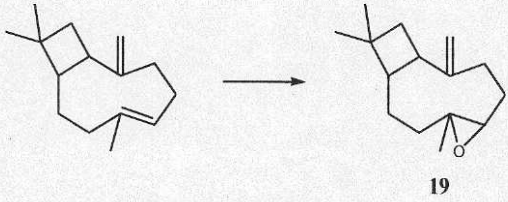


Figure 13. Caryophyllene oxide **19** is the only oxidation product identified in autoxidized β-caryophyllene.

The concentrations of caryophyllene oxide in the oxidized samples of β-caryophyllene were determined using GC, and the results are shown in Figure 14. The concentration of caryophyllene oxide increased rapidly in the beginning of the oxidation process and reached a concentration of about 40% after ca. 12 weeks.

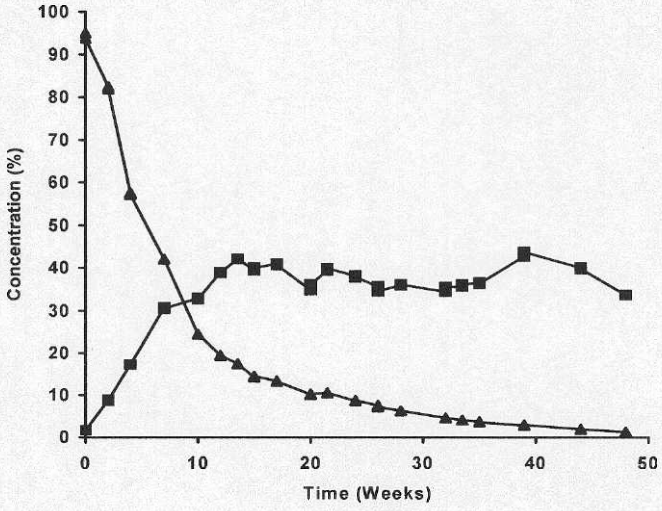
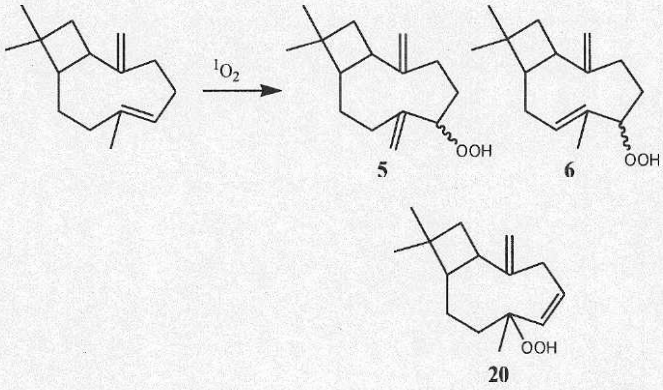


Figure 14. Concentrations of β-caryophyllene (▲) and caryophyllene oxide **19** (■), in air-exposed β-caryophyllene, over time

Hydroperoxides of β-caryophyllene were synthesized by photooxidation and used as reference compounds in the search for primary oxidation products in autoxidized β-caryophyllene. Photooxidation of β-caryophyllene would theoretically give the three hydroperoxides shown in Figure 15. The reactivity of double bonds towards singlet oxygen increases with the number of alkyl substituents,[99] and therefore photooxidation affects the endocyclic double bond of β-caryophyllene. Hydroperoxide **5** was the major hydroperoxide found while hydroperoxide **6** was found in lower amounts. Traces of another hydroperoxide were found but the minute amounts prevented the identification. The product distribution agrees with results in the literature where caryophyllene was photooxidized and the resulting hydroperoxides reduced to alcohols.[100] The alcohol corresponding to **5** was the major alcohol formed. The alcohol corresponding to **6** was also found, but the tertiary alcohol was not detected. In the present study, photooxidation also gave rise to a relatively high proportion of caryophyllene oxide.

The synthesized hydroperoxides were used as reference compounds in the search for primary oxidation products in air-exposed β-caryophyllene. However, no significant amounts of hydroperoxides could be detected at any air exposure time, but the epoxide, caryophyllene oxide **19**, was formed to a large extent. Caryophyllene oxide is a stable, crystalline compound and our results imply that the primary oxidation products are instantly decomposed to this epoxide.



5 **6** **20**

Figure 15. Photooxidation of β-caryophyllene would theoretically give the three hydroperoxides **5**, **6**, and **20**. Hydroperoxides **5** and **6** were identified in photooxidized β-caryophyllene.

The stability of hydroperoxides **5** and **6** from the photooxidation was investigated using HPLC by analyzing a sample of the hydroperoxides kept at room temperature for a week. No degradation of these hydroperoxides was seen, and no caryophyllene oxide could be detected. Therefore, we suggest that the caryophyllene oxide obtained in the photooxidation was formed by decomposition of the hydroperoxide **20**, that could not be found in photooxidized β-caryophyllene. When β-caryophyllene is autoxidized, hydroperoxides are formed according to a different mechanism and the product distribution is not necessarily the same as in the photooxidation.[54] A possible explanation for the absence of hydroperoxides in autoxidized β-caryophyllene is that the formation of hydroperoxide **20** is favoured in the autoxidation process, and due to the instability of this hydroperoxide, only caryophyllene oxide could be detected.

Effect of autoxidation on the allergenic activity of linalool, β-caryophyllene, and β-myrcene

Experimental studies (Papers I-III)

The effect of autoxidation on the allergenic activity of the studied terpenes was investigated using the FCAT method and the LLNA. Pure linalool and 10 weeks oxidized linalool were tested in guinea pigs. Pure linalool did not sensitize the animals, since no reactions to linalool were seen in the exposed animals. The animals induced with oxidized linalool became sensitized and a statistically significant response was found to the test concentrations 10.3, 5.1, and 2.6% (w/w).

Linalool, oxidized linalool, and some oxidation products of linalool were tested in mice according to the LLNA procedure. The results are presented in Figure 16. An EC3 value of 46.2% (3.0 M) was obtained for pure linalool. The response was just above the threshold for being judged as an allergen and no clear dose response was seen. Linalool does not have a structural alert but is known to cause irritation in high concentrations.[101-103] The effect observed for linalool is therefore considered to be due to its irritating effect.

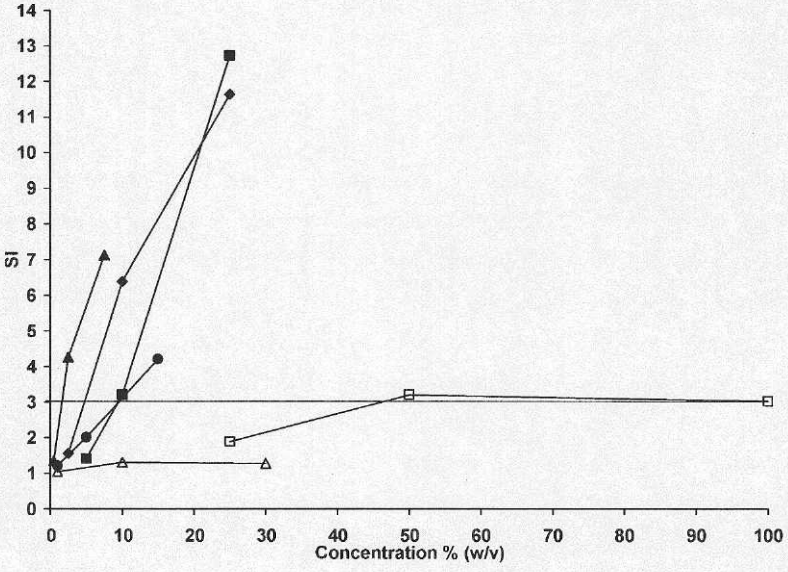


Figure 16. Dose response curves for linalool hydroperoxides **1** and **2** (▲), air-exposed linalool (45 weeks) (♦), air-exposed linalool (10 weeks) (■), linalool aldehyde **11** (●), linalool alcohol **8** (Δ), and pure linalool (□), tested in the LLNA.

The oxidized samples induced a clear proliferation. An EC3 value of 9.4% (0.6 M) was obtained for linalool air-exposed for 10 weeks, while the EC3 value for linalool air-exposed for 45 weeks was 4.8% (0.3 M). The hydroperoxides **1** and **2** were tested as a mixture, and were shown to be strong allergens with an EC3 of 1.6% (86 mM), which is in accordance with other hydroperoxides tested in the LLNA.[11,13] The α,β-unsaturated aldehyde **11** was also expected to be allergenic, and was therefore tested. It was shown to be a weaker allergen than the hydroperoxides with an EC3 value of 9.5% (0.56 M). Alcohols are not considered to be reactive enough for protein binding, unless metabolically activated, and therefore only the most prominent among the identified alcohols, alcohol **8**, was tested. It was shown to be a non-sensitizer, as expected. No sensitization studies were performed on the furan- and pyran oxides since they in a previous LLNA study were shown not to possess any substantial allergenic activity.[13]

The experiments show that the autoxidation greatly influences the sensitizing effect of linalool, and that the sensitizing effect is determined by the air exposure time and thus by the composition of the oxidation mixture. The hydroperoxides were the strongest allergens of the oxidation products tested. Hydroperoxide **1** was present in high concentrations in the oxidized samples of linalool. The concentration of the hydroperoxide was higher (15%) in the 45 weeks oxidized sample, than in the sample air-exposed for 10 weeks (4%), and the former sample possessed a higher sensitizing capacity. Based on this, hydroperoxide **1** is believed to be the major contributor to the increase in the sensitizing effect of the oxidation mixture obtained by autoxidation of linalool.

The allergenic activity of pure β-caryophyllene and its major oxidation product, caryophyllene oxide, was tested in a guinea pig experiment. The results show that β-caryophyllene lack allergenic activity, while caryophyllene oxide is an allergen of moderate strength. The sensitizing effect of the caryophyllene hydroperoxides obtained from the photooxidation, was evaluated using the LLNA. The hydroperoxides were tested as a mixture, and were shown to be strong allergens (EC3 0.6%, 25 mM). In contrast, a sample of β-caryophyllene air-exposed for 10 weeks was weakly sensitizing according to the LLNA, with an EC3 value of 26.2% (1.2 M). This is what could be expected, since the oxidation mixture contained large amounts of the moderately sensitizing epoxide (35%), but no hydroperoxides.

An EC3 value of 15.3% (1.1 M) was obtained for pure β-myrcene in the LLNA which is low compared to the EC3 values obtained for the other pure terpenes. It is known from the literature that β-myrcene is metabolized to epoxides[104] which might explain the sensitizing activity observed for pure β-myrcene. A sample of β-myrcene air-exposed for 10 weeks was also tested in the LLNA. It was shown that autoxidation increased the sensitizing effect and an EC3 value of 4.3% (0.3 M) was observed for a sample of β-myrcene air-exposed for 10 weeks.

Patch test study (Paper IV)

Oxidized linalool (air-exposed for 45 weeks), a hydroperoxide fraction of linalool, oxidized β-caryophyllene (air-exposed for 10 weeks), caryophyllene oxide, and oxidized β-myrcene (air-exposed for 12 weeks) were tested in 1511 consecutive dermatitis patients at six European dermatology clinics. The total number of reactions to the different test materials is presented in Table 1.

Table 1. Total number of positive patch test reactions to the oxidized terpenes and fractions/oxidation products, in 1511 consecutive dermatitis patients tested at six dermatology clinics.

Oxidized linalool 2.0% pet.	Linalool hydroperoxide fraction 0.5% pet.	Oxidized β-caryophyllene 3.0% pet.	Caryophyllene oxide 3.9% pet.	Oxidized β-myrcene 3.0% pet.	Total no. of patients with pos. reactions
20/1511 1.3%	16/1511 1.1%	8/1511 0.5%	2/1511 0.1%	1/1511 0.1%	31/1511 2.1%

In total, 47 reactions were observed in 31 patients to the oxidized fragrance terpene materials. Of the 1511 patients tested, 25 (1.7%) reacted positively to oxidized linalool and/or the hydroperoxide fraction of oxidized linalool. Testing with oxidized β-caryophyllene gave positive reactions in 8/1511 patients, of whom two also reacted to caryophyllene oxide. Only one of the 1511 patients showed a positive reaction to oxidized β-myrcene.

The hydroperoxide fraction of linalool gave a high frequency of positive patch test reactions (1.1%). Of the patients allergic to oxidized linalool, 64% reacted to the hydroperoxide fraction. This can be compared with previous studies in patients allergic to oxidized *R*-limonene, where the hydroperoxides gave positive reactions in 59% of the patients allergic to the oxidation mixture.

Concomitant reactions to FM, colophonium, and Myroxylon pereirae were observed significantly more frequently in patients reacting to the oxidized patch test materials compared to the total population in the study. Of these patients (n=31), 39% showed positive reactions to FM, 39% to colophonium, and 21% to Myroxylon pereirae. In the total study population (n=1511) the respective frequencies of positive reactions were 6.9% (FM), 3.8% (colophonium), and 4.6% (Myroxylon pereirae). In a previous patch test study, a high number of concomitant reactions to FM, colophonium, and Myroxylon pereirae were also observed in patients with positive reactions to oxidized limonene, compared to those not reacting.[55]

Fragrance related contact allergy was considered in patients who showed positive reactions to own perfumes or perfumed products, and/or to known perfume allergens (e. g. FM, Myroxylon pereirae, ingredients of FM, Lyral®, essential oils) and/or had certain or probable history for fragrance intolerance (according to answers in the questionnaire). Based on this, a correlation was detected in 18 (58%) of the 31 patients reacting to the oxidized fragrance terpene materials. In five of these 18 cases, no positive reaction to FM and/or Myroxylon pereirae was observed. Thus, the allergy to a common fragrance material could have been missed in these patients if only the standard screening markers for fragrance allergy had been used.

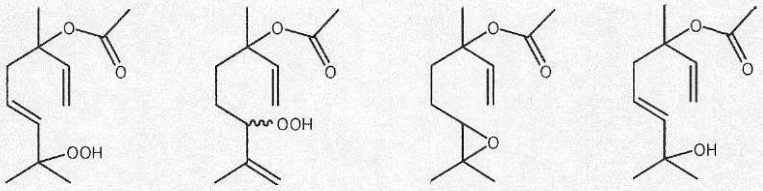
In a retest study, contact allergy to oxidized linalool was reconfirmed in three patients, who had previously shown allergic patch test reactions to oxidized linalool (oxidized linalool and/or the linalool hydroperoxide fraction). The patients were also tested with pure linalool in a dilution series with 20% as the highest concentration. No allergic reactions to non-oxidized linalool were observed.

Autoxidation of linalyl acetate and effect on skin sensitization (Paper V)

Linalyl acetate was air exposed in the same manner as described for the other terpenes. Samples were taken from oxidized linalyl acetate after 10, 16, 24, and 28 weeks to be analyzed with GC. The concentration of linalyl acetate decreased to 74% after 10 weeks, to 55% after 16 weeks, to 38% after 24 weeks, and to 24% after 28 weeks.

Air-exposed linalyl acetate was flash chromatographed to isolate and identify oxidation products. Since the structure of linalyl acetate is very similar to that of linalool, differing only by the hydroxyl group being acetylated in linalyl acetate,

similar oxidation products were expected to be formed. The hydroperoxides **3** and **4**, and the epoxide **14** were synthesized and used as reference compounds. The hydroperoxides **3** and **4** (Figure 17) were identified in oxidized linalyl acetate which is in agreement with the presence of hydroperoxides **1** and **2** (Figure 10) in oxidized linalool. The alcohol **21** (Figure 17), a secondary oxidation product from **3**, was also identified in air-exposed linalyl acetate.



3 **4** **14** **21**

Figure 17. Structures of synthesized reference compounds (**3**, **4**, and **14**) and oxidation products identified in air-exposed linalyl acetate (**3**, **4**, **14**, and **21**).

Another secondary oxidation product from the linalyl acetate hydroperoxide, an epoxide **14** (Figure 17), was found in oxidized linalyl acetate. The corresponding epoxide was not found in oxidized linalool, which was explained by the formation of the cyclic ethers, from attack of the hydroxyl group on the epoxide carbons (Figure 12). In the case of the linalyl acetate epoxide, ring formation is prevented because the hydroxyl group is acetylated, and consequently cannot attack the epoxide carbons.

The skin sensitizing potential of two samples of pure linalyl acetate (the experiment was repeated, lowering the concentrations to get an SI<3 for the lowest concentration), linalyl acetate air-exposed for 10 weeks, the linalyl acetate hydroperoxides **3** and **4**, and the linalyl acetate epoxide **14** was investigated using the LLNA. The results are shown in Figure 18.

Linalyl acetate was shown to be a weak allergen with EC3 values of 20% (1.0 M) and 25.4% (1.3 M) for the distilled sample. Esters show a great variety in sensitizing capacities depending on their structures, ranging from non-allergens to quite strong allergens (conjugated esters).[105] Autoxidation increased the sensitizing capacity to a great extent, and an EC3 value of 3.6% (0.2 M) was observed for the oxidized sample. The linalyl acetate hydroperoxides showed the same allergenic

activity as the oxidation mixture (EC3 3.6%, 0.2 M). The linalyl acetate epoxide **14** was inactive in the LLNA and failed to induce an SI of 3 even at the highest concentration tested (30%). The allergenic activity of epoxides is dependent on the structure of the epoxide. Epoxides that are highly substituted on the epoxide carbons show low allergenic activities, while less substituted, terminal epoxides show stronger allergenic activities.[13,106] The linalyl acetate epoxide is highly substituted and accordingly showed no contact allergenic activity. Based on the results from the LLNA studies, the linalyl acetate hydroperoxides were found to cause the increased allergenic activity of oxidized linalyl acetate.

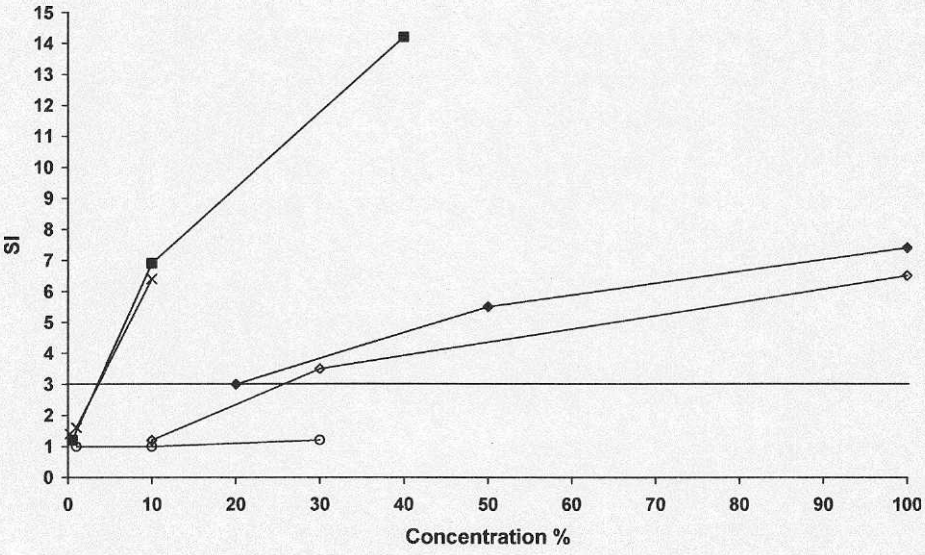


Figure 18. Dose response curves for air-exposed linalyl acetate (10 weeks) (■), linalyl acetate hydroperoxides **3** and **4** (×), linalyl acetate epoxide **14** (○), pure linalyl acetate (distilled) (◊), and pure linalyl acetate (♦), tested in the LLNA.

Autoxidation of lavender oil and effect on skin sensitization (Paper V)

Since our autoxidation studies so far involved pure terpenes, we were interested to see whether autoxidation of terpenes would take place also in an essential oil. Lavender oil was chosen, because linalool and linalyl acetate are the main constituents, and because also β-caryophyllene is present in the oil.

Lavender oil was air-exposed in the same way as the pure terpenes. The content of linalool, linalyl acetate, and β-caryophyllene, respectively, was quantified using GC after 0, 10, 16, 26 and 29 weeks. The non-oxidized sample contained mainly linalyl acetate and linalool (50 and 36%, respectively). The concentration of β-caryophyllene was 3%. The analyses showed that the concentrations of all three compounds decreased over time. After 10 weeks the oil contained 41% linalyl acetate, 28% linalool, and 0.3% β-caryophyllene. After 16 weeks the corresponding figures were 37, 24, and 0.1%, after 26 weeks 26, 16, and 0.1%, and after 29 weeks 24, 14, and 0.1%. The oxidation rates of the individual terpenes in lavender oil were approximately the same as when the pure terpenes were air-exposed.

Air-exposed lavender oil was also subjected to flash chromatography in order to see if the same oxidation products could be identified in the oxidized oil, as in the oxidation mixtures from the pure terpenes. Because lavender oil is a mixture of compounds to start with, it was not possible to isolate the oxidation products in completely pure form. Instead, fractions of enriched oxidation products were isolated and analyzed with HPLC, GC/MS and NMR spectroscopy using reference compounds. The hydroperoxides from both linalool (**1** and **2**) and linalyl acetate (**3** and **4**) were found in oxidized lavender oil together with the secondary oxidation products linalool oxide **18**, linalyl acetate epoxide **14**, and linalyl acetate alcohol **21**. Also the only isolated oxidation product from air-exposed β-caryophyllene, caryophyllene oxide **19**, was found in the oil.

The effect of autoxidation on the sensitizing effect of lavender oil was investigated, using the LLNA. Non-oxidized lavender oil gave an EC3 value of 35.9%. Lavender oil, that had been air-exposed for 10 weeks showed an increase in sensitizing potential with an EC3 value of 22.6%.

Even though lavender oil is a complex mixture of compounds, it was possible to study the autoxidation of some of the terpenes in the oil. Oxidation products could be isolated and identified, and the terpenes studied autoxidized in a similar way as when the pure terpenes were studied. Despite the complexity of the studied material, it was also possible to see an effect of the autoxidation on the sensitizing capacity.

GENERAL DISCUSSION

In these studies the impact of autoxidation on the allergenic activity of some frequently used fragrance terpenes was investigated. All terpenes studied autoxidized on air exposure, at room temperature but a difference in the autoxidation rate was observed since β-caryophyllene and β-myrcene were found to degrade faster than linalool. It was shown that autoxidation influenced the allergenic activity of all studied terpenes, but to different extents. This difference is striking for the two most thoroughly investigated terpenes, linalool and β-caryophyllene. The sensitizing capacity of linalool was markedly affected, while the effect for β-caryophyllene was much less pronounced. The dose response curves for the hydroperoxides and the oxidation mixtures of linalool and β-caryophyllene are shown in Figures 18 and 19. Hydroperoxides of both compounds were strong allergens and gave low EC3 values (86 mM for the linalool hydroperoxides and 25 mM for the caryophyllene hydroperoxides). The oxidation mixtures obtained from air exposure of linalool (10 and 45 weeks of air exposure) were found to contain high concentrations of hydroperoxides, and accordingly they produced relatively low EC3 values indicating a high sensitizing capacity. A 5-fold decrease in the EC3 value was observed for linalool oxidized for 10 weeks compared to that of pure linalool (EC3 3.0 M compared to EC3 0.6 M). However, in the oxidation mixture of β-caryophyllene, no hydroperoxides could be detected, and the oxidation mixture (10 weeks) showed only a weak sensitizing capacity, with an EC3 value of 1.2 M.

Linalyl acetate was not studied in detail and the concentrations of oxidation products were not determined. However, the degradation rate was approximately the same as for linalool and hydroperoxides could readily be isolated from air-exposed linalyl acetate. Also for linalyl acetate a 5-fold decrease in the EC3 value (EC3 1.0 M to 0.2 M) after 10 weeks of air exposure was observed. β-Myrcene polymerized quickly on air exposure and no oxidation products were identified, but the EC3 value decreased from 1.1 M to 0.3 M, after 10 weeks of air exposure.

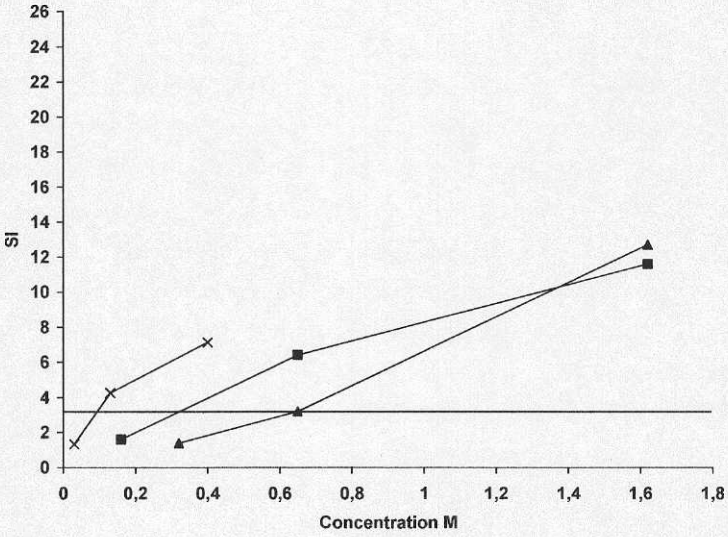


Figure 18. Comparison of the dose response curves, obtained from LLNA's, for the linalool hydroperoxides (×) and oxidation mixtures of linalool (linalool air-exposed for 10 weeks (▲) and linalool air-exposed for 45 weeks (■)). The molar concentration of oxidized linalool was calculated from the molecular weight of linalool.

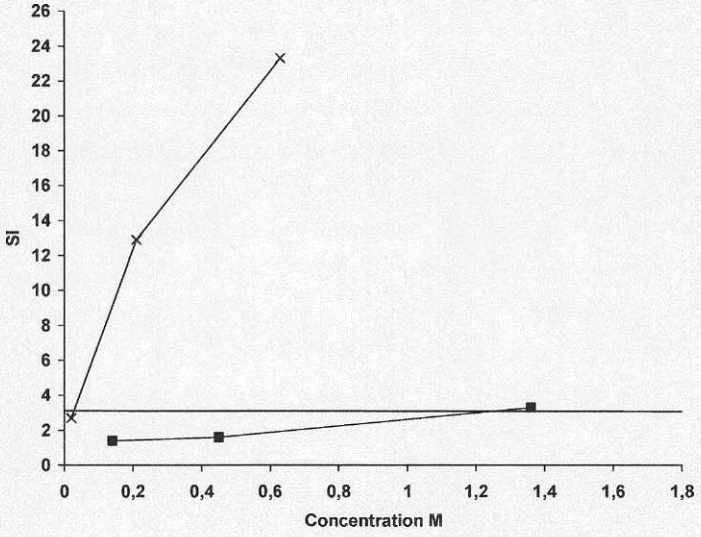


Figure 19. Comparison of the dose response curves, obtained from LLNA's, for caryophyllene hydroperoxides (×) and the oxidation mixture of *β*-caryophyllene (air-exposed for 10 weeks) (■). The molar concentration of oxidized *β*-caryophyllene was calculated from the molecular weight of caryophyllene oxide.

The results show that it is important to study the effect of autoxidation on the allergenic activity for each compound of interest. The formation of stable hydroperoxides seems to be essential for a significant increase in sensitizing capacity due to autoxidation. Not only the degradation of a compound needs to be investigated (*β*-caryophyllene was oxidized even faster than linalool and linalyl acetate) but also the composition of the obtained oxidation mixture, with main focus on the presence/absence of hydroperoxides. The previously studied Δ^3-carene and *R*-limonene were both shown to form stable hydroperoxides when autoxidized and accordingly they were also shown to be allergenic.[43,48,53]

To be able to determine the composition of the autoxidation mixture from an air-exposed terpene, it is important to have suitable analytical methods. GC is traditionally used for analyzing terpenes. However, hydroperoxides, the primary oxidation products of autoxidized terpenes and the most important sensitizers, are thermolabile which makes GC an inappropriate method to use if the aim is to investigate the composition from an allergenic perspective. Instead, straight-phase HPLC-methods were developed, using a silica column and mixtures of *n*-hexane and *t*-butyl methyl ether as mobile phases. However, the detection method is problematic when using HPLC. Terpenes are easily detected with the detectors commonly used for GC (e. g. FID and EI-MS) but other detectors are used together with HPLC. The MS-techniques used in LC/MS instruments e. g. electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are much softer than the EI-MS technique, and compounds are not as readily ionized. Since the terpenes investigated in this thesis are simple unsaturated hydrocarbons they are poorly ionized and thus give a low response in the LC/MS. UV detection was found to be the most suitable of the methods available and was chosen for the analyses of the oxidation products and the oxidation mixtures although the studied compounds have poor UV absorption properties. The HPLC-methods with UV-detection worked well for all oxidized terpenes investigated, and will contribute to the understanding of the oxidative decomposition of terpenes and its impact on the allergenic effect of the terpenes studied.

In the diagnosis of contact allergy, one can only detect an allergy to a compound if this particular compound is actually tested in the patient. Therefore, it is important to use the correct materials for patch testing. In previous patch test studies on linalool, autoxidation was not considered, and only a few cases of contact allergy to

linalool were reported.[64-66] In this study, when patch testing consecutive dermatitis patients with air-exposed linalool, which in the LLNA proved to be sensitizing, a relatively high frequency of positive reactions was observed (1.7% reacted to oxidized linalool and/or the hydroperoxide fraction of linalool). Our observations emphasize the need for testing using the actual compounds that patients come in contact with, and not only the ingredients originally applied in the commercial formulations. The importance of formation of stable hydroperoxides on the allergenic activity was evident also in the clinical study. Hydroperoxides were present in relatively high concentrations in oxidized linalool. Accordingly, oxidized linalool was found to be a frequent cause of contact allergy, while few reactions to oxidized caryophyllene and myrcene were observed. However, it is important to remember the generally higher exposure to linalool in the population.

To prove the clinical relevance of a positive patch test reaction, it is desirable to establish the presence of the allergen in products used by the patient. If the producer does not inform about the presence either by labelling or after direct questions analyses of the products used are necessary. This can be a difficult task, depending on the complexity and vehicle of the product, even for stable allergens that actually are added to the product. Methods based on bioassay-guided chemical fractionation, chemical analysis, and structure-activity relationship studies, have been developed.[107] These analyses are performed using GC/MS which is as previously mentioned not suitable for the analysis of unstable hydroperoxides. HPLC is instead the method of choice, with the detection problems already addressed. Since the identification of the compounds can not be performed only using UV some other spectroscopic method needs to be conducted, e. g. NMR spectroscopy. Furthermore, the oxidation products are probably present in very low concentrations, as they are formed from one of the many ingredients in a scented product. This makes the identification of hydroperoxides in products an even more difficult task compared to identification of other ingredients e. g. originally applied fragrance compounds.

Lavender oil is an essential oil with a relatively simple composition mainly containing the terpenes investigated in this thesis. It was possible to identify the oxidation products of linalool, linalyl acetate, and β-caryophyllene in air-exposed lavender oil since the concentrations of the original terpenes were high. Even though there are several reports of contact allergy to lavender oil in the literature, the allergen(s) have not yet been identified. Linalool and linalyl acetate are

mentioned as constituents of the oil, and possible allergens.[65,85,108] Also α-pinene is discussed as a possible allergen.[85] Considering structure-activity relationships for contact allergens, neither of these compounds should be the cause of contact allergy. We know from our studies that pure linalool and linalyl acetate lack or show weak sensitizing potential. α-Pinene is a simple unsaturated hydrocarbon and should not itself be sensitizing but need some sort of activation to become reactive. We have shown that the hydroperoxides of linalool and linalyl acetate are strong skin sensitizers, and that they are present in air-exposed lavender oil. They might therefore be true contributors to the allergenic activity of lavender oil.

Once an individual is sensitized to a compound, an immunological memory is created, and the allergy will persist throughout life. Thus, primary prevention is important to reduce the risk of sensitization. In a Danish study,[37] with focus on possible effects of preventive measures, the changes in prevalence of sensitization in consecutive dermatitis patients to common allergens between the years 1985-86 and 1997-98, were investigated. During this period a statutory order limiting the release of nickel from objects intended for close contact with the skin was adopted in Denmark. The effect of the regulation was seen in the youngest age group, which is the age group with highest frequency of contact allergy to nickel. In this group, the frequency decreased from 24.8% to 9.2%, which means a decrease of 63% calculated on the result from 1985-86 (Figure 20).

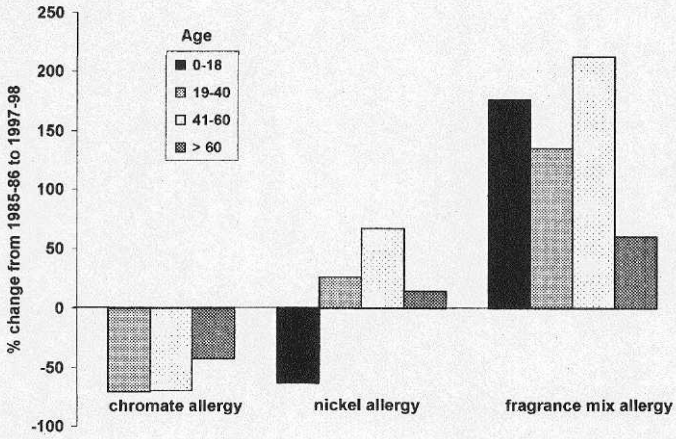


Figure 20. The change in chromate, nickel, and FM allergy in Denmark from 1985-86 to 1997-98. The change is illustrated by the percentage of the results from 1985-86 (From Johansen *et al.*[37]).

There was also an overall decrease of contact allergy to chromate, from 3.0% to 1.2%, among those of working age (Figure 20). This is explained by the addition of ferrous sulphate to cement in Denmark, reducing the amount of water-soluble chromate. On the other hand, contact allergy to FM increased from 4.1% to 9.9%, in all age groups. This can be explained by an increased use of scented products. Bearing in mind that only seven chemicals are used in the FM of all fragrance chemicals used in scented products and that the impact of autoxidation of common fragrance terpenes is not considered, the frequencies presented must be regarded as low estimates.

In order to develop effective preventive strategies it is necessary to know the true allergens that people come in contact with. A majority of cosmetic products, household chemicals, and chemicals in occupational use is scented. Thus, the exposure to fragrance chemicals is immense in the population. The use of fragrances in almost every product on the market, should be decreased to be able to reduce the frequency of contact allergy to fragrances. Furthermore, compounds with a low allergenic potential and a low content of oxidation/degradation products should preferentially be used.

CONCLUSIONS

In this thesis, the autoxidation of some commonly used fragrance terpenes and the effect of the autoxidation on their contact allergenic activities, were investigated. The terpenes linalool, β-caryophyllene, β-myrcene, and linalyl acetate were air exposed at room temperature and an oxidative degradation was observed for all the compounds. The autoxidation of β-caryophyllene and β-myrcene was faster than the oxidation of linalool and linalyl acetate. The complexity of the obtained oxidation mixtures differed for the different compounds. Oxidized linalool but also oxidized linalyl acetate, contained a large number of oxidation products whereas only one oxidation product was detected in oxidized β-caryophyllene. Oxidized β-myrcene polymerized quickly and could therefore not be studied in detail.

Hydroperoxides, a group of compounds with known skin sensitizing properties, were identified in air-exposed linalool. The hydroperoxides were the strongest allergens among the oxidation products of linalool tested. The major hydroperoxide was quantified in the oxidized samples and relatively high concentrations were detected. Accordingly, the sensitization experiments showed that autoxidation affected the contact allergenic activity of linalool to a great extent. Hydroperoxides were also identified in air-exposed linalyl acetate, and the allergenic activity was affected to the same extent as for linalool.

The epoxide caryophyllene oxide was the only oxidation product identified in oxidized β-caryophyllene. It was found to be a moderate sensitizer. Hydroperoxides of β-caryophyllene were synthesized and searched for, but could not be detected in air-exposed β-caryophyllene. Accordingly, oxidized β-caryophyllene showed only a weak sensitizing capacity.

Due to the fast polymerization of β-myrcene, no oxidation products were isolated and identified. β-Myrcene can be regarded as a prohapten in two ways; pure β-myrcene was found to be a weak allergen, which probably is due to metabolic transformation to epoxides, and the allergenic activity was increased because of autoxidation.

The results from the patch test study showed that oxidized linalool was a frequent cause of contact allergy, while only a few reactions to oxidized β-caryophyllene and

oxidized β-myrcene were recorded. This can be explained by the fact that the allergenic activity of linalool was affected to a greater extent by autoxidation, compared to the other compounds, and by the fact that linalool is more frequently used as a fragrance chemical.

Finally, lavender oil was air exposed in the same way as the pure terpenes to see whether autoxidation of the fragrance terpenes would take place also in an essential oil. The essential oils are often claimed to be protected from autoxidation by the presence of natural antioxidants. Linalool, linalyl acetate and β-caryophyllene were found to oxidize in the oil at approximately the same rates as when air exposing the pure terpenes. The same oxidation products could also be identified.

ACKNOWLEDGEMENTS

Så börjar det närma sig slutet på tiden som doktorand och det är dags att skriva de här raderna och tacka alla som har hjälpt mig på vägen. Så här i efterhand känns det på ett sätt som att tiden har gått ganska snabbt, men det har också hunnit hända väldigt mycket under de här åren. Det här arbetet påbörjade jag i Stockholm, på Arbetslivsinstitutet, Programmet för Hud och allergi och jag hade ju inte en aning om då att jag skulle avsluta det på Göteborgs universitet, i gruppen för Dermatokemi och hudallergi. Det är många personer som har bidragit till att jag har kunnat genomföra det här arbetet, och jag vill tacka er alla. Jag vill speciellt tacka:

Min handledare *Ann-Therese Karlberg*, för att jag fick möjligheten att göra det här arbetet. För att du har delat med dig av dina kunskaper, låtit mig utvecklas och arbeta självständigt samt för förtroendet att få presentera mina resultat i många både nationella och internationella sammanhang. Tack för ett bra samarbete, spännande diskussioner och en givande tid!

Mina medarbetare i "parfymprojektet"
Min biträdande handledare *Anna Börje*, för syntes- och NMR-hjälp. Och för att du, hjälpt mig att räta ut många frågetecken. Tack för den här tiden!
Mihaly Matura, för ett mycket givande samarbete och för att du delat med dig av din stora kunskap. För din otroliga entusiasm (som smittar av sig), alla dina idéer och för att du alltid, med glädje, hjälper till! Vilket team work vid inskickandet av reviderade versionen av patient-peket!

Mina tidigare arbetskamrater på ALI; *Anna, Janne, Gunnel, Malin, LiPing, Ulla, Lizbeth, Marie-Louise, Gunborg, Birgitta*. Som gjorde början på mitt arbete så trevlig och att det var så lätt att komma igång p g a all hjälp jag fick av er!
Ett speciellt tack till:
Kerstin, som introducerade mig till arbetet med auto-oxidation av terpener. Och som lärde mig allt om analyser. Och som omedelbart blev min vän. Tack för all hjälp och för att du gjorde att det var så roligt att åka till jobbet! Jag ska verkligen försöka ta mig i kragen och komma upp och hälsa på dig i vår.

Mina nuvarande arbetskamrater på GU; *Moa, Charlotte, Lina, Staffan, Petri, Lasse, Johanna.* Tack för all hjälp, värdefulla diskussioner, och för att det är så trevligt att

jobba med er. Jag kommer att sakna er! *Daniel W*, min apotekar-polare, för att det blev så trevligt sen du flyttade upp till oss på "nian". Och tack för alla "terapi-middagar"! Skulle ha skrivit ditt tack på dalmål, men vet inte riktigt hur man gör!
Kristin. Tack för att du är min vän och för allt roligt. För att det känns så bra att ha någon man verkligen kan prata med. För fjällresor och Turkiet och allt annat. Jag ser fram emot allt spännande vi har framför oss; Vasaloppet t ex!!!
Anna-Malin. Min förebild. Vad hade jag gjort utan dig? Så skönt att ha haft någon att ventilera allting med när det har varit jobbigt (pizza och öl-kvällarna!), och dela allting roligt med när det har gått bra. Jag hade aldrig fixat det här med göteborgs-flytten om inte du hade varit med. Tack för att du är en så bra vän! Jag lovar att jag ska komma upp och hälsa på dig också!

Elma och *Anna,* för väl utförda examensarbeten. Det var trevligt att ha er i gruppen.

Ett stort tack även till alla på läkemedelskemi, organisk kemi (GU) och organisk kemi (Chalmers). Ni har verkligen förgyllt göteborgstiden. Vad hade luncherna varit utan er på Chalmers! Och *Anders Dahlén*, det har verkligen varit apkul att lära känna dig!

Jag vill naturligtvis också tacka min familj; pappa *Påte*, mina syskon *Lisa* och *Stefan*, och framför allt min mamma *Pia* (många telefonsamtal har det blivit!). Tack för ert stöd och för att ni ALLTID tror på mig!

Det absolut största "tacket", vill jag ge till Christopher. Utan dig hade jag inte fixat det här. Tack för att du alltid finns där, för att du står ut med mig, för att du ger mig perspektiv på tillvaron och för att du flyttade med mig först till Uppsala och sedan till Göteborg. Tack för allt roligt och för all din kärlek! Jag älskar dig!

Tack för mig!

Maria

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ISBN 91-628-6446-7