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DOCTORAL DISSERTATION

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During the past few decades, linalool and limonene hydroperoxides have emerged as contact allergens as a result of high rates of positive patch test reactions worldwide. Including these two allergens in the baseline series for fragrance contact allergy screening in consecutive patients has been the subject of debate because it is difficult to establish the sources of allergen exposure and definite clinical relevance.

Two major problems that remain to be solved are the discrepancies of patch test results between fragrance mixes and their individual ingredients, and the assessment of clinical relevance in patients with contact allergy to hydroperoxides of linalool and hydroperoxides of limonene regarding uncertain sources of exposure.

The main objective of the work presented in this thesis was to improve the diagnostic procedures for fragrance contact allergy and allergic contact dermatitis, focusing on the hydroperoxides of linalool and hydroperoxides of limonene. Studies were carried out to determine the prevalence and demographics of fragrance contact allergy, and to update information on patch test results and patterns of simultaneous reactions. Repeated open application tests were also performed in patients with contact allergy to hydroperoxides of linalool.

The findings of these studies can be summarized as follows. Patients with single-fragrance contact allergy or who showed a weak reaction to the individual ingredients of the fragrance mixes were missed when patch tested with only fragrance mixes in the baseline series. The interpretation of patch test results using patch test preparations containing sorbitan sesquioleate remains problematic. Contact allergies to the hydroperoxides of linalool and hydroperoxides of limonene are increasing. The rates of contact allergies to these two culprits are significantly more common in a younger group than contact allergies to other fragrances. Repeated open application tests with creams containing hydroperoxides of linalool at the realistic concentrations reported in products rarely elicited skin reactions.

Key words: contact allergy, allergic contact dermatitis, fragrance, fragrance mix, linalool, limonene, hydroperoxides, patch test, epidemiology, repeated open application test.

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To my beloved family

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Thesis at a Glance

	Objectives	Method	Main findings
I	To determine the prevalence and trends of contact allergy to fragrances, to identify the factors associated with fragrance contact allergy, and to analyse the benefits and drawbacks of patch testing with the individual ingredients of the fragrance mixes	Retrospective analysis data from 2016 to 2020	The prevalence of fragrance contact allergy was 13.1%, of which 1.4% were diagnosed when tested with individual ingredients of fragrance mixes. Fragrance contact allergy was associated with higher age (≥ 40 y) and atopic dermatitis. More positive and stronger reactions to the individual ingredients were associated with positive patch test reactivity to the corresponding mix.
II	To determine the prevalence of sorbitan sesquioleate (SSO) sensitisation in consecutive dermatitis patients and concomitant patch test reactions to fragrance and non-fragrance test preparations containing SSO	Retrospective analysis of patients patch tested with SSO from 2016 to 2020	The prevalence of SSO contact allergy was 0.48%, and doubtful reactions were reported in 1.3% of the 3,539 patients. Both doubtful and weak positive reactions were common in patients with a history of atopic dermatitis. Simultaneous positive reactions to SSO and the test preparations containing SSO (fragrance mix I, *Myroxylon pereirae* resin and Oakmoss extract) were significantly more common in the patients with strong or extreme patch test reactions than in those with weak reactions to SSO (*P*-value = 0.018). Fragrance contact allergy cannot be ruled out because all the individual fragrance ingredients in the mixes are not always tested.
III	To determine the prevalence and any change in trends of contact allergy to oxidised terpenes in patients with dermatitis and to demonstrate the features of patch test reactions and clinical characteristics of patients with oxidised terpene allergy.	Retrospective analysis of patients tested with hydroperoxides (HPs) of linalool and HPs of limonene from 2013 to 2020	The prevalence of contact allergies to oxidised terpenes was 9.4%; 7.0% to HPs of linalool, 5.1% to HPs of limonene, and 2.7% to both. Trends of increasing contact allergy were observed in both. Patients showing exclusively positive reactions to HPs of linalool and HPs of limonene were significantly younger than those with fragrance allergies. Strong to extreme patch test reactions were significantly associated with the older age group (≥40 y).
IV	To investigate the patterns of simultaneous positive patch test reactions and the prevalence of multiple contact allergies in patients with contact allergy to linalool HPs and limonene HPs.	Retrospective analysis of data from patients tested with the Swedish baseline series and additional fragrance allergens from 2015 to 2020	The prevalence of multiple contact allergies was 9.8%. Patients with an exclusively positive patch test reaction to HPs of linalool showed a significantly higher likelihood of having multiple contact allergies. The number of simultaneous positive reactions increased significantly with increasing age. Patients with an exclusively positive reaction to HPs of linalool showed a higher total number of allergens with a significant simultaneous positive reaction than those with a positive reaction to HPs of limonene. Patients showing reactions to HPs of both were found to have multiple significant simultaneous positive reactions. Simultaneous reactions to fragrances and cosmetic-related allergens were common.
V	To perform repeated open application tests (ROATs) using realistic concentrations of linalool HPs in patients with a history of linalool hydroperoxide contact allergy, and to compare the results to those from other dermatitis patients with no contact allergy to linalool HPs.	A double-blind, controlled prospective clinical study. Patch testing (serial dilutions) and ROATs with four linalool HP creams were performed for 4 weeks in patients with and without HPs of linalool contact allergy.	Of the 47 participants, 31 had HPs of linalool contact allergy, and 16 were controls. The results did not indicate that the ROAT creams with realistic concentrations of HPs of linalool elicited a skin reaction. Only one HPs of linalool contact allergy patient showed a positive ROAT reaction in the area where the highest concentration of the cream (440 ppm) had been applied on Day 28.

Abstract

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List of Papers

Paper I

Sukakul T, Bruze M, Mowitz M, Antelmi A, Boonchai W, Dahlin J, Hamnerius N, Hauksson I, Lejding T, Svedman C. Simultaneous patch testing with fragrance markers in the baseline series and the ingredients of fragrance mixes: An update from southern Sweden. Contact Dermatitis. 2022 Jun;86(6):514-523. doi: 10.1111/cod.14072. Epub 2022 Mar 3. PMID: 35152428; PMCID: PMC9314710.

Paper II

Sukakul T, Bruze M, Mowitz M, Svedman C. Use of sorbitan sesquioleate in patch test preparations and patch testing with the substance – What do our results mean? Contact Dermatitis. 2023 Feb;88(2):134-138. doi: 10.1111/cod.14239. Epub 2022 Nov 1. PMID: 36305668.

Paper III

Sukakul T, Bruze M, Mowitz M, Antelmi A, Bergendorff O, Björk J, Dahlin J, Hamnerius N, Hauksson I, Isaksson M, Lejding T, Pontén A, Svedman C. Contact allergy to oxidised linalool and oxidised limonene: Patch testing in consecutive patients with dermatitis. Contact Dermatitis. 2022 Jan;86(1):15-24. doi: 10.1111/cod.13980. Epub 2021 Oct 10. PMID: 34561893.

Paper IV

Sukakul T, Bruze M, Mowitz M, Bergendorff O, Björk J, Dahlin J, Svedman C. Patterns of simultaneous contact allergies in patients with contact sensitization to oxidized linalool and oxidized limonene. Contact Dermatitis. 2023;1-9. doi:10.1111/cod.14445

Paper V

Sukakul T, Bruze M, Mowitz M, Kiuru A, Svedman C. Allergic contact dermatitis to linalool hydroperoxides; pitfalls in the diagnostic process - findings from a repeated open application test study. Manuscript, submitted October 2023.

List of important abbreviations

ACD	Allergic contact dermatitis
BOP	*Myroxylon pereirae* resin (balsam of Peru)
CAS	Chemical Abstracts Service
FCAT	Freund's complete adjuvant test
FM	Fragrance mix
GC-MS	Gas chromatography-mass spectrometry
GPMT	Guinea pig maximisation test
HICC	Hydroxyisohexyl 3-cyclohexene carboxaldehyde
HP	Hydroperoxide
Lim-1-OOH	Limonene-1-hydroperoxide
Lim-2-OOH	Limonene-2-hydroperoxide
Lin-6-OOH	Linalool-6-hydroperoxide
Lin-7-OOH	Linalool-7-hydroperoxide
LLNA	Local lymph node assay
ppm	Parts per million
ROAT	Repeated open application test
SSO	Sorbitan sesquioleate

Introduction

The skin

The main function of the skin is to protect the body from external agents such as microorganisms, temperature, light, and chemicals. Keratinocytes form an anatomical barrier in the skin as part of the innate immune mechanism.[1] One of the main functions of keratinocytes is to limit the absorption of chemicals to which the skin is exposed.[1] Immune cells found in the skin include dendritic cells, macrophages, mast cells, eosinophils, lymphocytes, neutrophils, and fibroblasts.

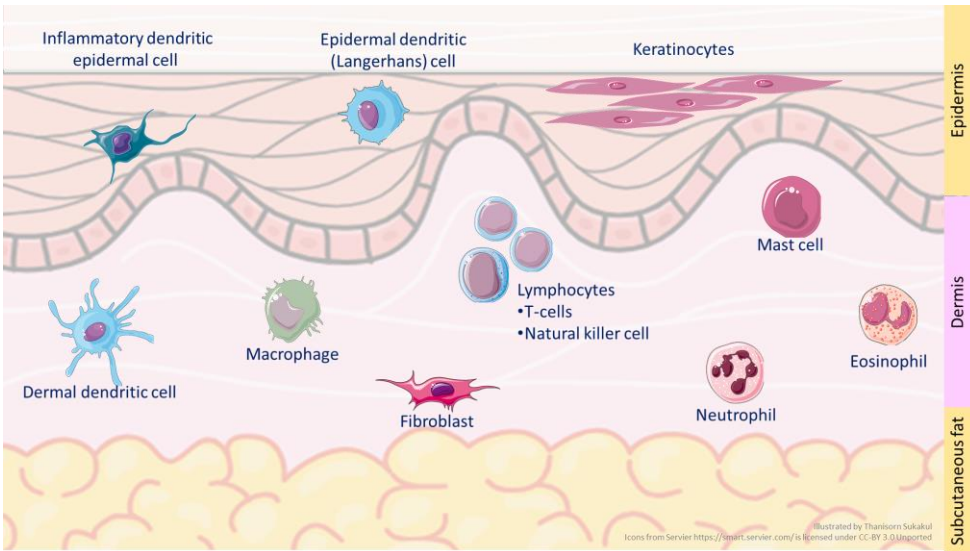


Figure 1. The structures and cells in the skin involved in the innate and adaptive immunity.

Contact allergy and contact dermatitis

Definitions

The terms used in this field are defined as follows.

"Hypersensitivity" has been proposed as the term defining reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by individuals.[2]

"Delayed-type hypersensitivity" refers to a hypersensitive reaction that occurs after antigen administration in a sensitised individual through T-cells, characterized by lymphocytic and mononuclear cell infiltration in skin histology.[2,3]

"Contact allergy", also known as contact sensitivity, is the altered immune status of an individual induced by a particular sensitizing substance, a contact allergen.[4]

"Contact dermatitis" is an inflammatory skin reaction caused by direct contact with noxious agents in the environment.[4] Contact dermatitis can be classified into four categories: allergic contact dermatitis (ACD), irritant contact dermatitis, photoallergic contact dermatitis, and phototoxic contact dermatitis.

"Allergic contact dermatitis" is used to describe or diagnose patients with skin manifestations of delayed-type hypersensitivity (type IV hypersensitivity) to a specific allergen.[4] Typical manifestations of ACD are skin erythema, infiltration, oozing, scales, and lichenification.[4,5]

"Irritant contact dermatitis" occurs when the skin is exposed to irritant chemicals or physical factors.[5] Skin injury activates the release of proinflammatory cytokines via the innate immune mechanism.[5] The clinical manifestations of irritant contact dermatitis can be dryness, erythema or those which are similar to ACD, as well as more severe forms, such as erosion, ulcer or skin necrosis (chemical burn) since the irritants can directly damage the keratinocytes and lead to a rapid response.[5,6]

"Cross-sensitivity" refers to a sensitivity reaction that occurs when an individual sensitised to one allergen, reacts when exposed to a structurally related substance that has not previously been encountered.[4,7]

Contact allergens: haptens, prehaptens, prohaptens

Contact allergens are chemicals that can induce contact allergy and usually have a low molecular weight of <500, but in exceptional cases up to 1,000.[4,8] Haptens are allergens that bind to proteins in the skin, causing contact sensitisation.[4] A hapten is a sensitizing chemical that can bind directly to the protein (protein-reactive). Prehaptens and prohaptens are chemicals that themselves are non-sensitizing or low-sensitizing.[8,9]

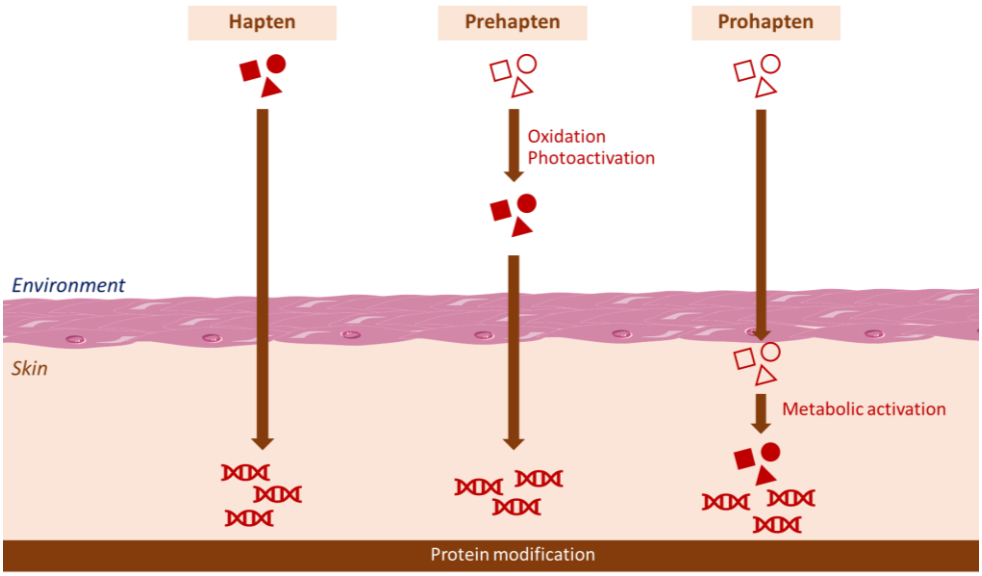


Figure 2. Hapten, prehapten and prohapten.

A prehapten is transformed into a hapten by chemical transformation, which occurs outside the skin, for example, autoxidation by exposure to air or photoactivation.[9,10] Examples of prehaptens that require air oxidation to cause contact allergy are linalool and limonene.[9] These chemicals are prone to autoxidation when exposed to air as the molecules have oxidizable allylic positions.[10,11] The main oxidation products are hydroperoxides (HPs).[11]

Prohaptens, such as eugenol or isoeugenol, are transformed into haptens in the skin (by bioactivation, i.e. a xenobiotic mechanism), usually via enzyme catalysis.[9,10] Geraniol and cinnamyl alcohol are examples of fragrance allergens that can exist as both prehaptens and prohaptens[10-12], while geranial can act as all three: a hapten, a prehapten, or a prohapten.[9]

Contact allergy processes

There are two phases of contact sensitisation: the induction or sensitisation phase and the elicitation phase (Figure 3). The induction phase occurs when the skin is exposed to an allergen inducing immunological responses in the individual by activating the skin's innate and adaptive immune mechanisms. This phase can occur without clinical manifestations. The elicitation phase occurs when renewed or continuous exposure to the allergen on a sensitised individual's skin causes an inflammatory reaction.[3]

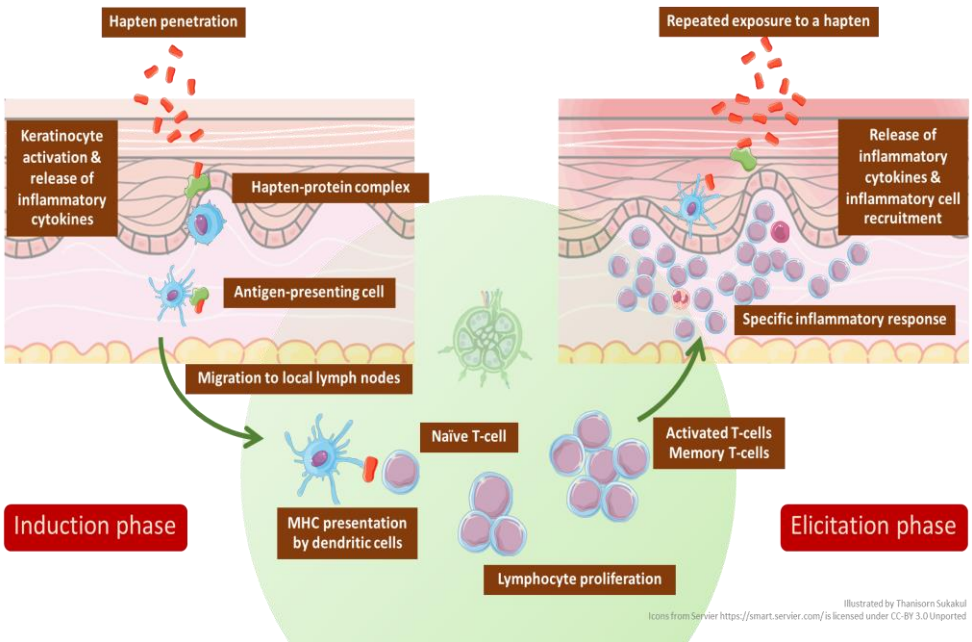


Figure 3. Contact allergy processes: induction phase and elicitation phase.

Induction phase

Skin sensitisation usually takes weeks to months in humans.[1,6] Chemical absorption or penetration through the skin activates proinflammatory cytokine production by keratinocytes leading to the release of danger signals and damage-associated molecular patterns (innate immune response).[13]

In this phase, the haptens activate nucleotide-binding oligomerization domain-like receptors and toll-like receptors, and activate the production of reactive oxygen species.[1,14] The following step is "haptenization", i.e. protein binding and the formation of a complete antigen (a hapten–protein complex).[13] The maturation of epidermal dendritic (Langerhans) and dermal dendritic cells then takes place, and these migrate to skin-draining lymph nodes.[1,13] The antigen-presenting dendritic cells in the skin present the allergen to naïve T-cell via major histocompatibility complexes (MHC) or CD1 molecules.[1,6]

Elicitation phase

The elicitation phase occurs when the skin has been sensitised by a particular sensitizing contact allergen through repeated exposure.[1,15,16] The innate inflammatory response triggers the release of cytokines and chemokines, as in the induction phase.[16,17] Memory T-cells, i.e., antigen-specific T-cells, are recruited and infiltrate the skin, whereas contact allergen-specific regulatory T-cells (Tregs) downregulate the immune response.[16]

This phase may take only a few days after repeated exposure, as demonstrated in patch testing. The dose required to elicit a response is lower than that in the sensitisation or induction phase.[18]

Diagnostic tests

Patch testing

Patch testing is the standard procedure used to diagnose contact allergy.[4,19] Contact allergy may present clinically in different ways, and is thus a differential diagnosis that should be taken into account in several clinical situations.[20] It is recommended that patch testing be performed in patients with suspected ACD, including dermatitis related to occupational exposure, dermatitis that does not improve with treatment, and other skin and mucous membrane eruptions, including nonimmediate adverse drug reactions.[4,21]

Baseline patch test series are sets of allergens used for routine patch testing in clinics. The baseline series used in Sweden have been used and adjusted by experts based on research evidence, and the test preparations included are usually similar to those in other European baseline series. The prevalence of at least one positive reaction to an allergen in the European baseline series in the general adult population reported in Europe during 2008-2011 was 27%.[22]

Patch test materials and procedures often differ between clinics, and there may also be differences in patch test reading criteria.[4,23,24] Appropriate dose, patch test skin site, and occlusion time have been recommended in the European Society of Contact Dermatitis patch test guidelines.[4]

The recommended doses of liquid and petrolatum-based preparations in different types of chambers are 29-30 µL/cm^2 and 36-40 mg/cm^2, respectively.[4] The preferred anatomical location is the upper back, and the chambers should be occluded for on Day (D) 0 for two days (48 hours).[4] Patch test readings can be performed from D2 to D7.[4]

The reading criteria are usually based on the classification of patch test reactions according to the International Contact Dermatitis Research Group.[4,19] The morphologies of a positive reaction are erythema, infiltration, papules, vesicles, and bullae.[4,19] The clinical relevance is evaluated after patch test readings, based on the patient's history, chemical exposure, and clinical course of dermatitis.[4]

Repeated open application testing

Repeated open application tests (ROATs), also known as "use tests" or "provocative use tests", are useful in both clinical practice and research. ROAT has been developed to help clinicians evaluate the clinical relevance of patch test reactions.[25,26] ROATs are used to determine the cause of dermatitis (both allergic and irritant reactions) by testing with consumer products (having a clinical relevance or not) and to determine whether the patients reacted to these products when the results of patch testing were negative or doubtful.[26]

ROATs are also used in research to investigate whether an allergy is of clinical relevance through exposure mimicking real life. Repeated open application testing can also be used to evaluate an eliciting dose and whether the concentrations permitted in products, mostly cosmetics, could elicit ACD in real-life exposure.[27-31] For example, ROATs have shown that low concentrations of formaldehyde, as allowed by the European Cosmetics Directive, could elicit ACD.[32]

ROATs are usually performed on the forearms or cubital fossa, for three to four weeks, by applying the test preparation once or twice daily.[26,33] This method has been used more often for leave-on than rinse-off products.[33] The size of the test area does not seem to affect the test results[34]; however, the dose per unit area (weight of substance/area) and total amount applied should be considered.[35,36]

The procedure for ROATs has been continuously improved and standardised.[26,34,36-40] The substances tested are mainly allergens contained in cosmetics, such as preservatives and fragrances.[41] However, the dose, size of the application area, test preparation vehicle, application frequency, site of application, and duration of studies have varied.[26-31,35-37,40,42-63] Many factors may affect the outcome of ROATs, including the site of application, exposure dose and time, and the chemical and physical properties of the test preparation, which will affect the percutaneous penetration, and thus the properties of the allergens.[25,62,63]

A sufficient dose and exposure time are necessary to elicit a response.[35] Test preparations at too low a concentration might not elicit a skin reaction in positive patch tested individuals within a 28-day study, however, the period of a ROAT may be longer.[57] It may also be easier to elicit a reaction when the ROAT is performed on non-healthy or eczematous skin.[55]

Moreover, methods of evaluation and outcomes also differ between clinics, and it has been suggested that the morphology and strength of reactions should be documented and reported.[39] Important clinical findings in a ROAT are infiltration (papules), erythema, and the area of erythema.[39] However, these morphologies cannot be used to distinguish between an allergic reaction and an irritant reaction.[64]

Several studies have reported a positive correlation between the intensity of patch test reactions and the percentage of patients who reacted positively to different doses of the repeated application of test preparations, but not all.[25,38,42,44,48-50,52,53,56,60] A stronger patch test reaction intensity may indicate a higher possibility of showing a positive and an earlier reaction in a ROAT.[43]

Based on previous studies on nickel and methyldibromo glutaronitrile, a relation was found that could be used to convert the patch test dose-response data into ROAT dose-response data for non-volatile compounds:

$$\text{Elicitation Dose } (ED_{xx}) \text{ for ROAT} = 0.0296 ED_{xx} \text{ patch test.}[37]$$

The relation for volatile compounds was different, and it was not possible to ascertain a corresponding conversion factor due to possible evaporation of the compound.[37]

Other diagnostic tests

Open and semi-open tests can be useful in some cases, especially when an immediate type of hypersensitivity (contact urticaria) is suspected.[4] Photopatch testing is performed when photoallergic or phototoxic contact dermatitis is one of the differential diagnoses.[4,65]

Fragrance

"Fragrance" and "perfume" are interchangeable terms. "Perfume" comes from "per fumus", meaning "through smoke" in Latin.[66] The materials used to make fragrances are usually obtained from plants through alcohol extraction, or are synthesized in the lab using chemical processes.[66] Fragrance chemicals in cosmetics are among the most common culprits in contact allergies.[67] More than 150 fragrance materials can cause contact allergy, mostly delayed-type hypersensitivity, including photoallergic contact dermatitis, while others may also cause immediate-type reactions.[68]

Fragrance allergens in patch testing

The baseline series contains several test substances that are used to screen for fragrance allergy. The most common fragrance allergen test preparations used are fragrance mix (FM) I, FM II, and *Myroxylon pereirae* resin (balsam of Peru, BOP) in petrolatum.[68]

"Perfume mixture" (nowadays called FM I) at a concentration of 16% (2% of each ingredient) in petrolatum was introduced for patch testing in 1977.[69] Common allergens were included in the mixture as one test preparation to effectively detect fragrance contact allergy in patients, making other areas of the skin available for patch testing with other preparations. FM I contains seven individual fragrance compounds, together with an extract of *Evernia prunastri* (Oakmoss absolute), and sorbitan sesquioleate (SSO), which is used as an emulsifier.[69-71] In the mid-1980s, the concentration of FM I was reduced from 16% to 8% (1% of each fragrance plus 5% SSO), and is widely used today (Table 1).[71,72] It has been reported that FM I at 8% could detect up to 80% of fragrance contact allergies in patients.[67]

FM II, at a concentration of 14%, was introduced and recommended for screening in the European Baseline Series in 2008 (Table 1), together with hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC, 5% in petrolatum), which is one of the ingredients of FM II.[73] FM II was introduced to improve screening by including more fragrances that are widely used and not previously included in patch testing. Including FM II in the baseline series led to the detection of additional fragrance contact allergy patients, up to 5%.[73] Later, in 2014, it was suggested that HICC should be removed from the baseline series as the positive reaction rate was low.[74]

Unlike the fragrance mixes, the concentrations of individual compounds in BOP are not clearly defined, and it can contain up to 250 compounds.[75] The compounds with the highest proportions in BOP are benzyl cinnamate, benzyl benzoate, cinnamic acid, benzoic acid, and coniferyl benzoate.[75] The BOP used for patch testing is dissolved in petrolatum at a concentration of 25%.[75] Some components in BOP are also included in FM I and FM II, but at different concentrations.[75]

Table 1. The ingredients of fragrance mixes I and II used in patch testing.

Fragrance mix I			Fragrance mix II		
Ingredient	Concentration (% in pet.)		Ingredient	Concentration (% in pet.)	
	Mix preparation	Individual preparations		Mix preparation	Individual preparations
Amyl cinnamal	1.0	2.0	Citral	1.0	2.0
Cinnamal	1.0	1.0	Citronellol	0.5	1.0
Cinnamyl alcohol	1.0	2.0	Coumarin	2.5	5.0
Eugenol	1.0	2.0	Farnesol	2.5	5.0
Evernia prunastri extract	1.0	2.0	Hexyl cinnamic aldehyde	5.0	10.0
Geraniol	1.0	2.0	HICC	2.5	5.0
Hydroxycitronellal	1.0	2.0			
Isoeugenol	1.0	2.0			
SSO	5.0	20.0			

Pet., petrolatum, HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde; SSO, sorbitan sesquioleate.

The substances included in the baseline series at different clinics and the rate of contact allergy may vary between clinics and over time.[76-94] Contact allergy to FM I almost always ranks the highest among fragrance test preparations, the rate being up to 22% in routine patch testing.[68,92,93] During the period 2009-2015, the rates of contact allergies to FM I and FM II in Sweden were 6.5% and 3.2%, respectively.[91] The rate of contact allergy to FM I has been reported to be increasing in recent decades in Denmark.[90] The prevalence of positive reactions in the general population in Europe has been reported to be up to 3.5% for FM I, 1.9% for FM II, and 1.8% for BOP.[75,95,96] Oakmoss absolute and HICC have been found to be the most common allergens in FM I and II, respectively.[72,91,92]

Since 2005, labels on cosmetic and household products in Europe must contain information on 26 fragrance ingredients, using the International Nomenclature Cosmetic Ingredient names.[9,97] The most recent amendment of the regulation was published in July 2023.[97] All 14 ingredients of FM I and FM II are among these ingredients.[98] The remaining 12 fragrance allergens that must be labelled can be tested separately using recommended test doses.[99]

When these 26 individual fragrance ingredients were tested from 2005 to 2007, after the legislation came into force, the prevalence of overall positive reactions to at least one of them was about 10% in patients referred for patch testing.[98] Clinical relevance was reported in about 60% of the patients with positive reactions.[98]

Among the 12 fragrance ingredients not included in the fragrance mixes, *Evernia furfuracea* (Treemoss absolute) was found to be the most common contact allergen.[92,98] Patch testing with additional fragrance materials has been found to be beneficial in detecting fragrance contact allergy.[94,100,101] About 20% of fragrance contact allergy patients would be missed if 26 additional individual fragrance substances were not tested concurrently with the FM I, FM II, and BOP.[100]

Sorbitan sesquioleate as an emulsifier in patch test preparations

SSO is an emulsifier widely used in cosmetics and topical medications.[102] It has been used as an emulsifier in many patch test preparations (Table 2), which could affect patch test results by giving false positive reactions, especially for FM I and BOP.[70,103,104]

The use of 20% SSO in petrolatum for patch testing could be useful when using other test preparations containing SSO.[105] However, the inclusion of SSO in baseline series and the interpretation of reactions are still being debated.

Table 2. Commercially available patch test preparations containing sorbitan sesquioleate.

SSO concentration	Allergen, concentration and vehicle	Manufacturer
5%	Ethylene urea, melamine formaldehyde mix, 5.0% pet.	CD
	Evernia furfuracea (Treemoss) extract, 1.0% pet.	SP
	Evernia prunastri (Oakmoss) extract, 2.0% pet.	CD
	Evernia prunastri (Oakmoss) extract, 1.0% pet.	SP
	Fragrance mix I, 8.0% pet.	CD & SP
	Glutaraldehyde, 0.5% pet.	CD
	Glutaraldehyde, 0.2% pet.	CD
	Myroxylon pereirae resin, 25.0% pet.	CD
2%	Decyl glucoside, 5.0% pet.	CD
1%	Alpha-amylcinnamic aldehyde, 1.0% pet.	SP
	Cinnamic aldehyde, 1.0% pet.	SP
	DMDM hydantoin, 1.0% pet.	CD
	Formaldehyde, 1.0% pet.	CD
	Hydroxycitronellal, 1.0% pet.	SP
	2-Hydroxyethyl methacrylate, 2.0% pet.	CD
	Isoeugenol, 1.0% pet.	SP
	Melamine formaldehyde, 7.0% pet.	CD
	Methylisothiazolinone + methylchloroisothiazolinone, 0.01% pet.	CD

Information obtained from info@smartpracticecanada.com and www.chemotechnique.se, September 2022. SSO, sorbitan sesquioleate; pet., petrolatum; CD, Chemotechnique MB Diagnostics AB, Vellinge, Sweden; SP, SmartPractice Canada, Calgary, Canada & SmartPractice Europe GmbH, Barsbüttel, Germany.

Fragrance terpenes

Terpenes are the most plentiful compounds in plants and animals, and more than 20,000 terpene structures have been reported to be used in fragrances or perfumes.[66,106] Terpene is a term used to denote volatile unsaturated hydrocarbon compounds that contain at least two units of isoprene – the basic C_5 unit (2-methyl-1,3-butadiene, C_5H_8).[106,107]

Terpenes have the chemical formula $(C_5H_8)_n$, where $n \geq 2$, and can be further classified by the number of carbons.[107] For example, terpenes containing two units of isoprene are called monoterpenes ($C_{10}H_{16}$), whereas terpenes containing three or four units of isoprene are classified as sesquiterpenes ($C_{15}H_{24}$) and diterpenes ($C_{20}H_{32}$), respectively.[107] In each group of terpenes, the isoprene units can be arranged in different ways (Figure 4a-e).

Terpenoids and isoprenoids are similar to terpenes in that they also comprise units of isoprene; however, they contain additional functional groups, usually oxygen-containing. It should be noted that the terms terpene, terpenoid and isoprenoid are used interchangeably.[108]

Figure 4a. Isoprene, C_5

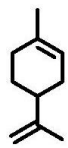


Figure 4b. Monoterpene, C_{10} (limonene – monocyclic monoterpene)

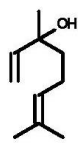


Figure 4c. Monoterpene, C_{10} (linalool – acyclic monoterpenoid)

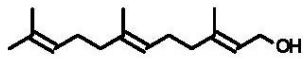


Figure 4d. Sesquiterpene, C_{15} (farnesol – terpenoid/isoprenoid)

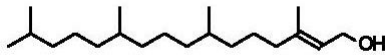


Figure 4e. Diterpene, C_{20} (phytol – terpenoid/isoprenoid)

Natural terpenes have been found to protect plants from animals and microorganisms, and can be growth inhibitors, deterrents, or toxins.[106] The benefits of terpenes to humans include their use as fragrances or flavourings, and their natural properties as herbal medicine since they have antioxidant and antimicrobial activities.[66,109,110] Monoterpenes and sesquiterpenes are among the numerous terpenes that are predominantly found in essential oils.[66] Examples of fragrance ingredients categorized as monoterpenes are menthol, linalool, and limonene, while α-bisabolol and farnesene are examples of sesquiterpenes.[107,109]

Linalool

Linalool (3,7-dimethyl-1,6-octadien-3-ol, $C_{10}H_{18}O$, Chemical Abstracts Service (CAS) no. 78-70-6) is a fragrance chemical originally found in plants such as lavender, rosewood, coriander, and jasmine.[111] It is widely used in processed food and consumer products, especially essential oils, perfumes, and cosmetics.[112] Lavender oil, an essential oil mixture of chemicals, contains mainly linalyl acetate and linalool.[113]

Linalool has two enantiomers: *R*-linalool (licareol, (-)-linalool, CAS no. 126-91-0) and *S*-linalool (coriandrol, (+)-linalool, CAS no. 126-90-9).[111,112] No allergic reactions to linalool have been observed in animal or *in vitro* tests.[114,115] Therefore, linalool is not considered to be allergenic.[114,115] Since linalool does not absorb ultraviolet radiation or visible light (wavelengths of 290 to 700 nm), it is not expected to be phototoxic or photoallergenic.[115] According to the European Commission (cosmetics regulation), products containing linalool must be labelled when it is present at greater than 10 ppm (0.001%) in leave-on products and 100 ppm (0.01%) in rinse-off products.[116]

Oxidised linalool

Linalool is an unsaturated hydrocarbon that can be oxidised when exposed to air.[111] It has been shown that the amount of linalool decreases with time when exposed to air due to autoxidation.[111,113,117] To produce air oxidation in previously published studies, non-oxidised substances have been stirred for 1 hour four times per day at room temperature.[117,118] The major oxidation products of linalool are HPs, mainly linalool-7-hydroperoxide (Lin-7-OOH) and linalool-6-hydroperoxide (Lin-6-OOH) (Figure 5), which cause contact allergy.[117]

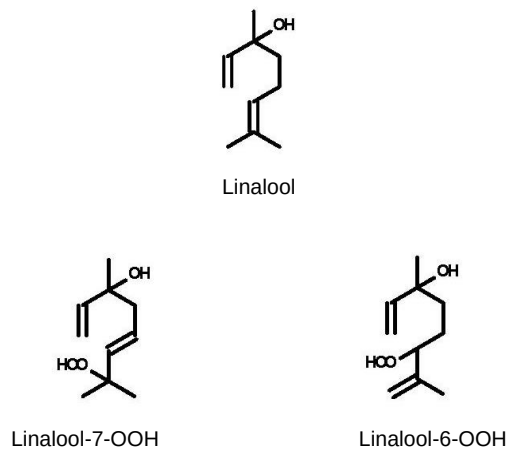


Figure 5. Chemical structures of linalool, linalool-7-hydroperoxide, and linalool-6-hydroperoxide.

The concentration of HPs of linalool has been found to increase after the exposure of lavender oil to air, and could be quantitatively detected using high-performance liquid chromatography after six weeks' exposure.[113] After exposure to air for ten weeks, oxidised linalool (82 ppm of HP) was found to sensitise guinea pig skin in a Freund's complete adjuvant test (FCAT).[111] The sensitizing potential has also been reported to increase with increasing air exposure time, according to a local lymph node assay (LLNA), which was used to estimate the concentration required to induce a stimulation index of 3 (EC3).[117] The EC3 value is the threshold for positive sensitisation; thus, the sensitizing potency is higher when EC3 is lower. The EC3 value of air-exposed linalool after 45 weeks (4.8) was found to be lower than that after 10 weeks (9.4), indicating that the allergenic potency increased over time.[117] When comparing to the oxidation process of *R*-limonene, the oxidation of linalool is slower.[111]

Patch testing

Patch testing with oxidised linalool has been performed in patients since about 2000. A large study (n=1,511) performed at six centres in Europe during 2002-2003 reported that 1.3% of the patients reacted positively to an oxidation mixture of 2.0% linalool in petrolatum, and that 1.1% reacted positively to 0.5% HPs of linalool in petrolatum, while no allergic reaction was observed when they were patch tested with 20% non-oxidised linalool in petrolatum.[119]

Patch testing was then carried out on 3418 patients in Sweden during 2005-2007 in order to identify the optimal concentration of oxidised linalool for use in patch testing of dermatitis patients.[120] The results suggested that 6.0% oxidised linalool in petrolatum, at a dose of 2.4 mg/cm^2 was appropriate.[120] The prevalence of positive patch test reactions to this dose was 5.3%.[120] It was also noted that at a higher concentration of 11.0% in petrolatum, the prevalence increased to 7.2%, while irritant reactions were rarely seen (<1%).[120]

The prevalence of positive reactions to oxidised linalool at different concentrations during different periods has varied considerably in previously published studies, from 0.8% to 20%.[121] A multicentre study, in which 2,900 consecutive dermatitis patients were patch tested with oxidised linalool at a concentration of 6.0% in petrolatum (corresponding to 1.0% HPs of linalool), was conducted in 2010-2011.[122] The prevalence of positive patch test reactions was reported to be 6.9% (range, 3-13%).[122] Doubtful reactions were reported to be more common (9.2%) than positive reactions, whereas irritant reactions were merely reported.[122] The prevalence of positive reactions to 6.0% oxidised linalool (1.0% HPs of linalool) in petrolatum has been continually reported from different clinics and countries.[94,101,123-127]

Repeated open application tests

A ROAT study over three weeks using air-oxidised linalool has previously been performed in Sweden (2010-2011) in six patients who had previously shown a positive reaction to oxidised linalool.[46] Two different kinds of preparations were used: creams (15% glyceryl stearate) and fine fragrances (96% ethanol) containing different concentrations of HPs of linalool.[46] Five of the six participants reacted to the cream which contained HPs of linalool from 560 ppm (n positive=2) to 5600 ppm (n positive =5), whereas four of them reacted positively to the fine fragrance preparations with contents of HPs of linalool of 560 ppm (n=1) and 1900 ppm (n=4), while none reacted to the lowest concentration (190 ppm).[46] The results of this study imply that oxidised linalool could elicit ACD in patients with a positive patch test reaction to 6.0% oxidised linalool in petrolatum.[46]

Chemical analysis

Chemical analysis has been performed to identify the HPs of linalool. The highest concentration of HPs of linalool detected in consumer products was about 420 ppm in an aftershave.[128]

Another study reported on the detection of HPs of linalool in hydroalcoholic and antiperspirant products, including samples recalled from consumers.[129] They detected very low amounts (14 µg/g) of HPs of linalool in aged fine fragrances.[129] It was concluded that the HPs of linalool might have originated from the raw fragrance material used in the products rather than being formed later when the products were stored. The amount of HPs was found to be much lower than the levels found to induce skin sensitisation in animal studies[129] and the amount used in the aforementioned ROAT study.[46]

Another study reported that the amount of HPs of linalool in 104 consumer products was also low compared to the sensitisation threshold determined in animal experiments.[130] In that study, the highest amount of HPs of linalool reported was 153 ppm in the same aftershave reported previously[128] (72 ppm of Lin-7-OOH and 81 ppm of Lin-6-OOH, analysed by GC-MS reduction method).[130] Therefore, the source of exposure to the HPs of linalool has yet to be elucidated, and the dose required to elicit ACD remains to be determined.

Limonene

Limonene (1-methyl-4-prop-1-en-2-ylcyclohexene, $C_{10}H_{16}$) is a terpene fragrance found in plants, especially fruits that have a pleasant lemon-like smell or citrus taste, such as lemons, oranges, and in Eucalyptus trees.[110,131] Limonene is widely used in consumer products, including essential oils extracted or cold-pressed from citrus oils, such as tangerine, grapefruit, bergamot, and peppermint.[131]

Limonene has two chemical forms: *R*-limonene ((+)-limonene, CAS no.5989-27-5), and *S*-limonene ((−)-limonene, CAS no. 5989-54-8). A racemic mixture of *R*- and *S*-limonene is known as dipentene (CAS no.138-86-3).[131] Examples of synonyms of limonene are cyclohexene, 1-methyl-4-(1methylethenyl)-, 4-Isopropenyl-1-methylcyclohexene, and p-mentha-1,8-diene.

According to the Human Health Safety Assessment, limonene has been found to be non-sensitizing and is not expected to be photoallergic or phototoxic.[132,133] As in the case of linalool, cosmetics and detergent products in the EU containing limonene must be labelled if they contain more than 10 ppm (0.001%) in leave-on products or 100 ppm (0.01%) in rinse-off products.[116]

Oxidised limonene

Air oxidation of limonene can also occur in a similar way as described for linalool. One of the early studies on air-oxidization of limonene demonstrated that the content of *R*-limonene decreased during exposure to air, and oxidised limonene was formed.[118]

It has been reported that oxidation products of *R*-limonene could be detected after five weeks of air exposure, including 1,2-limonene oxide (cis and trans forms), trans-carveol, cis-carveol, carvone, and HPs.[134] The presence of oxidised limonene substantially increased the sensitizing capacity, according to FCAT experiments and the guinea pig maximisation test (GPMT) study.[118]

The HPs, which are the primary oxidation products, have been found to be major skin sensitisers in the oxidised limonene mixture (Figure 6).[134,135] The sensitizing potencies of two major HPs of limonene, limonene-1-hydroperoxide (Lim-1-OOH) and limonene-2-hydroperoxide (Lim-2-OOH), have been studied using a modified murine LLNA, showing that Lim-1-OOH was more potent than Lim-2-OOH.[136,137] It has been suggested that the difference in radical formation of different HPs might affect the sensitizing potency.[137]

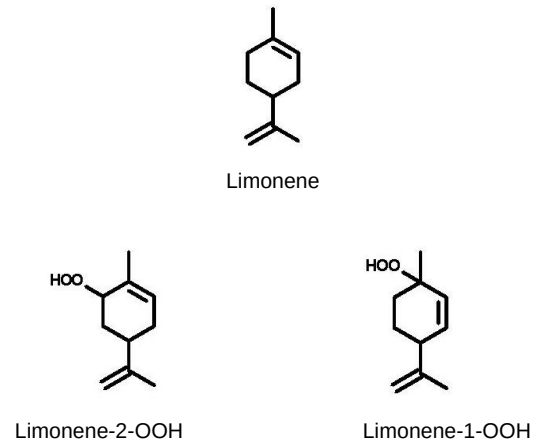


Figure 6. Chemical structures of limonene, limonene-2-hydroperoxide, and limonene-1-hydroperoxide.

It has thus been suggested that oxidised limonene test preparations that contain Lim-1-OOH, Lim-2-OOH, and carvone should be used for patch testing.[136] Oxidised linalool and oxidised *R*-limonene have been shown to be more irritant than the non-oxidised forms, and oxidised linalool has been reported to be less irritating than oxidised *R*-limonene at the same concentration.[138]

Patch testing

Patch testing with oxidised limonene was initially performed in consecutive dermatitis patients in Stockholm, Sweden (n=1318) and Leuven, Belgium (n=1482) in the early-to-mid 1990s.[134] In that study, oxidised *R*-limonene (2-5%), after air exposure for 10 and 20 weeks, and the *R*-limonene HP fraction (0.5% and 1%) were tested.[134] The prevalence of positive patch test reactions to oxidised *R*-limonene and the *R*-limonene HP fraction was found to be about 1-3% of the patients tested due to different test materials and concentrations of the test preparations used at the two centres.[134]

Later, in 1997-1999, patch testing was performed at four clinics in Europe with a 3.0% oxidised *R*-limonene mixture and a 0.5% *R*-limonene HP fraction, and the prevalence of positive reactions to any of the test preparations was reported to vary from 0.3% to 6.5% at the different clinics.[139]

A few years later, oxidised *S*-limonene was used for patch testing simultaneously with oxidised *R*-limonene, showing that not only oxidised *R*- but also oxidised *S*-limonene can cause contact allergy.[140] The overall prevalence of positive reactions to oxidised *R*- and/or oxidised *S*-limonene was reported to be 2.6%, and it was therefore suggested that patch testing with oxidised limonene at a concentration of 3% in petrolatum be used in dermatitis patients.[140]

In a multicentre study involving 2900 consecutive dermatitis patients in different continents, 3.0% oxidised *R*-limonene in petrolatum (corresponding to 0.33% HPs of limonene) was found to give positive patch test reactions in 5.2% of the patients.[141] As in the case of many patch-testing studies on oxidised linalool, doubtful reactions were commonly reported (7.0%), whereas irritant reactions were rarely seen (<1%).[141]

In concordance with the experimental studies, Lim-1-OOH elicited more positive patch test reactions than Lim-2-OOH when using the same concentration (0.5%).[142]

The prevalence of oxidised limonene contact allergy has been continually reported, and almost all studies report the prevalence together with oxidised linalool contact allergy.[94,101,124-127] The prevalence of contact allergy to oxidised limonene appears to have increased over time.[121] It has been suggested that the simultaneous positive reactions to HPs of linalool and HPs of limonene are caused by co-sensitisation, since no cross-reactions have been demonstrated in animal experiments.[143]

Repeated open application test

A ROAT study using oxidised limonene was conducted in Denmark and Sweden during 2017-2018.[44] Unlike the ROAT study of oxidised linalool[46], the ROAT preparations were prepared only in 80% ethanol solution.[44] The concentrations used were 140, 420, and 1,260 ppm HPs of limonene (doses of 0.33, 0.99, and 3.0 $\mu g/cm^2$).[44] All positive patch test patients (n=11) reacted positively to the highest concentration of the ROAT solution, whereas three patients (27%) reacted to the lowest concentration after a 3-week application period.[44] Two of 13 patients (15%) with doubtful patch test reactions also showed a positive reaction to the solution at the highest concentration.[44]

Chemical analysis

Gas chromatography-mass spectrometry (GC-MS) of consumer products showed that a shower oil contained 262 ppm of Lim-1-OOH and 141 ppm of Lim-2-OOH, whereas an Eau de Toilette contained Lim-1-OOH at 91 ppm and Lim-2-OOH at 36 ppm.[130] This is extremely low compared to the LLNA dose required to induce sensitisation (EC3) and the dose used in patch testing.[130]

Of 104 consumer products analysed, only four products contained more than 50 ppm (reporting limit) of the HPs, including Lin-6-OOH, Lin-7-OOH, Lim-1-OOH, and Lim-2-OOH.[130]

Rationale & Knowledge Gap

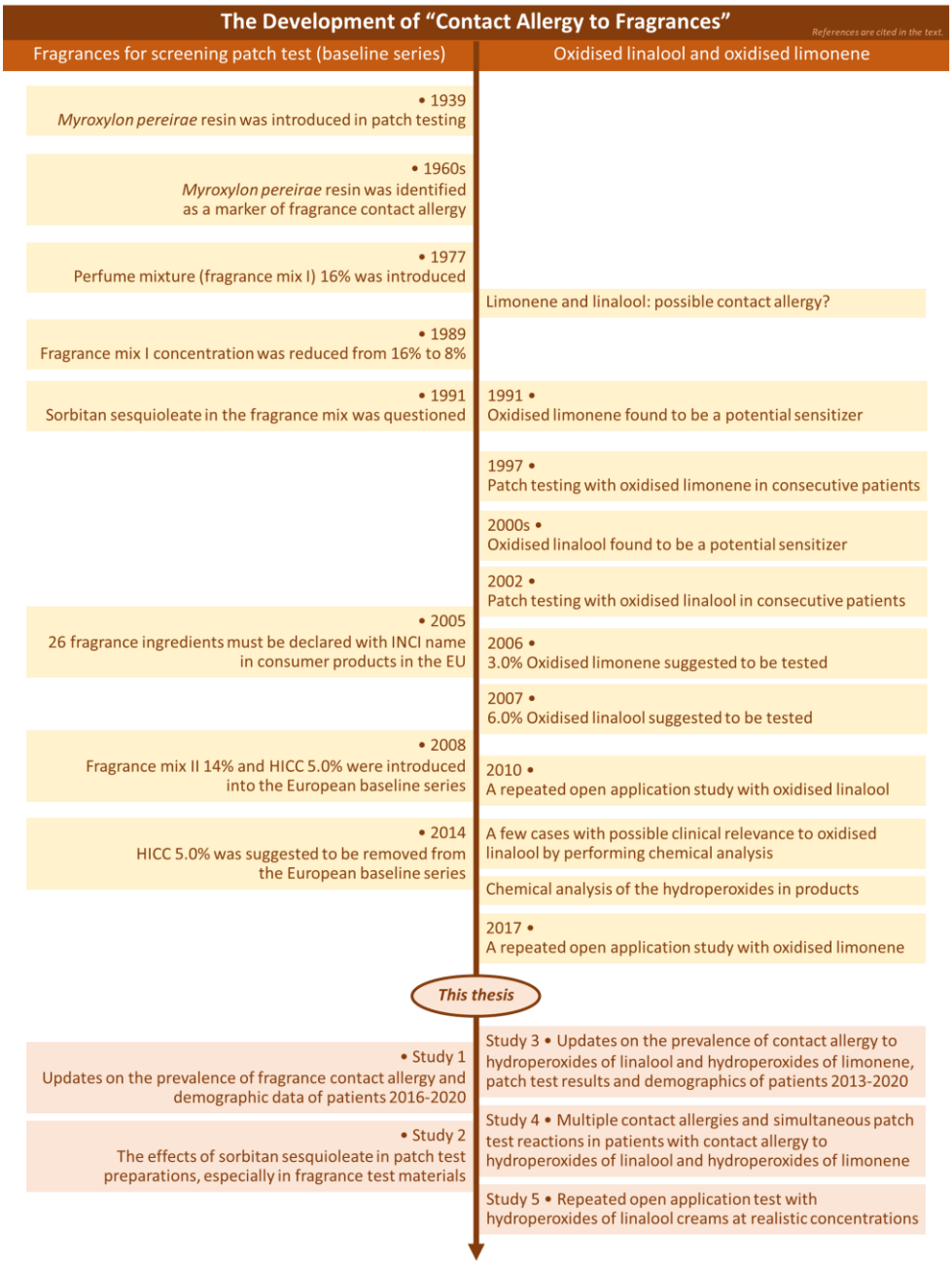
Fragrance contact allergy is common in dermatitis patients and may be overlooked in the general population. Although the European Commission has introduced legislation regarding the use of fragrances in cosmetics and household products, fragrance contact allergy rates have been shown to be similar or increasing during recent decades. Patch testing with fragrance allergens has been gradually improved and standardised over the past 50 years.

Linalool and limonene are the two most common fragrances used in cosmetic products on the European market, which have been labelled in about 30% of cosmetic products.[144] The role of linalool, limonene, and their oxidised products in contact allergy has been investigated from many perspectives, both *in vitro* and *in vivo*. Nevertheless, the reported results have led to further questions.

Some of the questions raised by the results from previous studies have been addressed in the work presented in this thesis.

1. Performing patch tests with fragrance mixes and interpreting the results remain complicated. Patch testing with the mixes can be not only false-negative but also false-positive from SSO in the mix. Many studies have recommended that dermatologists test patients with additional fragrance materials, mainly the individual ingredients of the mixes, since fragrance mixes are inefficient in screening fragrance contact allergies. However, patch testing with many individual fragrance allergens is impractical.
2. At the present time (2023), HPs of linalool and HPs of limonene are considered to be included in some baseline series but not in the European or Swedish baseline series. The rates of contact allergies to non-mix fragrances, mostly HPs of linalool and HPs of limonene, have increased over time. However, the clinical relevance of positive reactions to these HPs remains complicated. HPs in consumer products have rarely been reported in general clinical practice since chemical analysis of HPs is seldom performed. The concentrations of HPs, especially those of linalool recently reported in consumer products, have not been demonstrated to elicit dermatitis in patients.

A history timeline of fragrance contact allergy



HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde

Overall aim of this thesis

Prevention is the best cure for ACD. Identifying the allergens causing ACD is the key in preventing the exposure of patients to allergens. Since patch testing is the standard procedure for diagnosing contact allergy, which is the first step in the diagnosis of ACD, the main aim of this work was to improve the diagnostic patch test system for fragrance contact allergy. Recent trends concerning screening with fragrance contact allergies using fragrance allergen mixes and their ingredients, and HPs of linalool and HPs of limonene were investigated, with the aim of answering the following questions. *What and who should be tested? What have we missed? What could affect the patch test results?* The last but crucial part of this research focused on oxidised terpenes and their clinical relevance in contact allergy.

Study 1

The purpose of this study was to obtain up-to-date information on the prevalence and trends of contact allergy to fragrances from 2016 to 2020 in southern Sweden. Factors associated with fragrance contact allergy were identified, and the benefits and drawbacks of patch testing with individual FM ingredients were analysed.

Study 2

The aim of this study was to investigate the current prevalence of SSO sensitisation in consecutive dermatitis patients and concomitant patch test reactions to fragrance and non-fragrance test preparations containing SSO, in order to determine whether SSO could be useful for patch testing or affect patch test interpretation.

Study 3

This study was performed to determine the prevalence and trends regarding contact allergy to HPs of linalool and HPs of limonene in patients with dermatitis. The features of patch test reactions and clinical characteristics of the patients are reported.

Study 4

This study was carried out to investigate the patterns of simultaneous positive patch test reactions and the prevalence of multiple contact allergies in patients with contact allergies to HPs of linalool and/or HPs of limonene.

Study 5

In the final study, ROATs were performed using high but realistic concentrations of HPs of linalool in patients with a history of contact allergy to HPs of linalool, using dermatitis patients without contact allergy to HPs of linalool as controls.

General methodology

Patch testing

Patients attending the clinic are generally tested with the allergens included in the Swedish baseline series and the Malmö extended baseline series (Table 3). Fragrance allergens in the Swedish baseline series during the study period always comprised BOP (25.0% in pet.), FM I (8.0% in pet.), FM II (14.0% in pet.), and lichen acid mix (0.3% in pet.). The Malmö extended baseline series contained additional patch test preparations, in which allergens may be introduced or removed over time.

Table 3. A list of allergens in the Swedish baseline series and the fragrance allergens in the Malmö extended baseline series used in patch testing at the department during the study period (2013-2020).

Allergen	Preparation (%) in vehicle
Swedish baseline series	
Potassium dichromate	0.5 in pet.
para-phenylenediamine	1.0 in pet.
Thiuram mix	1.0 in pet.
Neomycin sulphate	20.0 in pet.
Cobalt(II) chloride hexahydrate	0.5 in pet.
Quaternium-15	1.0 in pet.
Nickel(II) sulphate hexahydrate	5.0 in pet.
Quinoline mix	6.0 in pet.
Colophonium	20.0 in pet.
Paraben mix	16.0 in pet.
Black rubber mix	0.6 in pet.
Sesquiterpene lactone mix	0.1 in pet.
Mercapto mix	2.0 in pet.
Fragrance mix II	14.0 in pet.
Epoxy resin	1.0 in pet.
Myroxylon pereirae resin	25.0 in pet.
4-tert-butylphenolformaldehyde resin	1.0 in pet.
Formaldehyde	2.0 in aq.

Allergen	Preparation (%) in vehicle
Swedish baseline series	
Fragrance mix I	8.0 in pet.
Phenol formaldehyde resin (PFR2)	1.0 in pet.
Diazolidinyl urea	2.0 in aq.
Methylchloroisothiazolinone/methylisothiazolinone	0.02 in aq.
Amerchol L-101	50.0 in pet.
Caine mix II	10.0 in pet
Lichen acid mix	0.3 in pet.
Tixocortal-21-pivalate	0.1 in pet.
Textile dye mix	6.6 in pet.
Budesonide	0.01 in pet.
Methyldibromo glutaronitrile	0.5 in pet.
Methylisothiazolinone	0.2 in aq.
Fragrance allergens in the Malmö extended baseline series	
Fragrance mix I ingredients	
Amyl cinnamal	2.0 in pet.
Cinnamal	1.0 in pet.
Cinnamyl alcohol	2.0 in pet.
Eugenol	2.0 in pet.
Evernia prunastri extract	2.0 in pet.
Geraniol	2.0 in pet.
Hydroxycitronellal	2.0 in pet.
Isoeugenol	2.0 in pet.
Sorbitan sesquioleate*	20.0 in pet.
Fragrance mix II ingredients	
Citral	2.0 in pet.
Citronellol	1.0 in pet.
Coumarin	5.0 in pet.
Farnesol	5.0 in pet.
Hexyl cinnamic aldehyde	10.0 in pet.
Hydroxyisohexyl 3-cyclohexene carboxaldehyde	5.0 in pet.
Hydroperoxides of linalool	1.0 in pet.
Hydroperoxides of limonene	0.3 in pet.
Carvone**	5.0 in pet.

aq., aqua; pet., petrolatum.

*as an emulsifier in fragrance mix I; **5.0% Carvone was introduced into the extended baseline series in 2017 and was excluded from data analysis in the studies presented in this thesis.

Patch tests at the clinic are conducted according to the guidelines of the International Contact Dermatitis Research Group and the European Society of Contact Dermatitis.[4,19] The allergens were purchased from Chemotechnique MB Diagnostics AB, Vellinge, Sweden.

Before 2018, 8-mm aluminium Finn Chambers were used in consecutive patch test patients, prepared by applying 20 mg (40 mg/cm^2) of petrolatum preparations or 15 μL of aqueous solutions. Finn Chambers Aqua (8-mm) were introduced in consecutive patients from January 2018, using the same doses of allergens as for Finn Chambers. Both chamber types were purchased from SmartPractice, Phoenix, AZ, USA.

I.Q. chambers (I.Q. Ultra and I.Q. Ultimate) purchased from Chemotechnique MB Diagnostics AB, Vellinge, Sweden, might be used instead of the Finn Chambers and Finn Chambers Aqua when patch tests were performed in branched clinics, such as in Halmstad and Växjö, Sweden. In these cases, 25 mg (39.06 mg/cm^2) of allergens in petrolatum preparations, or 20 μL of aqueous solutions, were applied. For fragrance allergens, the chambers are applied on the patient's back immediately after loading.

The chambers are left on the upper back for two days (D0 to D2), and patch-test reading is performed on D3 or D4, and on D7. The intensity of the reaction is evaluated as negative, doubtful, weakly positive (1+), strongly positive (2+), extremely positive (3+), or irritant. Any positive reaction on D3/4 or D7 is classified as a positive reaction, whereas negative, doubtful, and irritant reactions are considered negative in most statistical analyses. The highest reaction intensities are reported and analysed when combining two readings.

Statistical analysis

PASW Statistics for Windows (version 23.0; SPSS Inc., Chicago, Ill., USA) was used for statistical analysis. A descriptive analysis was used to report the numbers and percentages of clinical data and the prevalence of positive reactions to allergens in the first four studies in which data were analysed retrospectively. The Clopper–Pearson (exact) interval was used to report 95% confidence intervals for the prevalence. A two-sided Pearson chi-squared test or Fisher's exact test was performed to compare the proportion of patients in groups of patients in the studies. Continuous data (mean age differences) between the two groups were analysed using the independent t-test. Multiple logistic regression was used to examine the impact of factors associated with contact allergy, depending on the aim of each study. When *P*-values were reported, a *P*-value of less than or equal to .05 was considered to indicate statistical significance. Odds ratios were calculated to quantify the strength of the association between two events. Spearman's correlation was used to investigate associations between the intensity of reactions.

Study design

Retrospective studies (Studies 1-4)

The first four retrospective studies were performed using the electronic database (EKTA and DALUK[145] local database systems, obtained via QlikView, Pennsylvania, USA) at the Department of Occupational and Environmental Dermatology, Skåne University Hospital, Malmö, Sweden. The database contains the demographics of the patients (age, gender, history of atopy, localization of the rash, occupation), patch test series used, patch test results, and the dates on which patch tests were performed. All retrospective studies were approved by The Swedish Ethical Review Authority (Ethical Approval Number 2020-02190).

PART 1: Fragrance mixes: Studies 1 & 2

This part of the project was conducted to analyse data from patients tested with screening fragrance allergens and the ingredients of fragrance mixes from 2016 to 2020. A total of 3663 patients aged over 18 were registered in the database during this period. Patients who were not tested, or in whom patch test readings were not completed for the baseline series or individual ingredients of the fragrance mixes, were excluded. This resulted in data from 3539 patients being included in the analysis.

Study 1

The prevalence of and factors associated with contact allergy to fragrances were reported. Multiple logistic regression was then performed to identify significant factors related to fragrance contact allergy.

Comparisons were made as follows: 1) the number of patients with positive and negative reactions to fragrance mixes who had a positive reaction to individual FM ingredients, and 2) patch-test reactions to fragrance mixes between “weak” and “strong to extreme” reactions to the individual ingredients.

Study 2

The prevalence of, and demographics of patients with SSO contact allergy were analysed. The proportions of patients with different intensities of positive reactions to SSO were compared between patients ‘with’ vs ‘without’ a history of atopic dermatitis and ‘with’ vs ‘without’ simultaneous positive reactions to either FMI, BOP or Oakmoss extract. The proportion of doubtful and negative reactions to SSO in atopic vs non-atopic dermatitis patients was compared.

PART 2: Oxidised terpenes: Studies 3 & 4

These studies were carried out to investigate mainly contact allergies to HPs of linalool and HPs of limonene. Patients (n=5911) referred for patch testing due to dermatitis between 2013 and 2020 at the clinic were included.

Study 3

In this study, patients not tested with any of the following fragrance test preparations: FM I, FM II, BOP in the Swedish baseline series (except lichen acid mix), individual ingredients of the fragrance mixes, HPs of linalool, and HPs of limonene, or in whom patch test readings were not completed on both occasions, were excluded.

The terms used to describe various groups of patients in this study are as follows.

- Fragrance allergy patients: those who reacted to at least one fragrance allergen in the baseline series, FM I and FM II constituents, HPs of linalool, or HPs of limonene.
- Oxidised terpene allergy patients: those who showed at least one positive patch-test reaction to HPs of linalool and/or HPs of limonene.
- Exclusively oxidised linalool allergy patients: those who reacted positively to the HPs of linalool but no other fragrance allergy markers.
- Exclusively oxidised limonene allergy patients: those who reacted positively to the HPs of limonene but no other fragrances.

Trends of positive reactions and details of patch testing results were reported. The following comparisons were made in this study:

1) Demographics of patients with positive reactions to oxidised linalool and oxidised limonene vs negative reactions
2) Patients with exclusively positive reactions to oxidised linalool and oxidised limonene vs other patients with fragrance allergy
3) Patients in the oxidised terpene allergy group who had weak positive reactions vs those with strong to extreme positive reactions, and trends of reactions.

Possible associations between the intensity of reactions to oxidised linalool and oxidised limonene were investigated.

Study 4

In this study, only patients tested with all fragrance test preparations mentioned above in Study 3 and all other allergens in the Swedish baseline series were included. Since the textile dye mix was introduced into the Swedish baseline series in 2015, patients tested in 2013-2014 were also excluded.

Patients with oxidised terpene allergy were categorized as follows.

- Exclusively positive to HPs of linalool: those having a positive reaction to HPs of linalool but a negative reaction to HPs of limonene
- Exclusively positive to HPs of limonene: those having a positive reaction to HPs of limonene but a negative reaction to HPs of linalool
- Those having positive reactions to both HPs of linalool and HPs of limonene

"Multiple contact allergies" is the term used to refer to patients having three or more positive reactions to the allergens in the baseline series, HPs of linalool, and HPs of limonene. Patients with an allergy to formaldehyde and its releasers (diazolidinyl urea, quaternium-15) were considered to have one contact allergy, and those showing an allergy to isothiazolinones (methylchloroisothiazolinones, MCI; methylisothiazolinones, MI) were also classified as having one contact allergy in the statistical analysis.

The following comparisons were made in this study:

1) Demographic characteristics of patients with and without multiple contact allergies
2) The proportions of patients with and without multiple contact allergies in patients with exclusive contact allergy to either HPs of linalool or HPs of limonene
3) The proportions of patients having simultaneous positive reactions to other allergens between the three patient groups (exclusively positive to HPs of linalool, exclusively positive to HPs of limonene, and positive to both)

For simultaneous positive reactions, the proportions of patients with positive and non-positive reactions to the allergens tested were calculated in the three groups of patients. Odds ratios with 95% confidence intervals are reported.

A clinical study (Study 5)

Patients and study design

This study was a double-blind, controlled prospective clinical study carried out from March to May 2023. Patients over 18 years who had been patch tested with the Swedish baseline and extended baseline series were invited to participate. The study was approved by the Regional Ethics Review Board, Lund, Sweden (Approval No. 2019-04327).

A total of 48 previously patch-tested participants thus participated in the study. Initially, 30 patients who previously had a weak, strong, or extreme positive reaction to 1.0% HPs of linalool, constituted the contact allergy group. Another 18 age- and gender-matched patients, showing a negative reaction to all substances in the baseline and extended baseline series, including 1.0% HPs of linalool, were classified as the control group.

Before signing the informed consent, all patients were informed and allowed to ask questions about the background, aim, and procedures of the study (repeated patch testing and ROAT). The study design is illustrated in Figure 7.

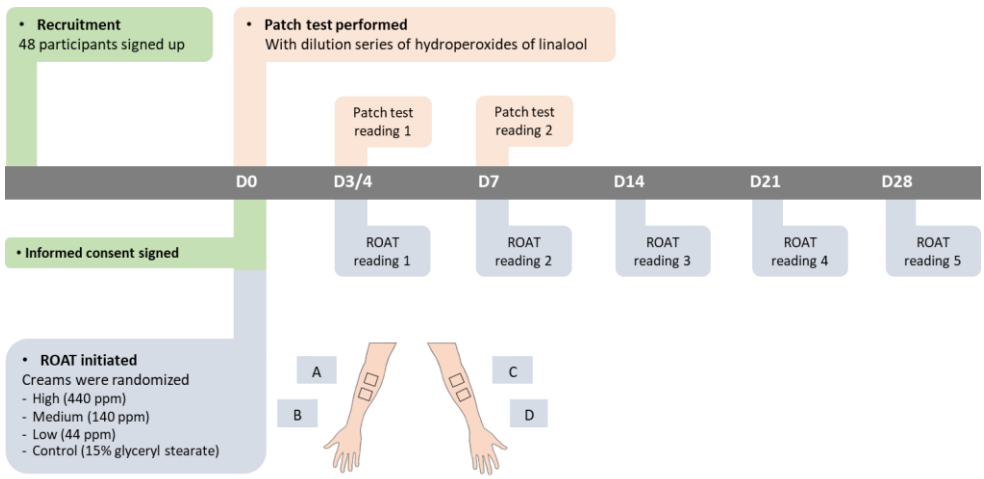


Figure 7. Study design. Illustration: Thanisorn Sukakul & Kajsa Davidson Källberg.

Patch testing with a serial dilutions of HPs of linalool in petrolatum, 100% petrolatum, and ROAT creams were initiated on the same day (D0) with ROATs (Figure 8). The concentrations of serial patch test dilutions of HPs of linalool were 1.0%, 0.32%, 0.1%, 0.032%, 0.01%, 0.0032%, 0.001%, 0.00032%, and 0.0001% w/w in petrolatum.

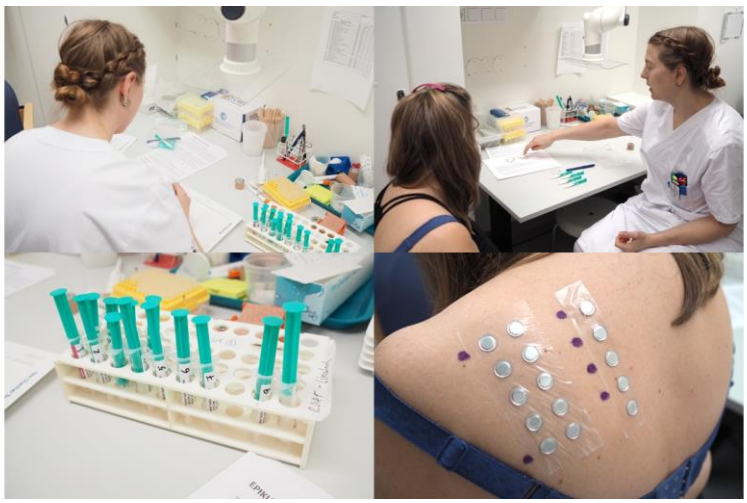


Figure 8. Patch testing with dilution series of hydroperoxides of linalool, repeated open application test creams, and 100% petrolatum.

According to the previous and recent patch test results, participants who had tested positive in the initial patch test were still included in the contact allergy group even if their reactions at retest were scored as doubtful or negative. If a patient reacted negatively to the initial patch test but reacted positively when retested, the patient would be allocated from the control group to the contact allergy group.

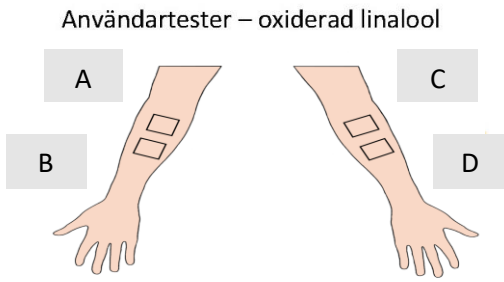
Repeated open application test

A 15% glyceryl stearate cream was used as the cream base and as a negative control. The linalool HP creams were prepared using 15% w/w glyceryl stearate in water. The final concentrations in creams were 440, 140, and 44 ppm HPs of linalool, representing the range of HPs of linalool reported previously in consumer products.[128,129,146,147]

The creams were analysed twice using GC-MS within a few days after preparation and 4-6 weeks after storage at +4 °C in 2-mL plastic syringes.

The ROAT was started on D0. The test syringes labelled A, B, C, and D were randomized using the Latin square method. The participants applied the cream to four areas on the flexural side of the forearms twice daily for four weeks (Figure 9, 10).

Figure 9. A repeated open application test procedure on Day 0.



1. Tvätta händerna med tvål.
2. Öppna spetsarna på sprutorna i den ordning som visas ovan.
3. Observera markeringarna på armarna och lägga plastrutan på huden.
4. Applicera den första krämen på huden i området med längden som visas på plastrutan.
5. Sprid krämen försiktigt på det fyrkantiga området med en fingerspets. Gnugga eller repa inte.
6. Gör samma steg för de andra 3 områdena. Tvätta gärna händerna!
7. Låt krämen absorbera och torka på huden i ca några minuter innan du bär en skjorta med långa armar.
8. Stäng spetsarna på sprutorna och förvara dem i kylen.
9. Tvätta händerna med tvål.

OBS!

- När du smörjer krämer, använd **INTE** samma finger.
- Var försiktig så att du inte applicerar fel kräm på fel område.
- Du får **INTE** tvätta underarmarna med tvål eller applicera andra krämer, fuktighetskrämer eller spraya dofter på testområdena.

Figure 10. Patient's information guide in Swedish by Thanisorn Sukakul, Anna Kiuru & Kajsa Davidson Källberg.

The participants were instructed to apply a 7–8 mm long string of cream from the syringes, corresponding to approximately 2.0 mg/cm^2, within the square areas marked on the patient's arm (3x3 cm^2). The amount applied was based on previous repeated open application test studies with cream test preparations and finger-tip unit measures (Table 4).

Table 4. The amount of creams applied in this study, previous repeated open application test studies and finger-tip unit measures.

Study	Cream (amount/area)	Amount mg/cm^2/application	Amount mg/cm^2/day
Study 5: HPs of linalool (44, 140, 440 ppm creams)	7–8 mm long string, twice daily over 3x3 cm^2	2.0	4.0
Bjorkman A, et al. Air-oxidised linalool elicits eczema in allergic patients - a repeated open application test study.[46]	0.1 mL twice daily over 10.2 cm^2	9.8	19.6
Hannuksela M, et al. The repeated open application test.[26]	0.1 mL twice daily over 5x5 cm^2	4.0	8.0
Isaksson M, et al. Repeated open application tests with methyldibromoglutaronitrile in dermatitis patients with and without hypersensitivity to methyldibromoglutaronitrile.[51]	0.5 cm long string twice daily over 5x5 cm^2	1.4	2.8
Isaksson M, et al. Repeated open application test with methylisothiazolinone in individuals sensitive to methylchloroisothiazolinone/ methylisothiazolinone.[29]	~50% of the area of the fifth fingernail over 5x5 cm^2	3.0	6.0
Uldahl A, et al. Clinical relevance of positive patch test reactions to lanolin: A ROAT study.[42]	7–8 mm long string 3 times per day over 3x3 cm^2	2.0	6.0
Long CC, et al. The finger-tip unit–a new practical measure.[148]	Fingertip unit (0.5 g over 286 cm^2)	1.75	3.5

The reactions on the forearms were read five times: on D3 or D4, and D7, D14, D21, and D28. The reactions were evaluated based on the morphology. Positive ROAT denotes the presence of erythema and infiltration in the same area, and possibly papules and vesicles in the affected area, covering at least 25% of the application area.[4,31]

Preparing test materials and chemical analysis of the test preparations

Test materials for patch tests and ROATs were prepared in-house at the department from the original substances, 1.0% HPs of linalool syringes, purchased from Chemotechnique Diagnostics, Vellinge, Sweden. Figure 11 demonstrates the preparation processes of the creams for ROATs.

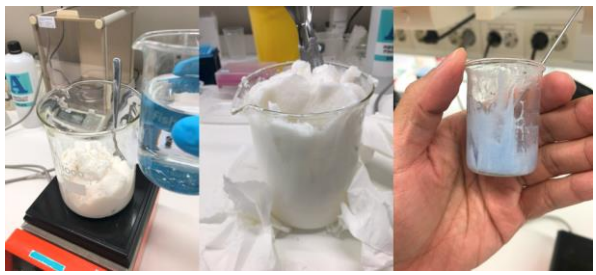


Figure 11a. Step 1) Cream base preparation: mix glyceryl stearate SE and water (final concentration: 15% glyceryl stearate w/w) & test for equal distribution of additional substance by mixing the cream with textile dye mix test preparation in petrolatum to observe whether the color can be blended.

Figure 11b. Step 2) Mix the cream base with petrolatum and the different amounts of hydroperoxides of linalool in petrolatum from patch test syringes for the final required amounts of the hydroperoxides: 44, 140, and 440 ppm; and the cream base with pure petrolatum as control.

Figure 11c. Step 3) Prepare the syringes and labelling. Cream preparations are filled in 2-ml plastic syringes. The syringes are labelled and stored at +4˚C.

Chemical analysis method for linalool hydroperoxides detection

In Study 5, the purchased patch test syringes and the creams prepared for the ROATs were quantitatively analysed. The GC-MS detection method in this study was performed as described in 'Standard operating procedure: HP detection in consumer products by the reduction-GC-MS method'.[149,150] The main HPs of linalool, Lin-7-OOH and Lin-6-OOH, are reduced to alcohols, using triphenylphosphine as a reducing agent. The alcohols are then detected and quantified by GC-MS. This method can be used to analyse hydroalcoholic fine fragrances and complex products, such as creams, lotions and deodorants.

The preparations were analysed with Method B: 'Reduction and extraction of HPs from complex consumer product bases with Extrelut NT method' as all the preparations were in complex forms (petrolatum and cream).

Figure 12. Preparing the repeated open application test creams for chemical analysis by using gas chromatography-mass spectrometry.

The analyses were performed on a GC-MS system consisting of an Agilent 8890 GC coupled to an Agilent 7250 GC/Q-TOF MS controlled by Mass Hunter 10.0 software (Agilent Technologies, Santa Clara, California, USA). The GC was equipped with a VF-WAXms capillary column (Agilent Technologies) with a length of 30 m, an internal diameter of 0.25 mm, and a film thickness of 0.25 µm. The carrier gas was helium (Air Liquide, Malmö, Sweden) at a flow rate of 1.2 mL/min. The injection volume was 1 µL, and the split ratio was 1:10.

The inlet temperature was 230 °C. The temperature programme of the column oven was as follows: isothermal at 50 °C for 2 minutes, raised by 2.5 °C/min to 160 °C, and thereafter raised by 20 °C/min, a final temperature of 240 °C. The transfer line temperature was 200 °C, the ion source temperature was 200 °C, and the quadrupole temperature was 150 °C. The electron energy was 70 eV. Electron-ionization mass spectra were collected in scan mode, recording ions with m/z from 25–500 with a spectral acquisition rate of 5 Hz.

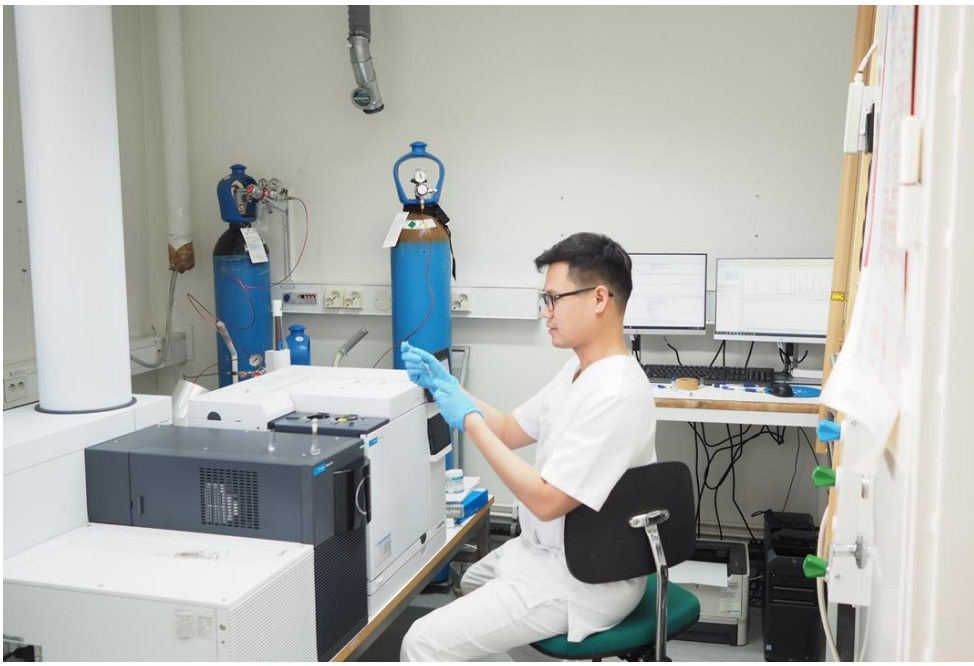


Figure 13. Chemical analysis of the preparations using gas chromatography-mass spectrometry. Photo: Anna Kiuru.

Calibration row samples for quantification were prepared from dilutions of Lin-7-OOH and Lin-6-OOH (Green Pharma S.A.S, Orléans, France), which were subjected to the same reduction procedure using triphenylphosphine (Sigma-Aldrich, Steinheim, Germany). Methyl caprylate (Sigma-Aldrich) was used as an internal standard.

Results

Study 1. Patch testing with fragrances in the baseline series and individual ingredients of the fragrance mixes

Of the 3539 patients included in the study, 2436 (68.8%) were female. The mean age was 44.4 ± 17.0 years, and 27.6% of patients had a history of atopic dermatitis. Common sites of lesions were the hands (33.3%) and face (21.5%).

Contact allergy to fragrances was diagnosed in 464 patients (13.1%), of which 48 patients (10.3%) reacted positively only to the ingredients of fragrance mixes in the extended baseline series but not the fragrance allergens in the baseline series (Figure 14). Being aged ≥ 40 years and having a history of atopic dermatitis were significant factors associated with fragrance contact allergy (Table 5).

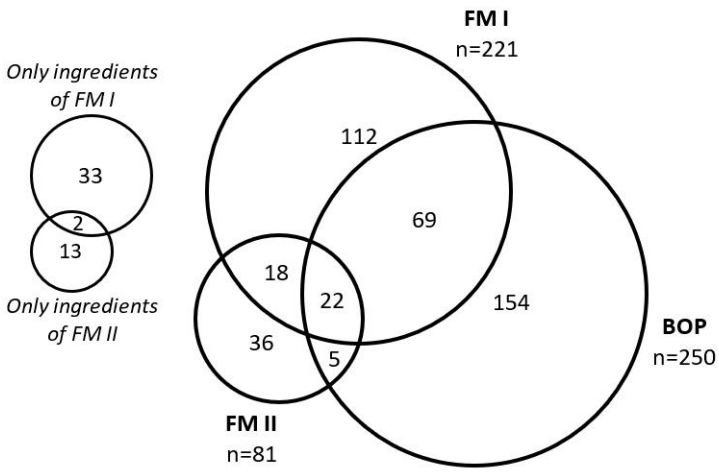


Figure 14. Number of patients with a positive patch test reaction(s) to fragrances.

Table 5. Prevalence and factors associated with fragrance contact allergy.

	Prevalence	Significant associated factors
Fragrance contact allergy	13.1%	Age group ≥40, atopic dermatitis
Fragrance mix I	6.2%	Age group ≥40, atopic dermatitis, female
Fragrance mix II	2.3%	Higher mean age
Myroxylon pereirae resin	7.1%	Age group ≥40
Only the ingredients of fragrance mix I and/or II	1.4%	Not applicable

Positive reactions to the ingredients of FM I and FM II were seen in 54.3% and 55.6% of the FM I and FM II contact allergy patients, respectively. Patients with a higher number of positive reactions to the individual ingredients of a mix were more likely to react positively and have a stronger positive reaction to their mixes. Patients with a stronger reaction to the ingredients of the mixes had a significantly higher probability of having a positive reaction to their mix (Table 6).

Table 6. Patients with positive reactions to fragrance mix I, fragrance mix II, and their individual ingredients.

	Positive to Fragrance mix I	Positive to Fragrance mix II
% positive to individual ingredients	54.3%	55.6%
Higher number of positive reactions to the individual ingredients	Reacted positively to their mix Stronger positive reaction to their mix	
A stronger reaction to the individual ingredients	Higher probability of having a positive reaction to the mix	

Study 2. Sorbitan sesquioleate and patch testing

Seventeen out of the 3539 consecutive dermatitis patients were found to have SSO contact allergy (0.48%). Seven of these 17 patients (41.2%) had a history of atopic dermatitis.

Doubtful reactions were reported in 46 patients (1.3%), and 45.7% of these had a history of atopy. Patients with a doubtful or weak positive reaction were significantly associated with having a history of atopy.

Simultaneous positive reactions to SSO and any of the mixes (FM I, BOP or Oakmoss extract) were significantly more common in patients with strong or extreme patch test reactions than in those with weak reactions to SSO (*P*-value = 0.018).

Since all individual ingredients of the mixes of BOP and Oakmoss extract had not been tested, fragrance contact allergy could not be ruled out when patients had simultaneous positive reactions to both SSO and FM I and/or BOP and/or Oakmoss extract (Figure 15).

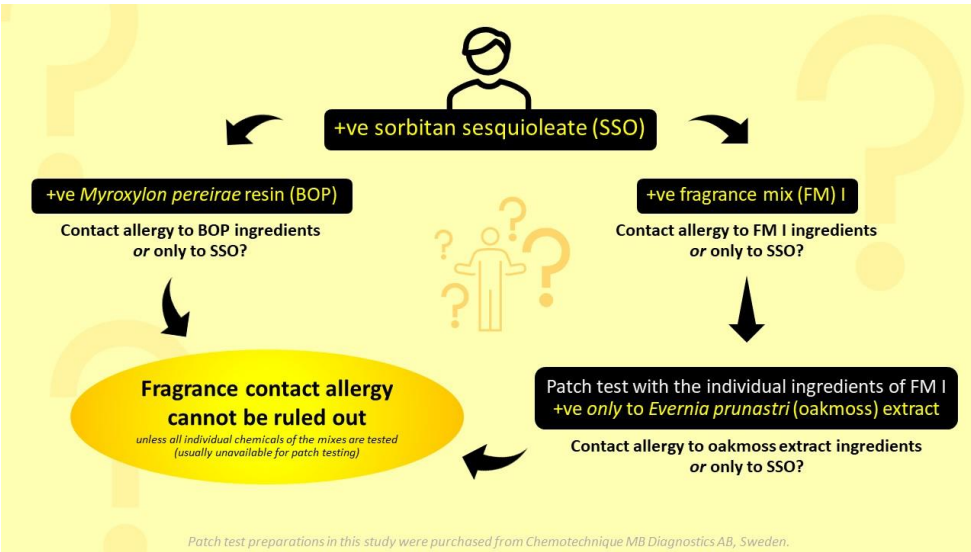


Figure 15. Interpretation of patch test reactions in sorbitan sesquioleate contact allergy patients.

Study 3. Prevalence of contact allergy to hydroperoxides of linalool and hydroperoxides of limonene

Of the 5773 patients in this study, 68.0% were female, and 24.2% had a history of atopic dermatitis. The mean age was 44.6 ± 17.0 years. The main sites of lesions were the hands and fingers (30.3%) and the face (18.0%).

The prevalence of contact allergy to the oxidised terpenes was 9.4%, which 403 (7.0%) reacted positively to HPs of linalool, 296 (5.1%) reacted positively to HPs of limonene, and 156 had simultaneous positive reactions to both (Figure 16).

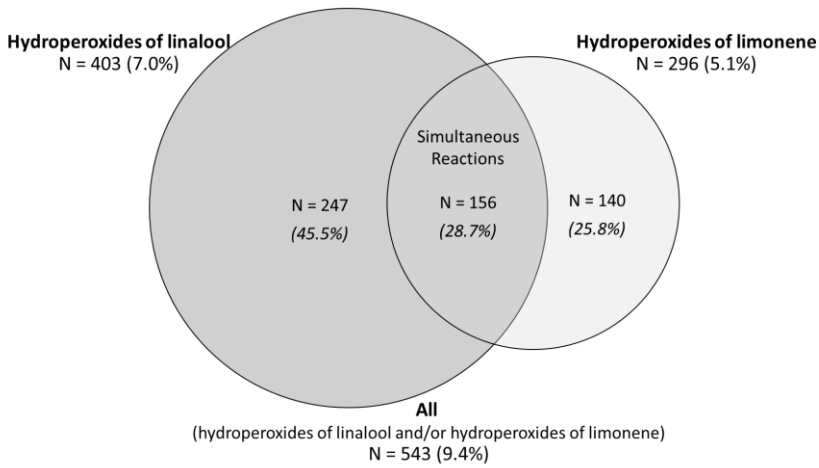


Figure 16. Number of patients with contact allergy to hydroperoxides of linalool and/or hydroperoxides of limonene.

The trends of positive reactions to HPs of linalool and HPs of limonene significantly increased during the study period, mainly the weak positive reactions, but not for strong to extreme positive reactions. Most of the positive reactions were reported on D3/4. Doubtful reactions reported on D3/4 (n=569 for HPs of linalool, 435 for HPs of limonene) became mostly negative on D7, and 1.4% and 1.6% of them became positive for HPs of linalool and HPs of limonene, respectively, on D7. Only 0.1% of negative patch test patients on D3/4 reacted positively on D7.

Patients who had an exclusively positive reaction to HPs of linalool or HPs of limonene were significantly younger than those with fragrance allergies. Patients in the older age group (≥40 y) were significantly associated with strong to extreme patch test reactions to HPs of linalool and HPs of limonene.

Study 4. Simultaneous contact allergies in patients with contact allergy to hydroperoxides of linalool and/or hydroperoxides of limonene

Following Study 3, patients who did not complete the test with all allergens in the baseline series were further excluded. Overall, 4192 patients (68.2% female) were included in the analysis.

Of these, 44.2% (n=1851) had at least one positive reaction to the allergens tested in the baseline series, HPs of linalool and/or HPs of limonene. The prevalence of contact allergy to the oxidised terpenes was 10.2% (n=428: 185 reacted positively to HPs of linalool alone, 114 reacted positively to HPs of limonene alone and 129 to both).

The prevalence of multiple contact allergies was 9.8%. The significant factor associated with multiple contact allergies was higher age. Patients with an exclusively positive patch test reaction to HPs of linalool had a significantly higher likelihood of having multiple contact allergies. The number of simultaneous positive reactions increased significantly with age.

Patients with an exclusively positive reaction to HPs of linalool showed a higher total number of allergens with a significant simultaneous positive reaction compared with HPs of limonene (22 vs. 9). Patients with reactions to both HPs of linalool and HPs of limonene were shown to have multiple significant simultaneous positive reactions to several fragrances and cosmetic-related allergens (n=29). Table 7 lists the allergens showing patterns of simultaneous positive reactions between patient groups.

Table 7. Patterns of simultaneous positive reactions between patient groups.

	Patient group		
	Exclusively positive to HPs of linalool	Exclusively positive to HPs of limonene	Positive to both HPs of linalool and HPs of limonene
Number of patients	185	114	129
Significantly have multiple contact allergies*	Yes	No	Not applicable
Number of significant simultaneous reactions**	22	9	29

	Patient group		
	Exclusively positive to HPs of linalool	Exclusively positive to HPs of limonene	Positive to both HPs of linalool and HPs of limonene
	Significant simultaneous positive reactions		
Fragrance allergens	BOP Lichen acid mix Fragrance mix I Fragrance mix II Eugenol Evernia prunastri Hydroxycitronellal Isoeugenol Citral Citronellol HICC	BOP Fragrance mix I Cinnamal Cinnamyl alcohol Evernia prunastri Isoeugenol HICC	BOP Lichen acid mix Fragrance mix I Fragrance mix II Amyl cinnamal Cinnamal Cinnamyl alcohol Eugenol Evernia prunastri Geraniol Isoeugenol Citral Coumarin Hexyl cinnamic aldehyde
Cosmetic-related allergens	Quaternium-15 Colophonium† Paraben mix SLM† MCI/MI MDBGN MI	Amerchol L-101	PPD Colophonium† Paraben mix Formaldehyde MCI/MI Amerchol L-101 MDBGN MI Sorbitan sesquioleate
Non-cosmetic-related allergens	Potassium dichromate Thiuram mix PTBFR Caine mix II	PFR2†	PTBFR PFR2† Caine mix II Tixocortal-21-pivalate Textile dye mix Budesonide

*Allergens in the baseline series and oxidised terpenes included, **Allergens in the baseline series, oxidised terpenes, and the individual ingredients of the fragrance mixes included. †may be considered as fragrance markers or fragrance-related allergens.

HP, hydroperoxides; BOP, *Myroxylon pereirae* resin; HICC, hydroxyisohexyl 3-cyclohexene carboxaldehyde; SLM, sesquiterpene lactone mix; MCI, methylchloroisothiazolinone; MI, methylisothiazolinone; MDBGN, methyldibromo glutaronitrile; PTBFR, 4-tert-butylphenol-formaldehyde resin; PFR2, phenol-formaldehyde resin; PPD, para-phenylene diamine.

Study 5. Repeated open application tests in patients with hydroperoxides of linalool contact allergy

The contents of syringes containing HPs of linalool (1.0%) purchased from Chemotechnique for this study were analysed. The preparations used in the ROATs were analysed twice using GC-MS: immediately after preparation (cream after preparation) and after 4-6 weeks (a random syringe). The measured concentrations were found to be ± 10% of the intended concentrations.

Of the 48 patients who agreed to participate, 47 (43 females) completed the study. The mean age (standard deviation) was 44.43 ± 13.39 years. Originally, 30 patients were classified as belonging to the contact allergy group, and 17 to the control group. The median (range) time from previous patch tests to retesting was 28 (4-87) months.

After re-patch testing, one patient who was initially classified as a control participant showed a positive reaction to the HPs of linalool and was therefore transferred to the contact allergy group for the analysis (Figure 17). Thus, 31 patients were classified as having contact allergy to HPs of linalool, and 16 were controls. The demographics of the participants (age, gender, history of atopic dermatitis, primary site of lesions) did not differ significantly between the groups.

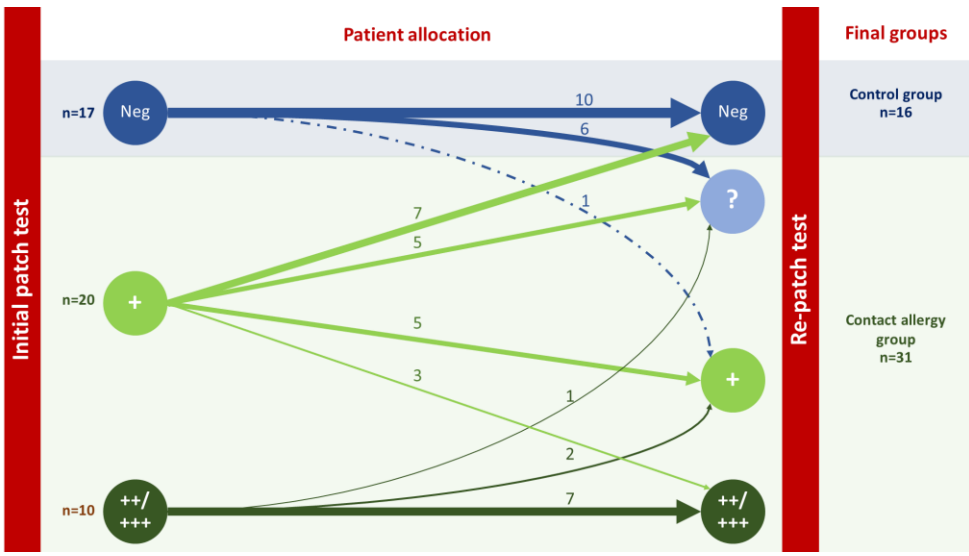


Figure 17. Patient allocation: initial patch test and re-patch test results.

Thirteen patients with a history of contact allergy to HPs of linalool showed non-positive patch test reactions at retesting, and 12 of these showed a weak positive reaction in their previous patch tests. The patients with a previous weak positive reaction showed a significantly higher proportion of having a negative reaction at retesting (*P*-value=0.009) (Table 8).

Table 8. Number of patients with different intensities of initial patch test reactions and positivity of patch test reactions at re-test.

	Initial patch test reaction (30 positive reaction patients)		
	Weak positive n (%)	**Strong to extreme n (%)**	**Total n (%)**
Reaction at re-test			
Negative	12 (60.0%)	1 (10.0%)	13 (43.3%)
Positive	8 (40.0%)	9 (90.0%)	17 (56.7%)
Total	20	10	30

On D21, two patients in the contact allergy group had skin erythema and a few papules in the area to which the 440 ppm linalool HP cream had been applied. However, the reactions were classified as negative since the area involved was less than 25%. The reaction in one of these two patients became positive on D28, while there was no progression of the reaction in the other patient. The only patient with a positive reaction in the ROAT also had a few papules on the area to which the medium-concentration linalool HP cream (140 ppm) had been applied, but this was classified as a negative reaction.

Discussion

The aim of the work presented in this thesis was to improve the diagnostic approach for fragrance contact allergy. The discussion is divided into two parts. The first part discusses fragrance mixes and their ingredients, while the second deals with contact allergy to HPs of linalool and HPs of limonene.

Patch tests with fragrance mixes and their individual ingredients

"Why do we test our patients with fragrance mixes?" – Patch testing with perfume ingredients was introduced to diagnose "perfume dermatitis" caused by cosmetics.[69] After many specific fragrance allergens had been found, Larsen and a perfumery chemist mixed eight common fragrance allergens into a "perfume mixture" to minimize the number of patch test chambers and spare the area of skin required for patch testing with other substances.[69,151] The concentration of their original perfume mixture was 16%, containing 2% of each ingredient mixed in petrolatum.[151] In 1979-1980, the prevalence of perfume mixture contact allergy was reported to be 6%.[151] At that time, cinnamic aldehyde was the most common culprit in the mix (4%),[151] rather than Oakmoss extract (1.2%), which is nowadays the most common culprit reported among FM I ingredients. It was also reported in the 1980s that simultaneous reactions to this perfume mixture and other fragrance-related allergens and essential oils were common.[152] The perfume mixture, with a concentration of 16% in petrolatum, was introduced in patch test screening in the 'standard' series.[151,152]

Later, in 1989, the irritant potency of the mixture at a concentration of 16% became the topic of discussion, and the concentration was reduced to 8% (1% of each ingredient).[71] The rates of positive reactions to the 8% and 16% fragrance mixes were found not to differ not significantly.[71] However, it should be noted that the two preparations were not tested simultaneously at the same period.[71] The 16% FM was tested in 1982-1983, while the 8% FM was tested in 1987.[71]

In the 1980s, individual ingredients of the FM (at 1%) were tested simultaneously with the 8% FM.[70,71] Patch testing with FM I (8%) was questioned as it showed a higher positivity than patch testing with the individual ingredients of the mix (1% each).[70] At that time, only 43% of the patients showing a positive reaction to the mix reacted to at least one constituent.[71] In the present work, 54% of the FM I contact allergy patients reacted to its ingredients, which is higher. The difference could be due to the use of higher concentrations of the individual ingredients tested recently (mostly 2%, except for cinnamal), different study periods, or different standardised patch testing and reading procedures. Although it has been demonstrated that "mixtures" of allergens could enhance the induction and elicitation of contact allergy in an animal study by increasing the response that produces memory T-cells[153], the concentrations of the ingredients in the FM preparations and the individual ingredient test preparations are not the same for patch testing. Therefore, it may not be possible to compare the results obtained from patch testing with the ingredients and the mixes with the results of the *in vitro* study.

Another possible cause of the discrepancy between testing with the mix and the individual ingredients could have been the presence of SSO, an emulsifier contained in the mix.[70] When 1% SSO was added to each 1% ingredient preparation of the mix, more allergic and irritant reactions were observed,[70,103] indicating that SSO could increase the reactivity of patch test reactions when using the mix. However, FM II does not contain SSO, but the discrepancy remains. Patch testing with 14% FM II has been recommended in the European baseline series since 2008.[73] Although the concentrations of the ingredients in FM II should be more standardised than those in FM I due to greater experience in the research field, the present work demonstrated that only 56% of the FM II contact allergy patients reacted to its ingredients, despite the fact that the individual ingredients of FM II were tested at twice the concentrations of those in the mix. Study 2 showed that it can be challenging to interpret the results of patch testing when all the single fragrance substances are not tested simultaneously with SSO and when the patients have a history of atopic dermatitis.

It has been a long discussion that fragrance contact allergy patients would be missed if the individual ingredients and other important fragrance allergens were not included in patch testing.[100] In the work presented in this thesis, it was shown that patients with fewer positive reactions and less intense positive reactivity to the ingredients were missed. These findings indicate that the concentrations of the fragrance mixes might be too low to detect patients who are allergic to one of the ingredients.

Overall, patch testing with fragrances and fragrance mixes has a long history and remains important. Despite this, there are still knowledge gaps leading to imperfections in patch testing. The concentrations of the test preparations used might not be accurately standardised due to previously limited information and experience. The concentrations of some fragrance patch test preparations could

perhaps be increased without causing irritation or active sensitisation, although this would be complicated and challenging. Furthermore, the use of SSO as an emulsifier might affect not only the interpretation of the patch test results but also the patch test reactivities.

Contact allergy to hydroperoxides of linalool and hydroperoxides of limonene

The discovery of HPs of linalool and HPs of limonene as potential contact allergens was an important advance in the diagnosis of fragrance contact allergy. Linalool and limonene were initially included in the testing of patients suspected of being exposed to the substances. They were considered important contact allergens until experimental studies on the sensitizing potential of non-oxidised linalool and limonene revealed that they are not potential allergens.[115,132] In contrast, oxidised linalool and oxidised limonene, mainly their HPs, have been found to be potent contact allergens.[111,118,135,136] Carbon- and oxygen-centred radicals were found to be involved in the formation of hapten–protein complexes causing contact allergy to the HPs.[136,137] Therefore, oxidised linalool and oxidised limonene were widely introduced in patch testing of suspected contact dermatitis patients. A dose of 6.0% oxidised linalool in petrolatum, equivalent to 1.0% HPs of linalool, and a dose of 3.0% oxidised limonene, containing 0.33% HPs of limonene, were suggested as standards in patch testing.[120,139,141]

There is no doubt that the HPs of linalool and HPs of limonene are contact allergens, but the sources of exposure remain obscure. Unlike other contact allergens, linalool and limonene are prehaptens, not potential allergens. No manufacturer intends to use allergenic oxidised products, such as HPs, in their products. The main problem has been the difficulty in evaluating the clinical relevance of positive reactions after patch testing, as there is no evidence that the amount of HPs in consumer products could elicit allergic reactions. However, the increasing prevalence of contact allergies to HPs of linalool and HPs of limonene underlines the importance of solving this issue.

"How are those sensitised to oxidised terpenes exposed?" - Since the levels of HPs in the investigated products have been reported to be low and could not be the cause of ACD, sources of exposure might not be originally from those products that had been analysed. When considering the demographics of patients with oxidised terpene contact allergy patients of the present work and previous studies[121,123,154], no specific site of lesions has been reported to be directly related to oxidised terpene contact allergies. Widespread skin exposure to the allergens could arise from the

extensive use of consumer products containing linalool and limonene that have not been analysed.

Studies 3 and 4 revealed some additional possibly important aspects. Patients with contact allergies to HPs of linalool and/or HPs of limonene were younger than patients with other fragrance contact allergies, and the allergic reaction might become more severe with age, as stronger positive patch test reactions were seen in older patients. Contact allergies to oxidised terpenes have also been reported to be common in children.[155,156] It may, therefore, be beneficial to investigate possible sources of exposure other than those previously studied, especially those related to young individuals.

Concomitant contact allergies to HPs of linalool and HPs of limonene and other fragrances/cosmetic-related allergens were also found to be common in this work. Other studies, including clinical and market survey studies, have reported similar findings.[130,144,157-159] Limonene and linalool have been found to be the most common fragrances labelled on all types of cosmetics.[144] Since cross-sensitisation between HPs themselves was reported to be unlikely[143], together with the simultaneous reactions reported in Study 4, patients could be co-sensitised to the HPs in consumer products, especially cosmetics.

Patch testing with specific amounts of HPs of linalool and HPs of limonene can elicit allergic reactions. However, it is still not known how patients are sensitised and elicited to HPs of linalool and HPs of limonene in daily life. Only a few cases have been reported that the rash could have been caused by the HPs of linalool in their cosmetic products.[146,147] From the research perspective, performing ROATs with HPs of linalool and HPs of limonene might help dermatologists determine whether repeated exposure could elicit the reaction. ROATs of both allergens have been studied.[44,46] However, the doses that could elicit reactions were considered high compared with the results of chemical analysis of products. While the patch test dose should be as high as it can elicit a weak positive reaction without causing active sensitisation, the dose used in a ROAT should be the dose that represents allergen exposure in real life.[50]

It is challenging to conduct a ROAT study. Several factors must be taken into account, including the quality of the test materials, the amount of ROAT preparations applied, and the frequency and duration of application. It should also be double-blinded, and a control group is required to allow the statistical comparison of patients with and without contact allergy. Study 5 was thus carefully planned and demonstrated that doses of HPs of linalool representing the allergen exposure in daily life could rarely elicit a positive ROAT reaction in four weeks. Therefore, the relationship between using products labelled “linalool”, which might contain only trace amounts of HPs of linalool, and clinical dermatitis in HPs of linalool contact allergy is still uncertain.

The limitations of performing ROATs are that the patient's sensitivity and the dose applied could affect the duration required to elicit positive test reactions.[57] Other factors that could affect the results may be vehicles, the anatomical site of application, the skin condition at the application site, and the kinetics of the test substance in the skin. Performing ROATs under different conditions, for example, over a longer period or on non-healthy skin with real-life doses, might elicit a positive reaction.

In conclusion, the clinical relevance of contact allergies to oxidised terpenes cannot be established unless it can be demonstrated that clinical dermatitis is related to allergen exposure in real life. Theoretically, it is possible to do more in clinical practice, such as analysing the products used by the patient and performing ROATs with HPs at realistic concentrations in patients diagnosed with contact allergy to the oxidised terpenes, in order to ascertain the relevant exposure of an individual, as in the case of other allergens. However, the most challenging problem is that the chemical analysis required to detect HPs is complicated, time-consuming, and requires experienced personnel and expensive equipment. It would be less problematic if there were more patch test clinics and laboratories that could perform chemical analyses of personal products used by patients. HPs of linalool and HPs of limonene should be included in patch testing of consecutive patients at some centres, principally to understand and initiate more research. Definitive positive clinical relevance should not be diagnosed in patients unless a positive reaction to a ROAT is demonstrated by applying the actual amount of HPs found in the patient's own products. Advising patients with contact allergy to HPs of linalool to avoid products labelled "linalool" is still not convincing.

Popular Scientific Summary

Skin allergy to fragrances has been a concern for decades, and unperfumed products have become increasingly popular. However, most people prefer a nice fragrance, so exposure to fragrances cannot be avoided. Some chemicals used to impart fragrance in products are more harmful to the skin than others. Patients with a skin rash caused by chemical exposure, including fragrances, are diagnosed as having allergic contact dermatitis, which can be confirmed by a process called patch testing.

Patch testing with suspected fragrance allergens is the gold standard in diagnosing fragrance contact allergy. Several common fragrance allergens have been combined to form mixtures that are used in patch testing in baseline patch test series for contact allergy screening in patients. Not only mixtures but individual fragrance compounds can also be tested. Each allergen is applied to the patient's skin in small patch test chambers that are left in place for 2 days, and the results on the tested area are evaluated by dermatologists afterwards.

Linalool and limonene are terpene fragrances used mainly in the consumer market. They are not potential allergens unless they are oxidised, usually by exposure to air. The major oxidation products causing contact allergy are hydroperoxides. These emerging contact allergens (hydroperoxides of linalool and hydroperoxides of limonene) have become more important since an increasing prevalence of contact allergies has been reported worldwide. However, no strong evidence has been found that the patient's rash is caused by hydroperoxides at the concentrations found in consumer products. It is difficult to estimate how much of the linalool and limonene contained in personal products is oxidised. Therefore, they have not been included in the baseline series for screening because it is difficult to establish the clinical relevance when patients react positively to the tests.

The main results of the work presented in this thesis were that fragrance contact allergy was more common among females and in patients aged 40 years and over. Roughly an additional 10% of the fragrance contact allergy patients were diagnosed by patch testing with individual ingredients of the fragrance mixes that are not included in the baseline series. This work showed that patients who could have been missed by screening with fragrance allergen mixtures in the baseline series had contact allergy to a single compound or showed a weak reaction to individual fragrance compounds. Some fragrance contact allergy cases might show a false-

positive reaction to a fragrance mixture because they have a contact allergy to the emulsifier used in the mixture. Therefore, patch testing with fragrance mixes in the baseline series should be further investigated and standardised.

An increasing trend in contact allergies to oxidised linalool and oxidised limonene was seen in the period 2013-2020, with about one-third of the patients having contact allergies to both. Patients with these contact allergies were significantly younger than patients with contact allergies to other fragrances. Patients with contact allergies to oxidised linalool and/or oxidised limonene also had simultaneous contact allergies to other fragrance and cosmetic-related allergens, indicating that the source of skin exposure to the allergens could be mainly from cosmetics. When performing a repeated open application study to mimic a real-life situation by asking the research participants to apply creams containing different concentrations of oxidised linalool reported in consumer products, the results showed that the cream with the highest concentration could elicit a rash in only one out of 31 patients who had contact allergy to linalool hydroperoxides, and none of the 16 negative controls. No statistically significant evidence was found that the rashes caused by oxidised linalool in contact allergy patients were elicited by the study creams containing realistic amounts of linalool hydroperoxides. Further investigations must, therefore, be performed to identify the sources of exposure to oxidised linalool and oxidised limonene.

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