National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Inhalation exposure to fragrance allergens

Are consumers at risk for respiratory allergies?

RIVM Report 340301004/2011

J. Ezendam | W. ter Burg | S.W.P. Wijnhoven

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Colophon

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This investigation has been performed by order and for the account of Food and Consumer Product Safety Authority, within the framework of Kennisvraag 9.1.2. Allergene stoffen in geurproducten voor consumenten

Abstract

Inhalation exposure to fragrance allergens
Are consumers at risk for respiratory allergies?

There is insufficient information available to assess if consumers are at risk for respiratory allergies when they inhale fragrance allergens. This is the conclusion of a study performed by the National Institute for Public Health and the Environment (RIVM) by order of the Food and Consumer Product Safety Authority.

Fragrances are used in numerous consumer products, including perfumes, cosmetics and household cleaning products. Twenty-six such fragrances are known potential causes of allergic contact dermatitis. An inventory of products by the RIVM has shown that the majority of these 26 fragrance allergens are present in air fresheners as well, leading to the exposure of consumers to these fragrance allergens also through inhalation. However, it is currently unknown whether this route of exposure represents a public health risk, such as eliciting allergic reactions in the airways. No validated methods are available to assess the health risks of inhaled fragrances. In addition, the concentrations of the fragrance allergens in air fresheners are unknown, making it impossible to estimate the degree of human exposure.

Inhalation studies in mice conducted by the RIVM do show that one of the five tested fragrance allergens appears to have an adverse effect on the immune system in the airways. Whether this effect represents a health risk to consumers is not clear. In addition, there is only limited evidence indicating that prolonged inhalation exposure to fragrance allergens in an occupational setting can cause respiratory allergies.

Keywords:
fragrances, inhalation exposure, consumer products, allergy, health risks

Rapport in het kort

Inademing van allergene geurstoffen
Lopen consumenten risico op luchtwegallergie?

Er is onvoldoende kennis beschikbaar om vast te stellen of consumenten allergische klachten aan luchtwegen kunnen krijgen als zij allergene geurstoffen in consumentenproducten inademen. Dit blijkt uit onderzoek van het RIVM, in opdracht van de nieuwe Voedsel en Waren Autoriteit (nVWA).

Geurstoffen komen voor in diverse consumentenproducten, zoals parfums, verzorgingsproducten en schoonmaakmiddelen. Van 26 geurstoffen is bekend dat ze een huidallergie kunnen veroorzaken. Een productinventarisatie van het RIVM heeft aangetoond dat deze 26 allergene geurstoffen eveneens bijna allemaal gebruikt worden in luchtverfrissers. Consumenten worden hierbij dus ook via de ademhaling blootgesteld aan deze geurstoffen. Onbekend is echter of deze vorm van blootstelling allergische reacties in de luchtwegen kan veroorzaken. Er zijn namelijk geen valide methoden beschikbaar om dit gezondheidsrisico vast te stellen. Bovendien zijn de gehaltes van de geurstoffen in de producten niet bekend, zodat het onmogelijk is om een schatting van de mate van blootstelling te maken.

Wel tonen inhalatiestudies met muizen die het RIVM heeft uitgevoerd aan dat 1 van de 5 onderzochte allergene geurstoffen een ongewenst effect op het immuunsysteem van de luchtwegen lijkt te hebben. Of dat ook een risico vormt voor de consument is nog niet duidelijk. Daarnaast zijn er geringe aanwijzingen dat mensen die tijdens hun werk langdurig allergene geurstoffen inademen allergische luchtwegklachten ontwikkelen.

Trefwoorden:
geurstoffen, inhalatieblootstelling, consumentenproducten, allergie, gezondheidsrisico’s

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Summary

The use of scented products, such as air fresheners, will lead to inhalation exposure to ingredients, such as fragrance chemicals, emitted from these products. Twenty-six such fragrance chemicals are known potential causes of allergic contact dermatitis, i.e. skin allergy. It is unknown if inhalation exposure to these fragrances can induce respiratory allergies in consumers. To evaluate if consumers are at risk when they use these products, two questions should be answered. First, is inhalation exposure to these fragrance allergens possible and to which extent? Second, will inhalation exposure to these fragrance allergens induce adverse immune effects in the airways?

To explore if human exposure is possible, a product inventory was performed to assess the presence and concentrations of the 26 fragrance allergens in scented consumer products. This inventory showed that of the 26 fragrance allergens, 20 were used as ingredients in scented consumer products. The most frequently used fragrance allergens were D-limonene, linalool, geraniol and citronellol. The information on concentrations of the fragrances in scented products was very limited and the exact exposure concentrations were therefore not assessed.

The respiratory Local Lymph Node Assay (LLNA) was used to assess if inhalation exposure could stimulate the immune system in the airways. In this model, isoeugenol, cinnamal, citral, methyl heptine carbonate and benzyl salicylate were tested. In the respiratory LLNA, isoeugenol was the only substance that increased lymphocyte proliferation in the mandibular lymph nodes, indicatory for respiratory sensitization. The respiratory LLNA is a short-term assay that only measures the induction phase of an immune response. To further investigate if isoeugenol could lead to respiratory allergy after repeated exposures, additional experiments were conducted with isoeugenol and cinnamal. In these studies, mice were sensitized through the skin and challenged with a single inhalation exposure. These fragrance allergens did not induce lung inflammation or impaired lung function.

There is limited human evidence that is in line with these mice studies. In a small human experiment it was shown that inhalation exposure to realistic concentrations of fragrance allergens does not lead to adverse respiratory effects in subjects with an existing skin allergy to this specific fragrance allergen. Remarkably, exposure to high, non-realistic concentrations aggravated the skin allergy in these volunteers. In addition, case studies show that occupational inhalation exposure to fragrance allergens can induce occupational asthma or rhinitis.

In conclusion, this project has shown that the use of scented consumer products leads to inhalation exposure to the majority of the 26 fragrance allergens. With the currently available data it was not possible to estimate the exact human exposure. The experiments in mice show that isoeugenol might lead to sensitization of the airways, but the effects of repeated exposures should be further explored to assess if this would pose a hazard for humans. The limited data from humans indicate that high dose and/or long-term exposure might lead to adverse effects. There are numerous knowledge gaps and uncertainties in the field of respiratory sensitization induced by chemicals, which makes it currently not possible to evaluate if the use of scented products would lead to health risks in consumers.

1 Introduction

Already in ancient times, people were attracted to products with a pleasant smell, such as perfumes. Nowadays, the selection of scented products extends beyond perfumes and fragrances are added to all kinds of consumer products, including cosmetics, cleaning products and air fresheners. The most common adverse effect that is induced by exposure to fragrances is allergic contact dermatitis, which is an allergic reaction induced after skin exposure. It is estimated that 1% of the general population suffers from a contact allergy to fragrances, making these chemicals the second most frequent cause of contact allergy after metals (Schnuch et al., 2002; Bruynzeel et al., 2005).

Besides skin exposure, the increased use of fragrances in air fresheners, cleaning sprays and room perfumes will lead to inhalation exposure. It is unknown if inhalation exposure to skin sensitizers, such as fragrances, can also induce allergic airway diseases. Classes of chemicals that can induce allergic airway diseases are isocyanates, acid anhydrides, reactive dyes (including hair dyes), and metal salts (Bernstein, 2003; Gezondheidsraad, 2008). Asthma induced by these substances is considered to be an important health problem in occupational settings. It is currently unknown if inhalation exposure to consumer products that contain sensitizers can lead to asthma. In an epidemiological study it was shown that frequent use of cleaning sprays in a household setting was associated with a higher risk on asthma. It was impossible to retrieve information on the causative agents from this study (Zock et al., 2007).

Respiratory and skin sensitizers are two different classes of compounds. According to the Dangerous Substances Directive (DSD) they have to be labelled with R42 or R43 risk phrases respectively. There is some human evidence that skin exposure to respiratory sensitizers is an important route of sensitization (Redlich & Herrick, 2008; Redlich, 2010). In animal studies it has been shown that skin exposure to respiratory sensitizers induced sensitization (Dearman et al., 1995; Vandebriel et al., 2000; Vanoirbeek et al., 2003; van Triel et al., 2011). In contrast, for skin sensitizers it is still a matter of debate whether inhalation of skin sensitizers can induce respiratory sensitization. There is no human evidence for this, but animal studies show that inhalation exposure to skin sensitizers can lead to sensitization of the airways and asthma-like symptoms (Garssen et al., 1991; Arts et al., 1998; van Triel et al., 2010). Other studies, however, fail to demonstrate that inhalation of skin sensitizers induced sensitization or respiratory symptoms (Farraj et al., 2004; Vanoirbeek et al., 2006; Henjakovic et al., 2008). In most of these studies strong skin sensitizers were used, such as dinitrochlorobenzene and picryl chloride, whereas effects of inhalation exposure to weak to moderate sensitizers, such as fragrance allergens, have not been studied.

The extrapolation of these animal data to human risks is hampered by lack of a validated animal model that can be used for hazard identification and characterization of respiratory sensitizers. Furthermore, there is still no consensus on the immunological mechanisms underlying respiratory sensitization. These differ from those involved in skin sensitization, which is a classical delayed-type hypersensitivity response. The clinical symptoms in skin allergy are elicited by cellular responses, involving T lymphocytes (Kimber et al., 2002). The immunological mechanisms of respiratory sensitization are not so

well understood. Some respiratory sensitizers, like metal salts and acid anhydrides induce type I or immediate-type hypersensitivity (Dykewicz, 2009). This immune reaction is mediated by IgE antibodies and leads to allergic asthma in the lower airways (Bernstein, 2003). However, for isocyanates it has been shown that in the majority of patients IgE is not involved (Bernstein, 1996). It has been suggested that delayed-type hypersensitivity responses can be involved in respiratory sensitization as well (Buckley & Nijkamp, 1994). These hypersensitivity responses can lead to allergic alveolitis or hypersensitivity pneumonitis in the upper airways as has been shown in occupational settings in which workers are exposed to high concentrations (Zeiss & Patterson, 1993; Sala et al., 1996).

The increase in consumer products intended to spread a pleasant smell, such as air fresheners, will lead to increased exposure to fragrances present in these products. To explore whether this inhalation exposure to fragrance allergens is a risk for consumers the Dutch Food and Consumer Safety Authority (nVWA) initiated this project. The aim of this project was to develop a risk assessment strategy for scented consumer products. Risk assessment of chemical substances relies on different pillars, including hazard identification, hazard characterization and exposure assessment. For respiratory sensitization, however, there is currently no risk assessment strategy available. Therefore, a more pragmatic approach was chosen to evaluate the risks associated with these consumer products. For hazard identification and characterization, experiments in mouse models were conducted and for exposure assessment the available literature and databases were searched to find data on presence and concentrations of fragrance allergens in scented consumer products. This report will summarize the outcomes of these studies and describe knowledge gaps.

2 Background

2.1 Overview of fragrance allergens

Specific and unique scents are developed by combining different fragrances. In the fragrance industry about 3,000 fragrance substances are used. Approximately 300-400 fragrances are of natural origin, i.e. balsams, essential oils, whereas the other fragrances are synthetically manufactured (Bauer et al., 1990). A small number of these fragrance chemicals have been identified as skin sensitizers, implying that they are able to cause allergic contact dermatitis. It is estimated that 1% of the general population suffers from a contact allergy to fragrances, making these chemicals the second most frequent cause of contact allergy after metals (Schnuch et al., 2002; Bruynzeel et al., 2005). In 1999, the Scientific Committee on Cosmetic Products and Non Food Products (SCCNFP, now known as the SCCS) has identified 24 fragrance chemicals that potentially could cause contact allergy. They composed two different lists, one list of most frequently reported and well-recognized skin sensitizers and a list with fragrances that are less frequently documented as skin sensitizers (see Table 1). Two botanical extracts, oak moss (*Evernia furfuracea*) and tree moss (*Evernia prunastri*), have been added to this list, resulting in a total of 26 fragrances associated with allergic contact dermatitis. The use of these fragrances is not restricted to specific limit values, but according to legislation these fragrances should be declared on the label of cosmetic products when the concentration exceeds a certain limit (EU Directive 2003/15/EC, 2003). On the labels of cosmetics, fragrances are listed as individual ingredients or labelled as 'perfume'.

Table 1 SCCNFP list of fragrance allergens[1]

Frequently reported sensitizers	**Less frequently reported sensitizers**
Amyl cinnamal	Anisyl alcohol
Amylcinnamyl alcohol	Benzyl benzoate
Benzyl alcohol	Benzyl cinnamate
Benzyl salicylate	Citronellol
Cinnamyl alcohol	Farnesol
Cinnamal	Hexyl cinnamaldehyde
Citral	Lilial
Coumarin	d-Limonene
Eugenol	Linalool
Geraniol	Methyl heptine carbonate
Lyral® (Hydroxymethylpentylcyclohexene carboxaldehyde)	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexe-1-yl)-3-buten-2-one (= γ-methylionone)
Isoeugenol	
Hydroxycitronellal	

[1] SCCNFP (1999) Opinion concerning fragrance allergy in consumers. SCCNFP/0017/98.

The clinical importance of these 26 fragrances has been investigated in a large European study. The German Information Network of Departments of Dermatology (IVDK) has assessed the frequency of fragrance allergy in more than 21,000 patients. Based on these clinical data, the 26 fragrances were categorized in three groups: (1) important sensitizers, (2) less important sensitizers and (3) rare sensitizers (Schnuch et al., 2007), as depicted in

Table 2. There are some discrepancies in the IVDK data compared to the SCCNFP lists. Some of the fragrances that are considered to be important sensitizers according to the SCCNFP, were of little clinical importance in the IVDK study. These differences illustrate the importance of monitoring the prevalence of fragrance allergy in a large cohort of patient to identify the most important sensitizing fragrances.

Table 2 Categorization of 26 fragrances to be labelled according to EU regulation[1]

Group 1: important sensitizers	**Group 2: less important sensitizers**	**Group 3: rare sensitizers**
Oak moss	Cinnamic alcohol	Benzyl alcohol
Tree moss	Citral	Linalool
Lyral®	Citronellol	Methylheptin carbonate
Hydroxycitronellal	Geraniol	α-Amyl-cinnamic aldehyde
Isoeugenol	Eugenol	α-Hexyl cinnamic aldehyde
Cinnamic aldehyde	Coumarin	Limonene
Farnesol	Lilial	Benzyl salicylate
	Amyl-cinnamic alcohol	γ-methylionone
	Benzyl cinnamate	Benzyl benzoate
		Anisyl alcohol

[1] Adapted from Schnuch et al., 2007

2.2 Skin sensitizing potential and potency

The risk of becoming sensitized depends on the exposure concentration and on the skin sensitizing potency, the latter being a metric for the intrinsic capacity of a chemical to induce sensitization (Basketter et al., 1999; van Och et al., 2000). Skin sensitizers can be categorized as weak, moderate and strong sensitizers based on either human data or data from animal models. The skin sensitizing potency can be determined in the mouse Local Lymph Node Assay (LLNA), a validated animal model for identification of skin sensitizers (OECD, 2000) and potency values derived from the LLNA correlate relatively well with human potency data (Gerberick et al., 2001; Griem et al., 2003; Schneider & Akkan, 2004). This is also the case for fragrance allergens, although a few discrepancies exist between human and LLNA data (see Table 3). The majority of fragrance allergens are weak sensitizers, with the exception of methyl heptine carbonate and isoeugenol, which are strong and moderate sensitizers respectively. Benzyl salicylate has a similar potency in the LLNA as isoeugenol, but in humans this compound is classified as a weak sensitizer. The fragrances cinnamal, oak moss, and hexyl cinnamaldehyde are classified as moderate human skin sensitizers. Coumarin is negative in the LLNA, but human data are available that this fragrance is a sensitizer (SCCP, 2006).

Table 3 Skin sensitizing potency of fragrances[1]

Fragrance	LLNA EC3 value	Human category
Methyl heptine carbonate	0.5	strong
Isoeugenol	1.5	strong
Benzyl salicylate	1.5	weak
Cinnamal	2.0	moderate
Oak moss	3.9	moderate
Farnesol	4.8	weak
Citral	5.6	weak
Anisyl alcohol	5.9	weak
Hexyl cinnamaldehyde	9.9	moderate
Eugenol	10.1	weak
Amyl cinnamal	10.6	extremely weak
Lyral®	17.1	weak
Benzyl cinnamate	18.4	weak
Lilial	18.7	weak
Cinnamyl alcohol	20.1	weak
3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	21.8	weak
Geraniol	22.4	weak
Amylcinnamyl alcohol	25	weak
Hydroxycitronellal	33	weak
Citronellol	43.5	extremely weak
Linalool	46.2	extremely weak
d-Limonene	69	weak
Tree moss	>20	moderate
Benzyl alcohol	>50	weak
Benzyl benzoate	>50	extremely weak
Coumarin	negative	weak sensitizer

[1] Adapted from Wijnhoven et al. (2008)

3 Presence and levels of fragrance allergens in scented consumer products

3.1 Product inventory scented products: approach

For determination of the presence and concentrations of the 26 allergenic fragrances in various products on the European market, different approaches were followed:

- Information on the fragrance allergens in scented products currently available on the Dutch market was obtained from the NVIC (Dutch National Poison Control Centre) database. The NVIC database contains information that was provided by the manufacturer of the products. The list contains the names and CAS numbers of ingredients in scented products for which inhalation exposure is likely to occur, together with ingredients and fractions of these ingredients. The database was searched for the 26 fragrance allergens by using their CAS numbers.
- Another source of information that was consulted was the website of Sara Lee (http://www.saralee-int.info/NL-NL/Our+Brands/AmbiPur; until April 2011).
- The information from the RIVM report 'Allergens in consumer products' by Wijnhoven et al., (2008) was used to identify additional information from European market surveys, conducted in the last 10 years. Information of scented products from these BEUC (The European Consumers Organisation) and Danish EPA market surveys are reported in the current product inventory.

3.2 Product inventory scented products: results

The results of the product inventory have been published before (Ezendam et al., 2009b) and are included in Appendix 2 and summarized below

3.2.1 Fragrance allergens in scented products available on the Dutch market

The NVIC database contains 113 scented products. Of these, 48 are air fresheners and room perfumes and the other products are intended for steam baths or saunas (mainly ethereal oils). The NVIC data show that the most frequently used fragrances in scented products (>40% of the products) were geraniol, linalool and citronellol. The fragrances cinnamyl alcohol, isoeugenol, amyl cinnamal, cinnamal, farnesol, benzyl cinnamate and oak moss were not frequently used (<10% of the products) in these scented products. Five fragrance allergens were not used in scented consumer products: anisyl alcohol, amyl cinnamyl alcohol, methyl heptine carbonate and tree moss.

The Sara Lee database contains 49 scented products. When all scented products were analyzed, it was shown that the most frequently used fragrances (present in >40% of the products) were limonene, linalool, geraniol, citronellol and α-isomethylionon. The fragrances anisyl alcohol, amyl cinnamyl alcohol, benzyl cinnamate, methyl heptine carbonate, oak moss and tree moss were not used as ingredients in these products.

3.2.2 Data from European market studies

The European Consumers Organisation (BEUC) has measured emission levels of different chemicals, including 11 fragrances, from 74 air fresheners in indoor air (BEUC, 2005). The fragrance that was measured the most was D-limonene and emissions ranged from 1–2003 $\mu g/m^3$. Emission of linalool was detected in almost 28% of the tested air fresheners, and concentrations ranged from 5-750 $\mu g/m^3$. The other fragrances that were emitted, although in a limited number of products were: lilial, cinnamal, coumarin, citral, benzyl benzoate, eugenol, benzyl alchohol, hydroxycitronellal and geraniol.

The Danish Environmental Protection Agency (EPA) has performed a market survey in different stores and supermarkets that sell air fresheners for use at home and in the car (Pors & Fuhlendorff, 2003). A total of 19 products were selected: 6 of these were car products and 13 were products for use at home. In these products the presence and concentrations of 24 fragrance allergens were measured. The presence of oak moss and tree moss was not assessed in this study. The most frequently used fragrances (in >50% of the products) were D-limonene, linalool, benzyl benzoate, hexyl cinnamal, eugenol, lilial, benzyl alchohol and benzyl salicylate. In this study, the concentrations of the fragrances were measured as well. Analyzing the individual products showed a large variation. In addition, some fragrance chemicals were used in higher concentrations than others. In general, cinnamal, isoeugenol, amyl cinnamal alcohol and methyl heptine carbonate were used in the lowest concentrations. The fragrances that were used in the highest concentrations were (in weight % of a product) : lyral (6.2%), linalool (3.9%), citral (2.6%), hexyl cinnamal (2.2%), and D-limonene (2.1%).

3.3 Scented products: product categories, location of use and applications

There are many different types of scented products available, which are listed below (Park et al., 2006). The way these products are used influence largely the way a subject may be exposed.

- *Room perfume in holders*

This is a large group of scented products, comprised of perfumes enclosed by a container, such as a glass disc or plastic flask, from which the scent is released slowly over time. The perfume can be a water-based or solvent-based liquid, a gel, or a solid soap-like substance.

- *Fragrant candles and wax*

Candles made of a fragrant wax, or sole wax. The scent is released by burning the candle or heating the wax.

- *Ethereal oils*

Fragrant oils that generally need heating before the scent is released fully. Candles or other warm objects such as lamps can heat the oils. Sometimes used as droplets in a bowl of heated water.

- *Fragrant sachets*

Bags of textile such as lace or cotton filled with scented products, such as lavender bags. The sachets can be placed in a room, but are usually placed between clothes and linen.

- *Potpourri*

Mix of (dried) flowers, fruits or other material, with natural scent or impregnated with perfume. The mix is placed in an open container.

- *Sprays*

Many scented products are available as aerosol spray cans or bottles. The product is often dissolved in volatile solvents, e.g. air fresheners, although some sprays can be water based.

- *Reed diffusers:*

Wooden sticks (reeds) are placed in a holder that contains a scented fluid. The scent is continuously released from the wooden sticks, without electricity or burning. The sticks have to be turned around every 3-4 days and scent is released during a period of three months.

- *Fragrant cardboards*

Pieces of cardboard, usually shaped as a leaf or other decorative figure, impregnated with perfume. They are commonly suspended from rear view mirrors in cars.

- *Toilet bowl rim hangers*

Container with grid, enclosing a fragrant solid, gel or liquid specifically designed to suspend from the toilet bowl rim. The scent is released by flushing the toilet.

- *Incense*

Cones or sticks of resin-like material that release the scent when burnt.

- *Ironing-perfumes*

A liquid perfume that can be added to the water container of a steam iron, the scent is released when the device is switched on.

- *Vacuum perfumes*

A ball that can be placed in the vacuum cleaner, the scent is released when the device is switched on.

The release pattern of the scented product ingredients differs per type of product. Some products release the scent without specific action, for example potpourri, fragrant sachets, scented sticks and passive room perfumes in holders. For other products, actions are needed to release the scent, for example for sprays or ironing and vacuum perfumes (see Table 4). Coincidentally, the scented products requiring an action or activity often have a peak release pattern (highest exposure directly after use that will subside rather rapidly), whereas the other products generate a more constant release pattern.

Table 4 Applications and scent release patterns

Product type	**Application type**	**Scent release pattern**
Room perfume in holders	Electric plug, ventilation, no specific action	Constant
Fragrant candles and wax	Heating, burning	Peak
Ethereal oils	Heating	Peak
Fragrant sachets	No specific action	Constant
Sprays	Spraying in the air	Peak
Potpourri	No specific action	Constant
Scented sticks	No specific action	Constant
Fragrant cardboards	No specific action	Constant
Toilet bowl rim hangers	Flushing	Peak/constant
Incense	Burning	Peak
Ironing-perfumes	Ironing	Peak
Vacuum perfumes	Vacuuming	Peak

3.4 Exposure assessment

Exposure assessment uses information from the product inventory together with the different product categories to derive default input parameters. For each product category information on the general composition with at least concentrations of fragrance materials and a scenario description are needed. The limitation of the product inventory is that there is limited information on the concentrations of fragrance allergens in the different products. Therefore, it is not possible to do an exposure assessment.

4 Hazard identification and characterization

4.1 Animal models for the identification of respiratory sensitizers

In the literature, several different animal models are described, including models in guinea-pigs, rats and mice (Arts & Kuper, 2007). However, none of these models is validated for hazard identification of respiratory sensitizers. In the models described in literature sensitization is induced by dermal, intratracheal, intranasal exposure as well. In most models, animals are challenged to measure airway responses, like bronchoconstriction (shortness of breath), cellular infiltrates in the lungs or airway pathology. Different methods are used for the challenge, including inhalation, dermal, intranasal and intratracheal exposure (reviewed in Arts and Kuper, 2007). Although inhalation is the preferred route of sensitization, because of the similarity with human exposure, this route is often not used as the route of sensitization in these models. The problem with inhalation studies is that they are labor intensive, time-consuming and specific expertise is required, for example to deliver the accurate dose. Also, it is unclear how many exposures are needed to induce sensitization and elicitation, and which parameters are predictive for respiratory sensitization.

Respiratory sensitizers are positive in the LLNA after dermal application (Basketter & Scholes, 1992; Kimber et al., 2007), showing that the LLNA can identify both respiratory and skin sensitizers. In order to distinguish skin from respiratory sensitizers additional cytokine profiling can be used. In general, skin sensitizers induce a Th1 response, associated with delayed-type hypersensitivity, whereas respiratory sensitizers induce a Th2 response, typical for immediate-type (IgE) hypersensitivity (Dearman et al., 1995; Vandebriel et al., 2000). This approach has not been validated and is not accepted for hazard identification of respiratory sensitizers.

Although the LLNA is able to identify respiratory sensitizers, the route of exposure is different from human exposure. To mimic a more relevant route of exposure a respiratory LLNA was developed, in which mice were exposed by inhalation for three consecutive days, followed by the assessment of cell proliferation in the mandibular lymph nodes. It was shown that both skin and respiratory sensitizers enhanced cell proliferation, suggesting induction of sensitization by inhalation. An important difference between the LLNA and the respiratory LLNA was the potency ranking of the different sensitizers, based on the dose-response curves obtained in these assays. In the respiratory LLNA the respiratory sensitizers were more potent than the tested skin sensitizers, whereas in the skin LLNA it was the other way around (Arts et al., 2008; De Jong et al., 2009). It is unknown if the potency rankings in these animal tests are representative for humans, since potency of respiratory sensitizers in humans is unknown.

4.2 Approach

In the current project, the respiratory LLNA was used to assess if fragrance allergens were able to sensitize the respiratory tract after short inhalation exposure. In this model only the *induction* of an immune response is measured. In the respiratory LLNA, five different fragrance allergens were tested. Fragrances were selected based on their skin sensitizing potency (see Table 3). The most potent skin sensitizers were chosen, because it can be expected that those are more likely to induce sensitization upon inhalation. Farnesol was

excluded because this fragrance was only present in a minority of the scented consumer products, whereas citral was used more frequently. The botanical extract oak moss was also excluded, because it is unknown if this extract is used in scented products (Ezendam et al., 2009b).
The following fragrance allergens were tested: cinnamal, methyl heptine carbonate, benzyl salicylate, isoeugenol and citral. A detailed description of the experimental design is included in Appendix 1.

4.3 Effects of fragrance allergens in the respiratory LLNA

Pilot experiments show that exposure to aerosols of isoeugenol and cinnamal dosed at 300 mg/m^3 resulted in toxic and lethal effects in mice exposed for 360 minutes per day (Ezendam et al., 2007). The results are summarized in Figure 1 and Table 5, excluding the groups in which toxic effects were visible. Exposure to cinnamal induced an significant two-fold increase of proliferation in mice that were exposed for 180 min/day. After exposure to isoeugenol, a dose (concentration x time)-dependent increase in proliferation was induced in all groups. When both fragrances are compared, isoeugenol increased proliferation significantly after shorter exposure time, i.e. at a lower dose and the increase in proliferation was higher compared to cinnamal. For isoeugenol, two experimental groups were excluded due to toxicity and it was decided to repeat the experiment with isoeugenol at lower concentrations (75 mg/m^3) and include three additional fragrances: benzyl salicylate, methyl heptine carbonate and citral. Cinnamal was not further tested, since only a small increase in cell proliferation was induced at a relatively high dose.

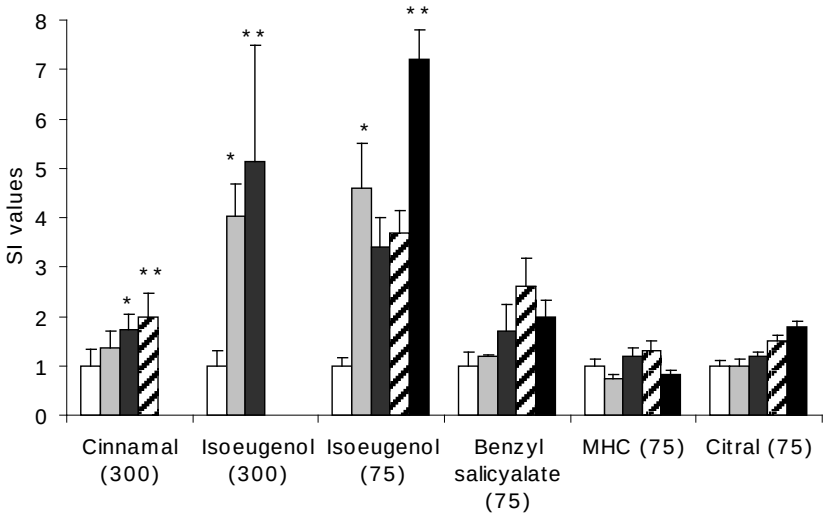


Figure 1: Stimulation indices in the mandibular lymph nodes in the respiratory LLNA.

Fragrances were tested in the following concentrations: 300 mg/m^3 (cinnamal and isoeugenol) or 75 mg/m^3 (isoeugenol, benzyl salicylate, methyl heptine carbonate (MHC) and citral). White bars represent the control groups exposed to acetone. Mice were exposed to the fragrances by increasing the exposure duration: 45 min/day (grey bars), 90 min/day (dark grey bars), 180 min/day (striped bars) and 360 min/day (black bars) for three consecutive days. Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NA: fragrances were toxic or fatal and these groups were excluded.

For all tested fragrances, the exposure concentration of 75 mg/m^3 did not induce any visible toxic effects. The only fragrance that significantly increased cell number and proliferation in the mandibular lymph nodes was isoeugenol. After 45 min/day exposure, cell proliferation was already significantly increased more than 4-fold compared to the control group. There was no clear dose (concentration x time)-response, since at the time points 90 min/day and 180 min/day SI values stayed on a plateau level of 3.5-fold increase. In the group that was exposed for 360 min/day cell proliferation increased further to a SI value of 7.2. Benzyl salicylate and citral increased cell proliferation in the mandibular LNs slightly, but these effects were not statistically significant. Exposure to benzyl salicylate induced a dose (concentration x time)-dependent increase of cell proliferation which peaked at the exposure time of 180 min/day, reaching a SI value of 2.6. This effect was not significant and longer exposure to benzyl salicylate did not increase cell proliferation further. After inhalation exposure to citral the maximum SI value that was reached after 360 min/day exposure was 1.8. Finally, methyl heptine carbonate did not increase cell proliferation in the mandibular LNs.

Table 5 Effects of inhalation exposure to fragrance allergens on the mandibular lymph nodes

Exposure duration	Cinnamal	Isoeugenol	Isoeugenol	Benzyl salicylate	Methyl heptine carbonate	Citral
min/day	300 mg/m^3	300 mg/m^3	75 mg/m^3	75 mg/m^3	75 mg/m^3	75 mg/m^3
Control	1.0 ± 0,32	1.0 ± 0.30	1.0 ± 0.17	1.0 ± 0.29	1.0 ± 0.13	1.0 ± 0.12
45	1.37 ± 0.34	4.04 ± 0.64*	4.6 ± 0.91*	1.2 ± 0.02	0.73 ± 0.09	0.98 ± 0.16
90	1.74 * ± 0.30	5.13 ** ± 2.35	3.4 ± 0.61	1.7 ± 0.53	1.2 ± 0.17	1.2 ± 0.08
180	2.00** ± 0.48	NA	3.7 ± 0.43	2.6 ± 0.57	1.3 ± 0.21	1.5 ± 0.11
360	NA	NA	7.2*** ± 0.61	2.0 ± 0.32	0.83 ± 0.09	1.8 ± 0.09

Results are shown as mean stimulation index (SI) ± SEM (n=6 per group). SI values were calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. Statistically significant differences were assessed with a one-way ANOVA with a Bonferonni's post hoc test. Asterisks depict significant differences from the control group: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NA: fragrances were toxic or fatal and these groups were excluded.

4.4 Potency in the respiratory LLNA

Arts et al. (2008) have described an approach to estimate the potency of chemicals in the respiratory LLNA (described in detail in Appendix 1). This approach is similar to the calculation of the EC3 value in the LLNA, which is used to estimate the skin sensitizing potency. Dose-response curves of the cell proliferation induced after three days of inhalation exposure were used to interpolate the ED3 value, which is the dose at which a SI value above 3 is

induced after three days of exposure. Only for isoeugenol at a dose of 75 mg/m^3, it was possible to calculate the ED3 value. The ED3 value that was found for isoeugenol was 923 μg.

Table 6 shows the ED3 values of skin and respiratory sensitizers that were tested in the respiratory LLNA (Arts et al, 2008). The concentration needed to induce a SI value of 3 is lower for isoeugenol compared to the respiratory sensitizers tested and much lower than the skin sensitizers that were tested. In the (skin) LLNA the potency of isoegenol is lower than for oxazolone and DNCB as well.

Unlike the EC3 value for skin sensitizers, which correlates relatively well with human potency (Griem et al., 2003), the correlation of the ED3 value of respiratory sensitizers with human data is unknown. This value illustrates, however, how much of a substance is needed to sensitize the respiratory tract in this particular model and is a measure to compare different compounds. It can be concluded that isoeugenol is a weaker sensitizer after inhalation exposure compared to respiratory sensitizers and other skin sensitizers.

Table 6 ED3 values of sensitizers in the respiratory LLNA[1]

Chemical	Class	ED3 value (μg)
hexamethylene diisocyanate	respiratory sensitizer	18
oxazolone	skin sensitizer	19
toluene diisocyanate	respiratory sensitizer	28
isophorone diisocyanate	respiratory sensitizer	44
Phtalic anhydride	respiratory sensitizer	63
trimellitic anhydride	respiratory sensitizer	156
dinitrochlorobenzene	skin sensitizer	173
isoeugenol	skin sensitizer	923

[1] Adapted from Arts et al. (2008)

4.5 Effects of inhalation of isoeugenol and cinnamal in dermally sensitized mice

To assess if subjects with an existing contact allergy for fragrances, i.e. who are sensitized via the skin, are at risk when they inhale the same fragrance, a different experimental approach was selected. A mouse model was used in which mice were sensitized by skin application and subsequently were challenged by inhalation. This approach is chosen to assess if inhalation exposure to fragrance allergens in subjects who are already sensitized to this fragrance via the skin is a hazard. In mice sensitized via the skin it is possible to measure if subsequent inhalation exposure to the same allergen is able to induce respiratory effects that indicate an allergic response. These include the measurement of shortness of breath (bronchial hyperreactivity) and airway inflammation.

In this experimental animal model isoeugenol and cinnamal were tested. Those compounds were selected since they have a similar skin sensitizing potency but they have different effects in the respiratory LLNA, in which isoeugenol induced a higher increase in cell proliferation at lower exposure levels than cinnamal (Ezendam et al., 2009a). The experimental model is described in detail in Appendix 1.

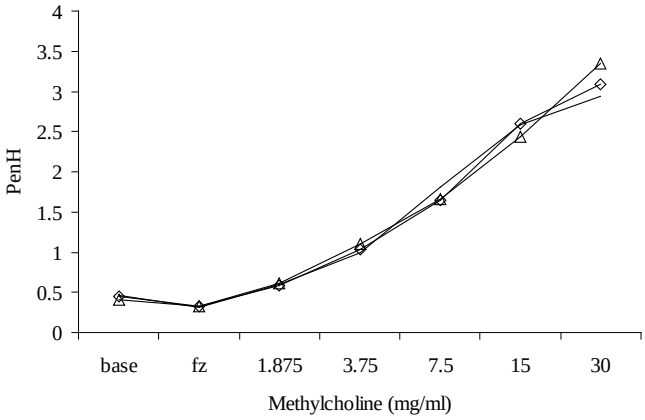
4.5.1 *Respiratory effects: airway hyperreactivity, lower airway inflammation and larynx pathology*

To assess if inhalation exposure to either isoeugenol or cinnamal has an impact on airway functioning, the responsiveness of the airways to a specific trigger was measured. To trigger the airways, mice were exposed to aerosols of methylcholine, which induces bronchoconstriction (shortness of breath). The effects of this methylcholine challenge were measured in a whole-body plethysmograph, in which several breathing parameters can be measured simultaneously (for a detailed description see Appendix 1).

Figure 2 shows airway responses after inhalation exposure to methylcholine. Methylcholine causes a dose-dependent increase of the PenH, a measure for airway hyperreactivity. There was no difference in airway hyperreactivity between mice that were sensitized and challenged with either isoeugenol or cinnamal compared to control mice. Hence, the inhalation challenge with these fragrances did not have an impact on the functioning of the airways.

To investigate if the inhalation challenge with isoeugenol or cinnamal caused an inflammatory response in the airways, a bronchoalveolar lavage was performed. The number of inflammatory cells in the lavage fluid was counted. It was shown that there was no increase in inflammatory cells in mice that were sensitized and challenged with isoeugenol or cinnamal compared to the control groups (data not shown).

A



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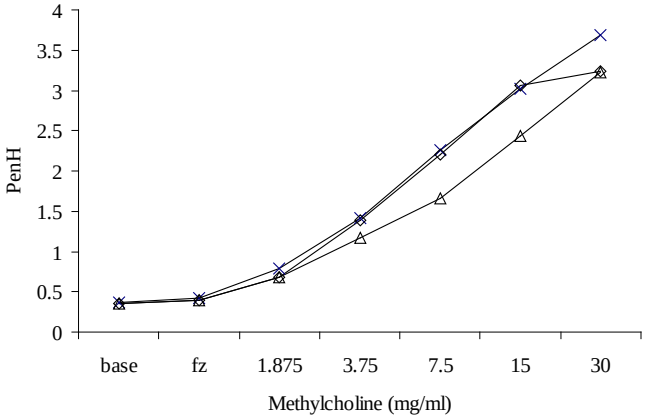


Figure 2: Airway hyperreactivity in response to methylcholine. The effects on inhalation challenge with isoeugenol (A) or cinnamal (B) on airway responses were assessed by measuring the PenH in unrestrained mice using a plethysmograph in response to a challenge with methylcholine aeorols. Airway responses were measured in controls (-x-), control mice challenged with isoeugenol or cinnamal (-◇-) and in sensitized and challenged mice (-Δ-).

The larynx has been shown to be a target organ after inhalation exposure to skin and respiratory sensitizers (Arts et al., 2008; van Triel et al., 2010). Table 7 shows the results of the histopathology of the larynx after exposure to isoeugenol and cinnamal. Histopathology showed no strong inflammatory changes or hyperplasia. In general, the histopathology changes that were found were very slight or slight. Furthermore, histopathological changes did not differ between naïve mice that were challenged with isoeugenol or cinnamal and sensitized mice that were challenged with isoeugenol or cinnamal. This shows that these changes were caused by irritation of the compounds rather than a specific immune response. If the latter would have taken place, the histopathological changes would only have occurred in the sensitized mice that were challenged with isoeugenol or cinnamal.

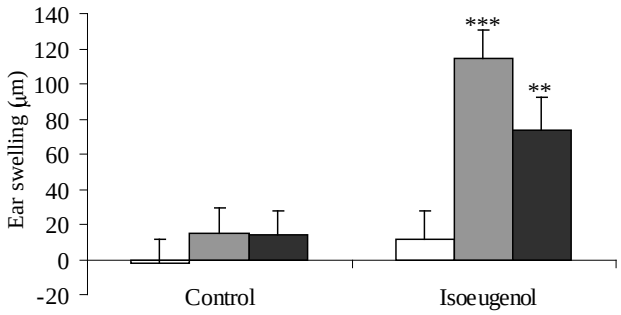
Table 7 Histopathological changes in the larynx: isoeugenol

Skin sensitization	Inhalation challenge	Inflammation (slight)	Hyperplasia (slight)
Isoeugenol			
AOO	Aceton	0	2/4
AOO	Isoeugenol	2/8	4/8
Isoeugenol	Isoeugenol	2/8	6/8
Cinnamal			
AOO	Aceton	1/4	1/4
AOO	Cinnamal	2/7	5/7
Cinnamal	Cinnamal	5/8	5/8

4.5.2 Parallel experiments to assess if the sensitization protocol induced sensitization

In this type of experiments it is important to confirm that the sensitization dose was sufficient to sensitize the mice. Otherwise it is impossible to translate the absence of any immunological effects in the airways to a hazard. A hallmark of type IV immune responses induced by contact sensitizers is the ability to induce ear swelling 24 to 48 hours after topical exposure on the ears in sensitized mice. Earlier ear swelling responses are indicative for skin irritation.

A



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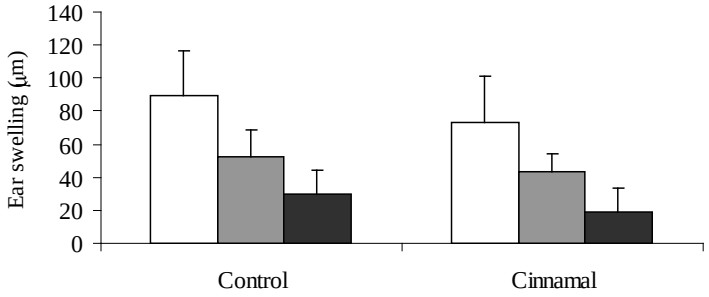

Figure 3: Ear swelling in mice sensitized with isoeugenol (A) or cinnamal (B). Mice were challenged with 20% isoeugenol or cinnamal by topical application on both ears. Ear swelling was measured in sensitized and non-sensitized control mice 6 hrs (white bars), 24 hrs (grey bars) and 48 hrs (dark grey bars) after challenge. Statistically significant differences with the control group are depicted with asterisks: ** $p<0.01$, *** $p<0.001$.

Figure 3 shows the results of the ear swelling experiments. Isoeugenol induced a significant ear swelling response 24 and 48 hours after challenge of sensitized mice and not in non-sensitized mice. There was no increase in ear swelling six hours after challenge with isoeugenol, showing that this compound did not induce skin irritation. Ear challenge with cinnamal resulted in an increase in ear swelling six hours after challenge, both in sensitized and non-sensitized mice. At the later time points, the ear swelling decreased in both sensitized and non-sensitized mice. These data show that cinnamal induced non-specific acute reaction, possibly by irritation.

Besides ear swelling, the proliferation in the auricular lymph nodes was measured 48 hours after ear challenge. For isoeugenol, the proliferation responses confirmed the results of the ear swelling test. Lymphocyte proliferation was only increased in sensitized mice challenged with isoeugenol. For cinnamal it was also shown that lymphocyte proliferation was only increased in mice that were sensitized and not in non-sensitized mice (see Figure 4). These results indicate that the sensitization dose of cinnamal did induce a specific immune response. The failure to detect this in the ear swelling assay, c be caused by the irritant properties of cinnamal. Possibly, the challenge dose was too high and effects induced by skin irritation masked the specific immune response. The proliferation in the auricular lymph nodes shows that these mice were sensitized as well.

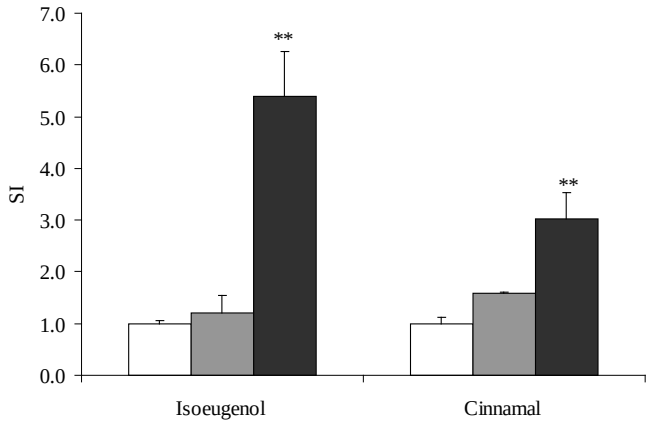

Figure 4: Cell proliferation in the auricular lymph nodes. Proliferation was expressed as the SI value, which was calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the control group. Cell proliferation was assessed in controls (white bars), control mice challenged with isoeugenol or cinnamal (grey bars) and in sensitized mice challenged with isoeugenol or cinnamal (dark grey bars). Statistically differences with the control group are depicted with asterisks: ** $p<0.01$

5 Exploring the possibilities of hazard identification without using experimental animal models

In the area of toxicology there is much pressure to find test methods that can reduce or replace the use of experimental animals in toxicology tests. This area is receiving more and more attention due to concerns in society on the use of experimental animals for scientific and safety purposes. Policy changes such as in the Cosmetics Directive, in which the use of experimental animals is banned completely in 2013 (2003/15/EC) and the EU legislation on chemicals (Registration, Evaluation, Authorization,and Restriction of Chemicals – REACH) have put even more pressure on the development of alternative test methods. In the area of skin sensitization many research projects on alternatives are going on. In contrast, development of non-animal test methods for the hazard identification of respiratory sensitizers has not received much attention.

5.1 Cell-based test methods

The development of *in vitro* alternatives to identify respiratory sensitizers is complicated for a number of reasons. First, the airways are complex and consist of many different cell types. Second, the biological mechanisms of respiratory sensitization are not fully understood and third not all respiratory sensitizers evoke the same type of immune response in the lungs (Verstraelen et al., 2008). Up to now, two cell lines are described that have been used for hazard identification of respiratory sensitizers: macrophage and bronchial epithelial cells. Pilot studies with a small number or respiratory sensitizers showed that gene expression profiles could be used to distinguish respiratory from skin and non sensitizers (Verstraelen et al., 2009a; Verstraelen et al., 2009b). However, larger studies validating these results with more respiratory and skin sensitizers are lacking and fragrance allergens have not been tested in these *in vitro* test systems.

5.2 Chemical reactivity assays

A different approach to predict respiratory sensitization potential is by using chemical reactivity measurements. One important hallmark of both respiratory and skin sensitizers is that they have to bind to proteins in order to induce sensitization. Hence, only substances with reactive groups can act as sensitizers and protein reactivity assays have been developed to assess these properties. In protein reactivity assays that include both respiratory and skin sensitizers it was shown that protein reactivity is a common feature for both skin and respiratory sensitizers (Gauggel et al., 1993; Gerberick et al., 2007). It is possible to distinguish respiratory from skin sensitizers when different substrates are included in the test system. Skin sensitizers selectively bind to cellular proteins and respiratory sensitizers to soluble proteins (Hopkins et al., 2005).

In addition, relationships between chemical structure and respiratory sensitization hazard have been studied by comparing chemical structures of respiratory sensitizers with control compounds (Karol et al., 1996; Cunningham et al., 2005; Jarvis et al., 2005; Enoch et al., 2009; Enoch et al., 2010). Strong correlations were found between the presence of multiple reactive groups and the ability to induce respiratory sensitization, suggesting that respiratory sensitizers bind proteins in more than one place, i.e. cross-link to proteins. This might be an important mechanism involved in respiratory sensitization.

However, some respiratory sensitizers do not have to cross-link to induce sensitization. Enoch et al. (2009) introduced the concept of 'reactivity threshold' which takes into account both electrophilic and cross-linking ability. The theory is that a highly electrophilic allergen can compensate for the lack of cross-linking and act as a respiratory allergen. The most common binding mechanism to proteins of respiratory sensitizers was by acylation (Enoch et al., 2010). In addition, respiratory sensitizers prefer binding to lysine, whereas skin sensitizers prefer binding to cysteine (Hopkins et al., 2005; Holsapple et al., 2006).

Differences in chemical reactivity towards proteins could be a possible explanation for the differential effects of isoeugenol in the respiratory LLNA compared to the other fragrance allergens. A literature review was conducted to find information on peptide reactivity and chemical structure of the five tested fragrance allergens. An overview is presented in Table 9. Natsch et al. (2007) used a high-performance liquid chromatography mass spectrometry (LC-MS) analysis to detect peptide depletion. Further characterization of the reaction revealed that isoeugenol, cinnamal and citral were depleted peptides by adduct formation. In addition, isoeugenol and cinnamal are also oxidized peptides. The other fragrances were not tested. The authors commented that isoeugenol has a very complex reactivity and further studies are required to fully understand the observed adducts (Natsch & Gfeller, 2008). Although peptide reactivity did not reveal differences between the tested fragrances, isoeugenol appears to be a complex chemical. This does not directly explain the differences observed in the respiratory LLNA, but illustrates that this fragrance is different from the others. The peptide reactivity assay was used to test several fragrance allergens. It was shown that isoeugenol, cinnamal and citral were very reactive towards cysteine, whereas benzyl salicylate showed no reactivity towards this peptide. Methyl heptine carbonate was not tested (Natsch et al., 2007).

The mechanistic applicability domain of these fragrances is also depicted in Table 8. For skin sensitizers five different domains were identified. Substances are grouped according to the way they react to the protein. Cinnamal and isoeugenol are both Michael Acceptors whereas citral is a Schiff base former (Aptula et al., 2005; Roberts et al., 2007). These mechanistic domains can therefore not be used to explain the differences between isoeugenol and the other fragrances in the respiratory LLNA.

Table 8 Chemical characteristics and peptide reactivity of fragrance allergens

Fragrance	**Peptide reactivity[1]**	**Cysteine reactivity[2] (% depletion)**	**Mechanistic applicability domain**
Isoeugenol	Oxidizing and adduct forming	100	Michael Acceptor
Cinnamal	Oxidizing and adduct forming	86 ± 12.5	Michael Acceptor
Citral	Adduct forming	94.8 ± 5.3	Schiff base
Methyl heptine carbonate	ND	ND	ND
Benzyl salicylate	ND	0%	ND

[1] Peptide reactivity was determined by measuring peptide depletion by LC-MS (Natsch et al. 2007); [2] Reactivity towards cystein was measured in the peptide reactivity assay using a LC-MS (Natsch et al., 2007); [3] Mechanistic applicability domain (Roberts et al., 2007, Aptula et al., 2005). ND: not done

6 Evidence for adverse effects in humans exposed to fragrance allergens by inhalation

Respiratory sensitizers that have been identified so far are classified based on human evidence. Hence, these compounds are usually identified when airway allergies, such as asthma occur in occupational settings. To our knowledge, there is no published data available on the occurrence of occupational rhinitis or asthma in the fragrance industry. In the literature there are limited data available on inhalation exposure to fragrance allergens in relation to respiratory allergies in humans and these studies are discussed below.

6.1 Case reports

Three case reports describe the occurrence of respiratory allergy due to occupational exposure to fragrance allergens. In the first, the clinical history of a saleswoman working in a perfumery is described. This woman suffered from respiratory distress at her work. The symptoms could be reproduced in the hospital by giving an inhalation challenge with different perfume brands, demonstrating that inhalation of these perfumes caused the respiratory problems. No further research was done to find the causative agent in these perfumes (Baur et al., 1999).
In the case of a hair dresser suffering from occupational eczema, rhinitis and asthma, the cause of the complaints seem to be related to occupational exposure, since this subject did not suffer from these problems in the past. To asses which causative agents were involved, different diagnostic procedures were performed, including inhalation challenges and patch testing. The respiratory symptoms could be reproduced after inhalation challenge with eugenol. The patch test for eugenol was negative in this subject, but patch tests for isoeugenol, cobalt chloride and potassium dichromate were positive. It can be concluded that the respiratory symptoms induced by eugenol were not caused by dermal sensitization, which implies that this subject was sensitized via the airways (Quirce et al., 2008).
In a worker who picked and handled citrus fruits, both allergic contact dermatitis and asthma was diagnosed. The symptoms were clearly associated with his work, since they disappeared when he stopped his normal work. After resuming his work, the symptoms reappeared again. The man had positive patch tests to limonene and citronellol, demonstrating sensitization. The occurrence of asthma was not further investigated with inhalation challenges with these fragrances allergens. It is therefore unclear if inhalation of these fragrances caused the respiratory symptoms (Guarneri et al., 2008).

6.2 Epidemiological studies

A few epidemiological studies found associations between fragrance contact allergy and respiratory symptoms. In a Danish study, questionnaires were used to find associations between fragrance exposure and respiratory symptoms. In the partipicants, the prevalence of hand eczema was determined by using questionnaires and performing patch tests with nickel and fragrances. This study showed that either a history of hand eczema or a perfume contact allergy were significantly associated with airway symptoms elicited by fragrance products. This association was not found in subjects sensitized to nickel (Elberling et al., 2004). In a recent study, similar associations were found. In this population-based study, associations between sensitization to fragrances or nickel and

bronchial hyper-responsiveness were assessed. Sensitization was assessed with a patch test for fragrance mix I or for nickel. To measure bronchial hyperresponsiveness, subjects inhaled methacholine, which induced bronchial hyperresponsiveness. Women that were sensitized to fragrances responded significantly more often to the methylcholine challenge than non-sensitized women. This was not found in women sensitized to nickel. The association between fragrance sensitization and bronchial hyperresponsiveness was not found when men sensitized to fragrances were included in the analysis (Schnabel et al., 2010).

These studies illustrate that inhalation of fragrances might be associated with symptoms of respiratory distress. Since these studies were not designed to assess causal relationships, it is difficult to conclude which specific ingredients and mechanisms are involved in these reactions. If these respiratory symptoms are caused by specific allergic reactions or by airway irritation is unclear.

6.3 Experimental human experiments

In an experimental pilot study, the causal relationship between inhalation of fragrance allergens and existing contact allergy to fragrances was investigated. In this study, patients with a contact allergy to isoeugenol (n=11) or hydroxyisohexyl-3-carboxaldehyde (HICC) (n=10) were exposed in environmental exposure chambers to either isoeugenol or HICC. Occlusive clothing was worn to prevent elicitation of contact allergy. As a negative control, inhalation exposures were also done with geraniol, a fragrance to which these subjects were not sensitized. After exposure lung function tests were performed to measure bronchoconstriction.

The inhalation studies showed that there were no significant changes in lung function but a tendency towards an increased bronchial hyperresponsiveness after exposure to any of the compounds, i.e. geraniol, HICC or isoeugenol. This indicates that inhalation of the fragrances induced a slight effect on the airways, but this was not specific for the allergen the patients were sensitized to. Patients did, however, respond with flare ups of pre-existing eczema when they inhaled the allergen they were sensitized to and this occurred despite the protective clothing. Hence, inhalation of these fragrance allergens caused elicitation of allergic skin responses. The skin effects were elicited after exposure to the high concentrations of the fragrances. When the subjects were re-exposed with more realistic concentrations, there were no skin symptoms elicited (Schnuch et al., 2009). Hence, this study suggest that inhalation of fragrances by subjects sensitized to these fragrance compounds, does not lead to respiratory symptoms and that only high dose exposure elicits allergic reactions in the skin.

The design of this study has similarities with the experiments in mice that were sensitized by skin exposure and subsequently challenged by inhalation. Similarly, in these experimental studies in mice no respiratory symptoms were elicited after inhalation of isoeugenol or cinnamal.

7 Summary of most important findings

This report provides an extensive overview of different product categories of scented consumer products and the most frequently used fragrance allergens in these products. The immune effects of inhalation exposure to a selection of fragrances with skin sensitizing potential were assessed in two different animal models. The most important findings are summarized below:

- Of the five tested fragrances, isoeugenol and cinnamal were the only fragrance chemical that induced a positive response in the respiratory LLNA. This indicates that these fragrances are able to induce sensitization upon inhalation exposure. The effects of cinnamal were induced after exposure to high toxic concentrations. Isoeugenol induced a positive response at non-toxic doses. The other tested fragrances had no significant effects on cell proliferation.

- The fragrances selected for the respiratory LLNA were all, with the exception of citral, stronger skin sensitizers, when the OECD classification is used, i.e. EC3 values ≤ 2%. In humans, the most potent skin sensitizer is methyl heptine carbonate, followed by isoeugenol, cinnamal, citral and benzyl salicylate (Table 3). Remarkably, the most potent skin sensitizer, methyl heptine carbonate, was negative in the respiratory LLNA. Furthermore, the only fragrances that induced a significant increase in cell proliferation were isoeugenol and cinnamal. Isoeugenol was more potent than cinnamal in the respiratory LLNA. In contrast, in the (dermal) LLNA the skin sensitizing potency of isoeugenol and cinnamal are comparable. These data suggest that the skin sensitizing potency does not predict the potency in the respiratory LLNA. That the potency is dependent on the route of exposure has been shown before in the respiratory LLNA (Arts et al., 2008).

- The respiratory LLNA only measures the induction phase of an immune response, i.e. sensitization. This model is not validated to predict if substances that are positive will induce respiratory allergy after repeated exposures. Since there is currently no validated animal model to predict the hazard of respiratory sensitization, follow-up studies were done in which mice were sensitized via the skin. The skin is often used as a route of exposure in experimental animals and might be a relevant sensitization route in humans as well. This approach can be used to assess the hazards of inhalation exposure to fragrance allergens in subjects who are sensitized to the same allergen by skin exposure. In these studies it was shown that inhalation challenge with isoeugenol or cinnamal in dermally sensitized mice did not elicit adverse effects in the upper or lower airways. It is important to note that the mice received only one inhalation challenge, which might have not been sufficient to elicit respiratory allergy. It is currently unclear which experimental protocol is optimal to elicit symptoms of respiratory allergy. For very potent skin sensitizers, such as picryl chloride, it has been shown that a single inhalation challenge is sufficient. For less potent sensitizers, multiple inhalation challenges might be needed to elicit airways responses. Due to these uncertainties the results of the experiments with isoeugenol and cinnamal should be interpreted with caution.

- In a small experiment in humans with allergic contact dermatitis caused by isoeugenol or HICC, it was shown that inhalation challenges did not elicit any allergen-specific adverse effects in the airways. Exposure to high concentrations of isoeugenol, HICC or geraniol slightly increased methylcholine-induced bronchoconstriction. In addition, in these subjects inhalation exposure to high concentrations resulted in allergen-specific aggravation of pre-existing skin dermatitis. Lower, more realistic concentrations did not have this effect on the skin. This shows that high concentrations of these fragrance allergens can lead to non-specific aggravation of respiratory symptoms and specific aggravation of pre-existing skin symptoms.

- Physical-chemical properties of the tested fragrance chemicals could not be used to explain the differential effect of isoeugenol in the respiratory LLNA.

- There is limited evidence from case studies that occupational exposure to fragrances can lead to asthma or rhinitis.

- The product inventory showed that 21 of the 26 fragrance allergens are used in scented products that are available on the Dutch market. The fragrances anisyl alcohol, amyl cinnamyl alcohol, methyl heptine carbonate, and tree moss were apparently not used as ingredients. Similar results were found in the Danish market survey, showing that 22 of the 24 chemical fragrance allergens were detected. Anisyl alcohol and farnesol were not used in scented products and the presence of the botanical extracts oak moss and tree moss was not assessed.

- There is a wide range of scented products available for consumers. The location of use differs between these products, which could have an effect on the exposure to the emitted ingredients. The scent release patterns differ also, both peak and constant exposure can occur.

- For dosimetry the most important variable for exposure is the product of exposure concentration (in mg/m^3) x exposure time (Cxt). The impact of peak exposure (short-term exposure at a high dose) versus chronic exposure to low doses is largely unknown. There is limited evidence for isocyanates, that peak exposure is a more important determinant for the risk on respiratory sensitization than the cumulative dose of exposure (Leroyer et al., 1998). Experiments in rats support these human data. It has been shown that high concentrations delivered to the respiratory tract during short exposure periods appear to bear a higher sensitizing potency than equal concentration product (i.e. the product of concentration x exposure time) during longer exposure periods (Pauluhn & Poole, 2011). If this holds true for other respiratory sensitizers is not known. Furthermore, there is insufficient insight in the effects of prolonged exposure to low doses, but for certain chemicals these might be important in the acquisition of sensitization as well. In occupational settings, asthma develops mostly in the first two years of employment, which might support a role for chronic exposure as well.

- There are some differences in the most frequently used fragrances per product type, but in general it can be concluded that the most frequently used fragrances in products for the Dutch market are D-limonene, linalool, geraniol, and citronellol. Fragrance allergens that are less frequently used in scented products are benzyl cinnamate, cinnamyl alcohol, cinnamal,

isoeugenol, and oak moss. The most frequently used fragrances in the Danish market survey were also D-limonene and linalool. In addition, benzyl benzoate, hexyl cinnamal and eugenol were detected in the majority of Danish products.

- The Dutch product inventories only provide information on the presence of fragrance allergens, but not on the concentration levels in the products or emitted from the product. In the Danish EPA study concentrations were measured and this study showed that concentrations can vary widely. These data are therefore not useful to determine exposure levels. The lowest levels were found for amyl cinnamyl alcohol, cinnamyl alcohol, cinnamal, isoeugenol, methyl heptine carbonate, benzyl cinnamate. The highest levels were found for lyral, linalool, citral, hexyl cinnamal and D-limonene. The BEUC study measured emission concentrations of 11 fragrance allergens from 74 air fresheners. It was shown that D-limonene was emitted from the majority of tested scented products. Other fragrances that were emitted, but by much less products, were linalool,lilial, cinnamal, coumarin and citral. Only a few air fresheners emitted detectable benzyl benzoate, eugenol, benzyl alcohol, hydroxitronellal and geraniol.

- The product inventory did not identify the weight fractions of the fragrance materials in a specific product. In the exposure assessment at least the weight fraction of the (total) fragrance materials is required to obtain an exposure estimate. An accurate estimate of exposure levels is therefore troublesome. The data in the VWA report, in which concentration levels of fragrances are measured in different air fresheners will be used in 2011 for exposure estimations using ConsExpo.

- It can be concluded that consumers who use scented products are exposed to the majority of fragrance allergens. However, the exact exposure concentrations remain unknown.

8 Knowledge gaps and conclusions

In this report the risks of inhalation exposure to fragrance allergens has been investigated. In experiments performed in the current project, isoeugenol was identified as a sensitizer in the respiratory LLNA. Compared to other respiratory and skin sensitizers tested in this assay, isoeugenol was less potent. It is currently not known if the potency estimates from the respiratory LLNA correlate to human potency of respiratory sensitizers. So, the results on potency should be interpreted with caution. The results of the respiratory LLNA can only be used as an indication for a stimulation of the immune system, but it is unknown if prolonged inhalation exposure would lead to adverse immune effects in the respiratory tract. Beside the lack of a validated model for hazard identification, many other knowledge gaps exist in the field of respiratory allergy that hampers the development of a risk assessment strategy. The most important knowledge gaps are listed below:

- The route of exposure for sensitization is still debated and studies suggest that sensitization can be induced via inhalation exposure, dermal exposure or both.
- Exposure variables that increase the risk on sensitization are not well studied. It has been shown for respiratory sensitizers that for dosimetry the product of concentration (in mg/m^3) x exposure time (C x t) should be used.
- The impact of short-term high dose (peak) exposure versus prolonged low dose exposure is largely unknown. There is limited evidence for isocyanates that peak exposure is more important than chronic exposure, but if this is true for other respiratory sensitizes is not known. Insight in the most relevant exposure variables is necessary in a risk assessment strategy.
- Chemical reactivity studies have revealed that differences exist between skin and respiratory sensitizers. It should be explored if the insight in chemical characteristics can be used to determine if skin sensitizers have the potential to sensitize the airways as well.
- An important hallmark of quantitative risk assessment is assessment of threshold levels, i.e. the EC3 value which is used as a point-of-departure as used in the skin LLNA. Threshold levels are important in a quantitative risk assessment and can be used to derive exposure limits to protect people for the induction of sensitization. It is believed that threshold levels do exist for respiratory sensitizers, but the exact levels are not known (Gezondheidsraad, 2008).

In conclusion, it is currently not possible to assess the risk of inhalation exposure to fragrance allergens. The numerous uncertainties and knowledge gaps in the field of chemical respiratory sensitization hamper the identification of newly emerging respiratory sensitizers and offer any protection to exposed subjects. Furthermore, it is not possible to assess risks associated with an increased use of all kinds of scented products that result in airborne exposure. The need to investigate possible risks of inhalation exposure to fragrance allergens has been noticed by others as well. The Research Institute for Fragrance Materials (RIFM) has launched a research initiative on evaluating the inhalation safety of fragrances, which focuses on respiratory sensitization. Results derived from these initiatives might in the near future enable a better assessment of risks associated with inhalation exposure to fragrance allergens.

Furthermore, in the area of respiratory sensitization a paradigm shift from the classical toxicological approach of risk assessment towards testing strategies without or with less experimental animals is needed. Especially since many years of research have not resulted in predictive animal models. At the RIVM, a new project is initiated called ‘A new framework for better risk assessment for human health without animal testing: An ASAT-like pilot for respiratory sensitization’. In the future, such a framework might be used to evaluate the risks associated with inhalation exposure to fragrance chemicals.

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Appendix 1: Experimental design of the inhalation studies in mice

Animals
Six to eight week old male BALB/c mice were obtained from the institute's own breeding colony. The animals were bred specific pathogen free (SPF) and kept in macrolon cages under conventional conditions. The mice were fed Hope Farms chow pellets (Woerden, the Netherlands) and water ad libitum during the whole experiment. The experimental setup of the study was examined and agreed upon by the institute's Ethical Committee on Experimental Animals, and all experiments were performed according to national legislation.

Fragrance chemicals
The following fragrances were used: cinnamal (purity >98%), methyl heptine carbonate (99% purity; CAS 111-12-6), benzyl salicylate (99% purity), and isoeugenol (98% purity) from Sigma (St. Louis, MO, USA) and citral (purity >95%) from Fluka (Buchs, Switzerland).

Experimental design respiratory LLNA
The respiratory LLNA was performed as described previously (Arts et al., 2008). In short, groups of male BALB/c mice (six animals per group) were exposed nose-only to one of the various test materials on three consecutive days for 45, 90, 180, or 360 min/day. Variation in exposure duration rather than in concentration was used to investigate the dose-response relationships. During exposure all mice were placed in restraining tubes which were connected to one of the two central exposure chambers for nose-only exposure. Mice that were exposed to the vehicle control were connected to the exposure chamber of the vehicle and mice that were exposed to the fragrance allergen were connected to the exposure chamber of the fragrance. All fragrances were nebulized in acetone to produce an aerosol of liquid droplets. In pilot experiments with isoeugenol and cinnamal, concentrations of 300 mg/m^2 were used (Ezendam et al., 2007). In the following experiments with iseugenol, citral, benzyl salicylate and methyl heptine carbonate, a concentration of 75 mg/m^3 were used (Ezendam et al., 2009a).

The animals were necropsied three days after the last exposure and the auricular and mandibular lymph nodes (LN) were excised, pooled for each animal, and suspended in 5 ml RPMI 1640 (Gibco, Life Technologies, Breda, the Netherlands) with 5% heat inactivated Fetal Calf Serum (FCS) (Integro, Zaandam, the Netherlands), 100 U/ml penicillin and 100 µg/ml streptomycin (standard medium). At the autopsy other lymph nodes (deep cervical, parathymic, and mediastinal lymph nodes) were macroscopically examined for lymph node enlargement to indicate possible cellular stimulation.

In all experiments, a parallel experiment was performed, in which the (skin) LLNA was performed for each fragrance allergen. In short, mice were topically exposed to 10% (v/v) isoeugenol, citral, benzyl salicylate or methyl heptine carbonate in acetone: olive oil 4:1 (AOO) on the dorsum of both ears (25 µl/ear) on three consecutive days. Control mice received the same treatment with the vehicle (AOO). Mice were necropsied three days after the last exposure and the auricular LNs were excised and were pooled for each animal and suspended in standard medium with 5% FCS.

Experimental design: inhalation challenge in dermally sensitized mice
In Table 10 an overview of the experimental groups is depicted. Mice were sensitized on three consecutive days by applying 50 µl of the test compounds on the shaved flanks. Cinnamal and isoeugenol were both applied in a concentration of 50% dissolved in AOO. Group 1 and 2 (controls) were sensitized with AOO only. At day 10, mice were challenged nose-only with cinnamal or isoeugenol in a concentration of 50 mg/m^3 for a period of 60 minutes. Group 1 was the negative control group and received a nose-only challenge with acetone alone. Group 2 was challenged with cinnamal or isoeugenol, similar to the sensitized mice. At day 11, airway responsiveness to methacholine was measured in the whole body plethysmograph. Briefly, airway responses were measured in conscious unrestrained mice using a plethysmograph (Buxco, EMKA, Technologies, France). The mice were exposed for 3 minutes to doubling doses of aerosolized methacholine ranging from 1.88 to 30 mg/ml. After each exposure, lung function was measured for three minutes. From the lung function parameters peak expiratory flow, tidal volume, expiratory time and frequency the enhanced pause (PenH) was calculated. The PenH crorrelates strongly to airway resistance in mice (Hamelmann et al., 1997).
At day 12 mice were sacrificed and bronchoalveolar lavage was performed and the lungs, trachea and larynx were collected for histopathological examination.

Two parallel groups (4 and 5) were included to determine if the sensitization dose applied induced sensitization. These mice were sensitized either with AOO or with isoeugenol or cinnamal, as described above. At day 10 an ear swelling test was performed. Mice were challenged topically on both ears with 25 µl of the test compounds in a concentration of 20% (v/v). Ear swelling was measured in duplicate with a digital micrometer before and 6, 24 and 48 hours after challenge. At day 12 mice were sacrificed and auricular lymph nodes were collected and cell proliferation was assessed.

Table 9 Experimental design

Group	**N=**	**Day 0, 1, 2** **Dermal sensitization**	**Day 10** **Nose-only challenge** **60 min**
1	8	AOO	acetone
2	8	AOO	Isoeugenol or cinnamal (50 mg/m^3)
3	8	Isoeugenol or cinnamal (50%)	Isoeugenol or cinnamal (50 mg/m^3)
			Ear challenge **Day 10**
4	4	AOO	Isoeugenol or cinnamal (20%)
5	4	Isoeugenol or cinnamal (50%)	Isoeugenol or cinnamal (20%)

Assessment of cell proliferation
Single cell suspensions were prepared in standard medium with 5% FCS under aseptic conditions by pressing the auricular and mandibular LN trough a 70 µm nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed in standard medium with 5% FCS (10 minutes, 300 g, 4°C) and resuspended in 1 ml standard medium with 10% FCS. A Coulter Counter (Z2, Coulter Electronics, Mijdrecht, the Netherlands) was used to count the cells. Then the concentration of the cell suspensions was adjusted to 1×107 cells/ml. Of each

cell suspension, 200 µl was seeded in triplicate in a U-bottom 96-well tissue culture plate (Greiner, Alphen aan den Rijn, the Netherlands). After addition of 10 µl/well (37 kBq methyl-3H-thymidine (specific activity 185 MBq/mmol, Amersham Biosciences, Buckinghamshire, UK) the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 during 20–24 h. The cells were harvested on glass-fiber filters (LKB-Wallac, Turku, Finland) using a multiple cell culture harvester (LKB-Wallac). The [3H]-thymidine activity was determined using a liquid scintillation counter (1205 Betaplate TM, LKB-Wallac). For further calculations the median of the triplicates was used. The [^{3}H]-thymidine incorporation is expressed per animal, being the measured counts per minute (cpm) times the cell number of the two LN and divided by the cell number in culture. The mean [^{3}H]-thymidine incorporation per experimental group ± SEM was calculated. Stimulation indices (SI) were calculated by dividing the [^{3}H]-thymidine incorporation of the experimental group with the mean [^{3}H]-thymidine incorporation of the vehicle group. The SI after respiratory exposure was calculated by using the nose-only vehicle group and the SI after dermal exposure by using the dermal vehicle group.

Assessment of sensitizing potency
In the LLNA skin sensitizing potency is defined as the concentration at which a 3-fold increase of proliferation is induced, the EC3 value. This value can be determined by analyzing dose-response data by nonlinear regression analysis, as described previously (van Och et al., 2000). In the respiratory LLNA it is possible to calculate potency as well, called the ED3 value (Arts et al., 2008). The potency in the respiratory LLNA was calculated by determining the duration of exposure that induced a 3-fold increase of cell proliferation. This was done by non-linear regression using PROAST software (Slob, 2002). The potency (ED3) was then calculated by using the mean actual concentration (mg/m^3), the duration of exposure (min) at which a 3-fold increase in proliferation was obtained, the mean body weight, and a standard ventilation rate of 1.5 liter/kg mice. Absorption was assumed to be 100%.

Statistical analysis
Statistical analysis was performed using one-way analysis of variance (ANOVA). Significant differences of the control group were determined with the Bonferroni post hoc test, using a significance level of p=0.05.

Inhalation exposure and atmosphere generation and analysis
In the respiratory LLNA inhalation exposure was done by nose-only exposure. Mice were placed in restraining tubes which were connected to one of the two central exposure chambers for nose-only exposure. Mice that were exposed to the vehicle control were connected to the exposure chamber of the vehicle and mice that were exposed to fragrances were connected to the exposure chamber of the fragrance.
In order to achieve higher concentrations than possible in the vapour phase, the fragrances were nebulized in acetone to produce an aerosol of liquid droplets. In the pilot experiments with isoeugenol and cinnamal, the concentration of the solutions used for nebulization was 5 vol%, resulting in a concentration of 300 mg/m^3 when nebulized. These doses were toxic to the mice and in the other experiments the exposure concentrations were reduced to 75 mg/m^3 for benzyl salicylate, methyl heptine carbonate, isoeugenol and citral. The fragrances were sampled on 47 mm Teflon filters at a flow rate of 1 litre/min for five minutes. The collected mass was determined gravimetrically immediately after sampling to minimize evaporations of the collected droplets and used for concentration calculations. The vapour in this mixture downstream of the filters was also

sampled on activated charcoal. In addition, the test atmosphere was sampled at a flow rate of approx 1 litre/min for five minutes on activated charcoal and these were used for wet chemical determinations and used to calculate the average actual concentrations during the exposures.
The actual air concentrations measured are presented in Table 11. The fluctuations of all test atmospheres were less than 10% as indicted by continuous mass concentration measurements using a Total Carbon Analyzer (TCA). The inlet of the TCA was heated to evaporate all droplets. Vaporization of cinnamal resulted in equal amounts of vapour and aerosols, vaporization of benzyl salicylate resulted predominantly in aerosols, whereas vaporization of methyl heptine carbonate resulted predominantly in vapour. Vaporization of isoeugenol and citral resulted exclusively in vapour (Ezendam et al., 2007; Ezendam et al., 2009a).

Table 10 Aerosol, vapour and total mass concentration of each fragrance exposure

	Mass mg/m^3	Vapour mg/m^3	Total mg/m^3
Benzyl salicylate			
Day 1	68	2	70
Day 2	75	2	77
Day 3	77	2	79
Acetone			2,740
Methyl heptine carbonate			
Day 1	4	76	80
Day 2	5	76	81
Day 3	6	76	82
Acetone			2,300
Isoeugenol			
Day 1	0	80	80
Day 2	0	80	80
Day 3	0	80	80
Acetone			1,500
Citral			
Day 1	0	80	80
Day 2	0	80	80
Day 3	0	80	80
Acetone			2,840

In the experiments in which dermal sensitized mice received a challenge by inhalation exposure, the same experimental set-up was used as depicted in Figure 1 (see section 4.3). For vehicle exposed mice, a small adjustable airflow controlled by a mass flow controller (MFC) (Bronkhorst Hi Tec, Ruurlo, the Netherlands) was fed to a temperature controlled acetone bubbler. This flow was diluted with air to the desired concentration. Total flow was 6 litres per minute (lpm). The challenge was performed with isoeugenol or cinnamal dissolved in acetone. The test solution was delivered by a motor driven syringe (TSE systems Model 540200, Chesterfield MO, USA) to a compressed air driven Spray nozzle (Schlick type 970/S, Coburg, Germany). The aerosol was further diluted with dilution air. Both conditioned (20^{o}C and 50%RH) airflows (nozzle and dilution) were controlled by mass flow controllers (Bronkhorst Hi Tec, Ruurlo, the Netherlands). In the mixing chamber all acetone was evaporated from the nebulized particles. Depending on the vapour pressure of the test substance the aerosol particles were completely or partially evaporated. The total flow was 24 l.

The test atmosphere was measured during exposure with the TCA as described above. The results are shown in Table 11.

Table 11 Mass concentrations of each fragrance exposure

	Mass (mg/m^3)
Cinnamal	55
Acetone	1,640
Isoeugenol	55
Acetone	1,590

Appendix 2: Presence of fragrance allergens in scented consumer products

Fragrance allergens in scented products available on the Dutch market
Two databases were used to obtain information on the presence of fragrance allergens in scented products that can be purchased in the Netherlands. The data from the NVIC database and the publicly available data from Sara Lee (http://www.saralee-int.info/NL-NL/Our+Brands/AmbiPur) are summarized in Table 12 and 13, respectively.

Table 12 NVIC database: fragrances used in scented products

Fragrances	% in all scented products
n =	113
Geraniol	54%
Linalool	46%
Citronellol	42.5%
D-limonene	38.1%
Eugenol	37.2%
Lilial*	34.5%
Citral	32.7%
Hexyl cinnamal	20.4%
Benzyl salicylate	19.5%
Coumarin	19.5%
Lyral*	17.7%
Benzyl benzoate	13.3%
α-Isomethylionon	13.3%
Hydroxycitronellal	12.4%
Benzyl alcohol	11.5%
Cinnamyl alcohol	9.7%
Isoeugenol	8.0%
Amyl cinnamal	6.2%
Cinnamal	5.3%
Farnesol	3.5%
Benzyl cinnamate	2.6%
Oak moss	0.9%
Anisyl alcohol	0%
Amylcinnamyl alcohol	0%
Methyl heptine carbonate	0%
Tree moss	0%

* INCI names Lilial: butylphenyl methylpropional and Lyral: hydroxyisohexyl-3-cyclohexene carboxaldehyde

The NVIC database contained 113 scented products. Of these, 48 were air fresheners and room perfumes and the other products were intended for steam baths or saunas. The NVIC data show that the most frequently used fragrances in scented products (>40% of the products) were geraniol, linalool and citronellol. Almost all fragrance allergens from the SCCP list are used as ingredients in the products, with the exception of anisyl alcohol, amyl cinnamyl alcohol, methyl heptine carbonate and tree moss. The fragrances cinnamyl alcohol, isoeugenol, amyl cinnamal, cinnamal, farnesol, benzyl cinnamate and oak moss were not frequently used (<10% of the products) in these scented products.

The Sara Lee database contained 49 scented products. It was shown that the most frequently used fragrances (present in >40% of the products) were limonene, linalool, geraniol, citronellol and α-isomethylionon. The fragrances anisyl alcohol, amyl cinnamyl alcohol, benzyl cinnamate, methyl heptine carbonate, oak moss and tree moss were never used as ingredients in these products.

Table 13 Frequently used fragrances in scented products[1]

Fragrances	% in all scented products	% in scented car products	% in sprays	% in electrical room perfumes	% in scented candles
n =	*49*	*12*	*15*	*18*	*4*
D-limonene	69.4%	83.3%	46.7%	77.8%	75%
Linalool	69.4%	83.3%	40%	83.3%	75%
Geraniol	53.1%	66.7%	13.3%	77.8%	50%
Citronellol	49%	41.7%	20%	77.8%	0%
α-Isomethylionon	42.9%	50%	13.3%	72.2%	0%
Citral	36.7%	50%	6.7%	61.1%	0%
Eugenol	32.7%	41.7%	0%	50%	50%
Benzyl alcohol	26.5%	0%	0%	66.7%	25%
Coumarin	26.5%	33.3%	6.7%	38.9%	25%
Benzyl benzoate	24.5%	16.7%	0%	44.4%	50%
Benzyl salicylate	22.4%	8.3%	13.3%	38.9%	25%
Hydroxycitronellal	20.4%	16.7%	6.7%	33.3%	25%
Lillial*	16.3%	8.3%	0%	38.9%	0%
Hexyl cinnamal	16.3%	0%	20%	27.8%	0%
Lyral*	16.3%	16.7%	0%	27.8%	25%
Cinnamyl alcohol	10.2%	8.3%	0%	22.2%	0%
Cinnamal	8.2%	8.3%	0%	11.1%	25%
Isoeugenol	6.1%	0%	0%	16.7%	0%
Amyl cinnamal	2.0%	0%	0%	11.1%	0%
Farnesol	2%	0%	0%	5.5%	0%
Anisyl alcohol	0%	0%	0%	0%	0%
Amyl cinnamyl alcohol	0%	0%	0%	0%	0%
Benzyl cinnamate	0%	0%	0%	0%	0%
Methyl heptine carbonate	0%	0%	0%	0%	0%
Oak moss	0%	0%	0%	0%	0%
Tree moss	0%	0%	0%	0%	0%

[1] Data are derived from the website of Sara Lee (http://www.saralee-int.info/NL-NL/Our+Brands/AmbiPur) * INCI names Lilial: butylphenyl methylpropional and Lyral: hydroxyisohexyl-3-cyclohexene carboxaldehyde

Data from European market studies

The European Consumers Organisation (BEUC) has measured emission levels of different chemicals, including 11 fragrances, from 74 air fresheners in indoor air (BEUC, 2005). The most often detected fragrance was D-limonene and emissions ranged from 1-2003 µg/m^3. In almost 28% of the tested air fresheners, emission of linalool was detected, and concentrations ranged from 5-750 µg/m^3. The other fragrances that were detected but in a limited number of products were: lilial, cinnamal, coumarin, citral, benzyl benzoate, eugenol, benzyl alchohol, hydroxycitronellal and geraniol (see Table 14).

Table 14 Fragrance emission by air fresheners

Fragrances n =	% in all products 74	Emissions ($\mu g/m^3$)
D-limonene	88.1%	1-2003
Linalool	27.6%	5-750
Lilial*	13.2%	2-310
Cinnamal	6.5%	3-146
Coumarin	5.3%	4-22
Citral	2.6%	2-48
Benzyl benzoate	1.3%	9
Eugenol	1.3%	16
Benzyl alcohol	1.3%	22
Hydroxycitronellal	1.3%	51
Geraniol	1.3%	40

Data are derived from an European study from the BEUC (2005)* INCI names Lilial: butylphenyl methylpropional and Lyral: hydroxyisohexyl-3-cyclohexene carboxaldehyde.

The Danish Environmental Protection Agency (EPA) has performed a market survey in different stores and supermarkets that sell air fresheners for use at home and in the car (Pors & Fuhlendorff, 2003). A total of 19 products were selected and 6 of these were car products and 13 were products for use at home. In these products the presence and concentrations of 24 fragrance allergens were measured. The presence of oak moss and tree moss was not assessed in this study.

In Table 15, the results are summarized and the most frequently used fragrances (in >50% of the products) were D-limonene, linalool, benzyl benzoate, hexyl cinnamal, eugenol, lilial, benzyl alchohol and benzy salicylate. When the products intended for use at home or in the car were analyzed separately, the distribution shifted slightly. In products for at home 22 of the 24 fragrance allergens were used, with the exception of anisyl alcohol and farnesol. In car products, 5 of the 24 fragrance allergens were not used, these products contained no anisyl alcohol, farnesol, benzyl cinnamate, methyl heptine carbonate, and isoeugenol.

In this study, the concentrations of the fragrances were measured as well. There was a large variation when the individual products were analyzed. In addition, some fragrance chemicals were used in higher concentrations than others. In general, cinnamal, isoeugenol, amyl cinnamal alcohol and methyl heptine carbonate were used in the lowest concentrations. The fragrances that were used in the highest concentrations were (in weight % of a product) : lyral (6.2%), linalool (3.9%), citral (2.6%), hexyl cinnamal (2.2%), and D-limonene (2.1%).

Table 15 Frequently used fragrances in air freshners from a Danish market survey

Fragrances n =	% in all products 19	Concentration range (ppm)	% in all products for at home 13	% in all car products 6
D-limonene	78.9%	41-21,000	84.6%	80%
Linalool	78.9%	970-39,000	84.6%	80%
Benzyl benzoate	68.4%	7.7-10,000	61.5%	100%
Hexyl cinnamal	68.4%	39-22,000	69.2%	80%
Eugenol	63.2%	11-9,000	61.5%	80%
Lilial*	57.9%	450-12,000	61.5%	60%
Benzyl alcohol	52.6%	7.7-10,000	53.9%	60%
Benzyl salicylate	52.6%	4.1-13,000	46.2%	80%
Citronellol	52.6%	190-18,000	61.5%	40%
α-Isomethylionon	52.6%	220-11,000	61.5%	40%
Coumarin	47.4%	15-13,000	46.2%	60%
Lyral*	47.4%	310- 62,000	53.9%	40%
Geraniol	42.1%	390-8,900	46.2%	40%
Citral	36.8%	200-26,000	38.5%	40%
Amyl cinnamal	26.3%	640-16,000	15.4%	60%
Hydroxycitronellal	26.3%	440-2,600	23.1%	40%
Cinnamal	15.8%	10-63	15.4%	20%
Isoeugenol	15.8%	23-120	23.1%	0%
Methyl heptine carbonate	15.8%	3.5-270	23.1%	0%
Benzyl cinnamate	10.5%	170-500	15.4%	0%
Cinnamyl alcohol	10.5%	19-230	7.7%	20%
Amylcinnamyl alcohol	5.3%	17-50	7.7%	0%
Anisyl alcohol	0%	--	0%	0%
Farnesol	0%	--	0%	0%
Oak moss	NA			
Tree moss	NA			

Data are derived from market survey conducted by the Danish EPA (Pors and Fuhlendorff, 2003)* INCI names Lilial: butylphenyl methylpropional and Lyral: hydroxyisohexyl-3-cyclohexene carboxaldehyde. NA: not assessed.

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