Contact allergy to fragrances with a focus on oak moss absolute

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2014

Contact allergy to fragrances

with a focus on oak moss absolute

LUND
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DOCTORAL DISSERTATION
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To be defended at Jubileumsaulan, Medicinskt forskningscentrum,
Jan Waldenströms gata 5, Skåne University Hospital, Malmö
Friday 3 Oct 2014 at 9.00 am.

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Document name
DOCTORAL DISSERTATION

Date of issue
October 3, 2014

Author(s)
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Sponsoring organization

Title and subtitle Contact allergy to fragrances with a focus on oak moss absolute

Abstract The exposure to fragrances is widespread and contact allergy to fragrance substances affects 1–4% of the general population. Many fragrance substances are volatile and it can therefore be suspected that they may evaporate from petrolatum patch test preparations applied in test chambers. In the first two papers included in this thesis the aims were to i) to investigate the stability of fragrance preparations in petrolatum when applied in patch test chambers, and ii) to investigate the patch test reactivity to samples of fragrance mix I (FM I) and fragrance mix II (FM II) when applied in test cambers 6 days in advance or immediately before the patch test occasion.

Oak moss absolute (OMA), an extract derived from the lichen *Evernia prunastri*, is a common cause of fragrance contact allergy. OMA contains several allergens, among them atranol and chloroatranol, which have been found to be strong allergens in humans. Therefore, the fragrance industry nowadays provides treated OMAs, where the content of atranol and chloroatranol has been reduced. The aims of studies III and IV in the thesis were to iii) compare the eliciting capacity of treated and untreated OMA samples in patch tests with dilution series and in repeated open application tests (ROATs), and iv) to investigate the reaction pattern in OMA-allergic subjects patch-tested with thin-layer chromatography (TLC) strips of treated and untreated OMA samples.

The findings were as follows: i) The concentrations of 4 of 7 substances investigated decreased by ≥20% within 8 h when stored in Finn chambers at room temperature. The decrease in concentration was slower when the test preparations were stored in a refrigerator. Statistically significantly more reactions were observed for the freshly applied sample of FM I than to the pre-loaded sample, demonstrating that FM I patch test prepared in advance may give false-negative reactions. No corresponding difference was observed for FM II. This is likely explained by differences in volatilities between the ingredients of FM I and FM II. iii) OMA-allergic subjects were statistically significantly less patch test reactive to the treated OMA sample than to the untreated sample. No significant difference was observed in the ROAT, though there was a significant difference in the time required to elicit a positive reaction. iv) The TLC patch tests indicate the presence of sensitisers other than atranol and chloroatranol in the untreated OMA sample. The studies on OMA indicate that the residual levels of atranol and chloroatranol and/or the presence of other sensitisers in the treated OMA samples may elicit allergic reactions in previously sensitised individuals.

Key words

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language

ISSN:1652-8220
Series: Lund University, Faculty of Medicine Doctoral Dissertation Series 2014:109

ISBN
978-91-7619-038-8

Recipient's notes

Number of pages 116

Price

Security classification

Contact allergy to fragrances

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Malmö 2014

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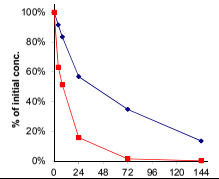
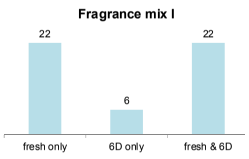
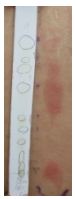
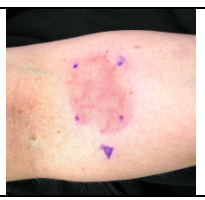
Lund University, Faculty of Medicine Doctoral Dissertation Series 2014:109
ISBN 978-91-7619-038-8
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2014

Thesis at a glance

Paper	Objective	Method	Illustration	Main findings/conclusions
I. Stability of fragrance patch test preparations applied in test chambers	To investigate the stability of petrolatum preparations of 7 fragrance mix I (FM I) ingredients when applied in patch test chambers stored at room temperature or in a refrigerator.	The content of the fragrance substances was determined using gel permeation chromatography and high-performance liquid chromatography.		The concentration decreased by ≥ 20% within 8 h in 4/7 preparations stored in Finn chambers at room temperature. The rate of evaporation from preparations stored in a refrigerator was slower for all substances investigated.
II. Fragrance patch tests prepared in advance may give false-negative reactions	To compare patch test reactivity to FM I and fragrance mix II (FM II) when applied in test chambers in advance and immediately before the patch test occasion.	795 consecutive dermatitis patients were patch-tested with duplicate samples of FM I and FM II. One sample of each mix was applied in the test chamber 6 days in advance, while the other was applied immediately before the patch test occasion.		Patch testing with pre-loaded fragrances may give false-negative reactions. Statistically significantly more reactions were observed for the freshly applied sample of FM I than for the pre-loaded FM I sample. No corresponding difference was observed for FM II.
III. Patch testing with serial dilutions and thin-layer chromatograms of oak moss absolutes containing high and low levels of atranol and chloroatranol	To compare the elicitation capacity of a treated oak moss absolute (OMA) with a reduced content of atranol and chloroatranol to that of an untreated OMA, and to investigate reactions to components of OMA separated by thin-layer chromatography (TLC).	15 OMA-allergic subjects were patch-tested with serial dilutions and TLC-strips of the treated and untreated OMA samples.		The elicitation capacity in patch tests was significantly lower for the treated OMA than for the untreated OMA. The TLC patch tests indicate the presence of sensitisers other than atranol and chloroatranol in OMA.
IV. Usage tests of oak moss absolutes containing high and low levels of atranol and chloroatranol	To compare the elicitation capacity of a treated OMA sample with a reduced content of atranol and chloroatranol to that of an untreated OMA sample in patch tests with serial dilutions and in a repeated open application test (ROAT).	15 OMA-allergic subjects and 16 controls underwent serial dilution patch testing and performed a ROAT with the treated and untreated OMA samples.		Statistically significantly more subjects reacted to the untreated than to the treated OMA in the patch tests. No corresponding difference was observed in the ROAT, although there was a significant difference in the time required to elicit a positive reaction.

List of publications

The thesis is based on the following papers, referred to in the text by their Roman numerals

I. **Stability of fragrance patch test preparations applied in test chambers**
Mowitz M, Zimerson E, Svedman C, Bruze M
British Journal of Dermatology 2012: 167: 822-7

II. **Fragrance patch tests prepared in advance may give false-negative reactions**
Mowitz M, Svedman C, Zimerson E, Bruze M
Accepted for publication in Contact Dermatitis

III. **Patch testing with serial dilutions and thin-layer chromatograms of oak moss absolutes containing high and low levels of atranol and chloroatranol**
Mowitz M, Zimerson E, Svedman C, Bruze M
Contact Dermatitis 2013: 69: 342-9

IV: **Usage tests of oak moss absolutes containing high and low levels of atranol and chloroatranol**
Mowitz M, Svedman C, Zimerson E, Bruze M
Acta Dermato-Venereologica 2014; 94: 398-402

Contents

Abbreviations

ACD	allergic contact dermatitis
D	day
FM I	fragrance mix I
FM II	fragrance mix II
GCMS	gas chromatography–mass spectrometry
GPC	gel permeation chromatography
HICC	hydroxyisohexyl 3-cyclohexene carboxaldehyde
HPLC	high-performance liquid chromatography
ICDRG	International Contact Dermatitis Research Group
IFRA	International Fragrance Association
INCI	International Nomenclature of Cosmetic Ingredients
MEC	minimum eliciting concentration¨
MP	Myroxylon pereirae
OMA	oak moss absolute
ROAT	repeated open application test
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
THF	tetrahydrofuran
TLC	thin-layer chromatography
w/v	weight/volume
w/w	weight/weight

1 Introduction

1.1 Contact allergy and allergic contact dermatitis

Contact allergy, also known as "delayed contact hypersensitivity or "type IV allergy" develops after exposure to sensitising substances—contact allergens. More than 4,300 substances are known to cause contact allergy (1), and once an individual is sensitised the allergy remains throughout life. Allergic contact dermatitis (ACD) is the clinical manifestation of contact allergy, and develops when an individual who is contact allergic to a certain allergen is exposed to this allergen in a dose exceeding that individual's threshold. By avoiding exposure to the allergen in question or to other chemically related substances, it is possible for a contact allergic individual to avoid developing ACD (2).

A contact allergen must be able to penetrate the skin barrier, and it must be able to react with proteins in the skin to form antigens. To penetrate the skin, it has to be a relatively lipophilic compound (log $P_{o/w}$ >1) (3) of low molecular weight, usually below 500 (4). Since these molecules are too small to act as antigens themselves, contact allergens are generally referred to as haptens (incomplete antigens). Many macromolecules, such as proteins, contain negatively charged nucleophilic functional groups. These groups can form covalent bonds with electrophilic compounds containing atoms that are positively charged. Most haptens have electrophilic properties. Some molecules are electrophilic in themselves, while others act as pro-haptens, which require metabolic conversion in the skin to obtain electrophilic properties. In addition, some molecules are pre-haptens, which are converted into haptens by air oxidation (2, 5).

When an individual becomes sensitised (the induction phase), the hapten binds to skin proteins, forming antigens that are taken up by antigen-presenting cells called Langerhans cells. These cells transport the antigens to the regional lymph nodes where they are presented to uncommitted T-cells, which become activated. The activated T-cells then release cytokines. This leads to the proliferation and differentiation of the T-cells into hapten-specific memory cells, with effector or memory function; these are released into the blood circulation. The induction phase requires from 4 days to several weeks. After re-exposure to the hapten (the elicitation phase), Langerhans cells present the antigen to the hapten-specific effector and memory cells, which become activated, proliferate, and induce a cascade of

inflammatory events in the exposed skin area. As a result, an eczematous reaction develops, usually within 1–4 days after exposure to the allergen (2). However, for some substances the elicitation phase can be longer—sometimes more than 2–3 weeks (6-9).

1.2 Patch testing

The method for establishing contact allergy, patch testing, was first developed by Jadassohn more than a hundred years ago and was later described in detail by Bloch in 1929 (10). The principle of patch testing is to re-expose patients with suspected ACD to the suspected allergen(s) under controlled conditions (Figure 1). An eczematous reaction at the test site indicates contact allergy. Although the patch test technique itself has remained substantially unchanged, developments have been made in standardization of the method with regard to allergens, vehicles, concentrations, doses, scoring etc. (11-15).

The allergens are dissolved or evenly distributed at appropriate concentrations in a vehicle and are then applied to the skin in small test chambers on adhesive tape strips, which are placed on the patient's back for 48 h. When patch test reactions are read, they are often scored according to the International Contact Dermatitis Research Group (ICDRG) criteria: (+), doubtful reaction; +, weak positive reaction; ++, strong positive reaction; or +++, extreme positive reaction (12). Paired readings of the patch test reactions on both day (D) 3/4 and D7 have been shown to be the most accurate (16). The overall incidence of reactions to allergens in the baseline series developing from negative or doubtful to positive between D3/4 and D6/7 have been reported to be 3–9% (16-19).

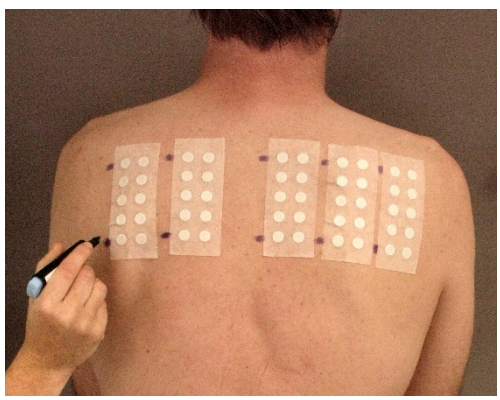


Figure 1. Patch tests applied to the back of a patient.

False-positive reactions are defined as reactions caused by irritation, but with a morphology indistinguishable from that of a true allergic reaction (12). To exclude the possibility that a reaction is false-positive, testing with serial dilutions of the test preparation and/or patch testing of controls may be performed (11).

False-negative reactions are defined as a failure to elicit a positive patch test reaction although the individual being tested indeed is allergic to the test substance (12). False-negative reactions may occur when patch testing with an insufficient dose, due to the choice of a concentration that is too low or to an unstable or unevenly distributed substance in the patch test preparation. Furthermore, in certain cases, testing in an improper vehicle or test chamber can cause the test substance to be converted into a non-sensitising product (20, 21).

Late patch test reactions are defined as reactions that are visible at the site of a previously negative patch test when inspected on D7 or later (12). Possible explanations for late patch test reactions may be a low degree of reactivity in the patient, a low test concentration, and/or slow penetration of the allergen. A late patch test reaction may be misinterpreted as a sign of active sensitisation.

Active sensitisation is an adverse effect of patch testing, defined as a negative patch test reaction followed by a flare-up reaction after 10–20 days, with a positive reaction on D3 at re-tests (11). Since some individuals may react to lower concentrations of an allergen later than D7 (6, 7), patch testing with serial dilutions should be performed when patch test sensitisation is suspected (15).

1.3 Fragrances

A fragrance ingredient can be defined as an ingredient used in the manufacture of fragrance materials for its odorous, odour-enhancing, or blending properties (22). Until the end of the nineteenth century, all fragrance ingredients were derived from natural sources such as plant extracts or animal secretions. Thereafter, the production of synthetic fragrance ingredients began, which reduced the cost and made the use of fragrances more widespread in society (23). The synthetic fragrance ingredients are often identical to naturally occurring substances, but the fragrance industry has also developed entirely new synthetic substances. Despite the advances made with regard to development of synthetic materials, extracts from plants and lichens are still important fragrance ingredients. Due to the complex blend of odoriferous substances in these extracts, they are not always easily interchangeable with synthetic substances (24). About 2,500 fragrance ingredients are currently in use, and the fragrance formula of a cosmetic product may consist of 10–300 different fragrance ingredients (22).

Today, the use of fragrances is ubiquitous—not only in cosmetic products mainly used for their scent, such as perfumes, eau de colognes, and aftershaves, but also in

soaps, lotions, detergents, cleaning agents, and in industrial products where they function as masking agents. Furthermore, people are exposed to fragrance substances in products where their main function is other than giving the product a pleasant scent, e.g. the use of natural extracts with antiseptic properties in topical medicaments and the use of eugenol in temporary cements used in dentistry (22).

1.3.1 Fragrance contact allergy

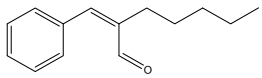
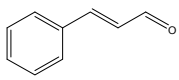
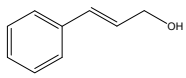
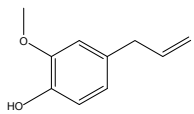
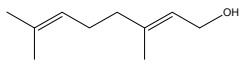
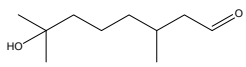
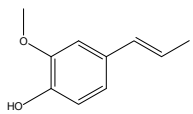
The clinical manifestations of fragrance contact allergy are usually eczematous reactions at the sites where products containing fragrance have been applied: on the neck, on the upper chest, in the face, behind the ears, and bilaterally in the axillae in the case of deodorants (25). Perfumes, deodorants, and scented lotions have been found to be the most common causes of fragrance contact allergy in women, while aftershave lotions and deodorants have been found to be the most common causes in men (25-27).

About 80 fragrance ingredients have been reported to act as contact allergens in humans (22). Two fragrance mixes (fragrance mix I, FM I, and fragrance mix II, FM II) containing 8 and 6 fragrance ingredients, respectively, are currently included in the European baseline series used for routine patch testing. The fragrance ingredients included in FM I and FM II are listed in Table 1 and Table 2. FM I has been used since the late 1970s (28), while FM II was introduced in in the European baseline series in 2008 (29). The baseline series also includes balsam of Peru (*Myroxylon pereirae,* MP) and colophony, which are considered to be markers of fragrance contact allergy, and a separate preparation of hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC, Lyral®)—one of the ingredients of FM II (29).

The 14 fragrance ingredients in FM I and FM II all belong to the group of 26 fragrance ingredients that, according to the Cosmetic Products Regulation of the European Union, must be declared with their International Nomenclature of Cosmetic Ingredients (INCI) names when they are present in leave-on products at levels of more than 10 ppm and when present in rinse-off products at levels of more than 100 ppm (30). The Scientific Committee on Consumer Safety (SCCS), an independent advisory committee to the European Commission, has suggested that the list of fragrance ingredients that have to be declared in the ingredient list of cosmetic products should be expanded to a total of 128 substances and extracts (31).

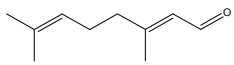
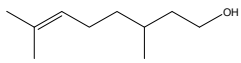
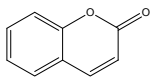
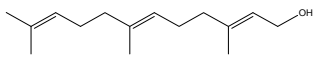
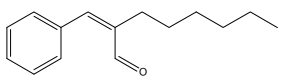
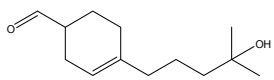
In studies collecting information from product ingredient labels and in chemical investigations of cosmetics, fragrance ingredients of FM I and FM II have been found to be commonly occurring in cosmetic products (32-35). Even so, the ingredients of FM I and FM II only represent a small fraction of the fragrance ingredients used in cosmetics. Therefore patch testing with the patients' own products is advisable (36).

Table 1. Fragrance ingredients of fragrance mix I

Ingredient	Molecular structure	CAS no.	Vapour pressure (Pa at 25°C)	Conc. in mix (% w/w)	Conc. in individual preparation (% w/w)
amyl cinnamal		122-40-7	0.31	1.0	2.0
cinnamal		104-55-2	3.6	1.0	1.0
cinnamyl alcohol		104-54-1	2.4	1.0	2.0
eugenol		97-53-0	1.3	1.0	2.0
oak moss absolute	–	90028-68-5	–	1.0	2.0
geraniol		106-24-1	1.7	1.0	2.0
hydroxycitronellal		107-75-5	0.43	1.0	2.0
isoeugenol		97-54-1	0.69	1.0	2.0

A number of fragrance terpenes have been found to auto-oxidize upon exposure to air forming strong contact allergens, and it is thought that these oxidised substances are the main allergens (37-40). It has therefore been suggested that oxidised forms of these substances should be used for patch testing. It has also been demonstrated that cinnamyl alcohol is oxidised into cinnamal, epoxy cinnamyl alcohol, and cinnamic acid when exposed to air (41).

Table 2. Fragrance ingredients of fragrance mix II

Ingredient	Molecular structure	CAS no.	Vapour pressure (Pa at 25°C)	Conc. in mix (% w/w)	Conc. in individual preparation (% w/w)
citral		5392-40-5	9.5	1.0	2.0
citronellol		106-22-9	2.4	0.5	1.0
coumarin		91-64-5	0.17	2.5	5.0
farnesol		4602-84-0	0.049	2.5	5.0
hexyl cinnamal		101-86-0	0.093	5.0	10.0
HICC*		31906-04-4	0.0039	2.5	5.0

*Hydroxyisohexyl 3-cyclohexene carboxaldehyde

The frequency of contact allergy in dermatitis patients has been reported to be 6–10% for FM I and 3–6% for FM II (Table 3). It has been found that 32–48% of patients who test positive to FM II do not react to FM I (42-44). In consecutively patch-tested dermatitis patients at our clinic in Malmö between 2009 and 2013, the frequency of contact allergy FM I and FM II was 6.1% (183 of 2,985) and 3.4% (101 of 2,985), respectively.

The prevalence of contact allergy to FM I in samples of the general population has been reported to be around 2% in adolescents (45) and between 1% and 4% in adults, depending on the age groups studied (46-49). In a recent European multi-centre study, where patch testing was performed in a random sample from the general population, the prevalence of allergic reactions to FM I and at least one reaction to any of the separate fragrance ingredients in the mix was 1.0%. The prevalence of allergy to FM II plus a single FM II ingredient was 1.6% (50).

In a multivariate analysis using data from 57,795 patients in a study investigating the association between occupation and contact allergic reactions to FM I, a high

occupational risk of fragrance contact allergy was found in masseurs, physiotherapists, metal furnace operators, potters and glass makers, and geriatric nurses (51). In an English study, patients working in the health care sector showed the highest frequency of FM I contact allergy. Healthcare workers and metal workers had statistically significantly higher rates of allergy to eugenol than other occupations. Significantly more reactions to cinnamal and cinnamyl alcohol were observed in food handlers (52).

Table 3. Frequency of contact allergy to fragrance mix I (FM I) and fragrance mix II (FM II) in consecutively patch-tested dermatitis patients reported in studies published between 2009 and 2014.

Reference	Location	Period	FM I		FM II	
			% pos	no. tested	% pos	no. tested
(44)	Leuven, Belgium	1990–2011	9.6%	13,114	6.0%	3,416
(53)	London, UK	2011–2012	6.4%	1,951	3.3%	1,951
(54)	Germany	2005–2008	6.6%	36,961	4.6%	35,738
(55)	Germany	2010–2012	8.2%	38,966	4.6%	38,966
(56)	North America	2007–2008	9.4%	5,079	3.6%	5,071
(57)	North America	2009–2010	8.5%	4,303	4.7%	4,307
(43)	Denmark	2005–2008	6.0%	12,302	4.5%	12,302

1.3.2 Other health aspects

Systemic contact dermatitis after oral intake of flavoured food containing fragrance-related substances has been reported, mainly in MP-allergic individuals (58-60). It has also been demonstrated experimentally that inhalation of isoeugenol may elicit systemic reactions in sensitised individuals (61).

Photoallergic reactions to fragrances are rare nowadays (62). Historically, they have mainly been associated with musk ambrette, to which many cases of photoallergic reactions were reported a few decades ago (63, 64). Musk ambrette is now banned from use in cosmetic products in the EU (30).

Some fragrance substances, e.g. cinnamal and cinnamyl alcohol, as well as MP may cause non-immunological contact urticaria (65-67). Immunological contact urticaria from fragrances appears to be rare, but it has been suggested in a case of contact urticaria from geraniol (68).

Inhalation of fragrances may cause respiratory symptoms, but the mechanism is unclear and the symptoms are difficult to substantiate objectively (61, 69). It has been found that individuals with fragrance contact allergy and/or hand eczema have more

frequent and more severe respiratory symptoms after exposure to volatile fragrance products (70).

1.4 Oak moss absolute

1.4.1 Contact allergy to lichens

Oak moss absolute (OMA, INCI name Evernia prunastri extract) is an extract used in perfumery that is derived from the lichen *Evernia prunastri*. A lichen is a composite organism consisting of a fungus and a photosynthetic partner, usually a green alga or a cyanobacterium, growing together in a symbiotic relationship. Contact allergic reactions to lichens are most often found for the *Evernia, Cladonia,* and *Parmelia* species and less frequently to the *Hypogymnia, Platismatia, Physconea, and Alectoria (Bryoria)* species (71). Occupational ACD caused by lichens have been reported in forestry workers, horticultural workers, and in lichen pickers, but also other outdoor activities, e.g. hunting, or contact with living trees, firewood, and wood dust may be associated with ACD from lichens (72-76). The main cause of lichen-related ACD is, however, the use of scented products containing extracts of oak moss and/or tree moss (*Evernia furfuracea*). Several substances found in lichens have been reported to be contact allergens, including atranorin, evernic acid, fumarprotocetraric acid and, usnic acid, atranol, and chloroatranol (76-80).

Photoallergic and photoaggravated contact allergic reactions have been demonstrated for crude lichens, extracts of lichens, and lichen-related substances and products (73, 81, 82). Lichens can also cause an airborne contact dermatitis, which may be misinterpreted as photodermatitis (81).

1.4.2 Frequency of oak moss absolute contact allergy

In a sample of the general population from five European countries, 1.0% (32 of 3,119) tested positive to 2.0% OMA in petrolatum (50). The frequency of OMA allergy in unselected dermatitis patients has been found to be around 2% in studies from various European centres (Table 4). In a selected German dermatitis population simultaneously patch-tested with OMA, tree moss absolute, and colophony, 5.7% (173 of 3,030) were positive to OMA tested at 1% in petrolatum (83). In Malmö, OMA has been tested in the baseline series since 2004. The frequency of OMA allergy in dermatitis patients patch-tested at our department between 2004 and 2013 is presented in Figure 2.

Table 4. Frequency of oak moss absolute allergy in unselected dermatitis patients.

Reference	Location	Period	Test conc. (% in pet.)	% positive	No. tested
(84)	Germany	2003–2004	1	2.2%	2,063
(35)	Gentofte, Denmark	2008–2010	1	2.1%	1,503
(53)	Great Britain	2011–2012	2	1.7%	1,951
-	Malmö, Sweden	2004–2013	2.0	1.7%	6,656

pet., petrolatum

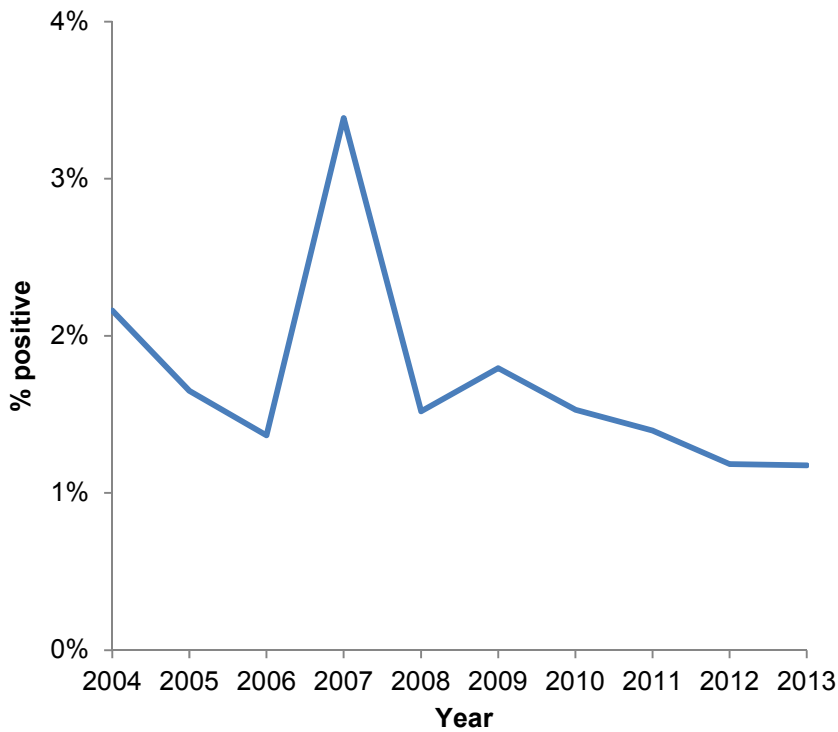


Figure 2. The frequency of contact allergy to oak moss absolute in Malmö between 2004 and 2013.

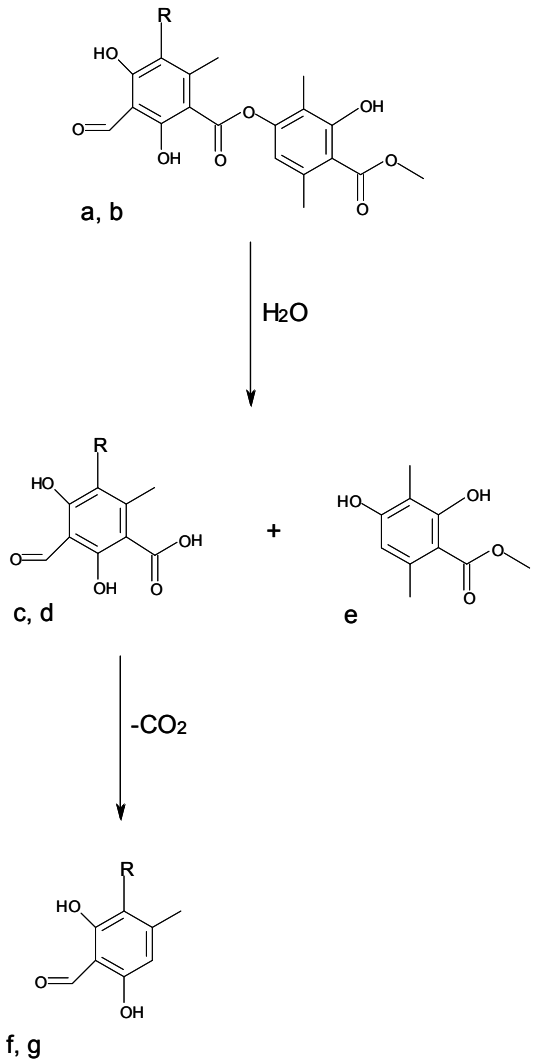


Figure 3 Decomposition of atranorin (a, R=H) and chloroatranorin (b, R=Cl) by hydrolysis, giving haematommic acid (c, R=H) and chlorohaematommic acid (d, R=Cl), together with methyl β-orcinol carboxylate (e). Haematommic acid and chlorohaematommic acid are further decarboxylated to atranol (f, R=H) and chloroatranol (g, R=Cl).

1.4.3 Processing of oak moss extracts

The raw material used for OMA is the lichen *Evernia prunastri* which is collected on oak trees in the south-central regions of Europe as well as in Morocco and Algeria. Each year, about 700 tons of the lichen are processed in France. After the harvest, the

lichen is desiccated and then humidified with water prior to the extraction procedure with organic solvents. The solvents used are either hexane or mixtures of hexane and more polar solvents, especially acetates. The crude solvent extracts, called resinoids, are further treated with ethanol in order to obtain the absolutes, which are then used in fragrance compositions. The absolutes may in addition be subjected to physical treatments such as discoloration with charcoal or vacuum distillation (85). The chemical composition of natural extracts is often complex, and more than 170 substances have been identified in oak moss extracts. Some of these substances are formed during the processing of the extracts. When the dried lichen is treated with water, phenyl benzoate derivatives such as atranorin and chloroatranorin are hydrolysed and further decarboxylation results in the formation of atranol and chloroatranol (Figure 3). The degradation of odourless phenyl benzoates is essential for the olfactory properties of OMA. Orcinol monomethyl ether and methyl β-orcinol carboxylate, which are considered to be the dominant odour constituents of OMA, are formed from evernin in a pathway analogous to the one described in Figure 3. The phenyl benzoates may also be degraded through a transesterification reaction with ethanol (80, 85, 86).

1.4.4 Tree moss

Apart from OMA, an extract made from *Evernia furfuracea,* commonly referred to as tree moss, is a commonly used fragrance ingredient. The INCI name of this extract is *Evernia furfuracea* extract. Although not included in any of the fragrance mixes, it has been demonstrated to be an important fragrance allergen. When tree moss growing on conifers is collected, some of the bark is included. Since the bark contains resin acids also included in colophony, concomitant patch test reactions to colophony and tree moss can be suspected. It has been reported that oak moss extracts have deliberately or unintentionally been mixed with tree moss extracts when used as fragrance ingredients. OMA contaminated with resin acids from tree moss extracts used in patch test preparations has been suspected to be a source of misdiagnosis of OMA allergy in patients positive to colophony (87). In later reports, the degree of concomitant reactions to OMA and colophony was not found to be high (88, 89). The degree of concomitant reactions could be suspected to be dependent on the content of resin acids in the OMA used for patch testing. However, no overall difference in reactivity was found in 119 consecutive patients tested with one OMA sample containing 0.05% resin acid and one OMA sample not containing any measurable amounts of resin acids (limit of detection not reported). Furthermore, when the reactions to OMA and FM I, both containing resin acids, and colophony were investigated in 885 consecutive patients, significant relationships between reactions to colophony and FM I and also between colophony and OMA were found. The relationship between positive reactions to colophony and to FM I was still significant when all reactions to OMA were disregarded (90).

The current International Fragrance Association (IFRA) standard on oak moss extracts states that "oak moss extracts used in fragrance compositions must not contain added tree moss, which is a source of resin acids" (91). According to the IFRA standard on tree moss extracts, the extracts must not contain more than 0.8% dehydroabietic acid, which would correspond to a total lichen acid content of 2% (92).

In a retrospective study on 3,030 patients who were simultaneously patch-tested with OMA, tree moss absolute, and colophony, two subgroups of tree moss-allergic subjects were identified by the authors. The first group contained those sensitised to (oxidised) resin acids, as indicated by a positive patch test to colophony. The second group contained those sensitised to common constituents of OMA and tree moss absolute, without reacting to oxidised resin acids/colophony (83).

1.4.5 Atranol and chloroatranol

Atranol and chloroatranol have been known for decades (93), but it was not until recently that they were identified as strong contact allergens in a study combining patch testing of OMA-allergic individuals with fractions of OMA, chemical investigations, and structure-activity relationship analysis of the substances identified. In addition, methyl β-orcinol carboxylate was identified as a weak allergen (80). As mentioned earlier, atranol and chloroatranol are degradation products formed during processing of the extracts. However, it has also been found that they are present to some degree also in the living lichen (94). Interestingly, already in 1979 Dahlquist and Fregert performed patch testing with a sample of hydrolysed atranorin, which likely would have contained atranol, haematommic acid, and methyl β-orcinol carboxylate. However, no reactions were observed in the three atranorin-positive subjects tested, and the authors concluded that this would indicate that atranorin itself was the sensitiser (77).

Chloroatranol has been found to cause allergic reactions at the ppb level in patch tests and at the ppm level in a repeated open application test (ROAT) (95). The elicitation capacity of chloroatranol has been found to be 2.2 times higher than that of atranol. However, the concentration of atranol in OMA is about twice as high as the concentration of chloroatranol (96). When comparing the challenge responses in mice sensitised to atranol, chloroatranol, or OMA, it has been found that OMA is more potent than chloroatranol, which, in turn is more potent than atranol (97).

In 2004, Rastogi et al. found atranol and chloroatranol in 27 of 31 products investigated, mainly perfumes. The median concentration in perfumes was 0.50 ppm for atranol and 0.24 ppm for chloroatranol. The authors concluded that these sources of exposure could explain the high frequencies of OMA contact allergy (98). In 2007, a significant decrease in the proportion of products containing chloroatranol was observed compared to the aforementioned study (99).

There have been several methods reported on how to reduce the content of sensitisers in OMAs—involving, for example chromatographic methods (100), treatment with amino acids (101) or binding to an insoluble polymer support (102). The two latter methods act towards the aldehyde function in atranol and chloroatranol, but there are also other aldehydes present in the oak moss extracts and it is also likely that their content is reduced when treated as mentioned above.

Nardelli et al. performed patch testing with a 1% petrolatum preparation of OMA treated with a polymer-based method, which reduced the content of atranol and chloroatranol to < 75 ppm and < 25 ppm, respectively. Still, 8 out of 14 oak moss-allergic individuals reacted to the treated sample and the authors concluded that the treatment in question "reduces the allergenic elicitation potential in previously sensitized individuals only to a minor extent" and that the residual amounts of atranol and chloroatranol are "unsafe for the consumer" (102).

According to the Cosmetic Products Regulation of the European Union, OMA has to be declared with its INCI name when present in leave-on products at levels above 10 ppm and when present in rinse-off products at levels above 100 ppm. The Cosmetic Products Regulation does not, however, regulate the levels of oak moss extracts in cosmetic products or the levels of chloroatranol and atranol in the extracts (30). According to the IFRA Standard on oak moss extracts, the maximum concentration that is allowed in skin contact cosmetic products is 0.1%. Since 2008, there has also been an IFRA restriction on the concentration of atranol and chloroatranol in oak moss extracts, which must not exceed 100 ppm each (91). Consumers using cosmetic products containing these extracts are exposed to atranol and chloroatranol in concentrations of 0.1 ppm or below. However, The IFRA standard on OMA is part of a self-regulating system for the fragrance industry members of IFRA. Thus, also other OMA qualities than those manufactured in accordance with the IFRA standard may be available on the market.

In 2004, the Scientific Committee on Consumer Products (SCCP), an independent advisory committee to the European Commission, recommended that atranol and chloroatranol should not be present in cosmetic products (103). When reviewing sensitisation data on treated and untreated OMA samples in 2008, the SCCP concluded that it appears to be possible to reduce the content of atranol and chloroatranol to < 2 ppm each. A cosmetic product containing 0.1% OMA would then contain atranol and chloroatranol at such levels that the risk of both induction and elicitation of allergic reactions would be low. However, the SCCP expressed a need of appropriate clinical testing with treated OMA samples in subjects who have previously been sensitised to OMA, in order to demonstrate a reduction in the elicitation capacity (104). In 2012, the SCCS adopted a new opinion on fragrance allergens in cosmetic products, which states that atranol and chloroatranol should not be present in cosmetic products (31).

2 Aims

The aims of the studies included in this thesis were to:

- Investigate the stability of petrolatum preparations of FM I ingredients when applied in patch test chambers.
- Compare the patch test reactivity to FM I and FM II samples applied in test chambers in advance and immediately before the patch test occasion.
- Compare the eliciting capacity of samples of OMA containing high and low levels of atranol and chloroatranol in patch tests with dilution series and in ROATs.
- Investigate the reaction pattern in OMA-allergic subjects patch tested with thin-layer chromatography (TLC) strips of OMA samples containing high and low levels of atranol and chloroatranol.

3 Materials and methods

3.1 Chemicals and patch test preparations

3.1.1 Study I

The FM I ingredients cinnamal, cinnamyl alcohol (Bedoukian, Danbury, CT, USA), hydroxycitronellal, eugenol (Firmenich Inc., Plainsboro, NJ, USA), amyl cinnamal, geraniol, and isoeugenol (International Flavors & Fragrances, Union Beach, NJ, USA) were prepared in petrolatum (Snow White Quality E; Apoteket Produktion & Laboratorier, Göteborg, Sweden) at our department in the concentrations given in Table 1. The FM I—consisting of the seven above-mentioned substances and in addition OMA (Robertet, Grasse, France)—was prepared in petrolatum together with 5.0% of the emulsifying agent sorbitan sesquioleate by Chemotechnique Diagnostics (Vellinge, Sweden). The concentrations of the individual fragrance ingredients in the mix were 1.0% and the same batches of the seven chemically defined substances were used as when they were prepared individually. Dichloromethane, heptane (Fisher Scientific, Loughborough, UK), tetrahydrofuran (THF) (Merck KGaA, Darmstadt, Germany), and 99.5% ethanol (Kemetyl AB, Haninge, Sweden) were used in the chromatographic investigations of the petrolatum preparations.

3.1.2 Study II

Petrolatum preparations of FM I and its individual ingredients, prepared as described above, were used for patch testing in study II. Petrolatum preparations of the FM II ingredients citral, hexyl cinnamal (Firmenich Inc., Plainsboro, NJ, USA), citronellol (Bedoukian, Danbury, CT, USA), farnesol (Symrise GmbH & Co. KG, Holzminden, Germany), coumarin (Rhodia Opérations, Aubervilliers, France), and HICC (International Flavors & Fragrances, Union Beach, NJ, USA) were prepared at our department in the concentrations given in Table 2. The FM II, containing each of the 6 above-mentioned substances at concentrations corresponding to 50% of the concentrations used in the individual preparations was prepared in petrolatum by Chemotechnique Diagnostics. The same batches of substances were used both in the individual preparations and in the FM II preparation.

3.1.3 Study III

Two samples of OMA provided by Robertet (Grasse, France) were used in study III. Sample A(III) was a traditional OMA containing, in total, 2.7% atranol and chloroatranol; sample B(III) had been treated by the manufacturer in order to reduce the content of atranol and chloroatranol to a total concentration of 0.0066% (66 ppm). Samples A(III) and B(III) were patch-tested in serial dilutions prepared in our laboratory. Two per cent (2.0% w/v) stock solutions of samples A(III) and B(III) in acetone (VWR International S.A.S, Fontenay-sous-Bois, France) were further diluted by a factor of √10 to the following concentrations: 0.63%, 0.20%, 0.063%, 0.020%, 0.0063%, 0.0020%, 0.00063%, 0.00020%, and 0.000063% (w/v). Eleven of the subjects with negative or doubtful reactions to sample B(III) at 2.0% were also tested with sample B(III) at 6.3% (w/v).

Atranol and chloroatranol (Laboratoire de Dermatochimie, University Louis Pasteur, Strasbourg, France) were each dissolved and diluted in acetone to a concentration of 0.010% (w/v). Subjects who reacted positively to the 0.010% preparations of atranol and/or chloroatranol on D3 or D4 were additionally tested with dilutions of atranol and/or chloroatranol at 0.0032%, 0.0010%, 0.00032%, and 0.00010% (w/v). Subjects who were negative to the 0.010% preparations of atranol and/or chloroatranol on D3 or D4 were tested with 0.050% (w/v) preparations of atranol and/or chloroatranol. Furthermore, all subjects were tested with 0.1% (w/v) petrolatum preparations of the lichen allergens atranorin, usnic acid, and evernic acid (Chemotechnique Diagnostics).

Pyridine (Acros Organics, Geel, Belgium) and N-methyl-N-(trimethylsilyl)-trifluoracetamide (Acros Organics) were used in the GCMS analyses.

3.1.4 Study IV

Patch test and ROAT solutions were prepared in our department in a vehicle similar to those used in fine fragrances. The vehicle consisted of 2.0% (v/v) diethyl phthalate (DEP) (Sigma Aldrich, Steinheim, Germany) and 98.0% (v/v) ethanol (95%; Kemetyl AB). A sample of a traditional, untreated OMA (sample A(IV)) was provided by manufacturer I. Solutions of sample B(IV) containing equal amounts of 3 IFRA-compliant OMA samples with reduced levels of atranol and chloroatranol (from manufacturers I, II and III) were prepared from solutions of the individual samples. Samples A(IV) and B(IV) were patch-tested in dilution series with the same dilution steps as in a previous ROAT study on eugenol (105). Stock solutions (2.0% w/v) of samples A(IV) and B(IV) were further diluted by a factor of 2 to the following concentrations: 1.0%, 0.50%, 0.25%, 0.13%, 0.063%, 0.031%, 0.016%, 0.0078%, 0.0039%, 0.0020%, 0.00098%, 0.00049%, 0.00024%, 0.00012% and 0.000061% (w/v). To improve the sensitivity an extra dilution step at 1.3% (w/v) was included

between 2.0% and 1.0%. In addition, pure DEP and ethanol as well as the mixture of DEP and ethanol (2:98) were included in the patch test series. ROAT solutions of samples A(IV) and B(IV) were each prepared at a concentration of 0.10% (w/v). Furthermore, a sample of the vehicle and a 0.00020% (w/v) dilution of sample A(IV) with atranol and chloroatranol concentrations in the same order of magnitude as in the 0.10% preparation of sample B(IV) were used in the ROAT.

3.2 Subjects

3.2.1 Study II

Between April 2009 and December 2010, 795 consecutive dermatitis patients (507 female, 288 male; mean age 47 years, range 16–88 years) were simultaneously patch-tested with an extended baseline series containing duplicate samples of FM I and FM II as well as preparations of the individual ingredients of the mixes.

3.2.2 Study III

Fifteen subjects (11 females, 4 males; mean age 50 years, range 25-67 years) who had been diagnosed with contact allergy to OMA at our department between 2006 and 2008 were enrolled in the study. The strength of the original patch test reaction to OMA was scored as + in 5 subjects, as ++ in 7 subjects, and as +++ in 3 subjects.

3.2.3 Study IV

Fifteen subjects (13 females, 2 males; mean age 54 years, range 34–68 years) who had been diagnosed with contact allergy to OMA at our department during the period 2007–2011 were enrolled in the study. The strength of the original patch test reactions to OMA was scored as + in 5 subjects, as ++ in 5 subjects, and as +++ in 5 subjects. The study required 6 visits to our department, which is why it was necessary to recruit volunteers living or working close to Malmö. Due to this limitation, 6 subjects who had previously participated in study III were included in this study. In addition, 16 controls (13 females, 3 males; mean age 55 years, range 31–69 years) without contact allergy to fragrances or MP were included in the study. Exclusion criteria for the study were ongoing dermatitis at any of the test sites and treatment with systemic corticosteroids.

3.3 Patch testing

The patch testing in studies II, III, and IV was performed with 8 mm Finn Chambers, (Smart Practice, Phoenix, AZ, USA). According to recommendations, 20 mg of petrolatum preparations were applied in the test chambers (13). When patch testing with solutions, 15 µl was micropipetted onto the filter papers in the test chambers. The patches were removed by the patients/volunteers after 48 hours. In studies II and III, the tests were read on D3 or D4 and on D7. In study IV, the tests were read on D3 and D7. The tests were scored according to the ICDRG (12).

In study II, all patients were simultaneously patch-tested with duplicate samples of FM I and FM II as well as preparations of the individual ingredients of the mixes. For each fragrance mix one sample was applied to the test chamber 6 days in advance ("6D sample"), while the other sample ("fresh sample") was applied to the test chamber immediately before the test chambers were applied to the patient's back. The pre-loaded test chambers were stored on trays, which were put in plastic bags. The plastic bags containing the trays were stored in a cupboard at room temperature. The separate ingredients of the mixes were patch-tested freshly applied in the test chambers.

In study IV, the positions of the patch test preparations on the back were randomised for each subject and the tests were read by the dermatologist without knowing which test preparation had been applied at each position.

3.4 Preparation of thin-layer chromatograms used for patch testing

Patch testing with TLC strips was performed according to the procedure described by Bruze et al. (106). Samples A(III) and B(III) dissolved in acetone were applied on TLC strips cut from a Merck TLC silica gel 60 F254 plastic roll (Merck, Darmstadt, Germany). On the basis of the strength of previous patch test reactions, different doses, ranging from 0.06 to 3.0 mg of OMA diluted in acetone, were applied to the TLC strips. When the TLC patch tests were negative or doubtful at the first reading, the subjects were additionally tested with TLC strips containing a higher dose of sample A(III) or B(III). The samples were eluted on the TLC strips with a mobile phase consisting of 86% (v/v) chloroform (Merck) and 14% (v/v) acetonitrile (VWR International, Leuven, Belgium). For each subject, the TLC strips were prepared in duplicate; one strip was used for patch testing and the other was used as a template when the tests were read. The TLC strips were inspected under ultraviolet light at 254 and 366 nm. The visualised spots were marked with a pencil. The TLC strips were prepared as close in time to the patch test as possible, normally on the same day

or on the day before. The TLC strips were wrapped in aluminium foil and stored in a refrigerator until they were used for patch testing.

3.5 Repeated open application tests

The ROAT is designed to imitate the daily exposure to topically applied products (107). It has been used in combination with serial dilution patch tests in several studies on fragrances in order to investigate the relationship between elicitation threshold doses in patch tests and ROATs (105, 108-111). In study IV, a ROAT using OMA samples A(IV) and B(IV) and patch tests with serial dilutions of the two OMA samples were performed. The design of the study is described in Figure 4. The study was conducted in a double-blind fashion. One dermatologist read the patch tests and another dermatologist read the ROAT. They were not informed whether the subject belonged to the OMA-positive group or the control group. Also, the dermatologists were not aware of each other's observations.

The ROAT was performed on four 3 × 3 cm sites, two on the lower volar aspects of each arm. The corners of the squares were marked with a surgical marker pen. The squares and the 8-ml polypropylene droplet bottles (Chemotechnique Diagnostics) containing the ROAT solutions were coded A, B, C, and D. The content of the bottles was randomised according to a Latin square table. Neither the dermatologist nor the volunteers knew which solution was applied to each of the 4 sites on the arms. The participants were instructed how to apply the solution and to allow the solution to dry before putting on clothing. Two droplets (about 40 mg) of each solution were applied twice daily, and the solutions were distributed evenly on the marked sites with the tip of the bottle. The ROATs were evaluated on D3, D7, D14, D21, and D28. The ROAT was regarded as positive when at least 25% of the test area was covered with erythematous infiltration, with or without papules and/or vesicles. The strength of the reactions were classified as weak, moderate, or strong (15). In cases of only spotty erythema or a few papules, the subjects were encouraged to continue the application until a more marked response was seen. When a reaction was graded as positive, the participant was instructed to stop application to the site at which the reaction had occurred and continue with application of the solutions to the other sites. Every week, the used bottles were exchanged for fresh ones containing the same solutions. The bottles were weighed before and after use in order to obtain an estimate of the amount applied to the test sites.

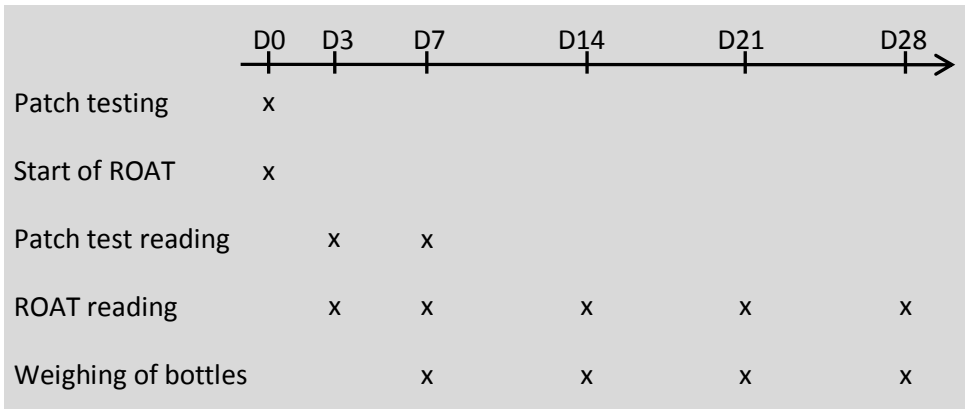


Figure 4. Design of study IV.

3.6 Chemical investigations

3.6.1 Stability investigations of petrolatum preparations

In study I, about 20 mg of the petrolatum preparations was applied in 8-mm Finn chambers stored at room temperature (23°C) or in a refrigerator (5°C). In addition, about 30 mg of the petrolatum preparation of cinnamal was applied in IQ chambers (Chemotechnique Diagnostics) stored under the same conditions. Duplicate samples of the petrolatum preparations were applied in test chambers for each individual reading. The samples were withdrawn and analysed at 0, 4, 8, 24, 72, and 144 h after application. When analysed, about 10–20 mg of each sample was carefully weighed into 10-ml volumetric flasks. The samples of cinnamal, cinnamyl alcohol, geraniol, hydroxycitronellal, isoeugenol, and FM I were dissolved in mixtures of heptane and 99.5% ethanol or heptane and THF in the same proportions as in the mobile phases used in the high-performance liquid chromatography (HPLC) system where these substances were analysed. The samples of amyl cinnamal and eugenol were dissolved in dichloromethane and analysed using gel permeation chromatography (GPC). The mean value of the duplicate samples analysed at each reading was calculated.

3.6.1.1 Gel permeation chromatography

The GPC system consisted of a P4000 quaternary pump, a UV6000 diode array detector, an AS3000 auto injector, an SN4000 control module, and software controlled by ChromQuest 4.1 and monitored by Spectral Analysis for ChromQuest (all from ThermoFinnigan, San Jose, CA, USA). Two GPC columns coupled in series were used for the separation. The first column was a PSS SDV column (4.6 mm,

internal diameter 250 mm) packed with styrene-divinylbenzene copolymer (SDV) granules 5 µm, 100 Å (Polymer Standards Service, Mainz, Germany). The second column was a Shodex KF401HQ (4.6 mm internal diameter 250 mm) packed with SDV granules 3 µm, 50 Å (Showa Denko, Kanagawa, Japan). Dichloromethane was used as the mobile phase and the flow rate was 0.3 ml/min. The injection volume was 20 µl. The detector operated in the 200- to 450-nm range and the chromatograms recorded at 285 nm and 281 nm were used for detection and quantification of amyl cinnamal and eugenol. Standards were prepared in petrolatum and were treated in the same way as the samples.

3.6.1.2 High-performance liquid chromatography

The analyses of amyl cinnamal and eugenol were performed using GPC. As the GPC method did not allow us to separate all of the substances studied from the petrolatum components, the rest of the analyses were performed using a chromatographic method based on HPLC. This method also improved the possibility of separating the ingredients in FM I.

The petrolatum preparations of cinnamal, cinnamyl alcohol, geraniol, hydroxycitronellal, and isoeugenol were analysed with a La Chrom Elite HPLC system consisting of an L-2200 autosampler, an L-2130 pump, and an L-2455 diode array detector (Hitachi High-Technologies Corporation, Tokyo, Japan). The system was controlled by EZ Chrom Elite software (Agilent Technologies, Santa Clara, CA, USA). The samples of cinnamal, cinnamyl alcohol, geraniol, hydroxycitronellal, and isoeugenol were eluted isocratically through a Phenomenex Luna column (4.6 mm, internal diameter 250 mm) packed with silica 5 µm, 100 Å (Phenomenex, Torrance, CA, USA). using a mobile phase consisting of heptane/ethanol 90:10 (v/v) for cinnamyl alcohol and isoeugenol, heptane/ethanol 97.5:2.5 (v/v) for geraniol, and heptane/THF 75:25 (v/v) for hydroxycitronellal. For analysis of cinnamal a gradient elution using mobile phases A (100% heptane) and B (heptane/THF 75:25) was used. The gradient started at 30% B and increased to 100% B in 4 min, and was then kept at 100% B for 4 min. The same gradient elution was also used when analysing cinnamal and cinnamyl alcohol in FM I. The injection volume was 20 µl and the flow rate was 2.0 ml/min. The detector operated in the 215- to 400-nm range and the wavelengths used for detection and quantification of the substances studied were 285 nm for cinnamal, 250 nm for cinnamyl alcohol, 215 nm for geraniol, 290 nm for hydroxycitronellal, and 260 nm for isoeugenol. When cinnamal was analysed in FM I, the wavelength used for quantification was 300 nm. Standard solutions were prepared by dissolving the pure substances in the mobile phase used for each substance. The repeatability of the HPLC method was determined by repeated injections of each petrolatum preparation. The coefficient of variation was 0.3% for cinnamal (9 injections), 0.6% for cinnamyl alcohol (10 injections), 0.3% for geraniol (10 injections), 2.8% for hydroxycitronellal (10 injections) and 1.0% for isoeugenol (10 injections).

3.6.2 Thin-layer chromatography

In study III, the TLC retardation factor values of atranol and chloroatranol were examined when they were applied as individual substances as well as in spiked OMA samples. The TLCs were prepared as described earlier.

Gas chromatography–mass spectrometry

The levels of atranol and chloroatranol in samples A(III) and B(III) were determined by gas chromatography–mass spectrometry (GCMS). Samples A(III) and B(III) and also atranol and chloroatranol standards were dissolved in pyridine and derivatised with N-methyl-N-(trimethylsilyl)-trifluoracetamide before being injected into the GCMS system. The system consisted of an Agilent 6890N gas chromatograph (Agilent Technologies) equipped with an HP-MSI 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 of Alphagaz 2 quality (Air Liquide, Malmö, Sweden) with a flow rate of 1.0 ml/min. The injection was splitless and the inlet was heated to 250°C. The injection volume was 1 μl. The temperature program was isothermal at 70°C for 3 min, was then raised by 8°C/min to a final temperature of 300°C, and was isothermal at this temperature for 10 min.

Electron-ionization mass spectra were recorded with a Jeol GCmate II mass spectrometer (Jeol Datum Ltd, Tokyo, Japan) in scan mode recording ions with m/z from 50 to 600 u, with a scan duration of 0.3 s and an interscan delay of 0.2 s. The temperature of the ion source was 250°C and the GCMS interface temperature was 250°C. The electron energy was 70 eV. The National Institute of Standards and Technology library of mass spectra was used for identification.

3.7 Data recording

Daluk, a computer-based registration system in which age, gender, and contact allergies are recorded (112), was used in study II to obtain data on the simultaneous patch testing with pre-loaded and freshly applied fragrance mixes and their constituents. Daluk was also used when we selected patients who would be asked to participate as volunteers in studies III and IV.

3.8 Ethics

Studies III and IV were approved by the Regional Ethical Review Board in Lund, Sweden and conducted in accordance with the ethical standards specified in the Declaration of Helsinki. Written informed consent was obtained from each participant.

3.9 Statistics

3.9.1 Study II

McNemar's test (two-sided) was used to compare the number of patients reacting to the fresh preparations and to the 6D preparations of FM I and FM II. Differences were considered significant at $p < 0.05$. McNemar's test was also used to compare the numbers of patients with stronger reactions to the fresh sample than to the 6D sample and vice versa. In these calculations, all doubtful reactions were regarded as negative. Differences were considered significant at $p < 0.05$.

3.9.2 Study III

McNemar's test (two-sided) was used to compare the number of OMA-allergic subjects reacting to the patch tests of samples A(III) and B(III). Differences were considered to be significant at $p < 0.05$. The minimum eliciting concentrations (MECs) were compared between samples A(III) and B(III). The positive patch test reactions were not always continuous. When the negative and/or doubtful reactions were followed by at least the same number of positive reactions, the lowest concentration giving a positive reaction was considered to be the MEC. Otherwise, the last positive concentration above the negative or doubtful reactions was considered to be the MEC (113). When the ratio between the MEC of sample B(III) and the MEC of sample A(III) was calculated for the subjects who tested totally negative for sample B, it was assumed that they would test positive if they were tested with a preparation of sample B(III) at a concentration corresponding to a step further up in the dilution series, i.e. the highest tested concentration multiplied by a factor of $\sqrt{10}$.

3.9.3 Study IV

Fisher's exact test (two-sided) was used when comparing the number of subjects and controls reacting positively to the patch tests and the ROATs. McNemar's test (two-sided) was used to compare the number of OMA-allergic subjects reacting to samples A(IV) and B(IV) in the patch tests and ROATs. McNemar's test was also used to compare the reactivity (expressed as MEC) to the patch tests of samples A(IV) and B(IV), and also for comparison of the time required for elicitation of a positive ROAT reaction. Differences were considered significant at $p < 0.05$. The positive patch test reactions were not always continuous. When the number of negative and/or doubtful reactions was followed by at least the same number of positive reactions, the lowest positive reaction was considered the MEC. If negative or doubtful reactions at 2.0% and 1.3% were followed by a positive reaction at 1.0% (as in subject 3), the latter was registered as the MEC. Otherwise, the last positive concentration above the negative or doubtful reactions was considered the MEC (113). In the calculations of the ratio of the MEC of samples A(IV) and B(IV), it was assumed that subjects who tested negative for to sample A(IV) and/or sample B(IV) would test positive to these samples at a concentration of 4.0% (w/v), i.e. the highest concentration in the dilution series multiplied by a factor of 2.

The correlation between the reactivity in the patch test of sample A(IV) and the reactivity in the ROAT of samples A(IV) and B(IV) was assessed using Spearman rank correlation. In these calculations, the patch test reactivity, expressed as the MEC, and the ROAT reactivity, expressed as the number of days until observation of a positive reaction, was ranked. The higher the reactivity, the lower the rank number. Subjects with negative patch tests and/or ROATs, i.e. those showing the lowest reactivity, were given the highest rank number.

4 Results

4.1 Study I

The concentrations of the FM I ingredients studied at the different storage times and under different conditions are presented in Figures 5–7. For all substances, the decrease in concentration was more rapid for the samples stored at room temperature than for the samples stored in a refrigerator. The concentrations of cinnamal, cinnamyl alcohol, eugenol, and geraniol in petrolatum preparations stored in Finn chambers at room temperature decreased to < 80% of the initial concentration within 8 h (Figure 5). Of the refrigerated samples, only the preparation of cinnamal showed a decrease in concentration to < 80% of the initial concentration within 24 h. The stability of cinnamal was slightly better when stored in IQ chambers than in Finn chambers. Nevertheless, the cinnamal concentration decreased to < 80% of the initial concentration within 4 h at room temperature and within 24 h in a refrigerator when stored in IQ chambers (Figure. 6). The observed decrease in concentration over time was lower when cinnamal and cinnamyl alcohol were analysed as ingredients in FM I than when they were analysed individually (Figure 7). No additional or growing peaks were observed in any of the chromatograms.

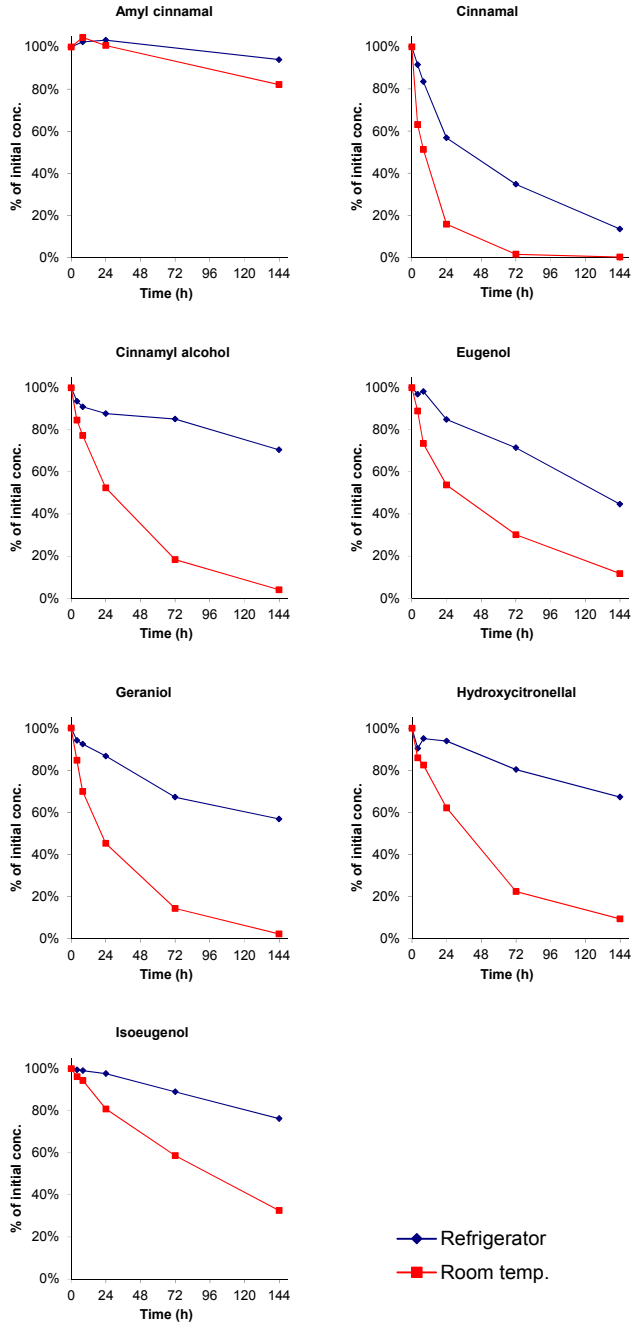


Figure 5. Concentrations of petrolatum preparations of amyl cinnamal, cinnamal, cinnamyl alcohol, eugenol, geraniol, hydroxycitronellal, and isoeugenol applied in Finn Chambers stored at room temperature or in a refrigerator.

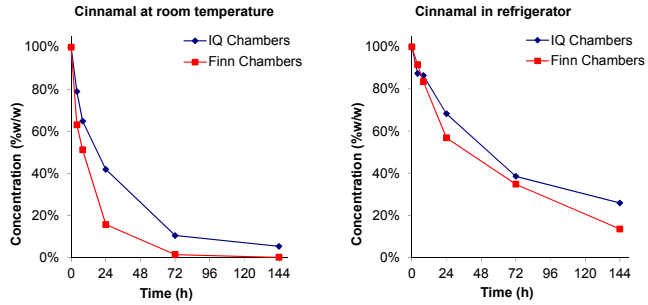


Figure 6. Concentrations of petrolatum preparations of cinnamal applied in Finn Chambers and IQ chambers stored at room temperature or in a refrigerator.

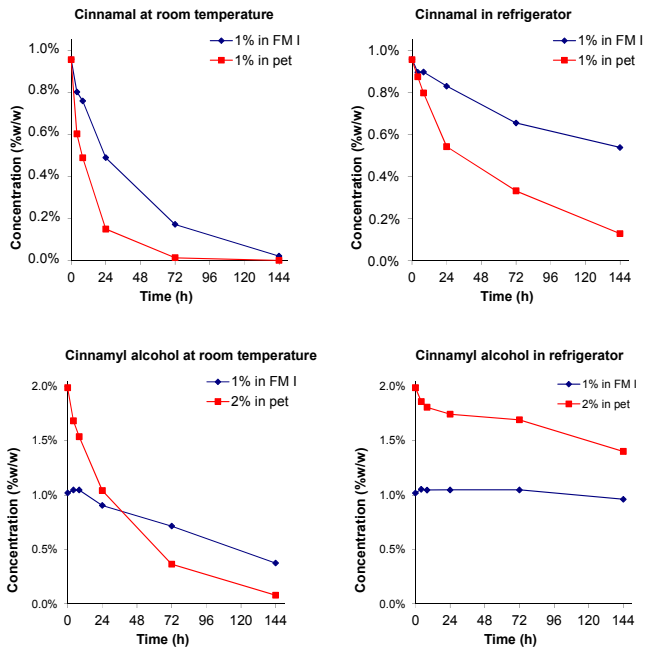


Figure 7. Concentrations of cinnamal and cinnamyl alcohol analysed in individual petrolatum preparations and as components of fragrance mix I (FM I). The test preparations were applied in Finn chambers and stored at room temperature or in a refrigerator.

4.2 Study II

The outcome of the patch tests with fresh and 6D samples of FM I and FM II is presented in Figure 8 and Table 5. There was a significant difference in the number of patients who reacted to the fresh sample and to the 6D sample of FM I ($p = 0.0037$). Twenty-two patients (2.8%) reacted exclusively to the fresh sample, 6 (0.7%) reacted exclusively to the 6D sample, and 22 (2.8%) reacted to both samples. Five of 22 patients who reacted exclusively to the fresh sample had a doubtful reaction to the 6D sample. Two of 6 patients reacting exclusively to the 6D sample had a doubtful reaction to the fresh sample. A significant difference was also observed when we compared the strength of the reactions to the fresh sample and the 6D sample of FM I ($p < 0.0001$). Twenty-five patients showed a stronger reaction to the fresh sample than to the 6D sample, 6 patients showed a stronger reaction to the 6D sample than to the fresh sample, and 19 patients showed reactions of equal strength to both samples.

No statistically significant difference was found between the numbers of patients who reacted to the fresh sample and to the 6D sample of FM II ($p > 0.3$). Nine (1.1%) reacted exclusively to the fresh sample, 6 (0.7%) reacted exclusively to the 6D sample, and 12 (1.5%) reacted to both samples; nor was any significant difference found when the strengths of the reactions to the fresh and 6D samples were compared ($p > 0.3$). All the patients who reacted exclusively either to the fresh sample or to the 6D sample of FM II showed + reactions, and all patients reacting to both samples showed the same degree of reactivity to both samples. Two of 9 patients who were exclusively positive to the fresh sample had doubtful reactions to the 6D sample, and 1 of 6 patients exclusively positive to the 6D sample had a doubtful reaction to the fresh sample.

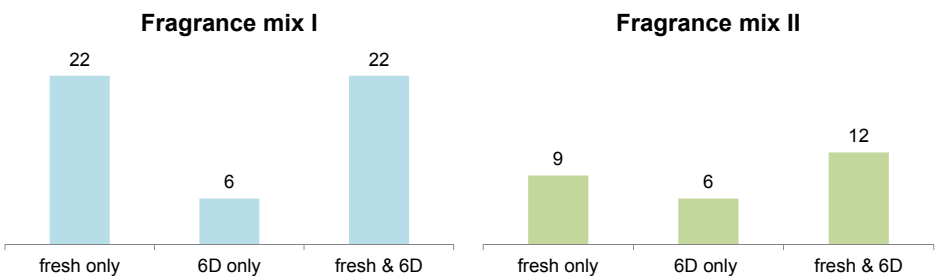


Figure 8. .Numbers of positive reactions to fragrance mix I and fragrance mix II applied in the test chambers 6 days in advance (6D) and immediately before the patch testing (fresh).

Table 5. Strength of patch test reactions in 65 subjects with a positive reaction to samples of fragrance mix I (FM I) and/or fragrance mix II (FM II) applied in the test chambers 6 days in advance (6D) and/or immediately before the patch test occasion (fresh)

	FM I		FM II	
Case no.	**fresh**	**6D**	**fresh**	**6D**
1–3	-	-	-	+
4–6	-	-	+	-
7–8	-	-	+	(+)
9–10	-	-	+	+
11	-	-	++	++
12	-	-	+++	+++
13	-	(+)	(+)	+
14	-	(+)	+	+
15–18	-	+	-	-
19	(+)	-	+	-
20–21	(+)	+	-	-
22–32	+	-	-	-
33–34	+	-	+	-
35	+	-	+	+
36–39	+	(+)	-	-
40–45	+	+	-	-
46	+	+	+	+
47	++	-	-	-
48	++	-	+	-
49–50	++	+	-	-
51	++	+	++	++
52–55	++	++	-	-
56–57	++	++	-	+
58	++	++	++	++
59	++	++	+++	+++
60	+++	-	-	-
61	+++	(+)	(+)	(+)
62–63	+++	+++	-	-
64–65	+++	+++	++	++

Thirty-eight patients reacted to at least one of the individual ingredients of FM I (Table 6). Twenty-nine of these patients also showed reactions to the fresh and/or 6D sample of FM I. In the 22 patients who reacted exclusively to the fresh sample of FM I, in total 8 reactions to the ingredients of the mix were observed in 6 patients. Four reactions were observed in 3 patients who reacted exclusively to the 6D sample and 34 reactions were observed in 20 patients who reacted to both samples of FM I. Nine reactions to the ingredients of the mix were observed in 9 patients who did not react to any of the FM I samples. Five of these were totally negative to the FM I samples, while 4 had doubtful reactions to the fresh sample and/or the 6D sample of FM I.

Twenty-six patients reacted to at least one of the individual ingredients of FM II (Table 7). Seventeen of these patients showed simultaneous reactions to the fresh and/or 6D sample of FM II. Among the 9 patients exclusively positive to the fresh sample of FM II, 7 reactions to the ingredients of the mix were observed in 5 patients. Two reactions were observed in 2 patients reacting exclusively to the 6D sample and 11 reactions were observed in 10 patients reacting to both samples of FM II. Nine reactions to the ingredients of the mix were observed in 9 patients not positive to any of the FM II samples. 4 of these were totally negative to the FM II samples, while 5 had doubtful reactions to the fresh and/or the 6D sample of FM II.

Table 6. Numbers of positive reactions to ingredients of fragrance mix I (FM I) and their distribution with regard to reactions to FM I samples applied to the test chambers 6 days in advance (6D) and immediately before the patch testing (fresh)

	FM I-positive			FM I-negative
Test preparation	**only fresh**	**only 6D**	**fresh and 6D**	
amyl cinnamal	0	0	0	0
cinnamal	1	1	6	3
cinnamyl alcohol	1	1	5	0
eugenol	0	0	4	1
oak moss absolute	3	1	9	1
geraniol	1	0	0	1
hydroxycitronellal	0	0	5	1
isoeugenol	2	1	5	2
Total no. of reactions (no. of patients)	8 (6)	4 (3)	34 (20)	9 (9)

Table 7. Numbers of positive reactions to ingredients of fragrance mix II (FM II) and their distribution with regard to reactions to FM II samples applied to the test chambers 6 days in advance (6D) and immediately before the patch testing (fresh)

	FM II-positive			FM II-negative
Test preparation	**only fresh**	**only 6D**	**fresh and 6D**	
citral	3	0	3	5
citronellol	1	0	0	0
coumarin	0	0	1	1
farnesol	3	1	1	1
hexyl cinnamal	0	0	1	0
hydroxyisohexyl 3-cyclohexene carboxaldehyde	0	1	6	2
Total no. of reactions (no. of patients)	7 (5)	2 (2)	11 (10)	9 (9)

4.3 Study III

The results of the patch tests with the dilution series of samples A(III) and B(III) are summarised in Table 8. All 15 subjects reacted to sample A(III) at concentrations of ≤ 2.0%, and 2 of 15 subjects reacted to sample B(III) at 2.0% ($p < 0.001$). Of the subjects with negative or doubtful reactions to sample B(III) at 2.0%, 4 of 11 showed a positive reaction when the test concentration of sample B(III) was raised to 6.3%. The MECs were 0.002% for sample A(III) and 2.0% for sample B(III). The ratio between the MEC of sample B(III) and the MEC of sample A(III) varied between 10 and 3,200 in the individual subjects. The numbers of subjects with positive reactions to the additionally tested individual substances were 7 for chloroatranol, 6 for atranol, 4 for atranorin, 2 for evernic acid, and 1 for usnic acid. Positive reactions to the TLC strips of sample A(III) were observed in 13 of 15 subjects. Only 1 of 11 subjects tested with TLC strips of sample B(III) showed a positive reaction. The reactions to the TLC strips of sample A(III) were distributed all over the area where the components of the OMA had migrated (Figures 9 and 10). The retardation factor values of atranol and chloroatranol observed when OMA samples spiked with atranol and chloroatranol were applied to the TLC strips differed slightly from those observed when atranol and chloroatranol were applied individually. Furthermore, the retardation factor values were affected by the amount of sample applied to the TLC strips. The approximate region where atranol and chloroatranol appeared on the TLC strips is marked in Figure 9. Eleven subjects reacted to spots within this region, and, in total, 11 subjects showed positive reactions to other areas of the chromatograms. On analysis by GCMS, approximately 2.5% (25 000 ppm) of atranol and 0.93%

(9300 ppm) of chloroatranol were found in sample A(III). Approximately 90 ppm of atranol and 20 ppm of chloroatranol were found in sample B(III).

Table 8. Patch test reactions to serial dilutions of sample A(III) and sample B(III) as well as individual test substances; the strongest reaction on either day D3/4 or day 7 is given

		Subject no.														
Sample	**Conc. (%)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
A(III)	2.0%	+++	++	++	NT	NT	++	NT	++	++	+++	+	+++	+	++	+
	0.63%	+++	++	+	+++	NT	++	NT	++	(+)	++	+	+++	-	++	-
	0.20%	++	++	+	+++	+	+	+++	+	+	+	+	++	-	++	-
	0.063%	+	++	-	++	+	(+)	++	+	-	-	(+)	+	-	+	-
	0.020%	+	++	-	(+)	+	-	-	(+)	-	-	-	+	-	+	-
	0.0063%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.0020%	-	(+)	-	-	+	-	-	-	-	-	-	-	-	-	-
	≤ 0.00063%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B(III)	6.3%	NT	NT	+	(+)	+	++	++	-	(+)	NT	-	NT	-	-	-
	2.0%	-	+	-	-	-	-	(+)	-	-	+	-	-	-	-	-
	0.63%	-	(+)	-	-	-	-	-	-	-	(+)	-	-	-	-	-
	≤ 0.20%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Evernic acid	0.1%	-	-	-	-	+	-	+	(+)	-	-	-	-	-	-	-
Usnic acid	0.1%	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-
Atranorin	0.1%	+	-	-	-	++	-	NT	+	-	-	-	-	-	-	-
	0.032%	+	NT	NT	NT	NT	NT	+++	NT	NT	NT	NT	NT	NT	NT	NT
	0.010%	(+)	NT	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	NT	NT
	0.0032%	(+)	NT	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	NT	NT
	0.0010%	-	NT	NT	NT	NT	NT	?	NT	NT	NT	NT	NT	NT	NT	NT
Atranol	0.050%	+	-	-	NT	++	+	NT	+	-	-	(+)	-	-	-	-
	0.010%	-	-	-	+	-	-	++	-	-	-	(+)	-	-	-	-
	0.0032%	NT	NT	NT	++	NT	NT	-	NT	NT	NT	NT	NT	NT	NT	NT
	0.0010%	NT	NT	NT	++	NT	NT	-	NT	NT	NT	NT	NT	NT	NT	NT
	0.00032%	NT	NT	NT	+	NT	NT	-	NT	NT	NT	NT	NT	NT	NT	NT
	0.00010%	NT	NT	NT	-	NT	NT	-	NT	NT	NT	NT	NT	NT	NT	NT
Chloroatranol	0.050%	++	-	-	NT	-	+++	NT	+	-	NT	(+)	++	(+)	-	-
	0.010%	-	-	-	++	-	+	NT	(+)	-	++	(+)	-	-	-	-
	0.0032%	NT	NT	NT	++	NT	NT	+	NT	NT	NT	NT	NT	NT	NT	NT
	0.0010%	NT	NT	NT	(+)	NT	NT	+	NT	NT	NT	NT	NT	NT	NT	NT
	0.00032%	NT	NT	NT	-	NT	NT	-	NT	NT	NT	NT	NT	NT	NT	NT
	0.00010%	NT	NT	NT	-	NT	NT	-	NT	NT	NT	NT	NT	NT	NT	NT

NT, not tested

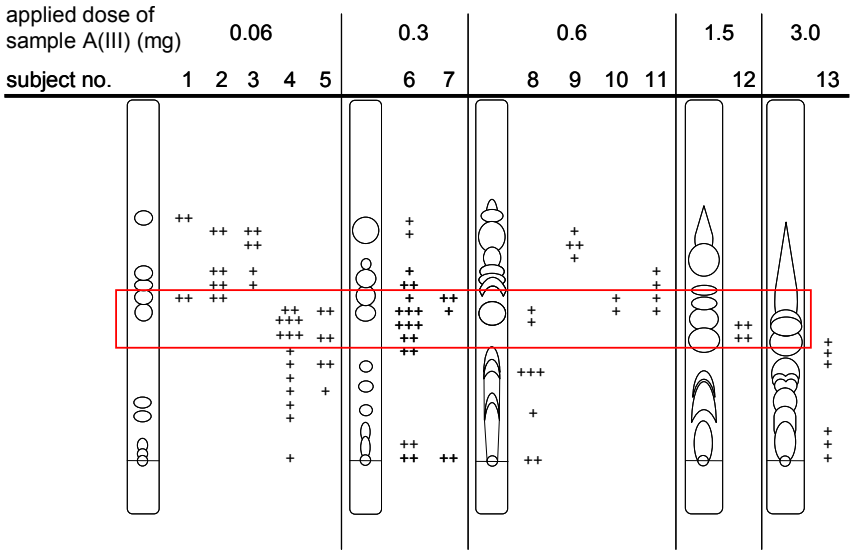


Figure 9. Distribution of positive reactions to thin-layer chromatography strips of sample A(III). The approximate area where atranol and chloroatranol appear on the chromatograms is marked in red.

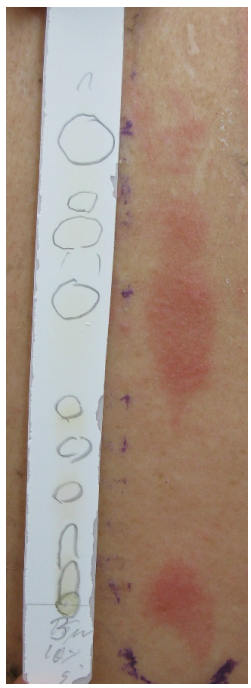


Figure 10. Positive reactions in subject 6 to components of sample A(III) on a thin-layer chromatography strip.

4.4 Study IV

4.4.1 Patch tests

The outcome of the patch tests is summarised in Table 9. Fourteen of 15 subjects of the OMA-allergic group reacted to at least one dilution of sample A(IV). No positive reactions to sample A(IV) were observed in the control group ($p < 0.001$). Eight of 15 subjects of the OMA-allergic group reacted to at least one preparation in the dilution series of sample B(IV), while no positive reactions were observed in the control group ($p < 0.001$). There was a statistically significant difference in the number of subjects who reacted to samples A(IV) and B(IV) ($p = 0.031$). Thirteen subjects were found to be more reactive to sample A(IV) and 2 were equally reactive to samples A(IV) and B(IV) ($p < 0.001$).

4.4.2 Repeated open application tests

The time required for elicitation of allergic reactions is given in Table 9. Figure 11 shows a positive ROAT to sample B(IV) in subject 12. The ROAT of sample A(IV) was positive in 11 of 15 subjects of the oak moss-allergic group and in none of the controls when tested at 0.10% ($p < 0.001$) and in 8 of 15 subjects and in none of the controls when tested at 0.00020% ($p < 0.001$). The ROAT of sample B(IV) at 0.10% was positive in 8 of 15 subjects and in none of the controls ($p < 0.001$). No reactions were observed for the vehicle, neither in the oak moss-allergic group, nor in the control group. There were no statistically significant differences between the number of subjects who reacted to samples A(IV) and B(IV) at 0.1% ($p = 0.25$), sample A(IV) at 0.10% and at 0.00020% ($p = 0.25$), or sample A(IV) at 0.00020% and sample B(IV) at 0.10% ($p > 0.3$). However, a significant difference was observed when comparing the number of days until observation of a positive reaction after exposure to sample A(IV) at 0.10% and sample B(IV) at 0.10%. Ten subjects were found to react earlier to sample A(IV) than to sample B(IV) and 5 were equally reactive to samples A(IV) and B(IV) ($p = 0.0020$). Similarly, there was a significant difference when comparing the reactivity to the 0.10% and 0.00020% preparations of sample A(IV). Eight subjects were more reactive to the 0.10% preparation and 7 were equally reactive ($p = 0.0078$). No statistically significant difference was found between the reactivity to sample A(IV) at 0.00020% and sample B(IV) at 0.1% ($p > 0.3$).

Figure 12 illustrates the relationship between the patch test reactivity to sample A(IV) and the outcome of the ROATs. Correlations were found between the MEC of sample A(IV) and the number of days until a positive reaction to the ROAT of sample A(IV) at 0.10% ($r = 0.85$, $p < 0.001$), the MEC of sample A(IV) and the number of days until a positive reaction to the ROAT of sample A(IV) at 0.00020%

(r = 0.76, p = 0.0011), and between the MEC of sample A(IV) and the number of days until a positive reaction to the ROAT of sample B(IV) at 0.10% (r = 0.86, p < 0.001).

The OMA-allergic subjects applied on average 140 (range 88–230) mg/day of the ROAT solutions to each test site and the controls applied on average 130 (range 86–230) mg/day.

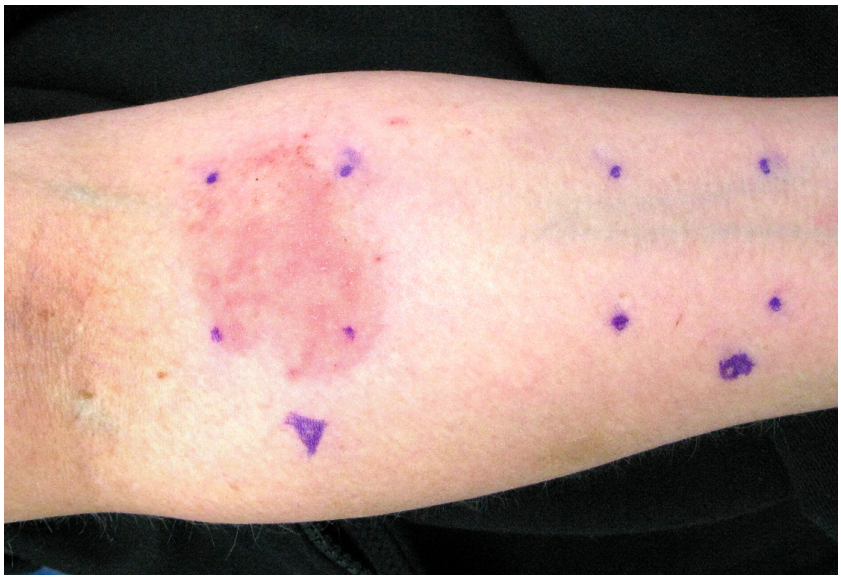


Figure 11. A positive repeated open application test to sample B(IV) 0.10% (w/v) in subject 12 on day 14.

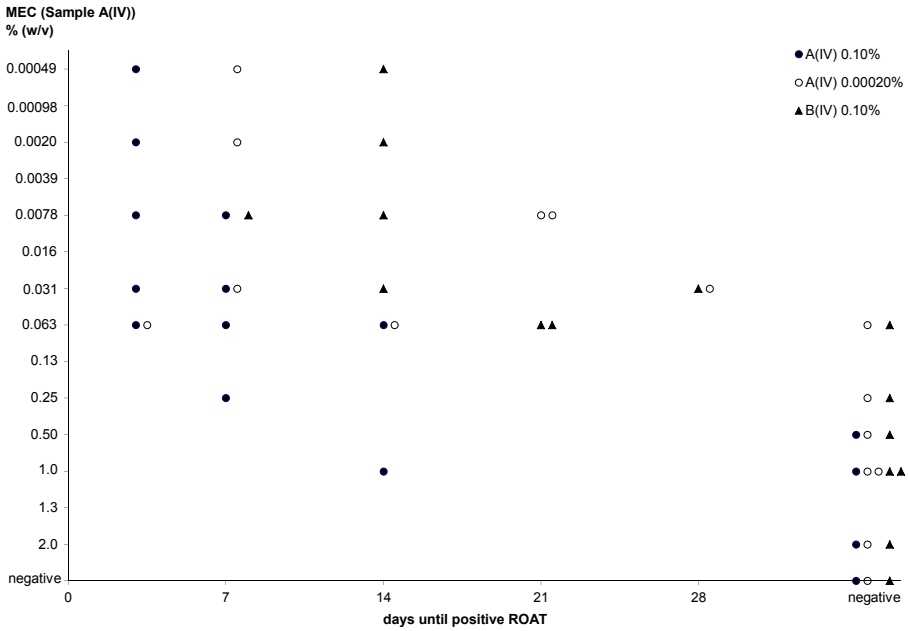


Figure 12. Relationship between the reactivity to the patch tests of sample A(IV) (expressed as the minimum eliciting concentration (MEC)) and the number of days until observation of a positive repeated open application test (ROAT).

Table 9. Patch test reactions of oak moss-allergic subjects to serial dilutions of samples A(IV) and B(IV)), and also the number of days until observation of positive repeated open application tests (ROATs). The concentrations of samples A(IV) and B(IV) are given as % (w/v), while the concentrations of diethylphthalate (DEP) and ethanol are given as % (v/v). The strongest reaction on either day 3 or day 7 is given.

Sample	Conc. (%)	Subject no.														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A(IV)	2.0	+++	+++	(+)[a]	+++	++	(+)	+++[a]	+++	++	+	-	++	+++	+[a]	+++
	1.3	+++	++	-	+++[a]	+++	(+)	+++[a]	++	++	(+)[a]	-	++	+++	+	+++
	1.0	+++	++	+	+++	++	+[a]	++[a]	++	++	-	-	++	+++	+	+++
	0.50	+++	++	-	++	++	+[a]	-	++	+	-	-	+++	++[a]	-	+++
	0.25	+++	++	-	+++	++	-	++[a]	++	+	-	-	++	++[a]	(+)[a]	+++
	0.13	+++	++	-	+	+	(+)	-	+	-	-	-	++	++[a]	-	+++
	0.063	++	++	-	+++	+	-	(+)[a]	+	+	-	-	++	+	-	+++
	0.031	++	++	-	+	-	(+)	+	(+)	(+)	-	-	++	+	-	+++
	0.016	+	++	-	-	-	(+)	(+)	(+)	(+)	-	-	++	(+)	-	+++
	0.0078	(+)[a]	+	(+)[a]	(+)[a]	-	-	(+)	-	-	-	-	+	-	-	+++
	0.0039	+	-	-	-	-	-	-	-	-	-	-	(+)	-	-	+
	0.0020	++	-	-	-	-	-	-	-	++	-	-	(+)	-	-	+
	0.00098	-	-	-	-	-	-	-	-	-	-	-	(+)	-	-	-
	0.00049	-	(+)	-	-	-	-	-	-	(+)	-	-	-	-	-	+
	0.00024	-	-	-	-	-	-	-	-	-	-	-	(+)	-	-	(+)
	≤0.00012	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B(IV)	2.0	(+)[a]	-	+[a]	++	IR	+	(+)	(+)	+	(+)[a]	-	+	-	-	+++
	1.3	-	+	-	+	-	-	-	-	+	-	-	(+)	-	-	+++
	1.0	-	-	+[a]	-	-	(+)	-	+	++	-	-	+	-	-	++
	0.5	-	IR	-	-	-	-	(+)	-	-	(+)	-	-	-	-	++
	0.25	+	-	-	-	-	(+)	(+)	-	-	(+)	-	-	-	-	+
	0.13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	0.063	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	(+)
	0.031	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.016	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)
	≤ 0.0078	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DEP	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ethanol	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
DEP/ethanol	2:98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		No. of days until positive ROAT/Strength of reaction[b]														
A(IV)	0.10	3/S	7/S	-	7/S	14/S	-	7/M	3/S	7/S	-	-	3/S	3/S	14/M	3/S
	0.00020	7/M	21/W	-	7/W	14/S	-	-	3/W	-	-	-	21/M	28/M	-	7/M
B(IV)	0.10	14/W	7/W	-	14/S	21/S	-	-	-	21/W	-	-	14/S	28/M	-	14/W
DEP/ethanol	2:98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

a, reactions on day 7

b, the reactions were graded as strong (S), medium (M), or weak (W)

5 Discussion

5.1 Studies I and II

Factors such as the purity, stability, and amount of patch test preparations applied may influence the outcome of the patch test (13, 114-118). We are usually careful when handling volatile solvents, e.g. acetone, as vehicles for patch test preparations in order to avoid evaporation of the solvent, which would result in an increased concentration of the allergen in the patch test solution. However, the allergens themselves may also evaporate from test preparations. Surprisingly, no-one appears to have considered this possibility and the significance of such an evaporation for the patch test result until our group started to explore this question some years ago (119). Since then, studies describing a decrease in concentration by evaporation from petrolatum preparations of acrylates, methacrylates, cinnamal, and eugenol have been published (120, 121).

A measure of the volatility of a substance is its vapour pressure, i.e. the pressure exerted by a vapour when the vapour is in equilibrium with its liquid or solid phase. The higher the vapour pressure, the more volatile the substance. The vapour pressure of a substance increases with increasing temperature. According to Raoult's law, the partial vapour pressure of a component in a mixture is equal to the vapour pressure of the pure component multiplied by its mole fraction in the mixture. This is valid for ideal solutions in which the forces between all molecules are equally strong. In non-ideal solutions, the partial vapour pressure of an individual component is also affected by interactions between the different molecules in the mixture. This effect is used in fragrance compositions, where fixative agents with low volatility are added in order to influence the molecular interactions in such a way that the evaporation rate of other substances is reduced (122). In study I, cinnamal—which has the highest vapour pressure among the substances studied—was the most volatile substance, while amyl cinnamal (with the lowest vapour pressure) showed no decrease in concentration over 24 h. The rate of evaporation of both fragrance compounds and acrylates/methacrylates from petrolatum preparations appears to be related to the vapour pressure of the pure substances (120, 121, 123).

Study I was conducted under circumstances that may prevail in a patch test clinic when the prepared test chambers are stored in a refrigerator or at room temperature several hours or days before they are applied on the back of the patient. The test

chambers were stored in the way that they usually are when prepared in advance at our department. Both the Finn chambers and the IQ chambers were stored on trays. No plastic covers were used on the Finn chambers, while the IQ chambers were covered with their built-in plastic covers.

Several of the test preparations analysed showed a rapid decrease in concentration after being applied in test chambers stored at room temperature. When the test chambers were stored in a refrigerator, the decrease in concentration was slower for all substances. In theory, the reduced content of the substances under study in the test preparations could be explained either by evaporation or by consumption in a chemical reaction, e.g. by oxidation. The observed decrease in concentration is most likely explained by evaporation of the substances from the petrolatum test preparations. Autoxidation of geraniol and cinnamyl alcohol have been reported (37, 41). However, several weeks of air exposure of undiluted geraniol were needed to convert geraniol into its oxidation products to an extent corresponding to the decrease in concentration observed within a working day in study I (37). The autooxidation of cinnamyl alcohol dissolved in ethanol was quicker, resulting in cinnamal as the most abundant oxidation product (41). Since cinnamal was the most volatile of the substances investigated in Study I, it is likely that it would have evaporated from the petrolatum preparation if it had been formed from cinnamyl alcohol, and would thus not have been detected in our chemical analyses. From our chemical analyses, there were no indications of new substances being formed in any of the samples.

We also analysed the concentrations of cinnamal and cinnamyl alcohol in preparations of FM I applied in Finn chambers, in order to compare the results with those observed when these substances were analysed individually. For both substances, the decrease in concentration was slower in FM I than in the individual petrolatum preparations. For cinnamyl alcohol, it is likely that this difference can be explained to some extent by the lower concentration in FM I (1.0% (w/w) as compared to 2.0% (w/w) when studied alone). Cinnamal was studied at a concentration of 1.0% (w/w), both in the individual petrolatum preparation and in FM I. However, a different kind of petrolatum was used in FM I than in the individual preparations prepared at our department. This might have affected the evaporation rate from the test preparations, as the physical and chemical properties of the two kinds of petrolatum may differ. The difference observed may also be explained by interactions between cinnamal and cinnamyl alcohol and other components in the FM I, resulting in a reduced evaporation rate. When comparing the stability of cinnamal in the preparation used in study I to that of a 1.0% preparation made from the same batch of cinnamal in petrolatum purchased from Chemotechnique Diagnostics and to that of a 1.0% cinnamal preparation in petrolatum purchased from Chemotechnique Diagnostics, the rate of evaporation was found to be lower in the two latter preparations. This indicates that the kind of

petrolatum used may affect the stability of petrolatum preparations of cinnamal (unpublished observations).

It is not uncommon that patients with positive reactions to FM I do not react to any of the individual ingredients when tested separately (44, 54). If our test preparations of FM I and cinnamal had been applied in test chambers at the same time a couple of hours before the actual patch test occasion, it would have been less likely that a patient with a weak allergy to cinnamal would react to the 1.0% preparation of cinnamal than to FM I, in which cinnamal is included in the same concentration. A deviation of ± 20% of the stated concentration in a test preparation is considered acceptable by commercial manufacturers (124). In study I, the concentration decreased by > 20% within 8 h in 4 of 7 preparations stored in Finn chambers at room temperature. Of the refrigerated samples, only the preparation of cinnamal had decreased in concentration by > 20% over 24 h. Preparations of cinnamal were also applied in IQ chambers equipped with a plastic cover. The stability of these preparations was slightly better than that of those applied in Finn chambers. Still, the cinnamal concentration in the petrolatum preparation applied in covered IQ chambers decreased by > 20% of the initial concentration within 4 h at room temperature, and within 24 h in a refrigerator.

In study II, we wanted to compare the patch test reactivity to fresh samples and to samples applied in the test chambers a couple of days in advance in consecutively patch-tested patients. Our patch test clinic usually runs on Mondays and Thursdays, and for practical reasons we chose to apply the samples 6 days in advance. The tests used on a Monday were prepared on the Tuesday of the previous week and the tests used on a Thursday were prepared on the Friday of the previous week.

A statistically significant difference was observed between the number of patients who reacted to the fresh sample and 6D sample of FM I. In the 22 patients who reacted exclusively to the fresh sample, altogether 8 reactions to the components of FM I were observed in 6 patients. The number of reactions for each component was too small in order to be able to draw any firm conclusions. Of the ingredients in FM I, cinnamal was found to evaporate most quickly from petrolatum preparations. When analysed by HPLC, the concentration of cinnamal in FM I was found to be about 2% of the initial concentration when stored in Finn chambers at room temperature for 6 days (123). It could be expected that patients who react (weakly) to cinnamal to a greater extent react to the fresh sample exclusively, but only one reaction to cinnamal was observed in the group that reacted to the fresh sample exclusively.

However, 6 of 7 patients who showed positive reactions to both cinnamal and the fresh sample of FM I showed ++ or +++ reactions to the latter. Although the concentration of cinnamal in the 6D sample was considerably lower in the 6D sample than in the fresh sample, it may still have been sufficient to elicit a positive reaction in patients with a strong allergy (125). Furthermore, 5 of 7 patients who showed positive

reactions to both cinnamal and the fresh sample of FM I also reacted to cinnamyl alcohol. Three of these patients were also positive to at least 1 other FM I ingredient.

Interestingly, we found no significant difference in the numbers of patients who reacted to the fresh sample and 6D sample of FM II. With the exception of HICC, the stabilities of petrolatum preparations of FM II ingredients applied in test chambers are not known. HICC has been found to evaporate only to a minor extent from petrolatum preparations applied in test chambers stored at room temperature (126).

With the exception of citral and citronellol, all the substances in FM II have vapour pressures lower than that of amyl cinnamal, which was the most stable of the FM I ingredients. The low vapour pressures of several of the substances in FM II may explain why the reactivity to the fresh sample and the 6D sample was essentially the same. Citral has the highest vapour pressure of the FM II ingredients. Of the 6 patients who reacted to both citral and the fresh sample of FM II, 3 reacted to the fresh sample only and not to the 6D sample. This may have been due to the high vapour pressure of citral, resulting in a fall in the concentration of citral in the 6D preparation of FM II to an extent that resulted in negative reactions. Further chemical investigations on the stability of citral would be of interest, especially since the vapour pressure of citral is 2.6 times that of cinnamal.

A limitation of study II was that we only compared the patch test reactivity to the fresh fragrance mix samples to that of samples that had been stored in test chambers for 6 days. At least with regard to FM I, it would also be of interest to examine the reactivity to samples applied to the test chambers a couple of hours or up to a day before to the patch test occasion, which would correspond to a more realistic situation in a patch test clinic. However, when preparing for larger studies or when sending pre-loaded test chambers by mail, it is not unlikely that the petrolatum preparations are applied to the test chambers several days in advance. In these situations, test chambers with a built-in protective cover, such as the IQ or IQ Ultra chambers (Chemotechnique Diagnostics) are usually preferred. However, the ability of the protective cover to prevent evaporation appears to be limited according to our analysis of petrolatum preparations applied in IQ chambers. The van der Bend transport container has been found to be the best alternative when storing or transporting single doses of petrolatum preparations of volatile allergens (121).

5.2 Studies III and IV

5.2.1 Patch tests

The eliciting capacity in patch tests was significantly lower for the treated OMA samples than for the untreated OMA samples, both in study III and in study IV. In study III, 2 of 15 subjects reacted to the treated sample tested at 2.0% and all 15 reacted to the untreated sample tested at the same concentration. In study IV the corresponding numbers were 8 of 15 for the treated sample and 14 of 15 for the untreated sample. The positive reactions to the treated OMA samples could be explained by reactions to the remaining low levels of atranol and/or chloroatranol, or by reactions to other allergens present in the samples.

When patch testing with a 1% petrolatum preparation of OMA treated with a polymer-based method that reduced the content of atranol and chloroatranol to < 75 and < 25 ppm, respectively, Nardelli et al observed positive reactions in 8 of 14 OMA-allergic subjects (102) . Although the total level of atranol and chloroatranol (< 1 ppm) in the 1% petrolatum preparation used in this study was similar to or lower than that in the 2.0% solution of sample B(III), containing, in total, 1.3 ppm atranol and chloroatranol, more reactions were observed in the Nardelli study. This might indicate that the method used by the manufacturer to reduce the content atranol and chloroatranol content in sample B(III) to a larger extent also reduces the content of other allergens.

The concentrations of atranol and chloroatranol were in the same order of magnitude in samples B(III) and B(IV) (unpublished GCMS investigations). Again, the difference in the proportion of positive reactions to these samples may therefore be explained by differences in concentrations of other allergens in the extracts. However, when comparing results from different studies, one must take into consideration all the possible differences between the studies regarding to the origin of the OMA, concentrations and vehicles used, time of patch test reading, and reactivities of the study populations to the untreated OMA samples. Six subjects participated in both study III and study IV. Of these, 4 reacted to sample B(IV) at concentrations of ≤ 2.0%, while none reacted to sample B(III) at 2.0%. This indicates a difference in allergen content between samples B(III) and B(IV), although both were manufactured in compliance with the IFRA standard.

In study III, the ratio between the MEC of sample B(III) and the MEC of sample A(III) ranged from 10 to 3,200 in the individual subjects. In study IV, the ratio between the MEC of sample B(IV) and the MEC of sample A(IV) ranged from 1 to 2,000. This variation might indicate the importance of allergens other than atranol and chloroatranol. If the difference in reactivity to the treated and untreated samples was a result of the different levels of atranol and chloroatranol, one would expect a

ratio of the MECs in the same order of magnitude as the ratio between the total concentrations of atranol and chloroatranol in the samples (about 400-500).

In study III, 6 of 15 subjects tested positive to atranol and 7 of 15 tested positive to chloroatranol. The ratio between atranol and chloroatranol in OMA has been reported to be about 2:1 (96). Using this ratio, the concentration of atranol calculated was 0.036% (360 ppm) and 0.88 ppm in the 2.0% preparations of sample A(III) and sample B(III), respectively. The corresponding concentrations of chloroatranol were 0.018% (180 ppm) in sample A(III) and 0.44 ppm in sample B(III). Sample B(III) was also tested at 6.3%, and the calculated concentrations of atranol and chloroatranol in this sample were 2.8 ppm for atranol and 1.4 ppm for chloroatranol.

Of the 6 subjects who tested positive to sample B(III), 2 tested negative to both atranol and chloroatranol, 1 tested positive to 0.05% (500 ppm) atranol, 1 tested positive to 0.05% (500 ppm) atranol and 0.01% (100 ppm) chloroatranol, 1 tested positive to 0.01% (100 ppm) atranol and 0.001% (10 ppm) chloroatranol and 1 tested negative to atranol and positive to 0.010% (100 ppm) chloroatranol. The latter subject was unfortunately not tested with chloroatranol at lower concentrations. The other 5 subjects who tested positive to sample B(III) were either totally negative to atranol and chloroatranol or did not react to atranol and/or chloroatranol at concentrations corresponding to those in sample B(III). The reaction pattern in these subjects is an indication of the importance of other allergens in sample B(III). It is also possible that the presence of other substances in OMA may increase the skin penetration of atranol and chloroatranol.

Three subjects who tested positive to atranol and/or chloroatranol tested negative to sample B(III) and 1 had a doubtful reaction. In these cases, it is likely that the concentrations of atranol and chloroatranol in sample B(III) were too low to elicit a positive reaction. Two of the negative subjects were, however, not tested with sample B(III) at 6.3% and it is possible that positive reactions would have been observed at this concentration. Interestingly, the subject with the doubtful reaction to sample B(III) at 6.3% showed the lowest MEC of atranol.

All 4 subjects who tested positive to atranorin and/or evernic acid also tested positive to atranol and/or chloroatranol. One subject tested positive to usnic acid but did not react to atranol or chloroatranol.

Our GCMS investigations of derivatised samples of A(III) and B(III) confirmed that the total concentrations of atranol and chloroatranol were in the same order of magnitude as the concentrations stated by the manufacturer. We observed slightly higher concentrations, which might be explained by the breakdown of atranorin/chloroatranorin into atranol/chloroatranol during storage of the samples, or by the heat in the GCMS analysis. However, the mass fragments used for identification and quantification of atranol and chloroatranol were not observed when we analysed atranorin treated with the derivatising agent.

5.2.2 Thin-layer chromatography patch tests

The patch tests with TLC strips of sample A(III) indicated the presence of oak moss allergens other than atranol and chloroatranol. We observed reactions in several regions of the chromatograms other than those where atranol and chloroatranol appeared. Chemical investigations of the spots giving positive reactions are needed in order to identify the substances responsible for the reactions. Only 1 subject reacted to the TLC strip of sample B(III). The spots giving positive reactions were located in the region where atranol and chloroatranol would be expected on the TLC strip. It is theoretically possible that more reactions would appear if a higher dose of sample B(III) were to be applied to the TLC strip. This was not done because the separation is impaired when too much of the sample is applied to the TLC strip.

5.2.3 Repeated open application tests

The ROAT solutions were applied to the test sites with droplet bottles. The daily dose of the ROAT solutions applied to each test site was approximately the same for the oak moss-allergic group (140 mg) and for the control group (130 mg). However, both groups consumed more of the solutions than intended 80 mg/day. It is likely that this difference to some extent could be explained by spill or leakage of the solutions from the bottles. A better degree of accuracy might have been achieved by the use of fixed-volume micropipettes (27), though this would require a large number of pipettes and careful instructions on how to handle the pipettes and tips. The study was terminated after 28 days, and it is possible that more positive reactions in the ROAT would have been observed if the exposure had been continued for a longer period of time.

The ROATs of samples A(IV) and B(IV) showed no statistically significant difference in the number of subjects who developed an eczematous reaction. However, a significant difference was observed when we compared the number of days until observation of a positive reaction after exposure to sample A(IV) at 0.10% and after exposure to sample B(IV) at 0.10%. Ten subjects reacted earlier to sample A(IV) than to sample B(IV) and 5 subjects developed reactions to both samples after the same period of time.

The ROAT was performed with two concentrations of sample A(IV), 0.10 % and a 500-fold dilution of the 0.10% preparation, i.e. 0.00020%, which reflects the ratio by which the content of atranol and chloroatranol is reduced in a treated OMA. Of the 9 subjects with a positive ROAT to either sample A(IV) at 0.00020% or sample B(IV) at 0.10%, one reacted to sample A(IV) exclusively and one reacted to sample B(IV) exclusively. The similarity in reaction patterns indicates that the residual levels of atranol and chloroatranol were to a great extent responsible for the allergic reactions. The levels of these substances were in the same order of magnitude, both in sample

A(IV) at 0.00020% and in sample B(IV) at 0.10%, while the levels of substances that are not affected by the treatment of the absolutes would be considerably higher in the 0.10% preparation of sample B(IV).

We found correlations between the patch test reactivity (expressed as the MEC) to sample A(IV) and the time required to develop a positive ROAT to sample A(IV) at 0.10%, sample A(IV) at 0.00020%, and sample B(IV) at 0.10%. The lower the MEC of sample A(IV), the less time required to develop a positive ROAT.

Three subjects (Nos. 1, 5, and 13) were positive in the ROAT of sample B(IV) but were not considered to be positive to the patch tests of sample B(IV). Subject 1 had a doubtful reaction at 2.0% and + reactions at 0.25% and 0.031%, but was not considered positive since there were several negative reactions above the first positive reaction in the dilution series. In subject No. 5, the patch test of sample B(IV) at 2.0% were interpreted as irritant, while no patch test reactions of any kind were observed for sample B(IV) in subject 13. In subjects 1, 5, and 13 the content of the bottles had been randomised in such a way that sample B(IV) and the vehicle were applied to one arm and the 2 dilutions of sample A(IV) were applied to the other. Thus, the risk of false-positive reactions to the ROAT of sample B(IV) due to spill of sample A(IV) or spreading of an eczematous reaction to sample A(IV) onto the area where sample B(IV) was applied could be ruled out.

6 Summary and concluding remarks

The evaporation of FM I fragrance ingredients from petrolatum preparations observed in study I is of clinical importance. Significantly more reactions were observed when patch testing with a fresh sample of FM I than with a sample applied in the test chamber 6 days in advance. No corresponding difference between the fresh sample and the sample applied in advance was not observed for FM II, possibly due to the low volatility of several of the ingredients of the mix. However, knowledge is lacking about the stability of most of the ingredients of FM II when present in petrolatum preparations. It cannot therefore be ruled out that single substances of the mix would evaporate to an extent that may affect the outcome of the patch test.

The patch tests performed in studies III and IV demonstrated that the OMA samples with reduced content of atranol and chloroatranol are significantly less capable of eliciting positive patch test reactions in OMA-allergic subjects. However, the reaction pattern when patch testing with serial dilutions of treated and untreated OMA and also with selected OMA allergens indicates that in addition to atranol and chloroatranol other allergens in OMA are of importance. The results of the TLC patch tests indicate the same, although only one subject reacted to the TLC test of the treated OMA sample.

No statistically significant difference in the number of subjects who were positive in the ROATs of the treated and untreated OMA sample could be observed, but a significant difference in the time until development of a positive ROAT was seen. Since the reactions to the treated OMA sample and to a 500-fold dilution of the untreated sample overlapped to a large extent, on can surmise that the residual levels of atranol and/or chloroatranol may be responsible to a large degree for the positive reactions to the treated sample.

In summary, our results indicate that both the low remaining levels of atranol and chloroatranol and the presence of other allergens in OMA can result in positive reactions to treated OMA samples in patch tests and in ROATs. We observed a statistically significant difference in the reactivity to the treated and untreated samples of OMA. It is therefore likely that the treated sample is also less able to induce sensitisation. Nevertheless, more than 50% of the OMA-allergic subjects reacted positively in a ROAT with a treated OMA at the maximum concentration allowed according to the IFRA standard. It can therefore be concluded that a cosmetic

product containing 0.1% treated OMA cannot be used without risk of developing a contact allergic reaction in subjects previously sensitised to OMA.

7 Popular scientific summary in Swedish

Kontaktallergier mot parfymämnen är vanliga och 1–4 % av normalpopulationen har i studier visat sig vara allergiska mot parfymämnen. Kontaktallergier diagnosticeras med ett lapptest, där beredningar av allergiframkallande ämnen placeras i testkammare på tejpremsor, vilka sedan appliceras på patientens rygg. Testerna får sitta på ryggen i två dagar och efter tre och sju dagar undersöker en hudläkare ryggen. Om patienten är allergisk mot ett ämne så uppkommer en eksemreaktion på den plats där detta ämne suttit. I svensk basserie för lapptestning ingår två mixer av parfymämnen, fragrance mix I (FM I) och fragrance mix II (FM II).

Då det inte är ovanligt att en patient vid ett och samma tillfälle testas med 50–100 olika ämnen är det ibland nödvändigt att förbereda inför en mottagning genom att i förväg applicera testberedningar i testkammare. Eftersom många parfymämnen är flyktiga kan det misstänkas att de avdunstar från testberedningar som placerats i testkammare i sådan utsträckning att testresultatet påverkas. Detta undersöktes i avhandlingens två första delarbeten. I studie I genomfördes kemiska analyser med hjälp av vätskekromatografiska metoder för att undersöka halten av 7 av parfymämnena från FM I i vaselinberedningar som placerats i testkammare som förvarats i kylskåp eller i rumstemperatur under olika lång tid.

I studie II jämfördes utfallet vid lapptestning av löpande patienter med FM I och FM II, då de var placerade i testkammare dels 6 dagar i förväg, dels i omedelbar anslutning till att lapptesterna sattes på patientens rygg.

Studie III och IV fokuserade på oak moss absolute (OMA), ett extrakt av en lav som är en av de parfymingredienser som i störst utsträckning orsakar kontaktallergi. Det finns flera kända allergen i OMA, bland dem atranol och kloratranol, vilka visats vara starka allergen som kan framkalla allergiska reaktioner även vid exponering för mycket låga halter. Av denna anledning har parfymindustrin tagit fram modifierade varianter av OMA där halterna av atranol och kloratranol är flera hundra gånger lägre än i traditionell OMA. I studie III och IV jämfördes de båda OMA-kvaliteternas förmåga att framkalla en allergisk reaktion hos försökspersoner med känd OMA-allergi. Detta gjordes genom lapptester med spädningsserier och genom användartester som simulerade en upprepad exponering för en parfymerad produkt innehållande OMA i högsta tillåtna koncentration enligt parfymindustrins frivilliga branschregler.

Dessutom undersöktes reaktionsmönstret vid lapptestning med tunnskikts-kromatografi (TLC)-remsor, på vilka komponenter i OMA hade separerats.

Resultaten av studie I visade att halterna av 4 av 7 ämnen minskade med ≥20% inom 8 timmar då vaselinberedningar förvarats i testkammare i rumstemperatur, men att avdunstningen var långsammare för samtliga ämnen då de förvarats i kylskåp. I studie II kunde en statistiskt signifikant skillnad påvisas mellan antalet patienter som reagerade för förberedda respektive nyupplagda testberedningar av FM I på så sätt att fler reagerade för de nyupplagda testberedningarna. För FM II observerades ingen signifikant skillnad, vilket troligen kan förklaras av att de flesta ämnena som ingår i FM II är mindre flyktiga än de som ingår i FM I. Eftersom vi har påvisat att parfymämnen kan avdunsta från testberedningar upplagda i testkammare i förtid och att lapptestning med dessa riskerar att ge falskt negativa reaktioner, är rekommendationen att applicera testberedningarna i testkammare i omedelbar anslutning till att testerna sätts på patientens rygg.

I studie III och IV påvisades att OMA-allergiska försökspersoner reagerade i statistiskt signifikant större utsträckning för de icke-modifierade OMA-proverna än för de modifierade när dessa lapptestades. I användartesterna kunde ingen signifikant skillnad påvisas mellan antalet försökspersoner som reagerade för modifierad och icke-modifierad OMA. Dock sågs en statistiskt signifikant skillnad när hänsyn togs till antalet dagar som krävdes för att utveckla en positiv reaktion i användartesterna. Antalet individer som reagerade tidigare för icke-modifierad OMA än för modifierad OMA var större än antalet individer som uppvisade ett omvänt mönster. Att några försökspersoner reagerade för de modifierade OMA-proverna kan bero på reaktioner mot antingen för de låga kvarvarande halterna av atranol och kloratranol eller mot andra allergen i OMA. Lapptestningen med TLC-remsor med uppdelade OMA-komponenter indikerar förekomsten av fler allergen än atranol och kloratranol. Då mer än hälften av de OMA-allergiska försökspersonerna reagerade vid användartest av modifierad OMA i högsta tillåtna koncentration enligt parfymindustrins branschregler, kan sänkningen av förmågan att framkalla kontaktallergiskt eksem i modifierad OMA inte bedömas vara tillräcklig för redan OMA-allergiska individer.

Acknowledgements

Many people have contributed in many ways to the work that have resulted in this thesis. I wish to express my sincere gratitude to:

My supervisor *Erik Zimerson* for generously sharing his vast knowledge in chemistry, for helping me to see things from a different perspective in situations when I have found it hard to carry the work forward, and for interesting discussions regarding, chemistry, science, and life in general.

My co-supervisor *Cecilia Svedman* for guidance regarding the dermatological aspects of the studies and for always being supportive and encouraging.

Magnus Bruze for giving me the opportunity to perform this research project, for his guidance and encouragement, and for always being available whenever I needed a piece of advice.

Lena Persson for skilful technical assistance and friendly collaboration in several of the studies, especially when we performed the liquid chromatographic analyses in study I.

Linda Rosén for preparation of the patch tests in study II.

Halvor Möller for constructive comments on the manuscripts and for helping me with the English grammar.

Henrietta Passlov for helping me with the cover design.

All colleagues at the Department of Occupational and Environmental Dermatology for creating a friendly environment, for many valuable comments during research seminars, and for helping me in many other ways during the years I have been working with my thesis.

Håkan Lövkvist for advice regarding the statistical calculations.

Alistair Kidd, Good Written English, for revising the English text.

My wife *Jessica* and my children *Henry* and *Beatrice* for their love and support.

All volunteers who participated in the studies.

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