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TITLE OF THE PROJECT

Fragrance chemical allergy: a major environmental
and consumer health problem in Europe

ACRONYM OF THE PROJECT

Fragrance Allergy

Contract No.

QLK4-CT-1999-01558

FINAL REPORT

SUMMARY

TITLE OF THE PROJECT			
Fragrance chemical allergy : a major environmental and consumer health problem in Europe			
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PROJECT CO-ORDINATOR			
Name Jean-Pierre LEPOITTEVIN		Title Professor	
Address Laboratoire de Dermatochimie, Clinique Dermatologique, CHU, 1 place de l'Hôpital, 67091 Strasbourg, France			
Telephone +33 (0)3 88 35 06 64		Telefax +33 (0)3 88 14 04 47	E-mail jplepoit@chimie.u-strasbg.fr
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<hr/>			
List of participants			
Principal Contractors			
No. 1 – ULPS: Co-ordinator. Professor Jean-Pierre Lepoittevin. Laboratoire de Dermatochimie, Clinique Dermatologique, CHU. 1, Place de l'Hôpital, 67091 Strasbourg, France. Tel : +33 3 88350664 ; Fax : +33 3 88140447 ; E-mail : jplepoit@chimie.u-strasbg.fr			
No. 2 – NIWL: Professor Ann-Therese Karlberg. National Institute for Working Life, 11391 Stockholm, Sweden. Tel : +46 31 7724726; Fax : +46 31 7723840; E-mail : ann-therese.karlberg@mc.gu.se			
No. 3 – UNIUK: Dr. David Basketter. SEAC Unilever Research. Colworth House, Sharnbrook MK 44 1LQ Bedford, United Kingdom. Tel. +44 123 4264776 ; Fax : +44 123 4222122 ; E-mail : David.Basketter@unilever.com			
No. 5 – UKBH: Professor Torkil Menné. Department of Dermatology, Gentofte Hospital, University of Copenhagen. Niels Andersens Vej 65, 2900 Hellerup, Denmark. Tel : +45 39 773200 ; Fax : +45 39 657137 ; E-mail : tomen@gentoftehosp.kbhamt.dk			
No. 6 – SKDO: Professor Peter J Frosch. Department of Dermatology, Dortmund and University of Witten/Herdecke. Beurhausstrasse 40, 44123 Dortmund, Germany. Tel: +49 231 5021550; Fax: +49 231 5021554; E-mail. P.Frosch@derma.de			
No. 9 – KCOLL: Dr. Ian White. St. John's Institute of Dermatology. St. Thomas' Hospital. Lambeth Palace Road, London SE1 7EH, United Kingdom. Tel : +44 207 9228076 ; Fax : +44 207 6200890 ; E-mail : ian.white@kcl.ac.uk			
Assistant Contractors			
No. 4 – NERI (to <i>principal contractor</i> No.1): Dr. Suresh Chandra Rastogi. National Environmental Research Institute. Frederiksborgvej 399, 4000 Roskilde, Denmark. Tel : +45 46 301200 ; Fax : +45 46 301114 ; E-mail : scr@dmu.dk			
No. 7 – OUH (to <i>principal contractor</i> No. 5): Professor Klaus E. Andersen. Department of Dermatology, University Hospital. 5000 Odense C, Denmark. Tel : +45 65 412700 Fax : +45 66 123819 ; E-mail : kea@dou.dk			
No. 8 – ULUND (to <i>principal contractor</i> No. 6): Dr. Magnus Bruze. Department of Occupational Dermatology, University Hospital. 205 02 Malmö, Sweden. Tel : +46 40 336516 ; Fax : +46 40 336213 ; E-mail : magnus.bruze@derm.mas.lu.se			
No. 10 – KUL (to <i>principal contractor</i> No. 9): Professor An Goossens. Department of Dermatology, University Hospital, KU Leuven. Kapucijnenvoer 33, 3000 Leuven, Belgium. Tel : +32 16 337860 ; Fax : +32 16 337012 ; E-mail : an.goossens@uz.kuleuven.ac.be			

Objectives

At the present time there is no treatment, other than symptomatic, for fragrance hypersensitivity reactions and the only means available to improve public health in this sector is prevention. The main objective of the project is the prevention of fragrance chemical allergy in non-sensitized (*primary prevention*) and in already sensitized (*secondary prevention*) individuals. The aim of the primary prevention part of the study is to create initiatives that regulate the exposure to fragrance chemicals so that induction of allergic contact sensitization does not take place. This includes the identification and validation of fragrance sensitizers including new emerging ones and insight to their sensitizing potential through predictive studies and QSAR analysis. The secondary prevention part of the study establishes measures aiming to avoid the elicitation of the skin disease in already fragrance sensitized individuals. It includes the standardisation of diagnostic methods to identify the individuals at risk, epidemiological clinical studies combined with exposure assessment, and cross-reactivity studies aiming at allergenic fragrance chemical substitution. The results of the different studies are compiled in a risk assessment-management model providing the basis for preventive measures.

Results and Milestones

Development of a method for the identification of fragrance sensitizers in complex mixtures: the model of oak moss. A new method has been developed and validated for the identification of sensitizers in complex mixtures using the model of oak moss. The method is based on the combination of chemical fractionation, patch testing, analytical chemistry and structure-activity relationships studies. Atranol and chloroatranol have been identified as major sensitizers in oak moss absolute. A minor sensitizer, methyl- β -orcinol carboxylate, has also been identified.

Allergenicity of fragrance terpenes with respect to their oxidation/degradation over time. The oxidative degradation at air exposure of fragrance terpenes has been studied for limonene, linalool, caryophyllene and myrcene. Different oxidation products have been identified in the oxidised samples e.g. hydroperoxides, alcohols and epoxides. The prevalence of sensitization to these oxidised compounds has been evaluated in the clinical network and showed that there is a risk of contact allergy in the population caused by the oxidised fragrance chemicals studied. It has also been shown that addition of anti-oxidants before air exposure makes these terpenes stable for some months up to more than a year, depending on the compound.

Sensitization potential of selected fragrance chemicals and development of QSAR studies. Reliable quantitative structure-activity relationships (QSARs) have been developed for two chemical families of common fragrance allergens: aliphatic aldehydes and α,β -unsaturated aldehydes. Knowledge derived from these QSARs is available to be incorporated into expert toxicity prediction systems.

Hand eczema and fragrance allergy. Hand exposure to fragrance allergens has been assessed by chemical analysis of domestic and occupational products. Based on this exposure assessment a new diagnostic screening series with 14 compounds has been established and tested on a large number of patients with hand eczema. 10% of the patients gave a positive test to one or more of the allergens in the new series.

Development of a new fragrance mix. A new diagnostic tool, the Fragrance Mix II (FM-II), has been developed and complements the currently used Fragrance Mix (FM-I). The FM-II is constituted by 6 new fragrance allergens: 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, citral, farnesol, citronellol, α -hexylcinnamic aldehyde and coumarin. Testing within the clinical network confirms that the new FM-II is a valuable tool for identifying subjects with a positive history of adverse reactions to fragrances. 30-50% of these patients would have been missed with the old FM-I.

The hand immersion study. The hand immersion technique has been used to assess relations that could exist between hand exposure to fragrance chemicals present in household products and chronic hand eczema. No relation could be evidenced for the 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde and hydroxycitronellal models.

Threshold dose-response studies of newly identified fragrance allergens. Three new fragrance allergens giving rise to significant problems in the consumer have been identified. Experimental studies in sensitized patients have shown that these allergens currently are used at unacceptable levels in consumer products causing significant disease in the consumer. This programme has provided data on no-effect levels that, if considered in risk assessment, will lead to safer environment and decreased disease. The results will be submitted to the EU-Commission for taking legislative action.

Allergenic fragrance chemical substitution: the case of isoeugenol. Testing within the clinical network with isoeugenol and its derivatives has suggested that isoeugenol ether derivatives may be a safer substitute than ester derivatives in isoeugenol allergic subjects. On another hand, the rate of isoeugenol allergy has not yet shown a consistent decline since the reduction in levels in cosmetic products. The knowledge gained will contribute to EU policy on fragrance allergen substitution.

Benefits and Beneficiaries

The *Fragrance Allergy* project provides operational scientific data and more effective methods for the diagnosis and risk assessment of fragrance chemical allergy, which are being used for preventive measures. The project brings a high degree of consumer protection as both induction and elicitation of fragrance contact dermatitis are prevented.

The principal beneficiaries are:

The eczema patients

The individual patients with contact dermatitis from fragrances benefit directly from the project because of the improved diagnostic technologies and increased knowledge on the occurrence of different fragrances in individual products and product types. In the long perspective they will benefit from safer perfumed products on the market.

The diagnostic technologies

The project has identified new significant contact sensitizers. This knowledge will be of public domain and will be the basis for the development of standardised patch test materials, that can be expected to be commercially developed. These products will be available for dermatologists on a world wide scale. The participating scientists in the project have earlier been and are currently involved in the development of such technologies on a non-profit basis.

The companies

The main bulk of fragrances for the world market is produced by a small number of large companies. Most industries (even the large multinationals) buy their fragrances from these main suppliers. The developed scientific technologies, based on controlled clinical experiments, will provide the fragrance manufacturers with new technologies which can be of major help in developing safer cosmetic products.

The European regulation

The results will be available for the development of alternative methods for predicting the sensitizing potential of fragrance chemicals and thus help to improve, and reinforce, European regulation about fragrance chemicals utilisation. The combination of the different studies will provide the EU legislator with scientific background for introducing either compulsive labelling of products, use concentration limitation or banning some of the most sensitizing fragrance substances. Data obtained on the most common fragrance sensitizers will supplement the EU's Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) guidelines on the restrictions to safe levels and labelling of the 26 fragrance allergens list included in the 7th Amendment to the European Cosmetics Directive (76/768/EEC).

Future Actions

A look ahead to the future should now be focused in the dissemination and use of the obtained results in order to improve the quality of life in the Community.

New fragrance allergy diagnostic tools have been developed. This, together with the identification and validation of fragrance sensitizers, including new emerging ones, will make possible from now to exactly elucidate the causative factors of the consumer contact allergy reactions due to scented products present in our environment. The identification of unknown significant contact sensitizers will be the basis for the development of standardised patch test materials that can be expected to be commercially developed. These products will be available for dermatologists on a world wide scale. The participating scientists have earlier been and are currently involved in the development of such technologies with several companies on a non profit basis. It is not our intention to apply for patents in this area. Also, the results will be available for the development of alternative methods for predicting the sensitizing potential of fragrance chemicals and thus help to improve, and reinforce, European regulation about fragrance chemicals utilisation. On another hand, the cosmetic manufacturers have the legal obligation to provide products that are safe. Fragrance ingredients have traditionally been excluded from the detailed ingredient labelling provisions within the EU Cosmetics Directive and are simply indicated by the name "perfume". Establishing new EU labelling guidelines for the most frequent and strong fragrance sensitizers will help the consumer to avoid contact with these compounds. Moreover, establishing safe level concentrations or banning some of the high fragrance sensitizers found in the project will lead to a decrease in the burden of fragrance induced skin disease in the European Community.

All results are being or will be published in peer reviewed journals such as Contact Dermatitis, Archives of Dermatological Research, The British Journal of Dermatology and Acta Dermatologica Venereologica. Most of the data have been already or will be presented at scientific meetings of the European Society of Contact Dermatitis, the European Academy of Dermatology and Venereology, the American Society of Contact Dermatitis and the World Congress in Dermatology. All knowledge will be of public domain.

**Fragrance chemical allergy:
a new risk assessment-management strategy**

Université Louis Pasteur, Strasbourg, France
National Institute for Working Life, Stockholm, Sweden
Safety & Environmental Assurance Centre, Unilever R&D Colworth, Bedford, United Kingdom
National Environmental Research Institute, Roskilde, Denmark
Gentofte Hospital, University of Copenhagen, Hellerup, Denmark
Dortmund Hautklinik-University of Witten/Herdecke, Dortmund, Germany
Odense University Hospital, Odense, Denmark
University Hospital, Malmö, Sweden
King's College, London, United Kingdom
University Hospital, Leuven, Belgium

Co-ordinator:

Jean-Pierre Lepoittevin
Laboratoire de Dermatochimie
Clinique Dermatologique, CHU
1, Place de l'Hôpital
67091 Strasbourg
France
Tel: +33 3 88 35 06 64
Fax: +33 3 88 14 04 47
E-mail: jplepoit@chimie.u-strasbg.fr

Introduction

Sensitization to fragrance materials remains a significant clinical problem. The prevalence in dermatitis patients seen by dermatologists world-wide is high. In most countries, the fragrance mix (FM-I) which is a composition of 8 commonly used fragrances to identify subjects with fragrance allergy, is among the "Top 5" of allergens, usually number 2 after nickel sulphate. Indeed, fragrance materials are major causes of allergic contact dermatitis. At least 35% of all allergic reactions to cosmetics are due to perfume ingredients, and approximately 1-2% of the unselected population is sensitized to fragrances (1-4).

Contact allergy is a type IV immunological reaction caused by low molecular weight substances that come in close contact with the skin (5). The clinical manifestation of contact allergy is eczema, which is an inflammatory skin disease characterised by erythema, induration and in some cases vesicles. At a later stage scaling and fissures may develop. In case of contact allergy to fragrance materials the face, neck, axillae and hands are the most affected areas (6, 7). Contact eczema may be a significant burden to the individual because itch, changed appearances, discomfort and functional limitations. Medical consultations, treatment with corticosteroids and in some individuals sick-leave is a consequence. Contact allergy is diagnosed by patch testing, where the patient under investigation is re-exposed to suspected allergens under controlled circumstances. International recommendations and standardisation for the patch test method, most common allergens and recording of the results exist (8).

The use of fragrances is ubiquitous. The main source of exposure is without doubt cosmetics and toiletries, being the European Union the biggest market. It is estimated that 95% of the female population and at least 75% of the male population come into daily contact with cosmetic products (9). However, fragrances are also widely used in other products such as household and occupational (dishwashing liquids and soaps, detergents, cleaners ...). Virtually everyone is therefore exposed continually to fragrances through contact with products present in our environment (1).

Fragrances may be of natural or synthetic origin. Natural fragrances are obtained, with few exceptions, from botanical sources. Balsams, essential oils, concretes and absolutes contain several hundred different chemicals, a few major and many minor ones, which are responsible for the complexity of the odour. Synthetic fragrances are well-defined organic compounds with a simple odour. A fragrance ingredient is defined by the International Fragrance Association (IFRA) as any basic substance, natural or synthetic, used in the manufacture of fragrance materials for its odorous, odour-enhancing or blending properties. Among thousands of chemical substances which have an odour, about 3000 (of which 300-400 are of natural origin) are used in the fragrance industry and are often combined to create characteristic scents (10). This way, a single perfume may contain from 10 to 300 different molecules (11).

The FM-I, which is included in the European standard patch test series as the indicator of fragrance allergy, does not detect all cases of contact allergy to fragrances, but estimates of between 50 and 80% (2-4). It seems clear nowadays that its 8 components (α -amyl cinnamic aldehyde, cinnamic alcohol, cinnamic aldehyde, eugenol, geraniol, hydroxycitronellal, isoeugenol, oak moss) do not adequately represent the thousands of chemical structures found in fragrances. The FM-I may consequently be missing cases of real fragrance sensitivity, leading to underestimates of the true frequency of allergy to fragrance ingredients. It is

therefore necessary to identify more fully the nature of the main fragrance chemicals causing contact allergy in the consumers and provide strategies for prevention.

In order to increase the safety standards of fragrance compounds a project entitled "Fragrance chemical allergy: a major environmental and consumer health problem in Europe", was supported by the 5th Framework Programme of the European Commission, under the Quality of Life and Management of Living Resources thematic programme (Contract QLK4-CT-1999-01558). The main goal of the project was the prevention of fragrance chemical allergy in non sensitized (primary prevention) and in already sensitized (secondary prevention) individuals. The aim of the primary prevention part of the project was to create initiatives that regulate exposure to fragrance chemicals so that induction of contact sensitization does not take place. This included the development of methods to identify new fragrance sensitizers as well as insight into their sensitizing potential through predictive studies and quantitative structure-activity relationships analysis (QSARs). The secondary prevention part of the project aimed at measures so that the already-sensitized individuals do not develop clinical symptoms. This included standardisation of diagnostic methods to identify individuals at risk, epidemiological clinical studies combined with exposure assessment and cross-reactivity studies aiming at fragrance chemical substitution.

This report describes the results of the different studies carried out in this project, conducted in parallel thanks to a strong collaboration between 10 European research centres specialised in allergic contact dermatitis and in environmental and occupational skin diseases. These studies are listed below together with their main objectives.

1. *Development of a method for the identification of fragrance sensitizers in complex mixtures: the model of oak moss.*
To develop and validate, using the model of oak moss, a new method of hazard identification in complex mixtures based on a combination of bioassay-guided chemical fractionation, chemical analysis and structure-activity relationships studies (SARs).
2. *Allergenicity of fragrance terpenes with respect to their oxidation/degradation over time.*
To investigate the influence of air exposure on the stability and allergenic activity of common fragrance terpenes.
3. *Sensitization potential of selected fragrance chemicals and development of QSAR studies.*
To develop QSARs for the prediction of the skin sensitization potential of common chemical families of fragrance allergens.
4. *Hand eczema and fragrance allergy.*
To identify the most important fragrance allergens present in domestic or occupationally used products with intended hand exposure. To establish the frequency of contact allergy to these fragrance chemicals in patients with hand eczema.
5. *The hand immersion study.*
To perform experimental exposure studies for fragrance allergens in relation to hand eczema.
6. *Development of a new fragrance mix.*
To evaluate a new fragrance mix in order to establish an improved diagnostic tool for fragrance allergy.

7. *Threshold dose-response studies of newly identified fragrance allergens.*
To establish dose-response data with the purpose of performing risk assessment for newly identified common allergens.
8. *Allergenic fragrance chemical substitution: the case of isoeugenol.*
To evaluate the prevalence of allergy to isoeugenol derivatives which may be being used as substitutes for isoeugenol itself and for which there are no guidelines on use.

All of these studies have been used to build a risk assessment-management strategy for fragrance chemicals providing the basis for preventive measures.

Materials and methods

General

All the clinical investigations were performed in eczema patients that were recruited from 1 or more of the 6 participating European departments of dermatology. All experiments involving human volunteers complied with the Helsinki Declaration in its latest version.

Patients were subjected to patch testing, which is an internationally standardised method of diagnosing contact allergy. It is performed by applying suspected allergens to the skin under standard conditions. The upper back was used for test. The allergens were applied in 8 mm aluminium chambers that were affixed with special tape (Finn Chambers[®] / Scanpore tape[®]). The chambers were left in place for 48 hours and readings of the results were done day 3 and 1 week after the application. An international scale of reading was used. Use testing was also performed in some of the studies. The patient repeatedly applied a product or a fragrance ingredient suspected of having caused a contact allergy reaction. The procedure simulated normal use situations of cosmetic products. A 7-14 day application period was used with 2 applications a day.

Development of a method for the identification of fragrance sensitizers in complex mixtures: the model of oak moss

Originally, a total of 40 patients with a previously diagnosed sensitivity to oak moss were recruited in 3 dermatological clinics. They were randomly patch tested with 5 different oak moss absolute samples (1% in petrolatum -pet.-) and asked to participate in subsequent testing with fractions. No significant differences in the elicitation potential were observed between the 5 samples and patients reacting positively reacted strongly to all the samples. Therefore, only 1 of the 5 oak moss absolutes was chosen for further chemical analysis.

Gel permeation chromatography of oak moss absolute was performed on a Sephadex[®] LH-20 column with a 1:1 mixture of dichloromethane-acetone as the eluent, to afford fractions F1-F5, that were patch tested on the patients. Initially, 30 of the 40 patients were volunteer subjects for the fractionation study. However, with time, some of them decided to quit the patch test procedure and did not follow the study until the end of the chemical fractionations. Fractions F1 and F4, the most eliciting ones, were further chemically re-fractionated by column chromatography on silica gel using a pentane-diethyl ether elution gradient to afford sub-fractions F1a-F1f and F4a-F4d respectively, that were patch tested on the left patients. In parallel, the chemical composition of each of the fractions and sub-fractions was analysed by gas chromatography-mass spectrometry (GC-MS) with a split-less injection. SARs studies allowed the identification of several potential sensitizers among the chemical constituents of the fractions that were also patch tested.

A mixture of atranol and chloroatranol was found to be the major elicitant of contact allergy to oak moss. In order to elucidate the individual eliciting potential of both compounds, atranol and chloroatranol were synthesised using classical organic methods and were patch tested pure and individually on 15 remaining patients.

Fractions and sub-fractions, as well as pure atranol and chloroatranol, were patch tested at 1% pet. equivalent to oak moss. This means that the patch test concentration of the fractions was calculated so that it corresponded to the amount of the fraction present in a sample of oak moss absolute 1% pet. The patch test concentration for other chemicals was 0.1% pet.

Allergenicity of fragrance terpenes with respect to their oxidation/degradation over time

Samples of *R*- and *S*-limonene, linalool, caryophyllene and myrcene were exposed to air at room temperature. The samples were kept in flasks, some were stirred 1 hour 4 times a day to mimic handling (12), while others were kept without stirring. The antioxidant butylated hydroxytoluene (BHT) was added to some samples. The oxidative degradation was monitored as a decrease in the concentration of original terpenes over time, using gas chromatography (GC) analysis with an on-column injection. Isolation and identification of oxidation products formed were performed using flash chromatography, GC-MS, ¹H-NMR and ¹³C-NMR spectroscopy. A suitable HPLC method for quantification of linalool hydroperoxide was developed. Oxidised *R*- and *S*-limonene were patch tested in 2411 consecutive dermatitis patients in 6 clinics. Oxidised linalool, a hydroperoxide fraction of linalool, oxidised caryophyllene, caryophyllene oxide and oxidised myrcene were tested in another 1343 consecutive patients in the same clinics. Non-irritating patch test concentrations were determined by pre-testing in a group of dermatitis patients.

Sensitization potential of selected fragrance chemicals and development of QSAR studies

Structures for 71 aldehydes were imported into the 2D-drawing package Chemdraw (Version 6, Cambridgesoft.). Log P values were estimated using ClogP (Version 4, BioByte Corp). Structures were energy minimised to generate 3D representations and physicochemical parameters calculated using Cerius 2 software (Version 4.6, Accelrys): LUMO (lowest unoccupied molecular orbital), dipole, heat of formation, HOMO (highest occupied molecular orbital), molar refractivity, number of hydrogen bond acceptor groups, number of rotatable bonds and molecular volume. Each aldehyde was then rationalised according to its reaction chemistry into 1 of 4 classes previously hypothesised (13). A cluster analysis was used to select subsets of 10 materials from the 2 classes where there was an abundance of chemicals. Schiff base (aliphatic) and Michael addition (α,β -unsaturated) aldehydes were targeted. A set of 10 aldehydes was selected from these classes for local lymph node assay (LLNA) testing. LLNA tests were conducted using a 4:1 acetone:olive oil vehicle to generate dose-response data for the aldehydes in order to determine EC₃ values (14). The EC₃ value (concentration of test substance which gives a threshold sensitization response) expressed as a weight percentage was converted into a molar basis by dividing the molecular weight. The negative logarithm of this molar EC₃, Log (1/EC₃), was used as a quantitative measure of sensitizing potential. Using the derived Log (1/EC₃) values it was investigated how the sensitization potential varied with the chemical reactivity and lipophilicity. Chemical reactivity was modelled using Taft σ^* values. The Taft σ^* constant for a substituent R is a measure of the inductive effect of R. The σ^* values used were taken from the extensive compilation by Perrin et al. (15). Lipophilicity was modelled by LogP values, computed using CLogP.

Hand eczema and fragrance allergy

As a first step 59 domestic and occupational products intended for hand exposure were subjected to GC-MS analysis. The products were analysed for 19 fragrance ingredients. The selection of these target substances was based on allergenic potency and total tonnage used. Based on the results obtained a patch test tray for hand eczema was established with 14 fragrance ingredients. As a second step the patch test tray for hand eczema was pre-tested in consecutive eczema patients to establish the optimal patch test concentration, followed by the application of the final patch test tray to consecutive hand eczema patients recruited in 3 different clinics. A total of 658 consecutive patients with hand eczema were invited to participate in the study after informed consent and permission from Ethical Committees. Prior to patch testing the patients filled in a questionnaire concerning demographic background variables, atopic manifestations and history of fragrance dermatitis.

The hand immersion study

The study was designed as a randomised, double-blind study using a paired design. A total of 21 patients previously diagnosed with hand eczema and who at a prior occasion had given a positive patch test to hydroxycitronellal or to 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde were included. None of the participants had any vesicles, fissures or eczema on the arms at the time of inclusion. Also use of steroids, UV treatments, or tar or PUVA were reasons for exclusions. The patient had 1 finger of each hand immersed in a solution with 10% alcohol, with or without the relevant fragrance ingredient, twice a day for 10 minutes during the study period of 2 weeks with 10 ppm followed by 2 weeks with 250 ppm. In case of appearance of visible clinical eczema on the hands participation was terminated before the end of the fourth week. All participants filled in a questionnaire about demographic background variables, atopic manifestations and history of fragrance dermatitis. Patients were evaluated at the beginning of the study, once a week during the 4 weeks of the study and, if necessary, before in the case of clinical eczema. Patients were evaluated according to a clinical scale and by laser Dobbler reading as done in an earlier study (16).

Development of a new fragrance mix

The fragrance mix II (FM-II) was constituted by 6 fragrance materials incorporated into the vehicle petrolatum at selected concentrations: 28%, 14% and 2.8%. 28% FM-II contained 5% 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, 2% citral, 5% farnesol, 1% citronellol, 10% α -hexylcinnamic aldehyde and 5% coumarin. The 14% FM-II had half of these constituents and the 2.8% FM-II one tenth. The homogeneous distribution of the 6 constituents in the mix was achieved by adding hard paraffin at 10% to white petrolatum. Then, the fragrances were cautiously warmed and mechanically stirred into the vehicle. This work was provided by courtesy of Hermal Laboratories (Reinbek, Germany). Stability of the materials in the mix was evaluated every 3 months. Stability values remained within the 90% limit after 6 months which is the requirement by German Pharmacy Law. At room temperature a separation of phases in the mix of the highest concentration was observed at 9 months despite of addition of hard paraffin. At 6°C this was not observed. Test preparations must therefore be kept in the fridge.

First, a pilot study was performed (1150 patients in 2 clinical centres) in order to obtain initial experience with the new mix in rather high concentrations. The possibility of strong irritant reactions to the mixes had to be excluded before distribution of the materials to the clinical centres. Second, the FM-II was evaluated in a bigger scale by a main patch test study in 6 clinical centres. In order to obtain results with the 3 concentrations of FM-II and the individual constituents it was decided that the 6 ingredients of FM-II 28% and FM-II 14% needed to be applied simultaneously with FM-II 28%, 14% and 2.8%. This added up to an extra set of 15 patches for every patient. If he/she reacted to FM-II 2.8% the constituents were tested additionally. This approach was found to be preferable to the often not followed procedure of calling back the patient for an extra testing with the constituents. A careful history regarding fragrance intolerance was taken on each patient using a 4-point scale. All centres were provided with a total of 66 syringes for the patch test study and 30 syringes for performing a repeated open application test (ROAT) in cases with a weak or doubtful patch test reaction to one of the mixes. The patient's own scented products were also patch tested, if a relationship with the dermatitis was suspected. If the patient's own product was patch test positive chemical analysis was performed in order to detect 1 of the 6 target substances of the FM-II. A total of 1272 patients participated in the study. GC-MS spectrometry on 20 commercial products was carried out.

Threshold dose-response studies of newly identified fragrance allergens

Three fragrance allergens of major importance found in other parts of the project were chosen for this study: 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, chloroatranol and hydroxycitronellal. A total of 45 patients previously diagnosed with allergy to 1 of the 3 allergens in question were included together with a control group of 17 individuals. Among them 3 were negative at re-patch testing and were subsequently excluded. The 3 allergens were tested in serial dilution under patch test conditions. 4-(4-Hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde and chloroatranol were tested at the upper back, while the lower arm was used for testing hydroxycitronellal to maintain the relevance to hand eczema. A concentration range was chosen including the diagnostic test concentration as well as realistic exposure concentrations. 4-(4-Hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde and chloroatranol were also tested on the volar aspect of the lower arm using a ROAT with 2 different concentrations, a minimum and a maximum exposure level. The first solution of allergen was applied twice daily on a test site of $3 \times 3 \text{ cm}^2$. On the other arm ethanol was applied as control. The first solution was applied for 14 days and if no reaction occurred the test was continued with the high concentration for another 14 days. The control subjects were tested in an identical manner. The effect measure was clinical symptoms quantified by a previously developed scale of reading (17). Dose-response curves from patch testing were drawn based on reactions scored as doubtful or greater. A logistic regression curve was fitted to the data and for comparison of groups the Fishers exact test was used. The chosen level of significance was $p < 0.05$.

Allergenic fragrance chemical substitution: the case of isoeugenol

A total of 2261 patients were patch tested with isoeugenol (1% pet.), *trans*-isoeugenol (1% pet.), isoeugenol acetate (1.3% pet.), isoeugenol benzoate (1.6% pet.), isoeugenol phenylacetate (1.7% pet.), isoeugenol methyl ether (1.1% pet.) and benzyl isoeugenol ether (1.5% pet.). Reactions were read on 2 occasions over 5 days. A positive reaction was regarded as at least a 1+ reaction on the ICDRG scale.

Results

Development of a method for the identification of fragrance sensitizers in complex mixtures: the model of oak moss

In order to select a clinically representative sample, 5 commercial oak moss absolutes from different producers were patch tested randomly in 3 clinical centres. No significant differences in the elicitation potential were observed between the 5 samples and patients reacting positively reacted strongly to all the samples. Therefore, only 1 of the 5 oak moss absolutes was chosen for further chemical analysis.

A gel permeation chromatography on Sephadex[®] LH-20 (molecular size based separation) afforded 5 fractions, F1-F5, that were patch tested at 1% pet. equivalent to oak moss on 30 volunteers. A relatively high number of patients gave a strong positive response to 1 or several of the fractions. Moreover, 6 patients gave clearly an extreme positive reaction to F4 and 4 patients to F1, with intense erythema, infiltration and vesicles. Severe reactions of this type were much less observed for F2, F3 and F5.

Further analysis was initially focused on fraction F4, which represented 40% (w/w) of oak moss absolute. The chemical composition of F4 was investigated by GC-MS. In parallel, a silica gel column chromatography of F4 (polarity based separation) gave 4 sub-fractions, F4a-F4d, which chemical composition was also studied by GC-MS and that were patch tested at 1% pet. equivalent to oak moss on 26 of the initial volunteers. Plus, these patients were patch tested with the main constituents of F4 (0.1% pet.), previously identified by GC-MS and classified as potential sensitizers by SARs analysis. Fraction F4d was the most eliciting one with a total of 17 patients having a positive reaction, followed by F4b with 14 positive patients and F4c with 12 positive patients. A total of 19 patients (73%) had a positive reaction to a mixture of chloroatranol-atranol (2:8), and among them 6 developed a very strong reaction with intense erythema, infiltration and coalescing vesicles. On another hand, 6 patients (23%) were positive to methyl- β -orcinol carboxylate. The patch test results of the fractions were consistent with the outcome of the patch tests realised with the GC-MS identified target fragrance sensitizers.

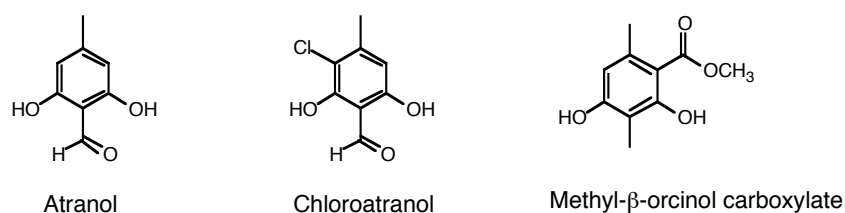


Figure 1: Chemical structures of atranol, chloroatranol and methyl- β -orcinol carboxylate

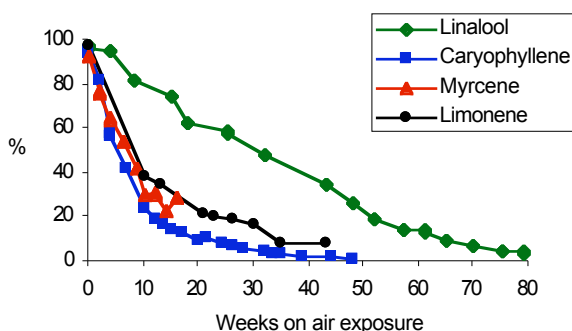
Based on these results, the mixture chloroatranol-atranol (2:8) was a strong elicitor of contact allergy to oak moss in already sensitized patients. As chloroatranol and atranol are not commercially available and in order to evaluate their eliciting potential separately, they were synthesised according to classical organic chemistry methods. Afterwards, they were patch tested at 1% pet. equivalent to oak moss in 15 of the 26 remaining patients. Both compounds had a strong eliciting potential. A total of 10 (67%) and 11 (73%) patients showed a positive reaction to chloroatranol and atranol respectively. No significant differences were

observed between the number of weak, strong and extreme reactions. However, the % (w/w) of chloroatranol in the oak moss sample was lower (0.9%) compared to the concentration of atranol (2.1%), which means that the patch test concentration, 1% pet. equivalent to oak moss, was lower for chloroatranol (90 ppm) than for atranol (210 ppm). Chloroatranol was therefore considered as a stronger elicitant compared to atranol.

In the same manner, a silica gel column chromatography of F1, which represented 21% (w/w) of oak moss, gave 6 sub-fractions, F1a-F1f, that were patch tested at 1% pet. equivalent to oak moss on 26 patients. F1e and F1f showed a strong eliciting potential. It appeared that GC-MS was not a suitable method for the analysis of their chemical composition due to the high temperature of injection. Further HPLC-UV studies using co-injection of reference compounds revealed the possible presence of atranorin and chloroatranorin in these fractions.

Allergenicity of fragrance terpenes with respect to their oxidation/degradation over time

The concentrations of all terpenes under study started to decrease immediately when exposed to air: 50% of the original compounds remained after 30 weeks for linalool, after 9 weeks for limonene, and after 8 weeks for caryophyllene and myrcene.



The antioxidant BHT prevented air oxidation of linalool for 1 year, of myrcene for 15 weeks, and of caryophyllene for at least 1 year.

Figure 2: Oxidative decomposition of fragrance terpenes at air exposure

Different kinds of oxidation products were identified in oxidised linalool. The major compound was identified as 7-hydroperoxy-3,7-dimethyl-octa-1,5-diene-3-ol (18). After 48 weeks of air exposure, 26% of linalool remained in the oxidised sample while the content of the major hydroperoxide was 15%. The major oxidation product identified in caryophyllene was caryophyllene oxide, but also formaldehyde was detected in the mixture. In the oxidised myrcene one epoxide (6,7-epoxymyrcene) was identified.

In a first test series and in a total of 2411 patients patch tested, 2.3% reacted to *R*-limonene oxidised for 10 weeks, 2.0% reacted to oxidised *S*-limonene and 1.6% to both. The results obtained showed variations between the test centres. A significant correlation with contact allergy to other fragrance materials was observed. In the second test series positive reactions were observed in 1.8% of in total 1343 patients tested. Most reactions were seen to linalool oxidised for 45 weeks (1.2%) and to its hydroperoxide fraction (1.0%).

Sensitization potential of selected fragrance chemicals and development of QSAR studies

A QSAR was developed for each the Michael addition aldehydes and Schiff base aldehydes (19). The equations of these QSARs are shown in Figure 3.

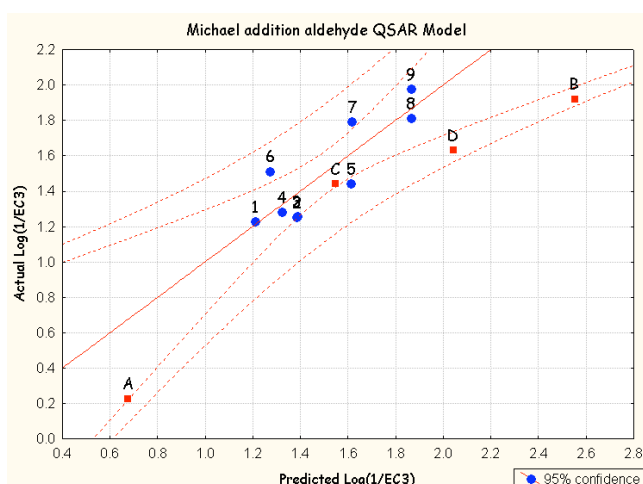
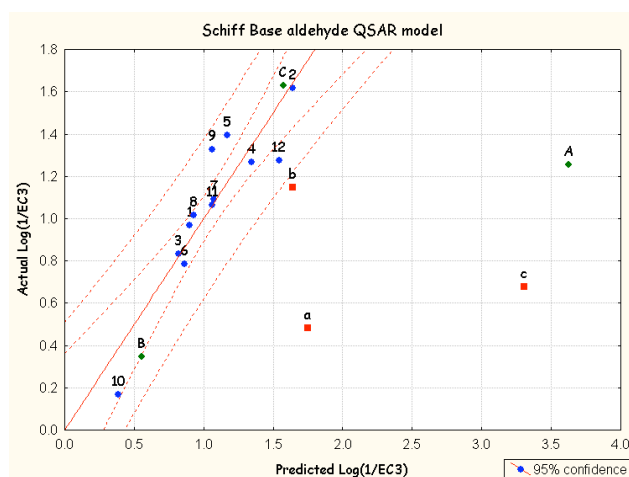
Michael addition aldehydes	Schiff Base aldehydes (13)
$\text{Log}(1/\text{EC}_3) = 0.54 + 0.17\text{LogP} + 0.49R\sigma^* + 1.31R'\sigma^*$	$\text{Log}(1/\text{EC}_3) = 0.25 + 0.28\text{LogP} + 0.86R\sigma^*$
$n = 9; R^2 = 0.741; s = 0.184; F = 4.77$	$n = 12; R^2 = 0.825; s = 0.17; F = 21.2$

Figure 3: QSARs equations for aldehydes

To increase the breadth of each QSAR and understand its domain applicability further aldehydes were identified for testing in the LLNA: 4 Michael addition aldehydes were selected for testing and 6 carbonyl containing compounds were tested to extend the Schiff base QSAR. Of these, 2 were 1,2-diketones which had been previously shown to be well predicted (20). One carbonyl containing ester was also included in this validation set. The results for these aldehydes are shown in Table 1 together with the plots highlighting the predictions made.

Table 1: Actual and predicted EC₃ values for Schiff base and Michael addition aldehydes

Schiff base aldehydes	Predicted EC ₃ (Actual EC ₃)	Michael addition aldehydes	Predicted EC ₃ (Actual EC ₃)
(1) cyclamen aldehyde	23.8 (20.5)	(1) 2-butyl-3-phenyl propenal	11.6 (11.2)
(2) glyoxal	1.3 (1.4)	(2) <i>trans</i> -2-hexenal	4.0 (5.5)
(3) hydroxycitronellal	26.0 (25.3)	(3) α -hexyl cinnamic aldehyde	8.9 (12.0)
(4) 2-methylundecanal	8.3 (10.0)	(4) amyl cinnamic aldehyde	9.6 (10.6)
(5) undec-10-enal	11.3 (6.8)	(5) (E,E)-2,4-heptadienal	2.7 (4.0)
(6) cis-6-nonenal	19.3 (23.1)	(6) methyl cinnamic aldehyde	7.8 (4.5)
(7) 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde	17.7 (17.1)	(7) <i>trans</i> -2-decenal	3.7 (2.5)
(8) para-tert-butyl- α -methylhydrocinnamic aldehyde	24.0 (19.7)	(8) cinnamic aldehyde	1.8 (2.0)
(9) α -methylphenylacetaldehyde	11.6 (6.3)	(9) 3-phenyl propenal	1.8 (1.4)
(10) diethyl acetaldehyde	41.0 (68.2)	(A) 2-methyl-2-butenal	17.8 (> 50)
(11) citral	13.2 (13.2)	(B) 2,3-diphenyl propenal	0.6 (2.5)
(12) farnesal	6.3 (11.7)	(C) hexa-2,4-dienal	2.7 (3.5)
(a) 4-nitrobenzaldehyde	2.7 (> 50)	(D) 5-methyl-2-phenyl-2-hexenal	1.7 (4.4)
(b) 4-chlorobenzaldehyde	3.2 (> 10)		
(c) 4,4'-dimethylbenzyl	0.1 (> 50)		
(A) 4,4'-dibromobenzyl	0.1 (20.5)		
(B) hexanal	27.9 (45.0)		
(C) methyl pyruvate	2.7 (2.4)		



The reactivity patterns of representative sensitizing fragrance aldehydes were studied. Hydroxycitronellal and citral were chosen as the representative compounds for the Schiff base and the Michael addition aldehydes, respectively. Both compounds were synthesised ¹³C labelled at the reactive sites and their interactions with amino acids studied by ¹³C-NMR. Only adducts of the Schiff base type were observed.

Hand eczema and fragrance allergy

A total of 59 domestic and environmental products intended for common contact with hands were analysed by GC-MS for 19 target fragrance substances (21). Product categories represented were liquid soaps and soap bars, soft/hard surface cleaners, fabric conditioners/laundry detergent for hand wash, dish wash, furniture polish, shampoo, stain remover and occupational environmental products. The 5 most frequently used fragrance chemicals were limonene (78%), linalool (61%), citronellol (48%), eucalyptol (41%), geraniol (41%) and α -pinene (39%). Similarly, the 5 highest concentrations were (mean values) for limonene (827 ppm), benzyl salicylate (801 ppm), hexyl salicylate (634 ppm), α -hexylcinnamic aldehyde (328 ppm) and citronellol (275 ppm). Based on the results, a patch test tray for hand eczema was established with 14 of the 19 fragrance ingredients (Table 2). A total of 10.2% of the patients with hand eczema had 1 or more positive patch test reactions to the series. Most prevalent were citral, hydroxycitronellal, 4-(4-hydroxy-4-methyl pentyl)-3-cyclohexene carboxaldehyde and eugenol. The FM-I in the standard series picked up 45.5% of the 10.2% positive to the new series. Further a co-relation between a positive reaction to the hand eczema fragrance series and other allergens from the standard patch test series was found, both fragrance chemicals and fragrance unrelated allergens.

Table 2: Hand eczema patch test tray results (658 patients)

Fragrance chemical (% pet.)	Positive (%)
1. citral (2%)	4.3
citral (1%) ⁽¹⁾	0.8
citral (0.5%) ⁽²⁾	0.3
2. hydroxycitronellal (5%)	3.0
3. 4-(4-hydroxy-4-methyl pentyl)-3-cyclohexene carboxaldehyde (5%)	2.1
4. eugenol (5%)	2.0
5. lillial (10%)	0.5
6. oxidised <i>l</i> -limonene (3%) ⁽³⁾	0.9
7. oxidised <i>d</i> -limonene (3%) ⁽⁴⁾	0.8
8. coumarin (5%)	0.5
9. geraniol (5%)	0.9
10. α -hexyl cinnamic aldehyde (10%)	0.5
11. benzyl salicylate (5%)	0.3
12. galaxolide (10%)	0.3
13. citronellol (5%)	0.3
14. benzyl benzoate (5%)	0.2

⁽¹⁾ 3/392 patients; ⁽²⁾ 1/392 patients; ⁽³⁾ 6/649 patients; ⁽⁴⁾ 5/649 patients

The hand immersion study

A total of 15 patients completed exposure according to the protocol and finalised the immersion either after 4 weeks or before in case of appearance of eczema on an immersed finger. Of these 13 had a positive patch test to hydroxycitronellal and 2 to 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde. 3 of the 15 (20%) developed clinical eczema on the fragrance exposed finger whereas 6 (40%) developed eczema on a placebo exposed finger.

Development of a new fragrance mix

Data obtained by patch testing 1272 patients with FM-II (28%, 14%, 2.8%) in 6 clinical centres are shown in Table 3. The mean values of positive results obtained were: 4% for FM-II 28%, 2.8% for FM-II 14% and 1.3%

for FM-II 2.8%. The corresponding figures for doubtful or irritant reactions were 8.3%, 6.3% and 1.6% respectively.

Table 3: Results of patch testing with FM-II

Centre	FM-I 8%		FM-II 28%		FM-II 14%		FM-II 2.8%	
	% + (n)	% ? (n)	% + (n)	% ? (n)	% + (n)	% ? (n)	% + (n)	% ? (n)
Dortmund (149 patients)	3.3 (5)	2.7 (4)	3.3 (5)	2.0 (3)	2.0 (3)	2.7 (4)	1.3 (2)	0.7 (1)
Gentofte (268 patients)	5.2 (14)	7.5 (20)	1.5 (4)	17.5 (47)	1.1 (3)	13.0 (35)	0.7 (2)	2.6 (7)
Leuven (274 patients)	6.6 (18)	0.4 (1)	6.9 (19)	1.4 (4)	5.5 (15)	2.2 (6)	2.2 (6)	2.2 (6)
King's College (293 patients)	4.8 (14)	1.7 (5)	3.4 (10)	3.4 (10)	2.4 (7)	2.0 (6)	1.4 (4)	0.7 (2)
Odense (151 patients)	10.6 (16)	22.5 (34)	4.0 (6)	23.8 (36)	2.6 (4)	17.2 (26)	2.0 (3)	2.6 (4)
Malmö (137 patients)	8.0 (11)	3.6 (5)	5.2 (7)	4.4 (6)	2.9 (4)	2.2 (3)	0	0
TOTAL: 1272 patients	6.1 (78)	5.4 (69)	4.0 (51)	8.3 (106)	2.8 (36)	6.3 (80)	1.3 (17)	1.6 (20)

n = number of patients; ? = doubtful

Reactions to FM-I 8% were in the range reported previously (22). Nearly half of the patients with a positive reaction to FM-II were negative to FM-I (20/51, 39.2%). For the total group of patients the corresponding figure was 1.5% (20/1272). The testing with constituents of the mix revealed 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde to be the main sensitizer, followed by farnesol, citral, α -hexylcinnamic aldehyde and citronellol. The results of the ROATs showed that even weak or doubtful reactions to 1 of the 3 FM-II concentrations were meaningful. 5/11 patients developed a positive reaction after open non-occlusive application to the forearm. 20 products of 11 patients who had shown positive reactions to FM-II and at least to 1 of the constituents were chemically analysed. It was shown that in several patients the product used contained the sensitizer the patient had reacted to upon patch testing.

Threshold dose-response studies of newly identified fragrance allergens

All 3 allergens, 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, chloroatranol and hydroxycitronellal, showed a significant dose-response pattern and reactions in a concentration range relevant to exposure to cosmetics or domestic products.

The range of reactions to 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde was from 6% to 6 ppm, to chloroatranol from 200 ppm to 0.0063 ppm and to hydroxycitronellal from 5% to 0.02%. Based on the dose-response curves the estimated eliciting dose for 50% of individuals was 662 ppm for 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, 0.2 ppm for chloroatranol (Figure 4) and 800 ppm for hydroxycitronellal. Figure 4 shows the percentage of sensitized individuals that react at patch testing to different concentrations of chloroatranol in ethanol (log10).

In the ROAT with 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde and chloroatranol, 94% to 100% reacted to realistic usage concentrations and no controls ($p < 0.001$).

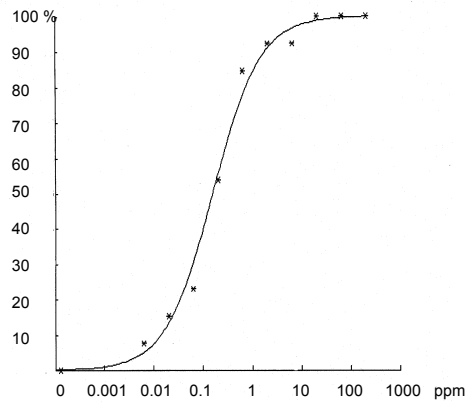


Figure 4: Percentage of sensitized individuals to different concentrations of chloroatranol

Allergenic fragrance chemical substitution: the case of isoeugenol

The frequency of positive reactions seen in all subjects tested (2261) were isoeugenol 1.77%, *trans*-isoeugenol 1.77%, isoeugenol acetate 0.84%, phenylacetate 0.71%, isoeugenol benzoate 0.18%, isoeugenol methyl ether 0.27% and isoeugenol benzyl ether 0.09%. Isoeugenol elicited concomitant positive reactions in 90% of those who reacted to *trans*-isoeugenol, in 68% of those positive to isoeugenol acetate, in 75% of those positive to isoeugenol benzoate, in 94% of those who reacted to phenylacetate; in contrast, none of those who reacted to methyl ether and benzyl ether were positive to isoeugenol. Concomitant reactions to derivatives in isoeugenol positive reactions were *trans*-isoeugenol 90%, isoeugenol acetate 32%, isoeugenol benzoate 7.5%, phenyl acetate 37.5%, isoeugenol methyl ether and isoeugenol benzyl ether were both 0%.

Discussion

Hazard identification is the first step in any risk assessment-management strategy for chemicals present in our environment. Methods to identify sensitizers are therefore basic tools for primary prevention. In the project, methods to identify fragrance sensitizers have been developed.

In addition to pure synthetic fragrance materials several natural extracts are still in use in the perfume industry. Among them oak moss absolute, prepared from the lichen *Evernia prunastri* (L.) Arch., is considered a major contact sensitizer and is therefore included in the FM-I used for diagnosing perfume allergy (23-25). The process of preparing oak moss absolute has changed during the last years and, even if several potential sensitizers have been identified from former benzene extracts, its present constituents and their allergenic status are not clear. In the course of previous investigations on fragrance chemical allergy we have developed a new approach for the identification of fragrance sensitizers present in commercial perfumes and eaux de toilette based on the combination of chemical fractionation, patch testing, chemical analysis and SARs (26, 27). Like commercial eaux de toilette and perfumes, a natural extract such as oak moss absolute contains several hundred different chemicals that are responsible for the complexity of the odour. First results of the above mentioned method applied to oak moss absolute showed that atranol and chloroatranol are strong elicitors in most patients sensitized to oak moss. Methyl- β -orcinol carboxylate was also found to elicit reactions in most patients. Oak moss absolute is traditionally prepared by extracting the harvested lichen with hydrocarbon solvents, followed by a subsequent treatment of the so called oak moss concrete with a mixture of alcohols (23). Freshly harvested oak moss has substantially no scent. The moss contains various types of depsides, which are non-volatile, odourless, polyfunctional diaryl derivatives, such as evernic acid, atranorin and chloroatranorin, among others (28). The characteristic oak moss fragrance is only developed after cleavage of the depsides during the treatment of oak moss concrete with alcohols, that gives volatile, scented, monoaryl derivatives (29). Our results indicate that monoaryl compounds derived from the decomposition of depsides are allergenic. Atranol and chloroatranol can be formed by transesterification and decarboxylation of atranorin and chloroatranorin during the alcohol treatment of oak moss concrete. Methyl- β -orcinol carboxylate, main responsible for the characteristic earthy-moss-like odour of oak moss products (30), is also a depside degradation product formed during the oak moss processing. Depsides still remaining in the absolute after treatment of the lichen with solvents may be present in the first fractions eluted from gel permeation chromatography of the oak moss sample which were also positive at patch testing. Bioassay guided chemical sub-fractionation of these fractions, analytical and SARs studies are currently undergoing.

Terpenes are another important category of naturally occurring fragrance chemicals used in scented commercial products. Of these terpenes, linalool from lavender oil and *R*-limonene from citrus oil are the most common fragrance chemicals found in domestic and occupational products (21). The EU classification of limonene as a sensitizer by skin contact (R43) due to formation of allergens when air exposed is based on our previous experimentally obtained data (12, 31). Clinical studies on *R*-limonene had supported this classification (32, 33). In this study, we have confirmed that not only *R*-limonene but also *S*-limonene are common causes of contact allergy. Furthermore, we have shown that the oxidative degradation seen for limonene at air exposure is also seen for other terpenes used as fragrance chemicals such as linalool,

caryophyllene and myrcene. In previous clinical studies we have shown that limonene itself does not give elicitation in individuals reacting to oxidised limonene (33). Parallel experimental studies have shown that linalool (18) and caryophyllene themselves are not allergenic, while oxidised linalool, its hydroperoxide fraction and caryophyllene oxide cause sensitization. Most patch test reactions were observed to oxidised limonene and linalool. This could, at least partly, be explained by their greater usage compared to that of the other fragrance chemicals studied. Based on these investigations, patch testing with oxidised limonene and linalool should be performed routinely on dermatitis patients with suspected fragrance allergy. A big difference was seen in the degradation of linalool compared to that of limonene, which makes it difficult to use limonene as a marker for the oxidative degradation of fragrance terpenes in general. On another hand, extensive studies are needed to be able to draw general conclusions regarding the effects of antioxidants on oxidative degradation, since no correlation was seen between the rate of degradation with and without the antioxidant BHT, when comparing the terpenes. The results imply that the EU regulation regarding limonene can be applicable on the chemicals studied, especially on linalool. Furthermore, it is important that the formation of allergens upon handling and storage of these fragrance chemicals is considered by the producers to prevent sensitization.

The second key step in a risk assessment-management strategy for fragrance sensitizers, once they have been identified, is the study and understanding of their potency (34). In the absence of validated *in vitro* models for the identification of skin sensitizers and for estimation of their potency, animal models still provide the foundation for risk assessment in this area of toxicology. The LLNA, which has undergone full international validation, offers a number of animal welfare advantages over the guinea pig methods, notably in reduction of animal numbers and in the refinement of their use. The method measures cell proliferation in lymph nodes draining the skin site to which the test substances have been applied. The potency descriptor is the EC₃ value or concentration of test substance which gives a threshold sensitization response (35-37). In this study the LLNA test was used to generate quantitative potency data on 2 common families of fragrance chemicals, aliphatic (Schiff base) and α,β -unsaturated (Michael addition) aldehydes. Physicochemical properties were then used in combination with LLNA EC₃ values to develop new QSAR models for prediction of the sensitization potential. QSARs objectively describe the interaction between chemicals and biological systems, and are now increasingly used in the prediction of a variety of toxicological endpoints, allergic contact dermatitis among them (38). Mechanistically based QSAR models hold the potential for becoming valid alternatives to extensive animal testing, particularly if they can be confirmed as robust predictors of fragrance allergy. The new QSAR models derived in this study for fragrance aldehydes were found to be fairly accurate. Plus, the understanding of aldehyde allergy was much improved in terms of risk assessment based on relative sensitization potential as well as in terms of understanding of the potential mechanisms of action.

Another basic element to establish a risk assessment-management strategy is the development and standardisation of new diagnostic methods for the secondary prevention of fragrance allergy.

Experimental studies in the area of hand eczema fragrance allergy had never been performed before. Population-based studies in Europe indicate a frequency of hand eczema of 7-10% (39). The identified risk factors for hand eczema are atopic dermatitis, contact allergy and exposure to wet-work. Co-relation

between hand eczema and contact allergy to certain haptens i. e. nickel, preservatives and rubber chemicals are well established in the literature. Also, fragrance allergy is of relevance to hand eczema. The currently used FM-I to diagnose allergy to fragrance ingredients has been established on the basis of exposure from cosmetic products. It has been suspected that products intended for hand exposure such as dishwashing and soap liquids, may contain very different fragrance ingredients. Therefore, appropriate diagnostic procedures are not performed by using the current FM-I diagnostic test. In this study, a new diagnostic tool for fragrance allergy in hand eczema patients was established based on chemical analysis of consumer products and information concerning allergen potency and industrial use. It was found that 10.2% of patients with hand eczema had 1 or more reactions to ingredients of the new patch test series with 14 fragrance ingredients. Less than half of these would have been picked up by the current diagnostic FM-I. That is to say that 5.6% of all the patients tested would have had an insufficient diagnostic work up if the 14 extra fragrances had not been included, probably adding to the chronicity of their disease. Among patients reacting to the fragrance hand eczema series 71.6% gave a positive fragrance allergy history compared to only 47.1% among those who did not react to the series. This finding underlined the clinical impression of the significance of these positive patch tests. Another significant finding in the study was the high number of reactions to citral which is a known contact allergen and also an irritant (21). As a complement, an experimental exposure model (the hand immersion study) simulating real life was established in order to describe thresholds for safe exposure. The allergens hydroxycitronellal and 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde were used as models as they were found in household products in the range of 15-140 ppm and 36-103 ppm respectively (21). There was no indication that exposure to a single potent fragrance chemical by hand immersion, simulating normal dishwashing, was able to make a flare of dermatitis in already sensitized individuals previously diagnosed with hand eczema. However, this observation does not exclude that patients with combined contact allergies, which simultaneously are exposed to irritants and prolonged exposure, might observe such a flare.

As it has been repeatedly mentioned in this report the currently used FM-I for patch testing with only 8 constituents does not identify all fragrance allergic patients. A new FM-II was evaluated as an improved diagnostic tool in patch testing. FM-II contained 6 fragrances of wide usage: 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, citral, farnesol, citronellol, α -hexyl cinnamic aldehyde and coumarin. FM-II was patch tested in 3 concentrations (28%, 14%, 2.8% pet.) on 1272 consecutive patients of 6 European dermatological centres. Positive reactions to FM-II were found in decreasing frequencies in regard to test concentration: 4% to FM-II 28%, 2.8% to FM-II 14%, 1.3% to FM-II 2.8%. 34.8% of the patients reacting to FM-II were negative to FM-I which produced positive reactions in 6.1% of the total population. Testing of constituents revealed 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, citral and farnesol to be the main sensitizers (4% to 1.6%). Patients positive to FM-II had a certain history of adverse reactions to scented products in 42.3%. The new FM-II identified additional patients sensitive to fragrances which would have been missed by using only the FM-I. The FM-II is therefore a candidate for inclusion into the European standard series for patch testing and thus improves the diagnostic value of this procedure.

In the context of secondary prevention it is also essential to establish dose-response data for newly identified common fragrance allergens. Exposure concentrations are determinant in induction as well as in elicitation of contact allergic reactions and thus dose-response information is also a key to perform risk assessment.

In the programme 3 allergens were found of major importance: 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde, chloroatranol and hydroxycitronellal. All of them are common in consumer products, cosmetics and/or domestic products, and in concentrations shown in this study to elicit allergic contact dermatitis under experimental conditions. The dose-response relationship for elicitation of these 3 major allergens was studied in order to evaluate the safety profile of current exposure in relation to allergic contact dermatitis.

4-(4-Hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde was shown to give positive reactions in 2% of consecutive eczema patients and has recently been included in the standard series in many clinics in Europe (40, 41). Moreover, it is considered the Top 1 sensitizer of the new FM-II diagnostic tool. The levels used currently of 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde in cosmetic products such as fine fragrances or deodorants are 10-1000 fold higher than the threshold demonstrated in this study (21, 40). This explains the high number of sensitized individuals in the Community and the need for a reduction in exposure concentrations. Currently there are no restrictions in usage of 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carboxaldehyde in cosmetic products.

Chloroatranol was identified as the major allergen in the natural extract oak moss absolute when developing a method for the identification of fragrance sensitizers in complex mixtures. Oak moss absolute accounts for 25-30% of reactions to the FM-I (25) and the identification of the major causative ingredient is a great advance for prevention of fragrance contact allergy. As described in the results section of this report chloroatranol elicited reactions to concentrations of allergen that are extremely low. 50% of sensitized individuals reacted to 0.2 ppm (0.00002%) and the lowest concentration of reaction in this experiment was 0.0063 ppm. This is to date the strongest allergen identified, which is a common part of consumer products such as cosmetics. Given this extreme potency methods should be developed to remove this substance from its natural mother compound, so it does not appear in consumer products.

Hydroxycitronellal was shown to be of major importance in patients with hand eczema, where it gave reactions in 3%. In the current study it was also shown that patients reacted to concentration levels present in consumer products.

Finally, always in the context of prevention to fragrance allergy, studies were conducted in order to evaluate the feasibility of fragrance chemical substitution in consumer products. The case of isoeugenol was chosen as a model. Isoeugenol, one of the constituents of the FM-I, is known to be an important fragrance allergen for consumers (42-46). Industry recommended that it should be used at 10% of its until recently suggested maximum levels in cosmetic products (reducing the maximum acceptable level from 0.2% to 0.02%), but without guidance on how it should be substituted (47). The objective was to look for cross reactivity to isoeugenol derivatives in isoeugenol allergic individuals. Such derivatives may be proposed as

replacements (allergen substitution) for isoeugenol. Potential cross reactivity in those individuals already sensitized to isoeugenol represents an important safety consideration which to date has not been adequately explored. 2261 subjects attending contact dermatitis clinics were patch tested to isoeugenol and to its esters and ether derivatives. Concomitant contact allergy between isoeugenol and its derivatives may occur through chemical cross reactivity or local skin metabolism of the derivatives. The data showed significant concomitant cross reactivity between isoeugenol and its ester derivatives but not to the ethers. This suggested ester substitution may be hazardous; the ethers may be a safer substitute assuming they are less allergenic in their own right. The question of fragrance chemical substitution must therefore be taken into consideration carefully. In parallel it was seen that the rate of isoeugenol allergy has not yet shown a consistent decline since the reduction in levels in cosmetic products.

Conclusion

At the present time, there is no treatment, other than symptomatic, for fragrance contact allergy reactions and the only means available to improve public health in this sector is prevention. As a result of the 5th EU Framework Programme "Fragrance chemical allergy" project, a risk assessment-management strategy for fragrance chemicals providing the basis for preventive measures has been built by compiling all the results and experiences obtained in the primary and secondary prevention studies carried out. This strategy is shown in Figure 5.

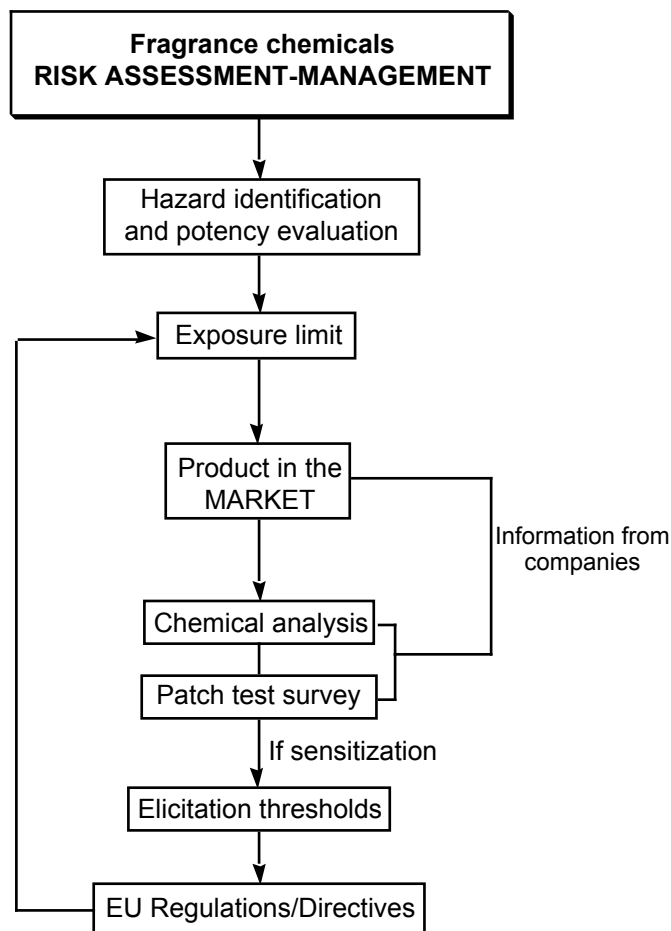


Figure 5: Risk assessment-management strategy for fragrance chemicals

Methods to identify fragrance sensitizers (hazards) are basic tools in primary prevention and need to be developed as a first step in any risk assessment procedure. Reliable tests allowing prospective recognition of allergens among the myriad chemicals used in fragrance composition rate high priority for the fragrance industry as well as for public health officials. Moreover, it is important to consider that certain fragrance chemicals can form sensitizers due to air oxidation at handling and storage. Second, the potency of the fragrance sensitizers identified needs to be assessed by predictive sensitization studies (i. e. QSARs) and an estimation of their exposure limit in consumer products needs to be performed before their release into the market.

When the process of allergen identification has failed or exposure limits used are inadequate, cases of contact allergy in the population will appear. Information about exposure to fragrance ingredients in consumer products is therefore essential for pinpointing possible contact allergens. The secrecy of the fragrance formula in marketed products is usually very well guarded by the fragrance industry to prevent imitations. Only on a case basis is it possible for the consumer to obtain information regarding the ingredients in a fragrance formula. However, actual developments in analytical chemistry have made it possible to determine the chemical composition of the formula. Moreover, patch testing of eczema patients is not only a diagnostic tool, but important for the monitoring of new causes of disease and failures in the risk management of recognised allergens. Thus, chemical analysis of commercial products combined with a patch test survey is a precious tool to acquire information about exposure to fragrance sensitizers in consumer products.

If sensitization to a specific fragrance ingredient is found, it is further necessary to perform studies on the doses in the products that induce and elicit contact allergy. Reliable information is complex to obtain for induction. Experimentation regarding induction (sensitization) thresholds is difficult to perform using animal tests under the actual legislation that bans animal testing and asks for the development and use of alternative test methods. For some allergens, information on sensitization thresholds under experimental conditions in human volunteers is available, although in the European Union such experiments are considered unethical (Directive 67/548/EEC). Eczema develops in already sensitized individuals if exposure exceeds a certain individual threshold of response. Elicitation thresholds can thus be determined easier by exposing sensitized individuals to a serial dilution of the allergen in question under controlled circumstances. In principle, lowering of exposure concentrations to individual fragrance allergens reduces the number of individuals who react on contact. However, depending on the product (i. e. cosmetics) the variations in exposure conditions are numerous. It is therefore necessary to define worst case scenarios to determine safer conditions of use.

The information acquired by performing elicitation threshold studies needs then to be used to create guidelines for the manufacturers to produce safer products. It constitutes the basis for decisions (EU regulations/directives) to improve consumer safety: product labelling of the most frequent sensitizing materials, guidelines concerning concentration limits for the use of certain fragrance materials, and even the exclusion of certain highly sensitizing fragrance materials from consumer products. Also a time limit must be considered for the usage of products containing fragrance materials that decompose to sensitizing chemicals unless adequately protected.

All aspects of this risk assessment-management strategy have been treated in the studies reported in the previous sections. New methods for the identification of sensitizers have been developed as well as new alternative methods for the predictive study of their sensitizing potential. Diagnostic methods have been standardised and new ones have been created to identify the individuals at risk. Moreover, epidemiological clinical studies have been performed in 6 European dermatological centres, combined with exposure assessment and cross reactivity studies aiming at allergenic fragrance chemical substitution. The results of these studies have provided data for the regulation by public authorities of fragrance ingredient exposure to prevent the development of contact sensitivity.

Exploitation and dissemination of results

This study provides basis for primary and secondary prevention of fragrance chemical allergy. The results can be exploited by medical doctors in diagnosing and advising people allergic to fragrances, by industry to produce less allergenic products and by regulatory authorities to set safety standards for fragrance allergens. The identification of unknown significant contact sensitizers will be the basis for the development of standardised patch test materials that can be expected to be commercially developed. These products will be available for dermatologists on a world wide scale. The participating scientists have earlier and are currently involved in the development of such technologies with several companies on a non profit basis. There is no intention to apply for patents in this area. Finally, the results will be available for the development of alternative methods for predicting the sensitizing potential of fragrance chemicals and thus help to improve, and reinforce, European regulation about fragrance chemicals utilisation.

All results are being or will be published in peer reviewed journals such as *Contact Dermatitis*, *Archives of Dermatological Research*, *The British Journal of Dermatology* and *Acta Dermatologica Venerologica*. Most of the data have been already or will be presented at scientific meetings of the European Society of Contact Dermatitis, the European Academy of Dermatology and Venereology, the American Society of Contact Dermatitis and the World Congress in Dermatology. All knowledge will be of public domain.

Publications

1. Rastogi S C, Heydorn S, Johansen J D, Basketter D A. Fragrance chemicals in domestic and occupational products. *Contact Dermatitis* 2001: 45: 221-225.
2. Sköld M, Börje A, Matura M, Karlberg A-T. Studies on the autoxidation and sensitizing capacity of the fragrance chemical linalool, identifying a linalool hydroperoxide. *Contact Dermatitis* 2002: 46: 267-272.
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4. Bernard G, Giménez-Arnau E, Rastogi S C, Heydorn S, Johansen J D, Menné T, Goossens A, Andersen K, Lepoittevin J-P. Contact allergy to oak moss: search for sensitizing molecules using combined bioassay-guided chemical fractionation, GC-MS and structure-activity relationship analysis (Part I). *Archives of Dermatological Research*, 2003, submitted.
5. Heydorn S, Johansen J D, Andersen K E, Bruze M, Svedman C, White I, Basketter D A, Menné T. A new fragrance hand eczema series. *Contact Dermatitis*, 2003, submitted (confidential until it has been published).

Publications on draft

At least 10 more scientific papers will be published during the next 5 years, some of them being already in preparation.

- Testing with a new fragrance mix: patch test results of a multicentre European study.
- Contact allergy to isoeugenol and its derivatives.

- Contact allergy to oak moss: search for sensitizing molecules using combined bioassay-guided chemical fractionation, GC-MS and structure-activity relationship analysis (Part II).
- Elicitation thresholds for the main sensitizers of oak moss.
- Analytical methods for the identification of atranol and chloroatranol in consumer products.
- Studies on interaction mechanisms of fragrance aldehydes with proteins.
- Results on the oxidative degradation products at air exposure of fragrance chemicals.
- European patch test study on air oxidised *R*- and *S*-limonene.
- European patch test study on air oxidised linalool, caryophyllene and myrcene.
- Results of the diagnostic series of fragrance allergens relevant to hand eczema.
- Results of the elicitation thresholds of 3 important fragrance allergens.
- Review paper summarising the Fragrance Allergy project.

Communications

1. Frosch P J, Pirker C, Johansen J D, Menné T, Rastogi S C, Bruze M, Andersen K E, Lepoittevin J-P, Goossens A, White I R. New markers for fragrance hypersensitivity. Poster presentation at the 5th Congress of the European Society of Contact Dermatitis, Amsterdam, The Netherlands, 11-13 May 2000. Published in *Contact Dermatitis* 2000: 42: Suppl. No. 2; pp. 51.
2. Sköld M, Börje A, Matura M, Karlberg A-T. Linalool, the most frequently used fragrance chemical, allergenic or not? *Forum för dermatologisk forskning*, 15th March 2001, Stockholm, Sweden.
3. Rastogi S C, Johansen J D, Menné T, Heydorn S, Basketter D, Andersen K E, Bruze M, Frosch P J, Giménez-Arnau E, Goossens A, Karlberg A-T, Lepoittevin J-P, White I. Contents of selected fragrances in household products analysed by gas chromatography – mass spectrometry. Poster presentation at the 24th International Symposium on Capillary Chromatography and Electrophoresis, 20-24 May 2001, Las Vegas, USA.
4. Sköld M, Börje A, Matura M, Karlberg A-T. Är linalool, den vanligaste fragransen i parfymerade produkter, allergiframkallande? (Is linalool, the most often used fragrance chemical, allergenic?). Oral presentation at the *Occupational Dermatology Meeting*, Stockholm, Sweden, 17th of May 2001.
5. Sköld M, Börje A, Matura M, Karlberg A-T. Luftoxidation av våra vanligaste doftämnen orsakar parfymallergi. (Air oxidation of our most often used fragrance chemicals causes allergy). Poster presentation at the *Swedish Physicians Yearly Congress*, Stockholm, Sweden, 28th-30th November 2001.
6. Bernard G, Giménez-Arnau E, Goossens A, Rastogi S C, Menné T, Andersen K E, Lepoittevin J-P. Development of a method for the identification of oak moss sensitizers. Oral presentation at the 17th Meeting of the European Research Group on Experimental Contact Dermatitis, Strasbourg, France, 18th-20th January 2002.
7. Sköld M, Börje A, Matura M, Karlberg A-T. Air exposure of linalool, the most frequently used fragrance chemical, creates allergenic oxidation products. Oral presentation at the 17th Meeting of the European Research Group on Experimental Contact Dermatitis, Strasbourg, France, 18th-20th January 2002.

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Actions taken towards an effective communication of the project and of already obtained results to a targeted audience as well as to a non-specialist audience

A 6 pages brochure covering the broad aim and approaches of the project, the participants, expected outputs and impacts, was prepared to help to publicise the project for a broad distribution. 200 copies were sent to the project European Community Scientific Officer for distribution.

A publication appeared in the popular scientific journal of the Université Louis Pasteur, Strasbourg, France : Ces fragrances qui nous agressent, *Ulp.sciences : le magazine de l'Université Louis Pasteur de Strasbourg*, n°2, January 2001, p. 18.

A poster communication was presented at the EC organised *Environment for Better Health Conference*, Denmark, 8-11 May 2003. Fragrance chemical allergy: a major environmental and consumer health problem in Europe.

Policy related benefits

The results of the study will provide guidelines for the manufacturers to produce safer products. They will be used as basis for decisions (new EU directives) to improve consumer safety: product labelling of the most frequent sensitizing materials, guidelines concerning concentration limits for the use of certain fragrance materials, and the exclusion of certain highly sensitizing fragrance materials from consumer products. The results will be available for the development of alternative methods for predicting the sensitizing potential of fragrance chemicals and thus help to improve, and reinforce, European regulation about fragrance chemicals utilisation. Data obtained on the most common fragrance sensitizers will supplement the EU's Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) guidelines on the restrictions to safe levels and labelling of the 26 fragrance allergens list included in the 7th Amendment to the European Cosmetics Directive (76/768/EEC).

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