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PhD thesis

Methylisothiazolinone: Contact Allergy and Antimicrobial Efficacy

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Abbreviations

ASM: American Society for Microbiology

CFU: Colony Forming Units

CLSI: Clinical and Laboratory Standards Institute

COLIPA: The European Cosmetic Association

ED: Eliciting Dose

FIC: Fractional Inhibitory Concentration

GMP: Good Manufacturing Practises

ICDRG: International Contact Dermatitis Research Group

MCI: Methylchloroisothiazolinone

MDBGN: Methyldibromo glutaronitrile

MET: Minimum Eliciting Threshold

MH: Mueller-Hinton

MI: Methylisothiazolinone

MIC: Minimum inhibitory concentration

PPM: parts per million (1ppm=0.0001%)

ROAT: Repeated Open Application Test

SAB: Sabouraud

SCCS: Scientific Committee on Consumer Safety

TSA: Tryptone Soya Agar

Table of Content

1.1 Summary (English)

Preservatives are one of the most common causes of contact allergy and an increasing prevalence has been demonstrated. Methylisothiazolinone (MI) used alone is a relatively new preservative, but has been used together with methylchloroisothiazolinone (MCI) for more than 30 years, and both are potent sensitizers. MI is permitted in cosmetics up to 100ppm in the EU. A few case reports on MI contact allergy have been published, but the prevalence of MI contact allergy is unknown.
Development of contact allergy is dose-dependent, so reducing the use concentrations of preservatives may lead to fewer cases of allergic contact dermatitis. However, the antimicrobial effects of preservatives are also dose-dependent. This means that a reduction in concentration to protect against contact allergy may cause the preservative to lose its antimicrobial effect, and the products will be at risk of contamination. A method to reduce the use concentration of allergenic preservatives without losing the antimicrobial efficacy could be to combine low levels of the more effective but allergenic preservative with a less effective but also less allergenic preservative. If this method proves effective, this could potentially lead to fewer cases of allergic contact dermatitis.

In this thesis the following was investigated:

- The antimicrobial efficacy of MI and other preservatives, either alone or in different combinations.
- The prevalence and cause of MI contact allergy in patch tested patients from Gentofte Hospital.
- The dose-response relationship of MI contact allergy in MI-allergic patients.

When the allergenic preservatives MI, diazolidinyl urea or MCI/MI were combined with phenoxyethanol, the use concentrations could be markedly reduced compared to if the preservatives were used alone. Phenoxyethanol (0.2%) in combination with MI (5ppm) was sufficient to preserve a cosmetic cream.
A total of 2536 eczema patients were patch tested with MI (2000ppm in aqua), and 1.5% of the patients developed a positive reaction. Exposure to MI was from cosmetic products, primarily rinse-off products and from occupational exposure with painters appearing to constitute a subgroup.

In the dose-response study it was tested if phenoxyethanol had any effect on the skin reactivity to MI in patients with MI contact allergy. In the patch test the threshold concentration for elicitation was 1.47 µg MI/cm^2, and phenoxyethanol had no influence on the reactivity. However, when MI was applied repeatedly on the skin simulating normal exposure to e.g. cosmetic products using a repeated open application test (RQAT), 2 (18%) patients reacted to 0.0105 µg MI/cm^2, which corresponds to a cream preserved with 5ppm MI.

On the basis of a previous safety evaluation 100ppm MI is considered safe in cosmetics in the EU. But the prevalence of MI contact allergy is already higher than that of other allergenic preservatives. Combinations of allergenic and non-allergenic preservatives could reduce the use concentration of preservatives and thereby potentially reduce the number of contact allergy cases. The prevalence of MI contact allergy is high, and the concentration needed to elicit a reaction is lower than the concentration considered safe by the EU. A solution may be to reduce the permitted concentration of MI and only use it in combination with other non-allergenic preservatives. This may also serve as a model for a more general approach to reducing allergy problems caused by allergenic preservatives.

1.2 Resumé (Dansk)

Konserveringsmidler er en af de hyppigste årsager til kontaktallergi, og prævalensen er stigende. Methylisothiazolinone (MI), brugt alene, er et relativt nyt konserveringsmiddel, men har været brugt sammen med methylkloroisothiazolinone (MCI) i mere end 30 år. Begge er sensibiliserende. I EU er det tilladt, at bruge op til 100 ppm MI i kosmetik. Der er publiceret enkelte case-reports om kontaktallergi over for MI, men prævalensen af MI kontaktallergi er ukendt.
Udvikling af kontaktallergi er dosisafhængigt, og en reduktion af koncentrationerne af konserveringsmidler kunne medføre færre tilfælde af kontaktallergi. Den antimikrobielle effekt af konserveringsmidler er dog også dosisafhængig. Det betyder, at en reduktion i koncentrationen af konserveringsmidler for at undgå kontaktallergi kan medføre, at konserveringsmidlet mister sin antimikrobielle effektivitet, og produktet derved risikerer at blive kontamineret.
Hvis man kombinerer konserveringsmidler og derved reducerer koncentrationen af et effektivt men allergifremkaldende konserveringsmiddel ved at bruge det sammen med et mindre effektivt men ikke allergifremkaldende konserveringsmiddel, kan man bibeholde den antimikrobielle effektivitet og samtidig reducere risikoen for allergi. Potentielt kan dette medføre færre tilfælde af kontaktallergi.

I afhandlingen blev følgende undersøgt:

- Den antimikrobielle effekt af MI og andre konserveringsmidler alene eller i forskellige kombinationer.
- Prævalens og årsager til kontaktallergi over for MI hos lappetestede patienter fra Gentofte Hospital.
- Dosis respons forhold af MI-kontaktallergi hos MI-allergikere.

Kombinationer mellem et af de allergene konserveringsmidler MI, diazolidinyl urea eller MCI/MI og det ikke-allergene phenoxyethanol viste, at kombinationerne var væsentligt mere effektive end konserveringsmidlerne alene. Phenoxyethanol (0,2 %) i kombination med MI (5 ppm) var tilstrækkeligt til at konservere en kosmetisk creme.

I alt 2536 eksem patienter blev lappetestet med MI (2000 ppm i vand) og 1,5 % af patienterne reagerede. MI-eksponering kom fra kosmetik, hovedsageligt rinse-off-produkter, og industrielle produkter, hvor malere udgjorde en større undergruppe.

I dosis-respons-studiet blev det undersøgt, om phenoxyethanol havde nogen indvirkning på de allergiske reaktioner over for MI hos MI-allergikere. I lappetest-forsøget var tærskelværdien for en allergisk reaktion 1.47 µg MI/cm^2. Phenoxyethanol havde ingen effekt på reaktionerne.

Da MI blev påført i en gentagen åben test (ROAT), der simulerer normal eksponering til f.eks. en creme, reagerede 2 patienter (18 %) på 0,0105 µg MI/cm^2. Dette svarede til en creme konserveret med 5 ppm MI.

Ud fra en tidligere sikkerhedsvurdering har EU besluttet, at 100 ppm MI er sikkert at bruge i kosmetik, men prævalensen af kontaktallergi er allerede højere end andre allergene konserveringsmidler. Hvis man reducerer koncentrationen af allergene konserveringsmidler ved at kombinere dem med ikke-allergene, kan man potentielt reducere antallet af tilfælde af kontaktallergi. Prævalensen af kontaktallergi over for MI er høj, og koncentrationerne der skal til at udløse en allergisk reaktion hos mange MI-allergikere er under den koncentration, som EU betragter som sikker. En løsning er, at reducere den maksimalt tilladte koncentration af MI og kun bruge det i kombination med andre ikke-allergene konserveringsmidler. Dette kan også bruges som en mere generel løsning på en reduktion af allergiproblemerne knyttet til allergene konserveringsmidler.

2 Background

2.1 Allergic contact dermatitis

Allergic contact dermatitis is a type IV immunological reaction consisting of 2 phases: sensitization and elicitation. The first phase, sensitization, is the induction phase in which exposure to an allergen above the tolerance threshold generates a T-cell mediated immunological memory of the allergen. In the second phase, elicitation, the T-cells react upon a re-exposure of the allergen and elicit the clinical response known as allergic contact dermatitis [1].

Allergic contact dermatitis is the clinical expression of contact allergy. The response is typically itching and erythema as well as infiltration, papules and vesicles [2]. Development of contact allergy is dependent on the sensitizing potential of the allergen, the exposure (dose per unit area), repeated exposures, solubility of the allergen, occlusion, vehicle and the permeability of the skin [3]. In Denmark studies have shown that between 7.3% and 12.9% of the adult general population has contact allergy [4;5]. Nickel is the individual contact allergen with the highest prevalence (approx. 6%), but groups of compounds such as fragrances and preservatives frequently used in cosmetics, household products and industrial products are also a common cause of contact allergy [5-9]. The first cases of allergic contact dermatitis to a new allergen often come from the industry where new compounds are introduced earlier than from consumer products owing to a less strict legislation. Often the workers also handle the allergens in higher concentrations compared to the end concentration in consumer products [10].

Allergic contact dermatitis may lead to sick leave and affect people's ability to work [11]. Skin diseases is the second most common reason for paid compensation for reduced ability to work in Denmark [12].

2.1.1 Diagnosis of allergic contact dermatitis

Contact allergy is diagnosed by patch testing. In patch testing the patient is exposed to a series of known allergenic chemicals and the clinical reaction is followed for up to 7 days. The recommended exposure and reading time for the patch test is: occlusion for 2 days followed by reading after D2, D3/4 and D7 [13]. Interpretation of the reactions should follow the International Contact Dermatitis Research Group (ICDRG), and include negative, irritative, doubtful (+?) and graduated positive reactions (+, ++, +++) (Figure 1) [14].

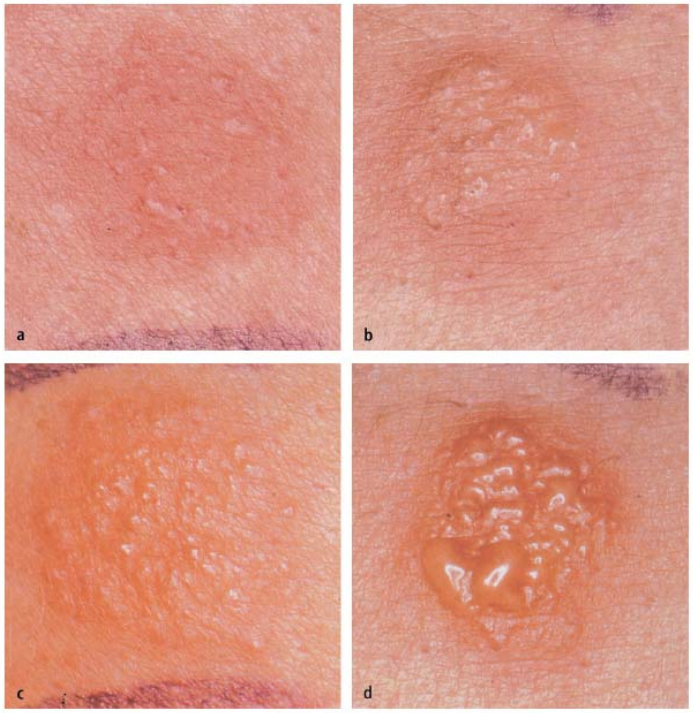


Figure 1: Positive reactions in the patch test. a and b=+, c=++ and d=+++ [13]

The European baseline series contains 26 different items. These are either allergens alone or combinations of allergens, such as the Paraben Mix, Fragrance Mix I and Fragrance Mix II [15]. Some of the most common allergens are represented in this series but it covers only a fraction of the 4350 chemicals described that can cause allergic contact dermatitis [16]. Other more specialized series are also tested if the patient is employed in a specific occupation, e.g. a hairdresser series for hairdressers or a bakery series for bakers [13]. The concentrations used in patch tests are a compromise between the concentration that will identify the greatest number of cases of allergy and the concentration that can cause irritation or sensitization [13]. Furthermore it is important to establish the relevance of a patient's positive patch test reaction, and whether the patients are exposed to the allergen at work or at home. This enables the patient to avoid future exposure to the allergen, but it also enables researchers to find possible coherences between patients with contact allergy to the same chemical.

2.1.2 Characterisation of contact allergy patients

Patients' characteristics are an important tool when differences in groups or subgroups are investigated. In patch test patients a common set of characteristics is the MOAHLFA index, which

includes the following demographic variables: Male, Occupational dermatitis, Atopic eczema, Hand eczema, Leg dermatitis/ulcers, Face dermatitis and Age above 40 [17]. The MOAHLFA index is used to stratify for the above mentioned confounding factors often seen in contact allergy patients. Sensitization rates differ among individual allergens in a patch test department, but also between patch test departments both nationally and internationally. A study on 40,000 dermatitis patients in the IVDK network showed that the different factors in the MOAHLFA index explained many of the variations among departments [17]. In general, hand dermatitis is often associated with an occupational exposure and face dermatitis is often associated with a cosmetic exposure [17]. These general observations can be different when investigators look at specific allergens: for instance hand dermatitis is almost as common as face dermatitis in fragrance allergic patients [18].

2.1.3 Dose-response relationship in contact allergy

In risk assessments of allergenic chemicals it is important to know what concentrations can cause sensitization and what concentrations cause elicitation. The concentration needed to sensitize and subsequently elicit an allergic reaction depends on many different factors and varies from individual to individual [3]. Site of exposure, the allergen's ability to penetrate the skin, single or repeated exposure and the dose per area of skin are some of the factors that influence the pattern of reactivity in allergic contact dermatitis [3]. Especially dose per unit area ($\mu g/cm^2$) is a key factor in sensitization as Friedmann and colleagues have shown with DNCB [3]. In general, the elicitation dose is lower than the sensitizing dose [3]. Furthermore there appears to be an inverse relationship between the sensitizing dose and the eliciting dose. Thus, high sensitizing doses equal low eliciting doses and vice-versa [19;20].

A compound sensitizing capability has for many years been assessed in animal tests such as the Buehler test, Guinea-Pig Maximization Test (GPMT) or Local Lymph Node Assay (LLNA) [21] and in humans in different versions of the Human Repeated Insult Patch Test (HRIPT). The results from the animal studies are extrapolated to humans to establish a concentration of the chemical that is safe to use. However, these safe levels have failed several times, and it is important to include human studies especially on already sensitized patients to define the Minimum Eliciting Threshold (MET) [22]. The MET can be determined by 2 different methods: the dose response patch test and the Repeated Open Application Test (ROAT). The dose-response patch test is a patch test with a serial dilution of a specific allergen on patients already sensitized to this allergen. The dose response

patch test follows the same standardised application and reading scheme as the normal patch test except that the reading scale is often expanded to include minor reactions [23;24]. Since repeated exposure is a key factor in contact allergy reactions, the ROAT is often preferred in MET determination. The ROAT was standardised by Hannuksela in 1986, and an alternative reading scale was suggested in 1998 [25;26]. Diagnostically the test can verify if a specific product (e.g. a cosmetic cream) is the reason for a patient's allergic reaction if no conclusion can be drawn from a patch test.

The result of the ROAT depends on several exposure conditions such as concentrations, frequency, duration and location [27;28]. The MET found in ROAT is often lower than that found in patch tests because of the repeated exposure [29]. On the basis of experiments with nickel and methyldibromo glutaronitrile (MDBGN) Fischer *et al* investigated if the relationship between the thresholds in patch test reactions and ROAT reactions were the same for the 2 allergens [23;24]. They developed the following model, which converts patch test data to ROAT data [29]:

$$ED_{xx}(\text{ROAT}) = 0.0296 \cdot ED_{xx}(\text{patch test})$$

In the model ED_{xx} (Eliciting Dose) is the dose that will elicit an allergic reaction in xx% of allergic individuals. If this model also applies to other allergens, it is possible to establish an eliciting threshold based on a dose-response patch test that includes repeated exposures. The ROAT is the most realistic test for MET determination, but it is also very time-consuming with applications twice a day for up to 3 weeks. If the model fits for other allergens, this could be an important tool in future risk assessments [29].

2.2 Preservatives

Preservatives are used in all sorts of products in which microorganisms can proliferate. Food, pharmaceuticals, industrial products, household products and cosmetics are some of the products that are at risk of contamination. In the 1960s almost 25% of cosmetic products were contaminated, and cases of infections caused by contaminated cosmetics were published [30;31]. This led to an increased focus on contaminated cosmetics and ways to prevent this. New preservatives were introduced, legislation was enacted, and a lot of research on preservative efficacy tests was conducted [30]. In 1972 3.5% of investigated cosmetic products were contaminated [30]. Today only a

few products are contaminated, and cases of infections caused by contaminated cosmetics are primarily seen in immunosuppressed patients [32-35].

In 1991 Anthony Fransway defined the perfect preservative as a "colorless, odorless, water soluble, nontoxic, nonallergenic, nonirritating chemical capable of inhibiting the growth of a broad spectrum of bacteria and fungi" [36]. So far no preservative fulfils all these demands.

2.2.1 Cosmetic preservatives

In the EU preservatives for cosmetic products are regulated [37]. At present there are 56 different preservatives permitted in cosmetics, and the preservatives can be added up to an individual maximum permitted concentration [37]. Industrial products such as paint, lacquers, glues, printing ink and cutting oils often require preservation as well [38].

The preservatives permitted in cosmetics in the EU have all been evaluated by an independent scientific committee, at present called the Scientific Committee of Consumer Safety (SCCS). The SCCS follows a guideline which incorporates the preservatives' chemical and physical properties and different aspects of toxicity, e.g. the preservatives' skin sensitization potential [39]. Even though preservatives are thoroughly evaluated by the SCCS prior to release in cosmetic products, some are frequent sensitizers, and preservatives have for several years been one of the most common causes of contact allergy to cosmetics [7;9;40]

Industrial products are not regulated to the same extent as cosmetic products, with respect to permitted preservatives and use concentrations. This is one of the main reasons why preservatives are introduced more quickly in industrial products and also why the first cases of contact allergy to new chemicals are often found here [10].

2.2.2 Use of preservatives and prevalence of contact allergy

Even though cosmetic manufactures have 56 different preservatives available, the market is dominated by a few preservatives, and among these we find some of the most frequent sensitizers. Voluntary registration of preservatives in cosmetics with the Food and Drug Administration (FDA) in the US showed that parabens, phenoxyethanol, formaldehyde releasers and methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) were the most frequently used preservatives in more than 27,000 individual registered products [41]. 5 of the 7 most commonly used preservatives were parabens (methyl-, propyl-, butyl-, ethyl and isobutylparaben), and the various

parabens were used in between 6% and up to at least 42% of the total number of registered cosmetic products. Phenoxyethanol is the third most used preservative and is found in 18% of the products [41]. Neither parabens nor phenoxyethanol are frequent sensitizers. Contact allergy prevalence in patch tested individuals ranges from 0.5% to 1.4% for the paraben mix [42-45] and 0.2% for phenoxyethanol [43]. Formaldehyde releasers frequently used in cosmetics are: imidazolidinyl urea, DMDM-hydantoin, diazolidinyl urea, quaternium-15 and 2-bromo-2-nitropropane-1,2-diol. The frequency of use ranges from 0.8% to 8% in cosmetic products [41]. Prevalence of contact allergy among patch tested individuals to the different formaldehyde releasers ranges from 0.5% to 1.6% in Europe [42-44]. In the US prevalence of contact allergy to the different formaldehyde releasers is higher than in Europe; especially quaternium-15 has a high prevalence of 10.3% in the US [45]. Besides the formaldehyde releasers used in cosmetic products many other releasers are available and are used in a wide variety of industrial products [46;47].

Formaldehyde itself is also permitted as a preservative, but its use is very limited [41]. However, it is a frequent sensitizer and the prevalence of contact allergy to formaldehyde is high. In Europe prevalences between 1.7% and 3.1% have been reported [42-44]. In the US the prevalence is much higher (9.0%) [45]. Contact allergy to formaldehyde is most probably associated with the use of formaldehyde releasers. In many cases individuals allergic to a formaldehyde releaser have a concomitant contact allergy to formaldehyde as well, and individuals with formaldehyde allergy are recommended to avoid all formaldehyde releasers [48;49].

Only one preservative available today (2-bromo-2-nitropropane-1,2-diol) was shown in one study to have an increasing trend in prevalence [43]. Prevalence of the other preservatives has been relatively stable for several years [42-44]. However, as the number of preservatives included in patch test series increases, the overall burden of contact allergy to preservatives increases as well [42]. Especially one preservative (MDBGN) has led to the rise in overall contact allergy to preservatives. A study from the European Environmental & Contact Dermatitis Research Group from 2002 showed that from 1991 to 2000 the prevalence of MDBGN contact allergy rose from 0.7% to 3.5% [44]. The SCCS initiated an evaluation of MDBGN and 4 years and 4 different opinions later MDBGN was banned from cosmetics in the EU from 2008 [50-53]. The effect of the regulation is documented in a Danish study, which showed that the prevalence of MDBGN contact allergy had decreased from 2003 to 2007 [54].

A few studies have investigated the use concentration of preservatives in cosmetics [55-57]. No products were preserved with concentrations above the maximum permitted concentration at the

time of the studies, but the range of use concentration was very wide and many of the investigated preservatives were used in concentrations at or just below their maximum permitted concentration. The studies did not include information on usage of preservative combinations in the products [55-57].

2.2.3 Isothiazolinones

Besides parabens, phenoxyethanol and formaldehyde releasers another preservative is also widely used in cosmetics, detergents and industrial products, namely methylchloroisothiazolinone (MCI) and methylisothiazolinone (MI) in a 3:1 combination. The chemical structures of MCI and MI are shown in Figure 2.

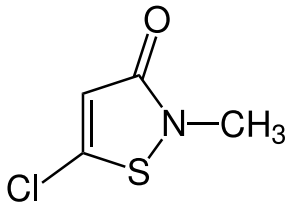
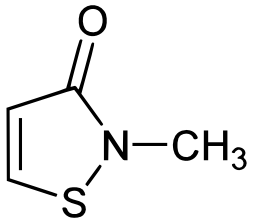


Figure 2: Methylchloroisothiazolinone



Methylisothiazolinone

MCI/MI was discovered in the late 1960s and has been used in cosmetics in Europe since the 1970s [58]. The first cases of MCI/MI contact allergy associated with cosmetics were reported in 1985 [59;60]. This was followed by investigations of the active ingredients (MCI and MI) and their allergenic potential [61-63]. These studies found that both MCI and MI were sensitizers. MCI is a stronger sensitizer than MI, but some individuals reacted to MI after exposure to MCI/MI [61-63]. Epidemiological studies describing the increasing trend in MCI/MI contact allergy in the 1980s led to a regulation in the maximum permitted concentration in both the EU and the US. In the EU the maximum permitted concentration was reduced from 30ppm to 15ppm in both rinse-off and leave-on products [64]. In the US safe concentrations of MCI/MI are 7.5ppm in leave-on products and 15ppm in rinse-off products [65]. The US safe concentrations are identical with the recommendations from the manufacturers of MCI/MI [58]. MCI/MI is currently undergoing safety evaluation by the SCCS in the EU [66].

Two studies, from Denmark and Sweden investigating the use concentrations of preservatives in skin creams and moisturizers found concentrations of MCI/MI ranging from 3.6ppm to 14.7ppm [55;57]. Despite the regulation of the maximum permitted concentration for MCI/MI from 30ppm to

15ppm and 7.5ppm respectively the prevalence of MCI/MI contact allergy has remained stable at approximately 2% for patch tested patients in Europe and a little higher in the US (approx. 3%) for many years [42-45].

In 2005 MI alone was approved for use in cosmetic products in the EU with a maximum permitted concentration of 100ppm [37]. With the present legislation the maximum concentration of MI permitted in cosmetic products has increased from 3.75ppm when combined with MCI to 100ppm when used alone. Before approval for cosmetic products MI was already used in many industrial products such as paints, lacquers, cutting oils and printing inks [67]. The first cases of isolated MI contact allergy caused by occupational exposure were published in 2004 and 2006, with one patient reacting to as low a concentration as 30ppm MI [68;69]. In 2010 the first cases of cosmetics-related contact allergy to MI were published [70]. The exposure patterns among industrial workers and consumers are very diverse. The concentrations in industrial products are often higher and the workers might handle the compounds in undiluted form, which can lead to chemical burns and subsequent sensitization [68;69;71]. Some of the patients described in the occupational cases had, besides MI contact allergy, concomitant reactions to MCI/MI or other isothiazolinones [69;72]. Allergic reactions to both MCI/MI and MI alone have been found in patients sensitized by MCI/MI. However, it is not known whether these were caused by cross-reactions between the 2 compounds or sensitization to both compounds [61;72]. When the primary sensitizer is MI, the pattern is often high reactivity against MI alone and lower reactivity to MCI/MI or MCI alone [68;69]. Again it is not known whether this is cross-reactivity or sensitization to both.

Only one study has investigated the prevalence of MI contact allergy and the authors found 41 out of 3983 (1.0%) patch tested patients who reacted to MI [43].

Benzisothiazolinone, octylisothiazolinone and dichlorinated octylisothiazolinone are preservatives for industrial products and known contact allergens [16;72]. They rarely cause contact allergy, and there is currently no evidence of cross-reactivity between these isothiazolinones and MCI and MI [72;73].

2.2.4 Antimicrobial efficacy of preservatives

Cosmetic products can be contaminated with all kinds of microorganisms capable of growing in the formulation. This means that the preservatives in the formulation must be able to withstand contamination from Gram-negative and Gram-positive bacteria as well as yeast and mould. MCI/MI

is one of few preservatives with a spectrum of activity against these 4 groups of microorganisms [74]. The efficacy of preservatives and other antimicrobials is measured as the Minimum Inhibitory Concentration (MIC). "The lowest concentration of agent that completely inhibits the growth of the test organism defines the MIC" [75]. This definition is not completely accurate since it is based on a visual definition of an endpoint (Figure 3). The MIC has obviously inhibited the growth of the microorganism, but it is not known whether it still proliferates in the media at a slower rate. This also depends on the antimicrobial effect of the agent. If it is a static antimicrobial, it will inhibit the growth of the microorganism without killing it, whereas a cidal agent kills the microorganism [75].

When measuring and comparing MIC values it is important to follow the same protocol for each microorganism tested. There are many factors that can cause variations in MIC values, primarily inoculum size, incubation time and growth media [75;76].

MIC tests for aerobic bacteria, filamentous fungi and yeast have been standardised by the Clinical and Laboratory Standards Institute (CLSI) in order to ensure low variability and comparable results from different departments [77-79].

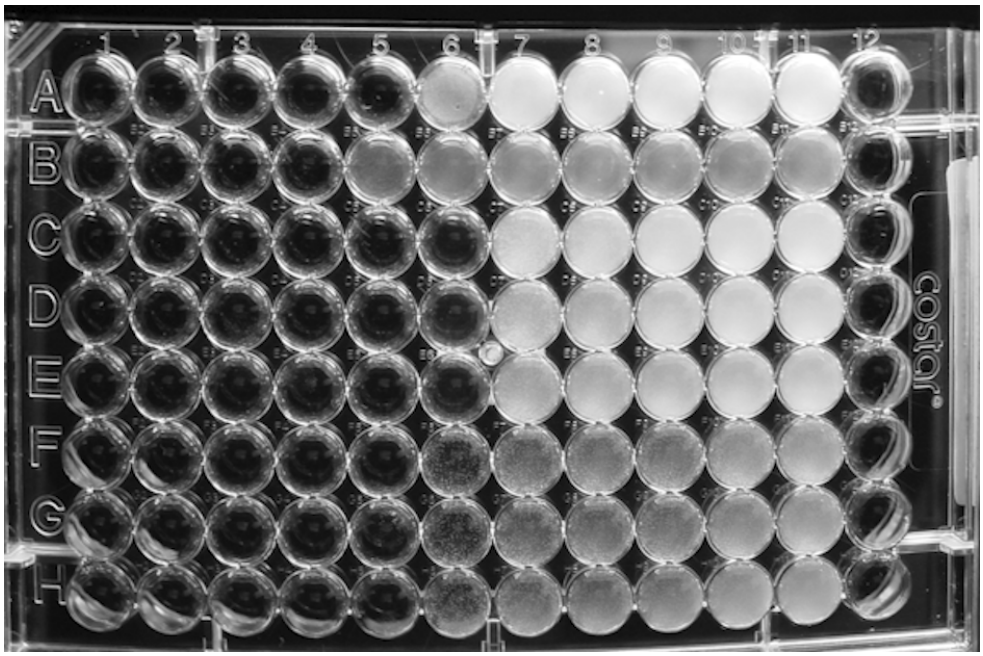


Figure 3: MIC test in a microtitre plate. Turbid wells indicate growth of microorganisms [80].

2.2.5 Combinations of preservatives

When 2 or more preservatives or other antimicrobials are combined with each other there are 3 different possible effects. 1) Synergy: The effect of the combined preservatives is greater than that of the individual preservatives used alone. 2) Antagonism: The effect of the combined preservatives is less than that of the individual preservatives used alone. 3) Additive effects: The effect of the combined preservatives is the same as that of the preservatives used alone [81]. Furthermore, the combination may also be active against a broader spectrum of microorganisms than when the preservatives are used alone.

The effect of combinations of preservatives can be investigated in a checkerboard assay [81]. This is a serial dilution assay in which 2 or more preservatives are combined in a series of concentrations from the MIC value or just above for each preservative and down to zero. On the basis of visible growth inhibition it is possible to calculate the Fractional Inhibitory Concentration (FIC), which indicates whether the combination is synergistic or not [81;82]. FIC is the sum of each preservative's MIC value obtained in combination with other preservatives divided by the MIC values of the preservatives used alone:

$$FIC = (MIC_{ab})/(MIC_a) + (MIC_{ab})/(MIC_b),$$

where MIC_{ab} is the MIC value of preservative a in combination with preservative b, and MIC_a and MIC_b are the MIC values of preservatives a and b used alone [81]. According to the American Society for Microbiology (ASM) the following FIC values indicate synergy (FIC≤0.5), indifference/additive effects (FIC >0.5 and ≤4), or antagonism (FIC>4) [82]. There are already many different combinations of preservatives available for cosmetic products [74], e.g. Neolone™ PE (MI and phenoxyethanol) and Germaben® II-E, which is a combination of diazolidinyl urea, methylparaben and propylparaben [83;84]. For Neolone™ PE there is also a recommendation on use concentrations, but for the allergenic constituent (MI) they do not differ from the recommendations for MI used alone [83;85]. Many different combination of preservatives have been tested, but the studies are old, and many of the combinations are not relevant in relation to current legislation and the use of preservatives today [81;86-88].

2.3 Microbiological quality of cosmetics

If cosmetic products are not adequately preserved, microbial contamination can cause alterations in the composition, odour or colour of the product. This can lead to an expensive withdrawal of the products [32]. In worse cases the contaminant is pathogenic and this can have serious consequences if the product is used by immunosuppressed individuals [35;89;90].

Manufacturers of cosmetics are obliged to comply with Good Manfacturing Practice (GMP) standards, but, GMP does not require sterility, so manufacturers add preservatives to minimise intrinsic contamination and especially to avoid consumer-based contamination during use [39]. If cosmetic products are not properly preserved or if a microorganism is resistant to the preservative, the products can become contaminated. There are 2 routes from which microorganisms can contaminate a cosmetic product. 1) Contamination during manufacturing with contaminated ingredients, sites in the production or personnel. This should be avoided by following GMP. 2) Consumers may introduce microorganisms during use. Adding preservatives should control proliferation and spoilage caused by introduced microorganisms. Preservatives are not intended to compensate for bad production facilities and lack of GMP compliance.

Besides legislation on what preservatives are permitted in cosmetics and their maximum permitted concentrations, there are also demands regarding the microbiological quality of cosmetic products as well as demands regarding their ability to withstand microbial contamination during use. In the EU these demands are listed in the SCCS (formerly SCCP) notes of guidance [39]. Here there are specific limits on the total viable count of aerobic mesophyllic microorganisms based on the type of product. Products for children under 3 years of age, eye products or products used on mucous membranes are subject to more stringent demands. The remaining products, such as creams, lotions, shampoos, liquid soaps, etc. must not contain more than 10^3 Colony Forming Units (CFU)/g or ml in 0.1 g or ml of the product [39]. Furthermore, the pathogenic bacteria *Staphylococcus aureus, Pseudomonas aeruginosa* and the pathogenic yeast *Candida albicans* must not be detectable in 0.5 g or ml of the product [39].

2.3.1 Efficacy of preservation

In order to test the product's ability to withstand consumer contamination all products have to pass a challenge test prior to marketing. The challenge test monitors a product's ability to eradicate an artificial contamination for at least 4 weeks. The SCCS recommends the challenge test setup from

either the European Pharmacopoeia or the US Pharmacopoeia [39]. At the moment there is no standardised challenge test available, and it is up to the manufacturer to decide on the details of the test to be used. The only requirement determined by the SCCS is the inclusion of the following microorganisms: *S. aureus, P. aeruginosa*, and *C. albicans* [39].

2.4 Objectives

The aim of this project was to study the antimicrobial efficacy and allergenicity of cosmetic preservatives with emphasis on the new preservative MI. The project consists of the following 3 studies:

- The antimicrobial efficacy of MI and other preservatives either alone or in different combinations (Study I).
- The prevalence and cause of MI contact allergy in patch tested patients from Gentofte Hospital (Study II).
- The dose-response relationship of MI contact allergy in MI-allergic patients (Study III).

Besides new information about MI, another perspective of the project was to suggest a new and general approach to preservation of cosmetic products in which allergenic preservatives are combined with non-allergenic preservatives to obtain sufficient preservation with lower concentrations of the allergenic preservatives.

3 Material and methods

3.1 Efficacy of cosmetic preservatives (Study I)

3.1.1 Microorganisms and preservatives

The following strains were used in all microbiological experiments: *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404). The preservatives used in the study were: Phenoxyethanol (Sigma Aldrich®), Diazolidinyl urea (Germall® II, Sigma Aldrich®), Methylchloroisothiazolinone/ methylisothiazolinone (Kathon™ CG 1.498% active ingredient, DOW) and Methylisothiazolinone (Neolone™ 950, 9.7% active ingredient, DOW).

3.1.2 Minimum inhibitory concentration

The MIC value of each cosmetic preservative was determined according to CLSI (formerly NCCLS) standard M7-A6 for *S. aureus* and *P. aeruginosa* [77]. MIC values for *C. albicans* and *A. niger* were determined according to CLSI M27-A2 and M38-A respectively [78;79]. All MIC tests were performed in 96-well microtitre trays in accordance with the standards for broth microdilution [77-79]. In order to mimic the storage conditions of cosmetics the incubation temperature was lowered from 35°C to 25±2°C. This slowed the growth of the microorganisms, so the incubation periods were changed to 48±2 hours for the bacteria and 72±2 hours for the yeast and mould.

3-5 colonies were selected from overnight cultures of *S. aureus* and *P. aeruginosa* on Mueller-Hinton (MH) Agar (OXOID), suspended in sterile saline (0.85%) and adjusted spectrophotometrically at 530 nm to match the turbidity of a 0.5 McFarland standard ($1x10^8$ CFU/ml). From the saline solution the microorganisms were further diluted in MH II broth (OXOID) to obtain a final inoculum in the well of approximately $5x10^5$ CFU/ml [77].

5 colonies were selected from overnight cultures of *C. albicans* on Sabouraud (SAB) agar plates (OXOID), suspended in sterile saline and adjusted spectrophotometrically at 530 nm to match the turbidity of a 0.5 McFarland standard ($1x10^6$ – $5x10^6$ CFU/ml). From the saline solution *C. albicans* was further diluted in RPMI 1640 broth medium (Sigma Aldrich®) to a final inoculum in the well of approximately $0.5x10^3$ – $2.5x10^3$ CFU/ml [79].

Spores from *A. niger* were proliferated for 5 days on SAB agar plates. New spores were harvested with sterile saline and a drop of Tween 20 (Sigma Aldrich®). Spores were counted in a Bürker Türk counting chamber and adjusted to a final inoculum in the well of $4x10^3$ – $5x10^4$ CFU/ml in RPMI 1640 broth medium [78].

The concentrations of phenoxyethanol tested were: 0.05%; 0.1%; 0.15%; 0.2%; 0.3%; 0.4%; 0.5%; 0.6%; 0.7%; 0.8%; 0.9% and 1%. The concentrations of diazolidinyl urea tested were: 0.03125%; 0.0625%; 0.1%; 0.125%; 0.2%; 0.3%; 0.4% and 0.5%. MCI/MI and MI are both effective at much lower concentrations; hence, the concentrations are given in ppm instead of %. The concentrations of MCI/MI tested were: 0.03125ppm; 0.0625ppm; 0.125ppm; 0.25ppm; 0.5ppm; 1ppm; 2ppm; 4ppm; 8ppm and 15ppm. The concentrations of MI tested were: 5ppm, 10ppm, 15ppm, 20ppm, 25ppm, 35ppm, 45ppm, 55ppm, 65ppm, 75ppm, 85ppm and 100ppm. For bacterial MIC determination the antimicrobial solutions were made in MH II broth, and for the yeast and fungi the solutions were made in RPMI 1640 broth [77-79]. MIC values were determined in at least 3 independent experiments. In the event of differences between MIC values the highest concentration found was considered the true MIC value.

3.1.3 Fractional inhibitory concentration

The possibility of synergy in combinations of preservatives can be determined as FIC values in a checkerboard assay [82]. On the basis of the MIC results obtained the following concentrations of phenoxyethanol (0%; 0.05%; 0.1%; 0.15%; 0.2%; 0.4%; 0.6%; 0.8% and 1%), diazolidinyl urea (0%; 0.03125%; 0.0625%; 0.125%; 0.25% and 0.5%), MCI/MI (0ppm; 0.03125ppm; 0.0625ppm; 0.125ppm; 0.25ppm; 0.5ppm; 1ppm; 2ppm and 4ppm) and MI (0ppm, 10ppm, 20ppm, 30ppm, 40ppm, 50ppm, 60ppm, 70ppm, 80ppm, 90ppm, 100ppm) were combined 2 or 3 together, and tested against the microorganisms. MCI/MI and MI were not combined in any of the experiments. The experimental setup followed the same guidelines as the MIC tests and the alterations mentioned in the previous section [77-79]. All combinations were tested at least twice.

3.1.4 Challenge test

A standard cosmetic cream with various concentrations of preservatives was purchased through Glostrup Pharmacy, Denmark. The composition of 1000g cream was: 5g polysorbate 80, 50g cetostearyl alcohol, 50g paraffin oil, 60g glycerol monostearate 40-50, 40g glycerol 85%, 70g

sorbitol and 725g water. The criteria for passing the challenge test were set in accordance with the European Pharmacopoeia "5.1.3 Efficacy of antimicrobial preservation for topical preparations" [91], and can be seen in Table 1. Passing the A criteria for bacteria demands a log 2 reduction (99%) of the inoculum after 2 days, a log 3 reduction (99.9%) after 7 days and no increase in CFU/g cream from day 7 to day 28. The B criterion is less strict and demands a 3 log reduction after 14 days and no increase from day 14 to day 28. For fungi the only difference between the A and B criteria is a 2 log or 1 log reduction after 14 days respectively. No increase in CFU/g cream should be found between day 14 and day 28. In the SCCP guidelines for cosmetic products it is not stated which of the criteria should be fulfilled [39].

Table 1: Passing criteria in challenge tests for topical preparations [91]

		Log reduction			
		D2	**D7**	**D14**	**D28**
Bacteria	A	2	3	—	NI
	B	—	—	3	NI
Fungi	A	—	—	2	NI
	B	—	—	1	NI

D: Days, NI: No increase

Each concentration/combination of preservatives was delivered in 5 containers with 50g of cream in each – one for each microorganism and one negative control. Furthermore a cream without preservatives was also included in the challenge test. Each cream was inoculated with a standardised suspension of each microorganism and incubated at 25ºC for 28 days. *S. aureus* and *P. aeruginosa* inoculums were prepared by overnight growth in MH II broth followed by centrifuging the bacteria and resuspension of the pellet in sterile saline. The suspension was adjusted spectrophotometrically at 530 nm to obtain a final inoculation in the cream of $10^5 – 10^6$ CFU/g cream. *C. albicans* followed the same protocol as the bacteria, except that it proliferated for 2 days in RPMI 1640 broth. *A. niger* was grown for 5 days on SAB agar before spores were harvested with sterile saline and a drop of tween 20. The spores were counted in a Bürker Türk chamber and adjusted to a final inoculum of $10^5 – 10^6$ CFU/g cream [91].

The number of CFU/g cream was determined on D0, D2, D7, D14, D21 and D28 for *S. aureus* and *P. aeruginosa*, and on D0, D7, D14, D21 and D28 for *C. albicans* and *A. niger*. 1ml cream was dissolved in 9 ml buffered NaCl-peptone solution (pH 7.0) and further diluted 10-fold in the peptone solution if necessary [92;93]. From the cream/petone solution 0.1ml was dispersed on

Tryptone soya agar (TSA) (OXOID) for bacteria and SAB agar for yeast and fungi. TSA plates were incubated for 1 day at 35°C, and SAB plates were incubated for 1-3 days at 25°C. Duplicate plates were made from each relevant dilution. It was attempted to have between 30 and 300 CFU per plate, which gives the most accurate counts, since CFU <30 is most probably exaggerated by dilution and CFU >300 are too numerous to count. Colonies were counted and CFU/g cream was calculated as the average of the duplicate plates. The number of CFU/g cream after 2 days for the bacteria and 7 days for the fungi was unknown. Hence duplicate plates from 10^{-1} to 10^{-4} cream/peptone solution were prepared for each cream on these test days. On the remaining test days the results from the previous test day were used as a guideline for the expected CFU/g cream and indicated the dilution that should be dispersed on the agar plates. An overview of the concentrations and combinations used in the challenge test are shown in Table 2.

Table 2: concentrations and combinations of preservatives in the challenge test

Phenoxyethanol (%)	Diazolidinyl urea (%)	MCI/MI (ppm)	MI (ppm)
0.8	-	—	—
0.8	0.25	—	—
0.8	0.25	3	—
0.8	0.25	1	—
0.8	0.125	—	—
0.8	0.125	3	—
0.8	0.125	1	—
0.4	—	—	—
0.4	0.25	—	—
0.4	0.25	3	—
0.4	0.25	1	—
0.4	0.125	—	—
0.4	0.125	3	—
0.4	0.125	1	—
0.4	0.1	—	—
0.4	0.1	—	50
0.4	0.05	—	—
0.4	0.05	—	50
0.4	—	3	—
0.4	—	1	—
0.4	—	0.5	—
0.4	—	—	50
0.4	—	—	30
0.4	—	—	15
0.4	—	—	5
0.2	0.1	—	—
0.2	0.1	—	50
0.2	0.05	—	—
0.2	0.05	—	50
0.2	—	3	—
0.2	—	1	—
0.2	—	0.5	—
0.2	—	—	50
0.2	—	—	30
0.2	—	—	15
0.2	—	—	5
—	0.5	—	—
—	0.37	—	—
—	0.25	—	—
—	0.25	3	—
—	0.25	1	—
—	0.125	3	—
—	0.125	1	—
—	0.1	—	50
—	0.05	—	50
—	—	8	—
—	—	6	—
—	—	4	—

3.2 Prevalence and cause of MI contact allergy (Study II)

3.2.1 Study population

From May 2006 to February 2010 all patients (n=2536) patch tested with the European Standard series and a supplementary series which included 2000ppm MI (in aqua) was included in the study. Patch tests were performed using Finn Chambers® (Epitest Ltd) on Scanpor Tape® (Norgesplaster A/S) in accordance with ICDRG recommendation (48 hours occlusion, and reading on D2, D3/4 and D7) [13]. A +, ++ or +++ reaction were considered as a positive reaction. Doubtful (+?), irritant and negative reactions were all considered as negative [14]. Patients with a positive patch test to MCI/MI prior to May 2006 and retested after this were registered as "not tested sensitized" for MI in our database. These patients (n=7) were not included in the study.

The patients' characteristics were registered for all patients in the MOAHLFA index. Furthermore patients with a positive MI patch test had their charts investigated for relevant exposure to MI, and the exact anatomical location of the dermatitis reaction (hands, face, scalp, arms, trunk, legs, feet, universal, other, absent).

3.2.2 Statistics

Data analyses were performed using the SPSS package (SPSS Inc. v.17.0). Differences in the MOAHLFA index between patients with and without MI contact allergy were investigated using the χ^2 test. Development in the trend of MI contact allergy between the different years was tested by χ^2 trend test (linear by linear association). Finally a one-sample Kologorow-Smirnow test was used to test for normal distribution of the age of MI-allergic patients.

3.3 Dose-response relationship in MI-allergic patients (Study III)

3.3.1 Test subject and control subjects.

Since 2005 MI has been part of a supplementary patch test series at Gentofte Hospital in either 1000, 1050, 1500 or 2000ppm (all in aqua), corresponding to 30, 31.5, 45 and 60 µg MI/cm^2 respectively. A total of 459 patients has been patch tested with 1000ppm MI, 410 patients with 1050ppm MI, 494 patients with 1500ppm MI, 375 patients with 2000ppm from Malmö and 2509 patients with 2000ppm from our own laboratory. There have been some overlaps in the concentrations inasmuch as some patients have been patch tested with up to 3 different concentrations of MI. All patients (n=52) with a +, ++ or +++ reaction to at least one of the concentrations were invited to participate in the study. Exclusion criteria were, age <18, eczema on the tested area, exposure to UV light within the preceding 3 weeks, systemic immunosuppressive therapy, pregnancy, breast-feeding and not being able to cooperate. Furthermore, patients with a positive patch test to MCI/MI from 2000 to 2005 were also invited to participate. 50 MCI/MI-allergic patients were invited and 5 agreed to participate. They were patch tested with 2000ppm MI prior to the study. Inclusion and exclusion criteria were the same. A total of 11 test subjects with MI contact allergy participated in the study, 2 women and 9 men, mean age 49.7, range 37-68.

Healthy volunteers were included in the study as a control group. Exclusion criteria were the same as for the test subjects. The control subjects were responders to a post on the website www.forsøgsperson.dk about the study. 14 control subjects participated in the study, 6 women and 8 men, mean age 27.5, range 20-44.

All test subjects and control subjects received written and oral information, signed a written consent form and received compensation for each meeting they attended. The study was performed in conformity with the Helsinki II Declarations and was approved by the local ethics committee (Region Hovedstaden H-2-2010-015)

3.3.2 Patch test

The patch test series consisted of 12 decreasing doses of MI in 10% ethanol and 90% aqua and the same 12 doses of MI combined with 9.26 µg phenoxyethanol/cm^2 in the same vehicle (Table 3).

The phenoxyethanol dose corresponds to a concentration of 0.4%. A control with phenoxyethanol but without MI was also included in the series.

15 µl of each dilution was applied to a filter disc placed in a small Finn Chamber® on Scanpore® tape. The patch test was occluded for 2 days, and reactions were read on D2, D3/D4 and D7. Readings from D3/4 were used in the statistical calculations. The following reading scale of reactions was used: 0 = no reaction; 1 = few papules with no erythema, no infiltration; 2 = faint erythema with no infiltration or papules; 3 = faint erythema with few papules and no homogenous infiltration; 4 = erythema, homogenous infiltration; 5 = erythema, infiltration and a few papules; 6 = erythema, infiltration and papules; 7 = erythema, infiltration, papules and a few vesicles; 8 = intensive erythema, infiltration and vesicles as suggested by Fischer *et al* [23;24]. The lowest concentration (minimum score =1) in a continuous line from 60 µg MI/cm^2 and downwards was defined as the threshold concentration. Patch tests were applied by nurses from the allergy laboratory. The patch tests were randomised and blinded for the reader. An example of reactions in the patch test is shown in Figure 4. The control subjects were only patch tested with one concentration of MI (60 µg MI/cm^2) and the control (0.4% phenoxyethanol).

Table 3: Doses in the patch test and the ROAT

Patch test dilution series* (µg MI/cm^2)	Equivalent ROAT doses (µg MI/cm^2)
60	—
30	—
15	—
8.82	3 weeks accumulated dose (0.21) in the ROAT
4.41	3 weeks accumulated dose (0.105)
2.94	1 week accumulated dose (0.21) in the ROAT
1.47	1 week accumulated dose (0.105) in the ROAT
0.441	3 weeks accumulated dose (0.0105) in the ROAT
0.21	Highest dose per application in the ROAT
0.147	1 week accumulated dose (0.0105) in the ROAT
0.105	Middle dose per application in the ROAT
0.0105	Lowest dose per application in the ROAT

* The same 12 concentrations were also applied with 9.26 µg phenoxyethanol/cm^2.

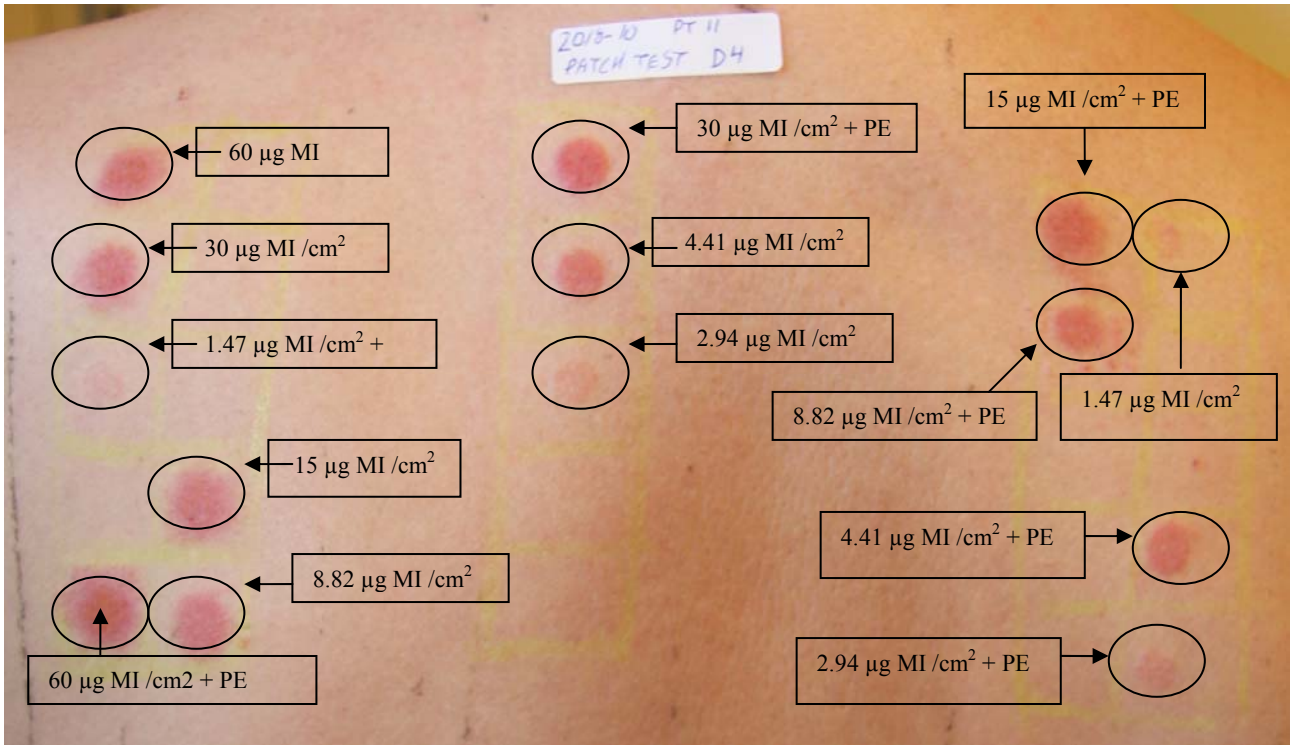


Figure 4: Reactions in the patch test at D4.

3.3.3 ROAT

Each participant in the study, i.e. the test and control subjects, had the volar aspect of their forearms divided into 4 areas of 9cm^2, 2 on each arm. The areas were numbered 1-4. Four bottles (numbered 1-4 corresponding to the number on the forearms), a 20 µl fixed volume pipette (Acura 815, 20 µl, Buch & Holm) and a box of pipette tips were handed out to the participants, and they were instructed thoroughly to apply 20 µl twice a day on each of the areas. Furthermore written instructions were handed out to each of the participants along with a telephone number they could call round the clock. Finally the participants were requested not to use any form of cosmetics on and around the exposed areas throughout the study. The ROAT mimicked a twice-daily use of a cream preserved with 100ppm, 50ppm and 5ppm MI in combination with 0.4% phenoxyethanol. With an exposure of 4.2 mg cream/cm^2/day from COLIPA [94], this corresponded to an exposure per application of 0.21 µg MI/cm^2, 0.105 µg MI/cm^2 and 0.0105 µg MI/cm^2, respectively and 9.26 µg phenoxyethanol/cm^2 in all of them (Table 3). The last bottle was a control containing 9.26 µg phenoxyethanol/cm^2. The vehicle was the same as in the patch test. The content of each bottle was randomised and unknown to the participants and the readers of the reactions. Readings were performed by the same nurse as in the patch tests together with Michael D. Lundov.

The ROAT was initiated on the same day as the patch tests, and reactions were read on D2, D3/D4, D7, D14 and D21 routinely and additionally if a reaction occurred between visits. After 21 days the experiment was terminated. Readings of reactions followed the ROAT reading scale developed by Johansen *et al* [26] and were based on involved area, erythema, number of papules and number of vesicles. If an area scored 5 or above, exposure to this area was terminated. The threshold concentration was the lowest concentration with a score of 5 or above, or the lowest concentration that gave a visible reaction still remaining at D21 if the exposure had not been terminated. As a control 5 sets of ROAT bottles were weighed before and after the 21 exposure days.

3.3.4 Statistics

Differences between the test subjects and the MI-allergic patients not participating were investigated with χ^2-tests with emphasis on reactivity in the diagnostic patch test and the MOAHLFA index.

Dose-response relationships often follow a logistic dose-response curve [23;24]. We used standard logistic regression analysis to estimate the dose-response relationship in the patch tests and the ROAT. The ED which predicts the dose that will elicit a reaction in 5%, 10%, 25%, 50%, 75%, 90% and 95 % of sensitized patients was calculated and a fitted dose-response curve was drawn. Comparison between the patch test reactions with or without phenoxyethanol was performed using the Wilcoxon ranked sums test, and correlations between the individual threshold doses were investigated by Spearman's ranked correlation coefficient. Differences in reactions to the same doses in the patch test and ROAT were investigated using McNemar's test. If the model for converting patch test data to ROAT data is used, 2 conditions have to be fulfilled. First, a positive correlation between the 2 test methods should be ascertained. Second, the dose-response curves have to be parallel. Spearman's ranked correlation was used to analyse the correlation between results from patch tests and ROAT performed on the same patients.

4 Results

4.1 Efficacy of cosmetic preservatives (Study I)

4.1.1 Minimum and fractional inhibitory concentrations

The MIC values determined for each preservative are shown in Table 4. MCI/MI was the only preservative effective against all 4 microorganisms. The other preservatives had MIC values at or above their maximum permitted concentration against 1 of the microorganisms. Phenoxyethanol (MIC=1%) was not effective against *S. aureus*. Diazolidinyl urea (MIC=0.5%) was not effective against *C. albicans*, and MI (MIC>100ppm) was not effective against *A. niger* (Table 4).

Table 4: MIC values of phenoxyethanol, diazolidinyl urea, MCI/MI and MI (n≥3)

	Phenoxyethanol (%)	Diazolidinyl urea (%)	MCI/MI (ppm)	MI (ppm)
Staphylococcus aureus	1	<0.03125	2	45
Pseudomonas aeruginosa	0.4	0.0625	2	15
Aspergillus niger	0.4	0.125	0.5	>100
Candida albicans	0.6	0.5	0.5	65

In Table 5 the mean and range of the obtained FIC values are shown. None of the combinations were antagonistic (FIC≥4), and the majority of the mean FIC values are below or just above 1. One combination (diazolidinyl urea/phenoxyethanol against C. albicans) did in some of the experiments fulfil the ASM demands for synergy (FIC≤0.5), and the mean FIC value was 0.55. The FIC values of some of the combinations could not be calculated owing to the microorganisms' sensitivity to the combinations. *S. aureus* and *P. aeruginosa* were highly susceptible to diazolidinyl urea and MI, respectively (Table 5).

Table 5: Range and mean FIC values in different combinations of preservatives (n≥2)

	C. albicans		*A. niger*		*P. aeruginosa*		*S. aureus*	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
DU* – MCI/MI	0.56 - 1.13	0.88	0.63 - 0.75	0.71	0.53 - 0.75	0.64	ND¶	ND
DU – PE‡	0.33 - 0.75	0.55	0.63 - 1.00	0.79	0.63 - 1.00	0.88	ND	ND
DU – MCI/MI – PE	0.66 - 1.23	0.85	1.00 - 1.75	1.30	0.66 - 1.13	0.93	ND	ND
MCI/MI – PE	0.75 - 1.17	0.98	1.00 - 2.38	1.79	0.50 - 1.00	0.75	0.45 - 1.30	0.88
DU – MI – PE	1.01 – 1.20	1.10	0.85 – 1.10	1.00	ND	ND	ND	ND
DU – MI	1.14 – 1.89	1.43	0.80 – 1.05	0.93	ND	ND	ND	ND
MI – PE	0.78 – 0.95	0.87	ND	ND	ND	ND	0.76 – 1.61	1.21

*DU: Diazolidinyl urea, ‡PE: Phenoxyethanol, ¶ND: not determined

4.1.2 Challenge test

48 different challenge test setups were tested (Table 2). Figure 5 shows the development in CFU/g cream for *C. albicans* and *P. aeruginosa* in creams preserved with different concentrations of diazolidinyl urea. *C. albicans* was inhibited but not killed by the preservative. Hence, it failed to pass the challenge test for all concentrations (Figure 5A). *P. aeruginosa* was eradicated to below the detection limit within 2 days in all concentrations (Figure 5B).

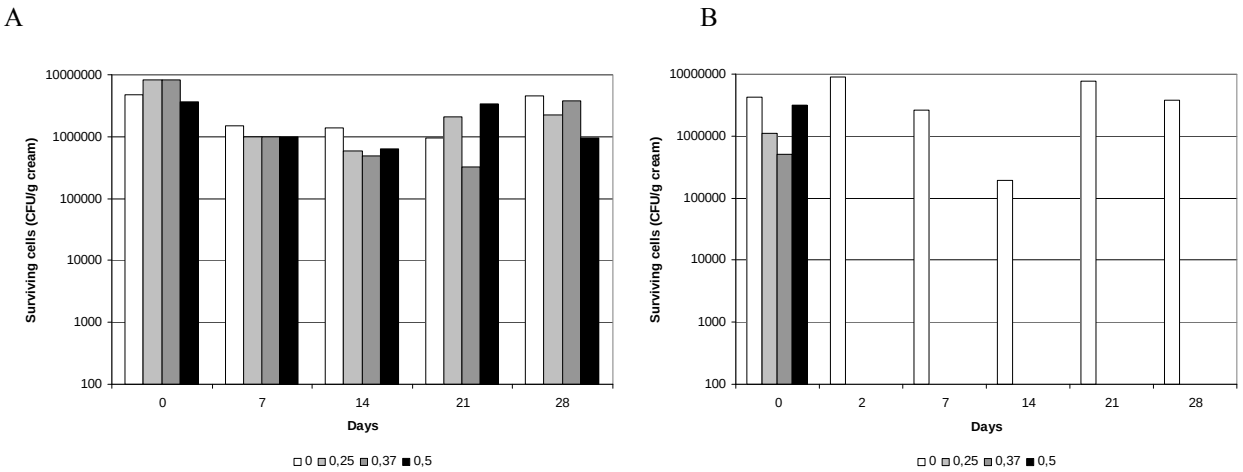


Figure 5: Challenge test result for *C. albicans* (A) and *P. aeruginosa* (B) in a cream with different concentrations (0, 0.25, 0.37 and 0.5 %) of diazolidinyl urea. Missing bars indicates CFU/g cream below the detection limit.

In the cream without preservatives the number of microorganisms remained constant during the 28 days of testing. This indicates inhibition by the cream itself. However, one cream inoculated with *P. aeruginosa* without preservatives was left in the refrigerator for 9 months. The result is shown in Figure 6. The cream clearly does not inhibit growth of *P.* aeruginosa. After 3 months in the refrigerator small green spots could be seen around the edge of the container (Figure 6B), and after 9 months the cream was overgrown with *P. aeruginosa* (Figure 6C). It is noteworthy that this cream was placed in the refrigerator at 4ºC, which slowed the growth of the microorganism. At room temperature or in a warm and moist bathroom faster growth rates can be expected.

Figure 6: A cream without preservatives inoculated with *P. aeruginosa* and left in the refrigerator. A: before the challenge test. B: After 3 months. C: after 9 months.

The highest concentration of diazolidinyl urea tested was 0.5%. This is both the MIC value of *C. albicans* and the maximum permitted concentration. It did not pass the challenge test against *C. albicans* (Table 6 and Figure 5A). 8ppm MCI/MI also failed to pass the challenge test against *C. albicans* (Table 6). This is a concentration 16 times higher than *C. albicans* MCI/MI MIC value (Table 4). When 0.4% and 0.8% of phenoxyethanol were used, 0.8% alone passed the A criteria while 0.4% barely passed the B criteria for both *S. aureus* and *C. albicans* (Table 6). Besides its antimicrobial properties phenoxyethanol is also a surfactant. This resulted in an almost liquid cream when preserved with 0.8% phenoxyethanol.

When the different preservatives were combined with each other, it was possible to lower the effective concentrations by several magnitudes. Table 7 shows challenge test results from combinations with 0.125% diazolidinyl urea, phenoxyethanol (0%, 0.4% and 0.8%) and MCI/MI (0ppm, 1ppm and 3ppm). Diazolidinyl urea (0.125%) combined with either MCI/MI (3ppm) or phenoxyethanol (0.4% and 0.8%) passed the challenge test, while 0.125% diazolidinyl urea combined with 1ppm MCI/MI failed (Table 7).

Table 6: Challenge test results with diazolidinyl urea, MCI/MI and phenoxyethanol

	Diazlidinyl urea (%)			Phenoxyethanol (%)		MCI/MI (ppm)		
	0.25	0.375	0.5	0.4	0.8	4	6	8
Staphylococcus aureus	P^a	P^a	P^a	P^b	P^a	P^a	P^a	P^a
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Aspergillus niger	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Candida albicans	F	F	F	P^b	P^a	F	F	F

P^a= passed level A criteria, P^b = passed level B criteria, F = failed both the A and B criteria of the European Pharmacopoeia [91].

Table 7: Challenge test result with 0.125% diazolidinyl urea in combinations with MCI/MI and phenoxyethanol

	Diazolidinyl urea 0.125%							
	MCI/MI 0ppm		MCI/MI 1ppm			MCI/MI 3ppm		
	Phenoxyethanol		Phenoxyethanol			Phenoxyethanol		
	0.4%	0.8%	0%	0.4%	0.8%	0%	0.4%	0.8%
Staphylococcus aureus	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Aspergillus niger	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Candida albicans	P^a	P^a	F	P^a	P^a	P^a	P^a	P^a

P^a= passed level A criteria, P^b = passed level B criteria, F = failed both the A and B criteria of the European Pharmacopoeia [91].

MI (50ppm) in various combinations with diazolidinyl urea (0%, 0.05% and 0.1%) and phenoxeythanol (0%, 0.2% and 0.4%) was also tested (Table 8). Both combinations without phenoxyethanol failed to preserve the cream while 50ppm MI in combination with phenoxyethanol passed the challenge test (Table 8).

Table 8: Challenge test result with 50ppm MI in combinations with diazolidinyl urea and phenoxyethanol

	Diazolidinyl urea (0%)		Diazolidinyl urea (0.05%)			Diazolidinyl urea (0.1%)		
	Phenoxyethanol (%)		Phenoxyethanol (%)			Phenoxyethanol (%)		
	0.2	0.4	0	0.2	0.4	0	0.2	0.4
Staphylococcus aureus	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Aspergillus niger	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Candida albicans	P^a	P^a	F	P^a	P^a	F	P^a	P^a

P^a= passed level A criteria, P^b = passed level B criteria, F = failed both the A and B criteria of the European Pharmacopoeia [91].

Phenoxyethanol (0.2% and 0.4%) was tested in combination with either diazolidinyl urea (0.05% and 0.1%), MCI/MI (0.5ppm, 1ppm and 3ppm) or MI (5ppm, 15ppm and 30ppm). The results are shown in Table 9 and Table 10. All combinations with 0.4% phenoxyethanol passed the A criteria of the challenge test except *S. aureus* against MCI/MI and MI (Table 9). In combination with 0.2% phenoxyethanol diazolidinyl urea (0.05% and 0.1%) and MCI/MI (0.5ppm) failed the challenge test (Table 10). The remaining combinations all passed either the A or B criteria (Table 10).

Table 9: Challenge test result with 0.4% phenoxyethanol in combination with diazolidinyl urea, MCI/MI or MI

	Diazolidinyl urea (%)		MCI/MI (ppm)			MI (ppm)		
	0.05	0.1	0.5	1	3	5	15	30
Staphylococcus aureus	P^a	P^a	P^b	P^b	P^b	P^b	P^b	P^b
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Aspergillus niger	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Candida albicans	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a

P^a= passed level A criteria, P^b = passed level B criteria, F = failed both the A and B criteria of the European Pharmacopoeia [91].

Table 10: Challenge test result with 0.2% phenoxyethanol in combination with diazolidinyl urea, MCI/MI or MI

	Diazolidinyl urea (%)		MCI/MI (ppm)			MI (ppm)		
	0.05	0.1	0.5	1	3	5	15	30
Staphylococcus aureus	P^a	P^a	P^b	P^b	P^b	P^b	P^b	P^b
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^b	P^b	P^b
Aspergillus niger	F	P^a	P^a	P^a	P^a	P^b	P^b	P^a
Candida albicans	F	F	F	P^a	P^a	P^b	P^b	P^a

P^a= passed level A criteria, P^b = passed level B criteria, F = failed both the A and B criteria of the European Pharmacopoeia [91].

4.2 Prevalence and cause of MI contact allergy (Study II)

4.2.1 Prevalence and characteristics of MI contact allergy

The overall prevalence of MI contact allergy between 2006 and 2010 was 1.5% (37/2536), with no significant (P_{trend}=0.88) increase or decrease in the period (Table 11).

Table 11: Prevalence of MI contact allergy among 2536 dermatitis patients patch tested from May 2006 to February 2010

		MI allergy		
Year	Tested total	total % (n)	men % (n)	women % (n)
2006	416	1.7 (7)	2.1 (3)	1.5 (4)
2007	655	1.1 (7)	1.8 (4)	0.7 (3)
2008	623	1.7 (11)	2.0 (4)	1.7 (7)
2009	749	1.3 (10)	1.3 (3)	1.4 (7)
2010	93	2.2 (2)	4.2 (1)	1.4 (1)
Total	2536	1.5 (37)	1.8 (15)	1.3 (22)

Dermatitis patients with a positive MI patch test were aged 18-81, mean 52.2 (P=0.9, one sample Kologorov-Smirnov test). When differences in the MOAHLFA index between dermatitis patients with a positive MI patch test and the rest of the group were investigated, MI contact allergy proved to be significantly more often associated with occupational dermatitis (P=0.03), hand eczema (P=0.002) and age above 40 (P=0.05) (Table 12). Among the 37 MI-positive patients, occupational dermatitis occurred significantly more often in men than in women (P=0.02). Hand (68%), face (30%), arms (30%) and legs (27%) were the most commonly affected areas in the MI patients. Concomitant reactions to MCI/MI in the MI-positive patients were found in 41% (15/37). Furthermore the MI-positive patients had significantly more frequent concomitant reactions to 3 or more allergens besides MI and MCI/MI (P< 0.001), primarily other preservatives and metals.

Table 12: MOAHLFA index among 2536 patch tested dermatitis patients stratified into MI patch test positives and MI patch test negatives

	MI-positive (n=37), % (n)	MI-negative (n=2499), % (n)	P-value*
Male	41 (15)	33 (812)	0.3
Occupational dermatitis	38 (14)	23 (570)	0.03
Atopic dermatitis	8.1 (3)	17 (429)	0.15
Hand dermatitis	68 (25)	42 (1041)	0.002
Leg ulcers	8.1 (3)	3.7 (93)	0.17
Facial dermatitis	30 (11)	23 (590)	0.39
Age > 40	81 (30)	65 (1632)	0.05

*χ^2-test except in 'Leg ulcers', where Fisher's exact test was used.

4.2.2 Exposure to MI

Relevant exposures to MI were found in 24 of the 37 MI-allergic patients (65%). The exposures were equally distributed between either cosmetics (32%) or occupational sources (30%) (Table 13). 1 patient was exposed to MI from a household product. 9 of the 12 cosmetic exposures were from rinse-off products, of which 7 were hair care products and 2 were liquid soaps. Leave-on products were creams (n=2), cleansing milk (n=1) and a suntan lotion (n=1). 5 of the 11 patients with occupational exposure were painters. 12 of the patients were exposed to MI alone, 10 were exposed to MCI/MI and 2 were exposed to both MCI/MI and MI (Table 13).

Table 13: Exposure to MI among the 37 MI-allergic patients

	Source			Substance(s)*		
	Men	Women	Total	MI	MCI/MI	Both
Occupational	—	—	11	—	—	—
Cleaning	1	1	—	1	1	—
Painters	4	1	—	3	1	1
Others[†]	3	—	—	—	3	—
Unknown	1	—	—	—	—	—
Cosmetics[§]	—	—	12	—	—	—
Leave-on	1	3	—	2	2	—
Rinse-off	4	5	—	5	3	1[$]
Unknown	—	1	—	—	—	—
Household products	—	1	1	1	—	—

*Based on products, ingredient labels or Material Safety Data Sheets brought in by the patients.
[†]Chemical factory, glue factory and cutting oils.
[§]2 patients had used both a leave-on and rinse-off product with either MI or MCI/MI in it.
[$]1 patient had used 2 different rinse-off products, 1 with MI and 1 with MCI/MI.

4.3 Dose-response relationship in MI-allergic patients (Study III)

4.3.1 Description of the test subjects

A total of 52 patients have had a positive patch reaction to at least 1 of the different concentrations of MI included in the patch test series since 2005. In this study 9 of the MI-allergic patients were eligible and agreed to participate in the study. Of the 52 patients with a positive diagnostic patch test for MI, 2 had reacted to 1000ppm (30µg MI/cm^2), 7 had reacted to 1050ppm (31.5 µg MI/cm^2), 4 had reacted to 1500ppm (45 µg MI/cm^2) and 43 had reacted to 2000ppm (60 µg MI/cm^2). 2 of the patients reacted to both 1500ppm and 2000ppm and 1 patient reacted to 1000ppm, 1500ppm and 2000ppm. All 3 of these patients had a + reaction to all concentrations. Of the 52 patients 33 had a + reaction in the diagnostic patch test: 4 of these participated in the study. 18 had a ++ reaction: 5 of these participated in the study. Finally 1 patient had a +++ reaction; this patient did not participate in the study. There were no statistical differences between the participants and non-participants reactions (Fisher's exact test P=0.25, omitting the 1 patient who had a +++ reaction). In the MOAHLFA index the only statistical difference found between the 2 groups was in gender with a higher proportion of men in the test subject group (Fisher's exact test P=0.023).

To include additional MI-allergic patients in the study MCI/MI-allergic patients patch tested prior to 2005 were invited. Approximately 40% of MCI/MI-allergic patients have a concomitant contact allergy to MI (Study I). 5 MCI/MI-allergic patients agreed to participate in the study and were patch tested with 60 µg MI/cm^2. 2 (40%) developed a positive reaction (+, ++ or +++) and were included in the overall study. Finally the study also included 14 healthy control subjects.

4.3.1 Patch test results

The highest concentration tested (60 µg MI/cm^2) elicited a reaction in all test subjects, but in 1 patient the reaction had disappeared at D4. The lowest threshold dose was 1.47 µg MI/cm^2, which more than half (54%) of the patients reacted to. The number and percentage of test subjects who reacted to the different patch test doses are shown in Table 14.

Table 14: Number and % of reactions to the doses in the patch test at day 3/4

	Reactions n (%)	
Patch test dose (µg MI/cm^2)	Without phenoxyethanol	With phenoxyethanol (9.24 µg /cm2)
60	10 (91)	10 (91)
30	10 (91)	10 (91)
15	10 (91)	10 (91)
8.82	10 (91)	10 (91)
4.41	8 (73)	10 (91)
2.94	7 (64)	6 (55)
1.47	6 (55)	6 (55)
0.441	0	0
0.21	0	0
0.147	0	0
0.105	0	0
0.0105	0	0

There were almost no differences in the visual scoring of reactions to the same doses with or without phenoxyethanol. But in 2 doses there were differences in the number of test subjects that reacted to the same dose with or without phenoxyethanol. 8 test subjects reacted to 4.41 µg MI/cm^2 while 10 reacted to the same dose with phenoxyethanol. 7 test subjects reacted to 2.94 µg MI/cm^2 while only 6 reacted to the same dose with phenoxyethanol (Table 14). On the basis of the test subject's reactions in the patch test it is possible to calculate the eliciting dose (ED_{xx}) in xx% of sensitized individuals (Table 15). The different ED_{xx} values are almost the same with or without phenoxyethanol, as can also be seen in the fitted dose-response curves, which are almost identical (Figure 7). The difference between the 2 series was not statistically significant (Wilcoxon's ranked sum test P=0.27). Furthermore there were no differences in the individual threshold doses with or without phenoxyethanol (Spearman's ranked correlation r_s=0.98 p=0.002).

None of the test subjects reacted to the control, and none of the control subjects reacted to MI (60 µg/cm^2) or the control.

Table 15: Calculated elicitation dose (ED) and 95% confidence interval (CI) in the patch test with and without phenoxyethanol

	Without phenoxyethanol		With phenoxyethanol	
	Dose (µg MI/cm2)	95% CI	Dose (µg MI/cm2)	95% CI
ED_5	0.20	0.012 – 0.54	0.23	0.016 – 0.58
ED_{10}	0.35	0.040 – 0.84	0.38	0.048 – 0.88
ED_{25}	0.82	0.20 – 1.8	0.84	0.22 – 1.7
ED_{50}	1.9	0.77 – 5.1	1.8	0.79 – 4.4
ED_{75}	4.4	2.0 – 21	4.0	1.9 – 16
ED_{90}	10	4.1 – 111	8.6	3.8 – 76
ED_{95}	18	6.3 – 362	15	5.6 – 227

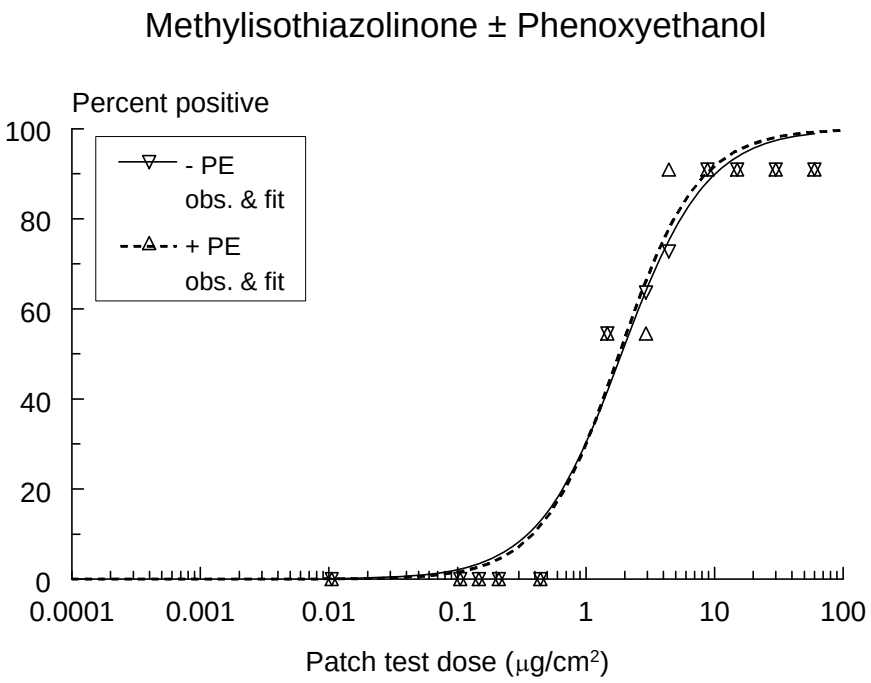


Figure 7: Fitted dose-response curves for MI ± phenoxyethanol.

4.3.2 ROAT

9 of the 11 test subjects followed the application scheme for all 21 days. One patient lost all the equipment and did not receive a new set until 4 days later. He did not develop any reaction to the exposures. 1 patient could only participate for 19 days as he was travelling the last 2 days. He had reacted to the 2 highest doses in the ROAT within the first 10 days, and had no reaction to the lowest dose after 19 days. 7 test subjects (64%) reacted to the highest and middle doses (0.21 µg MI/cm^2 and 0.105 µg MI/cm^2) within the 21 days and 2 (18%) reacted to the lowest dose (0.0105 µg MI/cm^2). None of the test subjects reacted to the control. After 21 days 3 of the 7 test subjects who reacted to the middle dose (0.105 µg MI/cm^2) had a clear visible reaction that scored below 5 points on the evaluation scale [26]; they were all considered positive and included in the calculations. None of the control subjects reacted to any of the doses or the control. Weighing 5 sets of ROAT bottles before and after the experiment showed that the participants had followed the instructions and had been exposed to the calculated values of MI and phenoxyethanol.

4.3.3 Comparison between the patch test and the ROAT

The frequency of reaction to the dose per application in the ROAT and the same dose in the patch test was compared (Table 16). In the highest and middle dose (0.21 and 0.105 µg MI/cm^2) the difference between the patch test and the ROAT was statistically significant (McNemar's test P=0.023).

Table 16: Comparison of the response frequencies in the doses identical in the patch test and the ROAT

Dose (µg MI/cm2)	Patch test response n (%)	ROAT response n (%)	P-values (McNemar's test)
0.21	0 (0)	7 (64)	0.023
0.105	0 (0)	7 (64)	0.023
0.0105	0 (0)	2 (18)	0.48

Figure 8 shows the fitted dose-response curves for both patch test and the ROAT. The ROAT dose-response curve is clearly displaced to the left, but not parallel with the patch test dose-response curve.

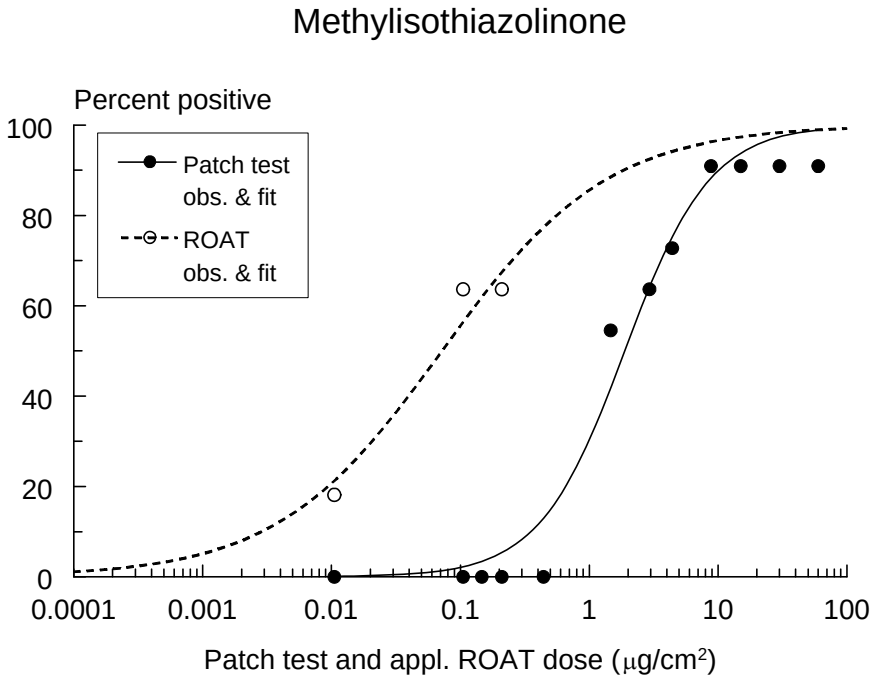


Figure 8: Fitted dose-response curves for patch test and ROAT.

One of the criteria for converting patch test data to ROAT is that the 2 dose-response curves are parallel. This was not the case, but Spearman's ranked correlation showed that the results were still correlated (r_s=0.64 P=0.043). Figure 9 shows the results when the patch test dose-response curve is converted into ROAT with the model (ED_{xx}(ROAT)= 0.0296 x ED_{xx}(patch test)). The calculated and observed ROAT dose-response curves are almost the same (Figure 8 and Figure 9). Based on the results from this study the model for converting patch test data into ROAT data would be:

$$ED_{xx}(ROAT)= 0.0362 x ED_{xx}(patch\ test).$$

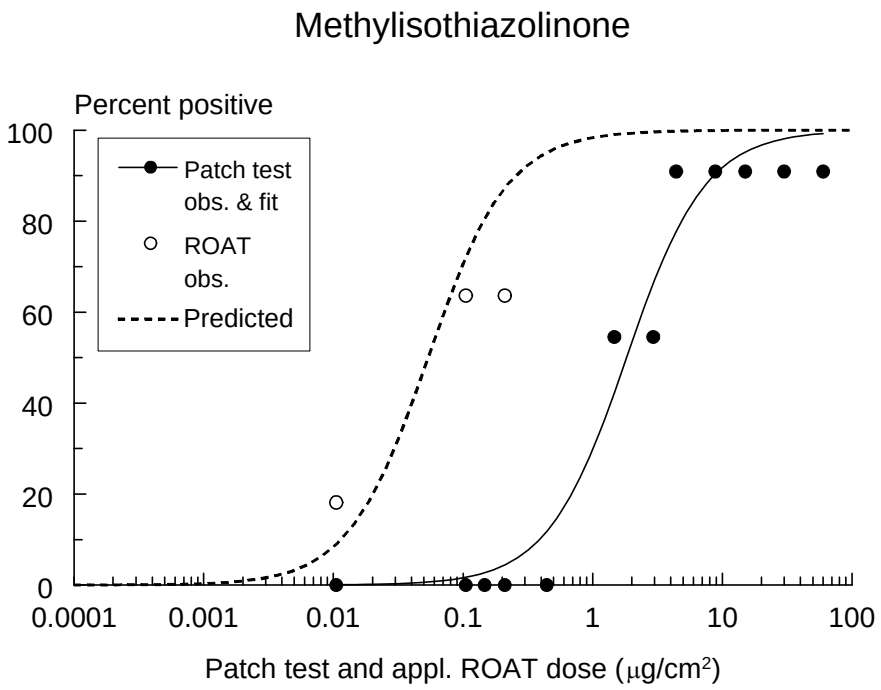


Figure 9: Fitted dose-response curve for patch test and calculated dose-response curve for ROAT based on the conversion model [29].

5 Discussion

5.1 Efficacy of cosmetic preservatives (Study I)

5.1.1 Minimum and fractional inhibitory concentrations

The MIC values found in this study differ in some cases from the MIC values found by the manufacturers [58]. In this study *A. niger* and *C. albicans* were the most susceptible (MIC=0.5ppm) to MCI/MI (Table 4). The manufacturer of MCI/MI reports MIC values of 9ppm and 5ppm, respectively [58]. Regarding the other preservatives the susceptibility of the different microorganisms is identical with that found by the manufacturer, e.g. diazolidinyl urea not being very effective against *C. albicans* and phenoxyethanol with reduced effect against *S. aureus* [95;96]. MI is primarily antibacterial [85], which is confirmed in this study (Table 4).

When various combinations of the preservatives were tested, the combinations were more effective than when the preservatives were used alone. None of the combinations were antagonistic according to the ASM standards [82]. Although none of them passed the ASM demand for synergy (FIC≤0.5), all the mean FIC values were close to 1 (Table 5), which corresponds to additive effects. This means that a combination of 2 preservatives with half of each preservative's MIC-value would still be effective.

Many different preservative combinations are available on the market, but the efficacy of these combinations and their frequency of use are not known. The available scientific information on synergy in preservative combinations is mostly confined to a 1985 review by Denyer *et al* [81]. The review is a collection of results with different preservatives, but many of the tested combinations are not representative of the use of preservatives in cosmetics today. Parabens appear to be synergistic with many different preservatives including phenoxyethanol and diazolidinyl urea [81]. Other studies have investigated MCI/MI or diazolidinyl urea in combinations with preservatives that are not permitted or not very frequently used in cosmetics today [86-88].

5.1.2 Challenge test

The challenge test results clearly showed that the different combinations were more effective than the preservatives used alone. 8ppm MCI/MI failed to pass the challenge test while 1ppm in combination with 0.2% phenoxyethanol or 0.5ppm and 0.4% phenoxyethanol passed the challenge

test (Table 6, Table 9 and Table 10). A 4-week ROAT study with MCI/MI showed that the elicitation threshold for leave-on cosmetics is around 2ppm. MCI/MI is the 9th most commonly used preservative in the US, and use concentrations in leave-on cosmetics from the EU range from 3.6ppm to 15ppm [41;55;57], which is a lot higher than the eliciting threshold for MCI/MI. Currently all cosmetic products in the EU can be preserved with up to 15ppm MCI/MI [37].

Diazolidinyl urea could be reduced 10 times from the maximum permitted concentration of 0.5%, which failed the challenge test, to 0.05%, which in combination with 0.4% phenoxyethanol passed the challenge test (Table 9). Diazolidinyl urea is the 10th most frequently used cosmetic preservative in the US [41]. In a ROAT a cream preserved with 0.05% diazolidinyl urea did not elicit any reaction in formaldehyde-allergic patients. The same cream with 0.3% diazolidinyl urea elicited a reaction in 7 out of 10 formaldehyde allergic patients. Among diazolidinyl urea sensitive patients 9 out of 10 reacted in the same ROAT when exposed to 0.15% [27]. The manufacturer of diazolidinyl urea recommends use concentrations from 0.1% to 0.3% [96]. The use concentrations found in products range from very small quantities and up to the maximum permitted concentration of 0.5% [57;97]. The small concentrations are probably residues from one or more of the ingredients used in the formulations. The most common use concentration appears to be 0.2% to 0.4% [57;97]. Again this is higher than the eliciting threshold.

50ppm MI in combination with diazolidinyl urea (0.1%) failed to preserve the cream, but when combined with 0.2% phenoxyethanol 5ppm MI passed the challenge test (Table 8 and Table 10). The maximum permitted concentration of MI in cosmetics is 100ppm, and the manufacturer recommends use concentrations between 50ppm and 100ppm [37;85]. Use concentrations of MI in cosmetics and frequency of use are not known, but on the basis of the permitted concentration, the recommended concentration and experience with the other preservatives, use concentrations ranging from 50ppm to 100ppm are most likely.

5.2 Prevalence and cause of MI contact allergy (Study II)

5.2.1. Prevalence and characterisation of MI contact allergy

The prevalence of MI contact allergy found (1.5%) is already at the same level or above the prevalence of other preservatives. The trend in preservative contact allergy from Gentofte Hospital is shown in Figure 10. If included in that study, MI would have been the 5th or 4th most prevalent preservative allergen at the same level as the formaldehyde releaser quaternium-15, which is the 4th

most common contact allergen [42]. Contact allergy to at least one preservative has significantly increased from 1985 to 2008, mainly owing to an increase in the number of allergens tested. However, it should be noted that the prevalence of the majority of the preservatives is relatively constant throughout the test years [42]. If MI were included in the study by Thyssen *et al*, the overall burden of preservative contact allergy would be even higher.

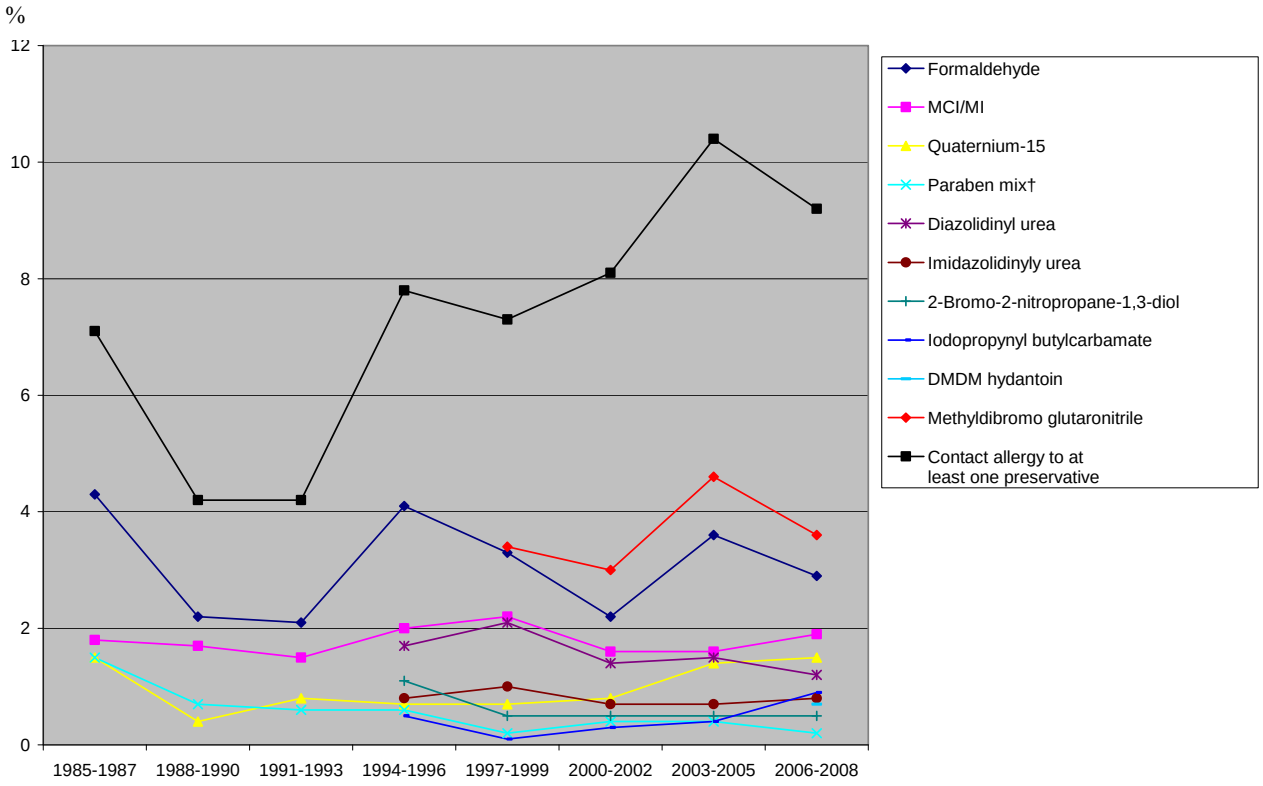


Figure 10: Temporal trends of preservative contact allergy in patch tested dermatitis patients (1985-2008) [42].

MI contact allergy was associated with occupational dermatitis, hand eczema and age above 40. Hand eczema is often associated with occupational dermatitis caused by exposure to irritants, e.g. wet work or occlusion from gloves [98]. The high correlation for hand eczema between the two groups (MI-allergic and non-MI-allergic) can be explained by the exposure pattern to MI found in the MI-allergic patients' medical charts. Exposures were mainly occupational (painters) and from rinse-off cosmetics. Both are associated with hand exposures.

Among the MI-allergic patients occupational dermatitis occurred significantly more often in men than in women. This distribution is also found in MCI/MI-allergic patients and indicates occupational exposures [17]. MI is a relatively new preservative and there is not much available information on the use of MI in industrial products. We know that MCI/MI is used in many

different products such as paint, glue, lacquers and printing ink [38]. It is plausible that MI has replaced MCI/MI in some products or is used together with MCI/MI. Paint products are often preserved with MI in combination with benzisothiazolinone and in some cases residues of MCI/MI used as a preservative in ingredients used in the final product. Use concentration of MI in paint ranges from 113 to 270ppm MI (personal communication from paint manufacturers). The frequent use of MI in paint is also seen from the fact that almost half of the MI-patients with occupational dermatitis were painters. Beside paint and paint-related products cosmetic rinse-off products were a major cause of relevant MI exposure. This indicates that the main type of cosmetic products preserved with MI is rinse-off products, primarily hair care products. The fact that rinse-off product constitute a large proportion of relevant exposure is noteworthy since the total applied and retained exposure to rinse-off products is a lot smaller than for leave-on products. This indicates that MI is a very potent allergen, and that its use in both cosmetic and industrial products should be followed closely.

Concomitant reactions to MCI/MI were seen in 40% of the MI-allergic patients. The patients with a concomitant reaction to MCI/MI could either have reacted to MI in the MCI/MI patch test or reacted to MCI as well. In a previous study patch test reactions to concentrations as low as 10ppm and 30ppm MI were seen [68;70]. The concentration of MI in the 100ppm MCI/MI patch test is 25ppm. There was no coherence between reactions to low doses (1.47 μg MI/cm^2, 49ppm) in the dose-response patch test and MCI/MI contact allergy. MI-allergic patients who reacted to the lowest dose in the patch test were not all MCI/MI-allergic and vice-versa. In an animal study it was shown that when MI was the primary sensitizer cross-reactivity to MCI was seen in some cases [62]. In this study it is not known what causes the concomitant reactions.

Besides high levels of concomitant MCI/MI contact allergy the MI-allergic patients were often polysensitized, especially to other preservatives and metals. A study on contact allergy to multiple preservatives without cross-reactivity potential has recently been published [99]. Polysensitization is probably due to a higher exposure to contact allergens since the sensitization threshold seems to be identical for polysensitized, monosensitized and controls, while the eliciting threshold could be lower in polysensitized patients [100]. The MI-allergic patients were also significantly older than the non-MI-allergic (age >40), which is also an indication of a longer lifetime exposure to allergens and hence an increase in prevalence of contact allergy with age [101].

5.3 Dose-response relationship in MI-allergic patients (Study III)

The MET in this study was 1.47 µg MI/cm^2 (49ppm). This dose elicited reactions in 54% of the test subjects. The next dose in the dilution series (0.441 µg MI/cm^2, 15ppm) did not elicit any reaction. Patch test reactions to 10ppm and 30ppm have been published previously [68;70].

All test subjects reacted to the highest dose applied (60µg MI/cm^2), but 1 test subject's reaction had disappeared by day 4. None of the control subjects had any irritant or allergic reactions to the tested dose (60 µg Mi/cm^2). This indicates that 60 µg MI/cm^2 (2000ppm) is the most suitable dose for diagnostic patch testing with MI.

Phenoxyethanol is a rare sensitizer with a prevalence of contact allergy of 0.2% [43]. It did not influence the reactivity in the patch test, and no subjects reacted to the control in the patch test or ROAT, which both contained phenoxyethanol. The fact that no reactions to the controls were seen, and that there were no differences in the patch test reactions with and without phenoxyethanol shows that the allergic reactions were caused by MI.

In the ROAT 2 (18%) test subjects reacted to the lowest dose (0.0105 µg MI/cm^2) while 7 (64%) reacted to the other 2 tested doses (0.105 and 0.21 µg MI/cm^2). The ROAT was designed to mimic usage of a cream preserved with 100ppm, 50ppm and 5ppm MI combined with 0.4% phenoxyethanol. As mentioned above, use concentrations in the range of 50ppm to 100ppm are very likely.

When the patch test reactions and the ROAT reactions were compared, the fitted dose-response curves were visually different (Figure 8). The ROAT results shifted to the left, which indicated that when exposed repeatedly MI-allergic patients reacted to lower doses than those used in the patch test. The 2 fitted curves were not parallel with each other, which is one of the criteria for fulfilling the conditions set for the model for converting patch test data to ROAT data [29]. However, the 2 curves were still correlated, and when calculated the conversion factor in this study was 0.0362. In the model suggested by Fischer *et al* the conversion factor is 0.296. This is based on 2 different studies with nickel and MDBGN, and the independent conversion factors for these 2 compounds were 0.0330 and 0.0265, respectively [29]. The factor of 0.0362 found in this study is fairly close to those for nickel and MDBGN when some of the differences between the studies are taken into consideration. The study setup was different and fewer patients participated in this study. However, it was not the main purpose of this study to validate the conversion model.

5.4 Methodological considerations

5.4.1 MIC and FIC determinations

The MIC values found in this study differed in some cases from the MIC values stated by the manufacturer or found in other studies [58;85;86;88;95]. There are several factors that influence MIC values. The size of the inoculum, incubation time, growth media and tested isolates are some of the more important factors [75;76]. Not all of this information is available for the MIC values stated by the manufacturer [58;85;95]. In this study we choose to use standardised protocols from CLSI [77-79]. This ensured that the only major difference in the MIC tests is the concentration of antimicrobial, thus enabling comparison between antimicrobials and microorganisms.

FIC values were determined according to ASM's recommendations [82], but still with the standardised protocols from CLSI. According to ASM, FIC≤0.5 =synergy, FIC>0.5 and ≤4 =additive effects and FIC≥4 =antagonism [82]. Others suggest that FIC<1 =synergy, FIC=1 =additive effects and FIC>1 =antagonism [102]. However, this narrow range is not suitable when testing several and low concentrations. In a combination of 2 or more preservatives a low concentration of one of the preservatives might not have any effect on the MIC value of the other preservative. This results in a FIC value >1 (=antagonism) if the narrow range is used. A wider range in FIC thresholds ensures that the calculated FIC values are based on the interactions between the combined preservatives.

5.4.2 Challenge test

The different combinations of preservatives found effective in this study are not guaranteed to work in other formulations and products. There are several factors that influence the preservative's stability and efficacy in cosmetic formulations [103]. Some ingredients added for other purposes are also antimicrobial or enhance the antimicrobial efficacy of preservative, e.g. essential oils or chelating agents (EDTA) [74;103]. The different effects exerted by the physicochemical composition of the product and the ingredients in the formulation on the efficacy of preservatives are one of the main reasons why all products must pass a challenge test prior to marketing. Even small alterations in the formula can change the efficacy of the added preservatives. In this study we included a cream without preservatives in each set of challenge tests to ensure that the reduction in CFU was caused by the added preservatives and not by the formulation itself. We did not detect a significant increase or decrease in the number of CFU in the cream without preservatives in the test period, but as

shown in Figure 6 extensive growth of *P. aeruginosa* occurred within 3 and 9 months of storage in the refrigerator.

The EU guidelines for safe cosmetics do not recommend any specific challenge test or pass/fail demands [39]. However they do require that *P. aeruginosa*, *S. aureus* and *C. albicans* are part of the challenge test, but the exact setup for the challenge test is decided by the manufacturer. We chose to use the challenge test setup from the European Pharmacopoeia and included *A. niger* as recommended by the European Pharmacopoeia [91]. These 4 microorganisms cover Gram-negative and Gram-positive bacteria, yeast and mould. They are all potential pathogens and representative of some of the most frequent contaminants of cosmetic products [32;104-106]. We also chose that the B criteria which are more easily achieved than the A criteria should be sufficient for passing the challenge test [91]. In the European Pharmacopoeia the B criteria are accepted if there is an increased risk of adverse reactions [91]. This could for instance be contact allergy. Our intention was to show the efficacy of the preservatives using a test with the minimum requirements that would still be accepted by the EU on the basis of the EU's own recommendations [39]. Alterations to the challenge test could be inoculation with mixed cultures instead of pure cultures, re-inoculation during the challenge test or use of microorganisms isolated from cosmetics [107;108].

5.4.3 Patch test and ROAT

A reaction to a dose of 0.105 µg MI/cm^2 which mimicked the use of a cream preserved with 50ppm MI does not necessarily mean that the patient will react to a product preserved with 50ppm or higher. The exposure is calculated based on an average consumer exposure model for a hand cream by COLIPA [94]. This was considered the most suitable exposure model. Use of more or less cream would of course change the reaction pattern. Finally the vehicle also influences the reactivity in already sensitized individuals [109]. The vehicle used (ethanol-aqua) was chosen for several reasons. We wanted to be able to compare patch test results with ROAT, and therefore identical vehicles in both were required. Aqua-ethanol is used in the model for conversion from patch test to ROAT [29], and aqua is the vehicle for MI patch test [43;70]. Comparison between previous and future studies is also desirable. Furthermore the exact exposure can be more precisely measured when applied with a fixed volume pipette than with a cream.

As in the dose-response studies by Fischer *et al* [23;24] we also chose to include weak reactions in both the patch test and the ROAT. An irritant reaction could be interpreted as an allergic reaction, but all

threshold values were based on a reaction to the lowest dose in a line of reactions from 60 µg MI/cm^2 and downwards, and we did not see any gaps in the line of reactions in the test subjects. In the original ROAT evaluation scheme only reactions above 5 points are considered positive [26]. In the studies by Fischer *et al* all reactions were included as positive [23;24]. At day 21 in the ROAT 3 patients had a clear positive reaction to 0.105 µg MI/cm^2 but did not score 5 points. These were considered positive. All patch test and ROAT reactions were read blinded by a trained nurse from the allergy laboratory. 9 previously diagnosed MI-allergic patients participated in the study. Besides a preponderance of men they were representative for all the MI-allergic patients. The two MCI/MI-allergic patients patch tested positive for MI allergy were included to get as many participant as possible. The number of test subjects is low and this affects the calculated ED values, which all have large confidence intervals. It was, however, not possible to recruit any more patients to the study.

6 Conclusions and future perspectives

Combinations of preservatives were more effective in preserving a standard cosmetic cream than the preservatives used alone. Combinations of preservatives could reduce the use concentrations of the individual preservatives and thereby potentially reduce the risk of sensitization and elicitation of allergic contact dermatitis. Especially concerning the allergenic preservatives MCI/MI, diazolidinyl urea and MI manufacturers of cosmetics should be aware that use concentrations of preservatives could be reduced by combining preservatives. This could be achieved either by legislation in the EU (e.g. lowering the maximum permitted concentrations) or voluntarily by the industry (e.g. through recommendations by COLIPA).

MI is a relatively new preservative but the prevalence of contact allergy is already higher than many other preservatives which have been on the market for more than 25 years. Both cosmetics and industrial products caused MI contact allergy and the development in contact allergy prevalence should be closely followed. When exposed to different doses of MI equivalent to the assumed use concentrations the majority of MI-allergic patients developed allergic reactions. Some also reacted to a very low dose of MI. We suggest that the permitted concentration of MI in cosmetics should be reduced, and that use of MI in industrial product should be restricted. If the prevalence of MI contact allergy increases without regulation of the permitted concentrations, there is a considerable risk that MI will cause an epidemic of contact allergy and eventually need to be banned from cosmetic products.

7 References

(1) Rustenmeyer T., van Hoogstraten I.M.W., von Blomberg B.M.E., Scherer S. Mechanisms in Allergic Contact Dermatitis. In: Frosch PJ, Menné T, Lepoittevin J-P, editors. Contact Dermatitis. 4th ed. 2006. p. 11-43.

(2) Johansen J.D., Frosch P.J., Menné T. Allergic Contact Dermatitis in Humans - Experimental and Quantitative Aspects. In: Frosch PJ, Menné T, Lepoittevin J-P, editors. Contact Dermatitis. 4th ed. 2006. p. 189-98.

(3) Friedmann P.S. The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. Br J Dermatol 2007: 157(6):1093-102.

(4) Thyssen J.P., Uter W., Schnuch A., Linneberg A., Johansen J.D. 10-year prevalence of contact allergy in the general population in Denmark estimated through the CE-DUR method. Contact Dermatitis 2007: 57(4):265-72.

(5) Thyssen J.P., Linneberg A., Menne T., Nielsen N.H., Johansen J.D. Contact allergy to allergens of the TRUE-test (panels 1 and 2) has decreased modestly in the general population. Br J Dermatol 2009: 161(5):1124-9.

(6) Uter W., Ramsch C., Aberer W., Ayala F., Balato A., Beliauskiene A., Fortina A.B., Bircher A., Brasch J., Chowdhury M.M., Coenraads P.J., Schuttelaar M.L., Cooper S., Corradin M.T., Elsner P., English J.S., Fartasch M., Mahler V., Frosch P.J., Fuchs T., Gawkrodger D.J., Gimenez-Arnau A.M., Green C.M., Horne H.L., Jolanki R. et al. The European baseline series in 10 European Countries, 2005/2006--results of the European Surveillance System on Contact Allergies (ESSCA). Contact Dermatitis 2009: 61(1):31-8.

(7) de Groot A.C., Bruynzeel D.P., Bos J.D., van der Meeren H.L., van J.T., Jagtman B.A., Weyland J.W. The allergens in cosmetics. Arch Dermatol 1988: 124(10):1525-9.

(8) Nielsen N.H., Linneberg A., Menné T., Madsen F., Frolund L., Dirksen A., Jorgensen T. Allergic contact sensitization in an adult Danish population: two cross-sectional surveys eight years apart (the Copenhagen Allergy Study). Acta Derm Venereol 2001: 81(1):31-4.

(9) Goossens A., Beck M.H., Haneke E., McFadden J.P., Nolting S., Durupt G., Ries G. Adverse cutaneous reactions to cosmetic allergens. Contact Dermatitis 1999: 40(2):112-3.

(10) Thyssen J.P., Johansen J.D., Menné T. Contact allergy epidemics and their controls. Contact Dermatitis 2007: 56(4):185-95.

(11) Cvetkovski R.S., Rothman K.J., Olsen J., Mathiesen B., Iversen L., Johansen J.D., Agner T. Relation between diagnoses on severity, sick leave and loss of job among patients with occupational hand eczema. Br J Dermatol 2005: 152(1):93-8.

(12) Anon. Arbejdsmarkedets Erhvervssygdomssikring, Årsrapport 2009. 2010 Apr 16.

(13) Wahlberg J.E., Lindberg M. Patch Testing. In: Frosch PJ, Menné T, Lepoittevin JP, editors. Contact Dermatitis. 4th ed. Springer: 2006. p. 365-90.

(14) Wilkinson D.S., Fregert S., Magnusson B., Bandmann H.J., Calnan C.D., Cronin E., Hjorth N., Maibach H.J., Malalten K.E., Meneghini C.L., Pirila V. Terminology of contact dermatitis. Acta Derm Venereol 1970: 50(4):287-92.

(15) Bruze M., Goossens A., Gruvberger B. Recommendation to include methyldibromo glutaronitrile in the European standard patch test series. Contact Dermatitis 2005: 52(1):24-8.

(16) de Groot A.C. Patch Testing. Test Concentration and Vehicles for 4350 chemicals. 3rd ed. Wapserveen: acdegroot publishing; 2008.

(17) Schnuch A., Geier J., Uter W., Frosch P.J., Lehmacher W., Aberer W., Agathos M., Arnold R., Fuchs T., Laubstein B., Lischka G., Pietrzyk P.M., Rakoski J., Richter G., Rueff F. National rates and regional differences in sensitization to allergens of the standard series. Population-adjusted frequencies of sensitization (PAFS) in 40,000 patients from a multicenter study (IVDK). Contact Dermatitis 1997: 37(5):200-9.

(18) Nardelli A., Carbonez A., Ottoy W., Drieghe J., Goossens A. Frequency of and trends in fragrance allergy over a 15-year period. Contact Dermatitis 2008: 58(3):134-41.

(19) Friedmann P.S., Moss C., Shuster S., Simpson J.M. Quantitative relationships between sensitizing dose of DNCB and reactivity in normal subjects. Clin Exp Immunol 1983: 53(3):709-15.

(20) Scott A.E., Kashon M.L., Yucesoy B., Luster M.I., Tinkle S.S. Insights into the quantitative relationship between sensitization and challenge for allergic contact dermatitis reactions. Toxicol Appl Pharmacol 2002: 183(1):66-70.

(21) Nielsen E., Ostergaard G., Larsen J.C. Sensitization. Toxicological Risk Assessment of Chemicals: A practical Guide. 1 ed. New York: Informa Healthcare USA: 2008. p. 118-24.

(22) Menne T., Wahlberg J.E. Risk assessment failures of chemicals commonly used in consumer products. Contact Dermatitis 2002: 46(4):189-90.

(23) Fischer L.A., Johansen J.D., Menne T. Nickel allergy: relationship between patch test and repeated open application test thresholds. Br J Dermatol 2007: 157(4):723-9.

(24) Fischer L.A., Johansen J.D., Menne T. Methyldibromoglutaronitrile allergy: relationship between patch test and repeated open application test thresholds. Br J Dermatol 2008: 159(5):1138-43.

(25) Hannuksela M., Salo H. The repeated open application test (ROAT). Contact Dermatitis 1986: 14(4):221-7.

(26) Johansen J.D., Bruze M., Andersen K.E., Frosch P.J., Dreier B., White I.R., Rastogi S., Lepoittevin J.P., Menne T. The repeated open application test: suggestions for a scale of evaluation. Contact Dermatitis 1998: 39(2):95-6.

(27) Zachariae C., Hall B., Cottin M., Cupferman S., Andersen K.E., Menné T. Experimental elicitation of contact allergy from a diazolidinyl urea-preserved cream in relation to anatomical region, exposure time and concentration. Contact Dermatitis 2005: 53(5):268-77.

(28) Zachariae C., Lerbaek A., McNamee P.M., Gray J.E., Wooder M., Menné T. An evaluation of dose/unit area and time as key factors influencing the elicitation capacity of methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) in MCI/MI-allergic patients. Contact Dermatitis 2006: 55(3):160-6.

(29) Fischer L.A., Voelund A., Andersen K.E., Menné T., Johansen J.D. The dose-response relationship between the patch test and ROAT and the potential use for regulatory purposes. Contact Dermatitis 2009: 61(4):201-8.

(30) Orth D.S. Basic Microbiology and Overview of Cosmetic Microbiology. Insights into Cosmetic Microbiology. 1 ed. Carol Sream, IL: Allured Books: 2010. p. 1-27.

(31) Brannan D.K. Cosmetic preservation. Journal of the Society of Cosmetic Chemists 1995: 46(4):199-220.

(32) Lundov M.D., Zachariae C. Recalls of microbiologically contaminated cosmetics in EU from 2005 to May 2008. Int J Cosmet Sci 2008: 30(6):471-4.

(33) Wong S., Street D., Delgado S.I., Klontz K.C. Recalls of foods and cosmetics due to microbial contamination reported to the U.S. Food and Drug Administration. J Food Prot 2000: 63(8):1113-6.

(34) Molina-Cabrillana J., Bolanos-Rivero M., varez-Leon E.E., Martin Sanchez A.M., Sanchez-Palacios M., Alvarez D., Saez-Nieto J.A. Intrinsically contaminated alcohol-free mouthwash implicated in a nosocomial outbreak of Burkholderia cepacia colonization and infection. Infect Control Hosp Epidemiol 2006: 27(11):1281-2.

(35) Alvarez-Lerma F., Maull E., Terradas R., Segura C., Planells I., Coll P., Knobel H., Vazquez A. Moisturizing body milk as a reservoir of Burkholderia cepacia: outbreak of nosocomial infection in a multidisciplinary intensive care unit. Crit Care 2008: 12(1):R10.

(36) Fransway A.F. The problem of preservation in the 1990s: I. Statement of the problem, solution(s) of the industry, and the current use of formaldehyde and formaldehyde-releasing biocides. Am J Contact Dermatitis 1991: 2(1):6-23.

(37) Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (Consolidated version), European Commission, (2008).

(38) Flyvholm M.A. Preservatives in registered chemical products. Contact Dermatitis 2005: 53(1):27-32.

(39) The SCCP's Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation 6th revision. 2006 Dec 1.

(40) Orton D.I., Wilkinson J.D. Cosmetic allergy: incidence, diagnosis, and management. Am J Clin Dermatol 2004: 5(5):327-37.

(41) Steinberg D.C. Voluntary Registration of Cosmetics and 2007 Frequency of Preservative Use. Cosmetics and Toiletries® magazine 2008: 123(10):47-52.

(42) Thyssen J.P., Engkilde K., Lundov M.D., Carlsen B.C., Menné T., Johansen J.D. Temporal trends of preservative allergy in Denmark (1985-2008). Contact Dermatitis 2010: 62(2):102-8.

(43) Schnuch A., Uter W., Lessmann H., Geier J. Contact allergy to preservatives. Results of the Information Network of Departments of Dermatology (IVDK) 1996-2007. Interdisciplinary Journal of Allergy and Environmental Medicine 2008: 17:631-8.

(44) Wilkinson J.D., Shaw S., Andersen K.E., Brandao F.M., Bruynzeel D.P., Bruze M., Camarasa J.M., Diepgen T.L., Ducombs G., Frosch P.J., Goossens A., Lachappelle J.M., Lahti A., Menné T., Seidenari S., Tosti A., Wahlberg J.E. Monitoring levels of preservative sensitivity in Europe. A 10-year overview (1991-2000). Contact Dermatitis 2002: 46(4):207-10.

(45) Zug K.A., Warshaw E.M., Fowler J.F., Jr., Maibach H.I., Belsito D.L., Pratt M.D., Sasseville D., Storrs F.J., Taylor J.S., Mathias C.G., DeLeo V.A., Rietschel R.L. Patch-test results of the North American Contact Dermatitis Group 2005-2006. Dermatitis 2009: 20(3):149-60.

(46) Flyvholm M.A., Andersen P. Identification of formaldehyde releasers and occurrence of formaldehyde and formaldehyde releasers in registered chemical products. Am J Ind Med 1993: 24(5):533-52.

(47) de Groot A.C., Flyvholm M.A., Lensen G., Menné T., Coenraads P.J. Formaldehyde-releasers: relationship to formaldehyde contact allergy. Contact allergy to formaldehyde and inventory of formaldehyde-releasers. Contact Dermatitis 2009: 61(2):63-85.

(48) de Groot A.C., White I.R., Flyvholm M.A., Lensen G., Coenraads P.J. Formaldehyde-releasers in cosmetics: relationship to formaldehyde contact allergy. Part 2. Patch test relationship to formaldehyde contact allergy, experimental provocation tests, amount of formaldehyde released, and assessment of risk to consumers allergic to formaldehyde. Contact Dermatitis 2010: 62(1):18-31.

(49) Lundov M.D., Johansen J.D., Carlsen B.C., Engkilde K., Menne T., Thyssen J.P. Formaldehyde exposure and patterns of concomitant contact allergy to formaldehyde and formaldehyde-releasers. Contact Dermatitis 2010: 63(1):31-6.

(50) Opinion of The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Concerning Methyldibromo Glutaronitrile Colipa n P77 (SCCNFP/0585/02). 2002 Jun 4.

(51) The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers Opinion Conserning Methyldibromo Glutaronitrile Colipa n P77 (SCCNFP/0806/04). 2004 Apr 23.

(52) Scientific Committee on Consumer Products SCCP Opinion on Methyldibromo glutaronitrile (sensitisation only) COLIPA n 77 (SCCP/0863/05). 2005 Mar 15.

(53) Scientific Committee on Consumer Products SCCP Opinion on Methyldibromo glutaronitrile (sensitisation only) COLIPA n 77 (SCCP/1013/06). 2006 Jun 20.

(54) Johansen J.D., Veien N., Laurberg G., Avnstorp C., Kaaber K., Andersen K.E., Paulsen E., Sommerlund M., Thormann J., Nielsen N.H., Vissing S., Kristensen O., Kristensen B., Agner T., Menne T. Decreasing trends in methyldibromo glutaronitrile contact allergy--following regulatory intervention. Contact Dermatitis 2008: 59(1):48-51.

(55) Rastogi S.C. Analytical control of preservative labelling on skin creams. Contact Dermatitis 2000: 43(6):339-43.

(56) Rastogi S.C. A survey of formaldehyde in shampoos and skin creams on the Danish market. Contact Dermatitis 1992: 27(4):235-40.

(57) Gruvberger B., Bruze M., Tammela M. Preservatives in moisturizers on the Swedish market. Acta Derm Venereol 1998: 78(1):52-6.

(58) ROHM and HAAS. KATHON^{tm} CG A safe, effective, globally approved preservative for rinse-off products. 2006; http://www.rohmhaas.com/assets/attachments/business/pcare/kathon_for_personal_care/kathon_cg/tds/kathon_cg.pdf

(59) de Groot A.C., Liem D.H., Weyland J.W. Kathon CG: cosmetic allergy and patch test sensitization. Contact Dermatitis 1985: 12(2):76-80.

(60) de Groot A.C., Liem D.H., Nater J.P., van Ketel W.G. Patch tests with fragrance materials and preservatives. Contact Dermatitis 1985: 12(2):87-92.

(61) Bruze M., Dahlquist I., Fregert S., Gruvberger B., Persson K. Contact allergy to the active ingredients of Kathon CG. Contact Dermatitis 1987: 16(4):183-8.

(62) Bruze M., Fregert S., Gruvberger B., Persson K. Contact allergy to the active ingredients of Kathon CG in the guinea pig. Acta Derm Venereol 1987: 67(4):315-20.

(63) Basketter D.A., Gilmour N.J., Wright Z., Walters T., Boman A., Lidén C. Biocides: Characterization of the Allergenic Hazard of Methylisothiazolinone. Cutaneous and Ocular Toxicology 2003: 22(4):187-99.

(64) Rastogi S.C. Kathon CG and cosmetic products. Contact Dermatitis 1990: 22(3):155-60.

(65) [Anon]. Final Report on the Safety Assessment of Methylisothiazolinone and Methylchloroisothiazolinone. Journal of the American College of Toxicology 1992: 11(1):75-128.

(66) SCCS (Scientific Committee on Consumer Safety), Opinon on the mixture of 5-chloro-2-methylisothiazolin-3(2H)-one and 2-methylisothiazolin-3(2H)-one. 2009 Dec 8.

(67) SPIN, Substances in Preparations In Nordic Countries. 2010 April 9; http://195.215.251.229/Dotnetnuke/

(68) Isaksson M., Gruvberger B., Bruze M. Occupational contact allergy and dermatitis from methylisothiazolinone after contact with wallcovering glue and after a chemical burn from a biocide. Dermatitis 2004: 15(4):201-5.

(69) Thyssen J.P., Sederberg-Olsen N., Thomsen J.F., Menné T. Contact dermatitis from methylisothiazolinone in a paint factory. Contact Dermatitis 2006: 54(6):322-4.

(70) Garcia-Gavin J., Vansina S., Kerre S., Naert A., Goossens A. Methylisothiazolinone, an emerging allergen in cosmetics? Contact Dermatitis 2010: 63(2):96-101.

(71) Nielsen H. Occupational exposure to isothiazolinones. A study based on a product register. Contact Dermatitis 1994: 31(1):18-21.

(72) Isaksson M., Bruze M., Gruvberger B. Cross-reactivity between methylchloroisothiazolinone/methylisothiazolinone, methylisothiazolinone, and other isothiazolinones in workers at a plant producing binders for paints and glues. Contact Dermatitis 2008: 58(1):60-2.

(73) Alexander B.R. An assessment of the comparative sensitization potential of some common isothiazolinones. Contact Dermatitis 2002: 46(4):191-6.

(74) Steinberg D.C. Preservatives for Cosmetics. 2nd ed. Carol Stream, IL: Allured Publishing Corporation; 2006.

(75) Madigan M.T., Martinko J.M., Parker J. Microbial Growth Control. Brock Biology of Microorganisms. 10th ed. Upper Saddle River, NJ 07458: Pearson Education: 2003. p. 695-726.

(76) Schuurmans J.M., Nuri Hayali A.S., Koenders B.B., ter Kuile B.H. Variations in MIC value caused by differences in experimental protocol. J Microbiol Methods 2009: 79(1):44-7.

(77) NCCLS. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standards - Sixth Edition. NCCLS Document M7-A6. 2003. Report No.: 6th.

(78) NCCLS. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. NCCLS document M38-A. 2002.

(79) NCCLS. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standards - Second Edition. NCCLS document M27-A2. 2002. Report No.: 2nd.

(80) Kuper K.M., Boles D.M., Mohr J.F., Wanger A. Antimicrobial susceptibility testing: a primer for clinicians. Pharmacotherapy 2009: 29(11):1326-43.

(81) Denyer S.P., Hugo W.B., Harding V.D. Synergy in preservative combinations. Int J Pharm 1985: 25(3):245-53.

(82) Moody J. Synergism Testing: Broth Microdilution Checkerboard and Broth Microdilution Methods. In: Isenberg HD, editor. Clinical Microbiology Procedures Handbook. 2nd ed. Washington DC: ASM Press: 2004. p. 5.12.1-5.12.23.

(83) ROHM and HAAS. NEOLONE^{tm} PE A safe, Broad Spectrum Preservative for Leave-on Products Based on Methylisothiazolinone and Phenoxyethanol. 2006; http://www.rohmhaas.com/assets/attachments/business/pcare/neolone/neolone_pe/tds/neolone_pe.pdf

(84) Germaben® II-E. 2008; http://online1.ispcorp.com/en-US/Pages/ProductDetail.aspx?BU=Personal%20Care&l1=Trade%20Name&l2=Germaben®&prodName=Germaben®%20II-E&prdId=20003

(85) ROHM and HAAS. NEOLONE^{tm} 950 A safe, Broad Spectrum Bactericide for the Preservation of Leave-on Products. 2006; http://www.rohmhaas.com/assets/attachments/business/pcare/neolone/neolone_950/tds/neolone_950.pdf

(86) Pons J.L., Bonnaveiro N., Chevalier J., Cremieux A. Evaluation of antimicrobial interactions between chlorhexidine, quaternary ammonium compounds, preservatives and excipients. J Appl Bacteriol 1992: 73(5):395-400.

(87) Llabres C.M., Ahearn D.G. Antimicrobial activities of N-chloramines and diazolidinyl urea. Appl Environ Microbiol 1985: 49(2):370-3.

(88) Nicoletti G., Boghossian V., Gurevitch F., Borland R., Morgenroth P. The antimicrobial activity in vitro of chlorhexidine, a mixture of isothiazolinones ('Kathon' CG) and cetyl trimethyl ammonium bromide (CTAB). J Hosp Infect 1993: 23(2):87-111.

(89) Becks V.E., Lorenzoni N.M. Pseudomonas aeruginosa outbreak in a neonatal intensive care unit: a possible link to contaminated hand lotion. Am J Infect Control 1995: 23(6):396-8.

(90) Itin P.H., Frei R., Lautenschlager S., Buechner S.A., Surber C., Gratwohl A., Widmer A.F. Cutaneous manifestations of Paecilomyces lilacinus infection induced by a contaminated skin lotion in patients who are severely immunosuppressed. J Am Acad Dermatol 1998: 39(3):401-9.

(91) European Pharmacopoeia 6.0. General Texts; 5.1.3. Efficacy of Antimicrobial Preservation. European Pharmacopoeia 6.0. 2007. p. 528-9.

(92) European Pharmacopoeia 6.5. Methods of analysis; 2.6.12 Microbiological examination of non-sterile products: Microbial Enumeration Tests. European Pharmacopoeia 6.5. 2007. p. 4769-73.

(93) European Pharmacopoeia 6.5. 2.6.13. Microbiological examination of non-sterile products: test for specified micro-organisms. 6.5 ed. 2010. p. 4773-7.

(94) QRA Expert Group. Dermal Sensitization Quantitative Risk Assesement (QRA) for fragrance ingredients. 2006.

(95) Galaxy Surfactants Ltd. When Protection is Paramount.... 2009; http://www.in-cosmetics.com/ExhibitorLibrary/142/Phenoxyethanol_3.pdf

(96) ISP S.l. Germall® II Preservative for Personal Care Products . 2008; http://online1.ispcorp.com/Brochures/Germall%20II.pdf

(97) Cosmetic Ingredient Review. Annual Safety Assessment Review. 2007.

(98) Diepgen T.L., Coenraads P.J. The epidemiology of occupational contact dermatitis. Int Arch Occup Environ Health 1999: 72(8):496-506.

(99) Statham B.N., Smith E.V., Bodger O.G., Green C.M., Clodagh K.M., Ormerod A.D., Sansom J.E., English J.S.C., Wilkinson S.M., Gawkrodger D.J., Chowdhury M.M.U. Concomitant contact allergy to methylchloroisothiazolinone/methylisothiazolinone and formaldehyde-releasing preservatives. Contact Dermatitis 2010: 62:56-7.

(100) Bangsgaard N., Carlsen B.C., Johansen J.D., Menne T., Skov L. Susceptibility and reactivity in polysensitized individuals following controlled induction. Contact Dermatitis 2010: 63(1):10-4.

(101) Carlsen B.C., Menne T., Johansen J.D. 20 Years of standard patch testing in an eczema population with focus on patients with multiple contact allergies. Contact Dermatitis 2007: 57(2):76-83.

(102) Berenbaum M.C. A method for testing for synergy with any number of agents. J Infect Dis 1978: 137(2):122-30.

(103) van Doorne H. Preservative Stability and Efficacy: Formulation Influences. In: Denyer SP, Baird RM, editors. Guide to Microbiological Control in Pharmaceuticals and Medical Devices.Boca Ranton, FL 33487-2742: CRC Press: 1990. p. 349-66.

(104) Mislivec P.B., Bandler R., Allen G. Incidence of fungi in shared-use cosmetics available to the public. J AOAC Int 1993: 76(2):430-6.

(105) Tran T.T., Hitchins A.D. Microbial Survey of Shared-Use Cosmetic Test Kits Available to the Public. Journal of Industrial Microbiology 1994: 13(6):389-91.

(106) Jemec G.B.E., Renneberg J., Wulf H.C. Microbiology of Sunscreens in use. Occupational and Environmental Dermatosen 1997: 45(6):275-7.

(107) Orth D.S. The Keys to Succesful Product Preservation. Insights into Cosmetic Microbiology. 1 ed. Carol Sream, IL: Allured Books: 2010. p. 27-111.

(108) Russell A.D. Challenge testing: principles and practice. International Journal of Cosmetic Science 2003: 25(3):147-53.

(109) Schnuch A., Uter W., Dickel H., Szliska C., Schliemann S., Eben R., Rueff F., Gimenez-Arnau A., Loffler H., Aberer W., Frambach Y., Worm M., Niebuhr M., Hillen U., Martin V., Jappe U., Frosch P.J., Mahler V. Quantitative patch and repeated open application test-

ing in hydroxyisohexyl 3-cyclohexene carboxaldehyde sensitive-patients. Contact Dermatitis 2009: 61(3):152-62.

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Low-level efficacy of cosmetic preservatives

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Synopsis

Preservation using combinations of preservatives has several advantages. This study shows that the concentration of some of the most frequently used allergenic preservatives can be markedly lowered when they are combined with phenoxyethanol. The antimicrobial efficacy of cosmetic preservatives and known allergens of various potency [diazolidinyl urea, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), methylisothiazolinone (MI) and phenoxyethanol] was tested alone and in various combinations of two or three preservatives together. The preservatives were tested for minimum inhibitory concentration (MIC) values and possible synergy using fractional inhibitory concentration. MCI/MI was the only preservative showing low-level MIC against all four tested microorganisms: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*. Different combinations of the preservatives indicated additive effects against the microorganisms. No combination of preservatives showed any inhibitory action on each other. Challenge tests with different concentrations and combinations were performed in a cosmetic cream. Diazolidinyl urea and MCI/MI alone were ineffective against *C. albicans* in a challenge test at concentrations up to 16 times higher than the observed MIC values. When combining phenoxyethanol with either one of the allergenic preservatives diazolidinyl urea, MCI/MI or MI, the cosmetic cream was adequately preserved at concentrations well below the preservatives' MIC values as well as 10–20 times below the maximum permitted concentrations. By using combinations of preservatives, effective preservation can be achieved with lower concentrations of allergenic preservatives.

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Résumé

La conservation utilisant les combinaisons de conservateurs a plusieurs avantages. Cette étude montre que la concentration de certains des conservateurs allergènes le plus fréquemment utilisés peut être manifestement diminuée quand ils sont utilisés en association avec le phenoxyethanol. L'efficacité antimicrobienne de conservateurs cosmétiques et connus comme allergènes à des degrés divers [diazolidinyl urée, methylchloroisothiazolinone/methylisothiazolinone (MCI/MI), methylisothiazolinone (MI) et phenoxyethanol] a été évaluée pour chacun séparément et dans diverses combinaisons de deux ou trois ensemble. Les conservateurs étaient évalués par l'estimation de leur MIC (concentration minimale inhibitrice) et les possibles synergies en utilisant la Concentration Inhibitrice Fractionnaire. MCI/MI était le seul conservateur montrant une MIC plus basse face aux quatre microorganismes testés : *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* et

Aspergillus Niger. Les différents mélanges de conservateurs ont montré un effet additif face aux microorganismes. Aucun mélange de conservateurs n'a montré aucune action inhibitrice. Les challenges tests avec différentes concentrations et avec les mélanges ont été effectués dans une crème cosmétique. Seuls diazolidinyl urée et MCI/MI étaient inefficaces face à *Candida albicans* dans un challenge test à des concentrations jusqu'à 16 fois plus importantes que les valeurs de MIC observées. En combinant le phenoxyethanol avec chacun des conservateurs allergènes diazolidinyl urée, MCI/MI ou MI, la crème cosmétique a été correctement protégée avec des concentrations en conservateurs bien au-dessous des valeurs de MIC comme 10 à 20 fois au-dessous du maximum des concentrations autorisées. En utilisant des mélanges de conservateurs, une protection efficace peut être obtenue avec des concentrations de conservateurs allergènes plus faibles.

Introduction

Microbiological contamination can cause spoilage of cosmetic products, ultimately leading to consumers being infected by the microorganisms [1]. To avoid proliferation of existing microorganisms or in-use contamination, manufactures add preservatives to their products. As many preservatives cause allergic contact dermatitis, it is important that cosmetic manufacturers use the lowest possible concentrations of preservatives, without losing the antimicrobial effect. Development of allergic contact dermatitis is dose dependent; accordingly, lowering the preservative concentration may lead to safer products and fewer cases of allergic contact dermatitis to preservatives.

Although there are several cosmetic preservatives available, both alone and in pre-prepared combinations, the market is dominated by a small number [2]. Phenoxyethanol, formaldehyde releasers and methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) are among the most frequently used cosmetic preservatives. In 2007, phenoxyethanol was the third most used cosmetic preservative, found in almost 20% of voluntarily registered products in the United States [2]. MCI/MI and the formaldehyde releaser diazolidinyl urea are used in approximately 5% of cosmetic products, which, respectively, corresponds to the 9th and 10th most frequently used cosmetic preservative [2]. In 2005, methylisothiazolinone, the MI part of MCI/MI, was permitted as a preservative in cosmetic products in the EU [3]. In 2007, MI alone appeared to constitute only a small percentage of the total preservative consumption [2].

MCI/MI, diazolidinyl urea and MI are all frequent causes of allergic contact dermatitis, with prevalences of slightly below 2% for all three preservatives [4, 5]. Phenoxyethanol rarely causes allergic contact dermatitis, despite its frequent use [6]. Recommendations, legal limits and concentrations for preservatives vary, which indicates that some products may be over-preserved [7]. Additionally, there is a general lack of information on whether cosmetic preservatives are used alone or in combination with other preservatives. Combinations of preservatives can potentially have synergistic or additive effects against microorganisms, and this has several advantages. Firstly, the concentrations needed for sufficient preservation of a cosmetic product can be lowered. Development of allergic contact dermatitis is dose dependent, so this could potentially lead to fewer allergic reactions. Secondly, the optimal combination of preservatives is also effective against a wider spectrum of microorganisms. In this study, the antimicrobial efficacy of the preservatives diazolidinyl urea, MCI/MI, MI and phenoxyethanol was investigated. The minimum inhibitory concentrations (MICs) of the preservatives alone and in various combinations against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* were determined. A series of challenge tests was used to investigate the preservatives' antimicrobial efficacy in a cosmetic cream to verify that combinations of preservatives are at least as effective as the use of single preservatives.

Materials and methods

Minimum inhibitory concentration

The efficacy of the cosmetic preservatives phenoxyethanol (Sigma Aldrich®, Schnelldorf, Germany), diazolidinyl urea (trade name Germall® II; Sigma Aldrich®, Schnelldorf, Germany), MCI/MI in a 3 : 1 mixture (trade name Kathon™ CG, kindly supplied by Rohm and Haas, Antwerp, Belgium) and MI (trade name Neolone 950, kindly supplied by Rohm and Haas,) was tested against *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853), *C. albicans* (ATCC 10231) and *A. niger* (ATCC 16404).

The minimal inhibitory concentration (MIC) of each preservative was determined according to CLSI (former NCCLS) standard M7-A6 for *S. aureus* and *P. aeruginosa* [8]. The MICs of *C. albicans* and *A. niger* were determined according to CLSI M27-A2 and M38-A, respectively [9, 10]. The only differences from the protocols were the incubation temperature and duration. The temperature was 25 ± 2°C for all microorganisms because this is the temperature used in the challenge tests, and because of the lower temperature, the incubation period was 48 ± 2 h for the bacteria and 72 ± 2 h for the yeast and mould. The tested concentrations of diazolidinyl urea were as follows: 0.03125%, 0.0625%, 0.1%, 0.125%, 0.2%, 0.25%, 0.3%, 0.4% and 0.5% (w/w). MCI/MI was tested in the following concentrations: 0.000003125%, 0.00000625%, 0.0000125%, 0.00005%, 0.0001%, 0.0002%, 0.0004%, 0.0008% and 0.0015% (w/w). The tested concentrations of phenoxyethanol were as follows: 0.05%, 0.1%, 0.15%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1% (w/w). MI was tested in the following concentrations: 0.0005%, 0.001%, 0.0015%, 0.002%, 0.0025%, 0.0035%, 0.0045%, 0.0055%, 0.0065%, 0.0075%, 0.0085% and 0.01% (w/w). MIC values were determined in at least three independent experiments, and in the case of differences between two or more MICs, the highest concentration was used.

Fractional inhibitory concentration

The antimicrobial efficacy of preservatives in combinations was tested for synergistic, additive/indifferent or antagonistic effects. Based on the MIC values obtained, the following concentrations of diazolidinyl urea: 0%, 0.03125%, 0.0625%, 0.125%, 0.25% and 0.5%; MCI/MI, 0%, 0.000003125%, 0.00000625%, 0.0000125%, 0.000025%, 0.00005%, 0.0001%, 0.0002% and 0.0004%; MI, 0.001%, 0.002%, 0.003%, 0.004%, 0.005%, 0.006%, 0.007%, 0.008%, 0.009% and 0.01%; and phenoxyethanol, 0%, 0.05%, 0.1%, 0.15%, 0.2%, 0.4%, 0.6%, 0.8% and 1% were combined two or three together and tested against the microorganisms. Inoculum preparation, inoculum size, incubation temperature and incubation time were the same as in the MIC tests. Indications of synergy or antagonism were calculated as fractional inhibitory concentrations (FICs) for combinations of two or three preservatives as described by ASM [11]. Briefly, FIC is the sum of each preservative's MIC value obtained in combination with the other preservatives divided by the MIC for the preservative alone. FICs ≤0.5 indicate synergy, >0.5–≤4 indicate indifference or additive effects and >4 indicate antagonism. Experiments were carried out at least twice.

Challenge tests

A cosmetic cream with various concentrations of preservatives was purchased through Glostrup Pharmacy, Denmark. The composition of 1000 g cream was as follows: 5 g polysorbate 80, 50 g cetostearyl alcohol, 50 g paraffin oil, 60 g glycerol monostearate 40–50, 40 g glycerol 85%, 70 g sorbitol and 725 g water. The cream was challenge tested according to the monographs in the European Pharmacopoeia [12, 13]. The different combinations of tested concentrations are shown in Table I.

The cream was inoculated with a standardized suspension of one of the microorganisms corresponding to 10^5–10^6 CFU g^{-1} cream and then incubated at 25°C for 28 days. Samples were drawn on days 0, 2, 7, 14, 21 and 28 for *S. aureus* and *P. aeruginosa*, and on days 0, 7, 14, 21 and 28 for *C. albicans* and *A. niger*. The number of microorganisms (CFU g^{-1} cream) was determined by dissolving 1 mL cream in 9 mL buffered NaCl–peptone solution (pH 7.0) [14] and further diluted if necessary. A volume of 0.1 mL cream/peptone solution was dispersed on tryptone soya agar (Oxoid, Greve, Denmark) for bacteria and on Sabouraud dextrose agar (Oxoid) for yeast and mould and incubated according to the monographs [12, 13]. Counts were made in duplicate for each dilution. The criteria for passing the challenge test for bacteria are either a 2 log reduction after 2 days and a 3 log reduction after 7 days (A criteria) or a 3 log reduction after 14 days (B criteria). Furthermore, no increase in CFU g^{-1} cream must be found after 28 days (both A and B criteria). For *C. albicans* and *A. niger*, a 1 log (B criteria) or 2 log (A criteria) reduction after 14 days and no increase in CFU g^{-1} cream after 28 days is accepted [12]. A cream without preservatives was inoculated and included in each challenge test as a positive control. To test for intrinsic contamination in each challenge test, a cream was not inoculated but otherwise followed the same sampling procedure as the other creams.

Table I Concentration (w/w) and combinations of preservatives investigated in the challenge test

Diazolidinyl urea (%)	MCI/MI (%)	MI (%)	Phenoxyethanol (%)
0.5	–	–	–
0.37	–	–	–
0.25	–	–	–
0.25	0.0003	–	0.8
0.25	0.0003	–	0.4
0.25	0.0003	–	–
0.25	0.0001	–	0.8
0.25	0.0001	–	0.4
0.25	0.0001	–	–
0.25	–	–	0.8
0.25	–	–	0.4
0.125	0.0003	–	0.8
0.125	0.0003	–	0.4
0.125	0.0003	–	–
0.125	0.0001	–	0.8
0.125	0.0001	–	0.4
0.125	0.0001	–	–
0.125	–	–	0.8
0.125	–	–	0.4
0.1	–	0.005	0.4
0.1	–	0.005	0.2
0.1	–	0.005	–
0.1	–	–	0.4
0.1	–	–	0.2
0.05	–	0.005	0.4
0.05	–	0.005	0.2
0.05	–	0.005	–
0.05	–	–	0.4
0.05	–	–	0.2
–	0.0008	–	–
–	0.0006	–	–
–	0.0004	–	–
–	0.0003	–	0.4
–	0.0003	–	0.2
–	0.0001	–	0.4
–	0.0001	–	0.2
–	0.00005	–	0.4
–	0.00005	–	0.2
–	–	0.005	0.4
–	–	0.005	0.2
–	–	0.003	0.4
–	–	0.003	0.2
–	–	0.0015	0.4
–	–	0.0015	0.2
–	–	0.005	0.4
–	–	0.005	0.2
–	–	–	0.8
–	–	–	0.4

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; MI, methylisothiazolinone; –, Preservative not present in formulation.

Results

The MIC values of the preservatives are shown in Table II. All MIC values were below the maximum permitted concentration in cosmetics, except phenoxyethanol against *S. aureus* (1% permitted), diazolidinyl urea (0.5% permitted) against *C. albicans* and *A. niger* against MI (0.01% permitted). MCI/MI was effective against all microorganisms at low concentrations (highest MIC values was 0.0002%). Diazolidinyl urea was more effective against bacteria than against *A. niger* and *C. albicans*. Phenoxyethanol was the weakest preservative, with MIC values close to or at the maximum permitted concentration (1%) (Table II).

The mean and range of the FIC values obtained are shown in Table III. Not one of the tested combinations was antagonistic (FIC >4), according to the levels set by ASM [11]. Almost all the mean FIC values were below or slightly above 1 (Table III). None of the combinations had a mean FIC value below 0.5, which indicates synergy. However, diazolidinyl urea combined with phenoxyethanol had a mean FIC value of 0.55 and a range between 0.33 and 0.75 against *C. albicans* (Table III). Some FIC values were not determined because of the microorganisms' sensitivity to the combinations.

The antimicrobial effect of the preservatives in the cosmetic cream was tested in a series of challenge tests with different concentrations of preservatives (Table I). Diazolidinyl urea and MCI/MI used alone failed against *C. albicans*. Phenoxyethanol alone just passed the B criteria for 0.4%, whereas 0.8% passed the A criteria set by the European Pharmacopoeia [12] (Table IV). However, 0.8% phenoxyethanol altered the composition of the cream and it became more fluid.

Tables V and VI show the results of two series of challenge tests where two concentrations of diazolidinyl urea (0.125% and 0.25%) were tested in combination with three concentrations of MCI/MI (0, 0.0001% and 0.0003%) and phenoxyethanol (0%, 0.4% and 0.8%), respectively. The majority of the combinations passed the challenge test, but three did not; they could not eradicate *C. albicans* and did not contain phenoxyethanol. Table VII presents the results of challenge tests with 0.005% MI in combination with diazolidinyl urea and phenoxyethanol. A combination of MI with diazolidinyl urea but without phenoxyethanol failed the challenge test, whereas MI together with 0.2% and 0.4% phenoxyethanol passed the A criteria (Table VII).

The lowest concentrations of combined preservatives tested are shown in Tables VIII and IX. The

Table II Minimum inhibitory concentrations values of diazolidinyl urea, MCI/MI, MI and phenoxyethanol (w/w)

	Diazolidinyl urea (%)	MCI/MI (%)	MI (%)	Phenoxyethanol (%)
Staphylococcus aureus	<0.03125	0.0002	0.0045	1
Pseudomonas aeruginosa	0.0625	0.0002	0.0015	0.4
Aspergillus niger	0.125	0.00005	>0.01	0.4
Candida albicans	0.5	0.00005	0.0065	0.6

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; MI, methylisothiazolinone.

Table III Range and mean FIC values of combinations of the cosmetic preservatives against microorganisms

	Candida albicans		*Aspergillus niger*		*Pseudomonas aeruginosa*		*Staphylococcus aureus*	
	Range	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**
DU–MCI/MI	0.56–1.13	0.88	0.63–0.75	0.71	0.53–0.75	0.64	ND	ND
DU–PE	0.33–0.75	0.55	0.63–1.00	0.79	0.63–1.00	0.88	ND	ND
DU–MCI/MI–PE	0.66–1.23	0.85	1.00–1.75	1.30	0.66–1.13	0.93	ND	ND
MCI/MI–PE	0.75–1.17	0.98	1.00–2.38	1.79	0.50–1.00	0.75	0.45–1.30	0.88
DU–MI–PE	1.01–1.20	1.10	0.85–1.10	1.00	ND	ND	ND	ND
DU–MI	1.14–1.89	1.43	0.80–1.05	0.93	ND	ND	ND	ND
MI–PE	0.78–0.95	0.87	ND	ND	ND	ND	0.76–1.61	1.21

FIC ≤0.5 = synergy, FIC >0.5 and ≤4.0 = additive/indifference, FIC >4.0 = antagonism.
DU, diazolidinyl urea; MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; PE, phenoxyethanol; MI, methylisothiazolinone; ND, not determined; FIC, fractional inhibitory concentrations.

Table IV Challenge test results with diazolidinyl urea, MCI/MI and phenoxyethanol

	Diazlidinyl urea (%)			MCI/MI (%)			Phenoxyethanol (%)	
	0.25	**0.375**	**0.5**	**0.0004**	**0.0006**	**0.0008**	**0.4**	**0.8**
Staphylococcus aureus	P^a	P^a	P^a	P^a	P^a	P^a	P^b	P^a
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Aspergillus niger	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Candida albicans	F	F	F	F	F	F	P^b	P^a

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; P^a = passed level A criteria; P^b = passed level B criteria; F = failed both A and B criteria of the European Pharmacopoeia [12].

challenge test failed when 0.05% and 0.1% diazolidinyl urea were combined with 0.2% phenoxyethanol (Table IX) but passed when combined with 0.4% (Table VIII). MCI/MI combined with phenoxyethanol was effective at 0.0001%/0.2% (Table IX) and 0.00005%/0.4% (Table VIII), respectively. When preserved with 0.0005% MI and 0.2% phenoxyethanol, the cream passed the B criteria (Table IX). The remaining concentrations tested all passed the challenge tests. The cream without preservative could not eradicate any of the tested microorganisms, and no microorganisms

Table V Challenge test results with 0.25% diazolidinyl urea and different concentrations of MCI/MI and phenoxyethanol

	Diazolidinyl urea 0.25%							
	MCI/MI 0%		**MCI/MI 0.0001%**			**MCI/MI 0.0003%**		
	Phenoxyethanol		**Phenoxyethanol**			**Phenoxyethanol**		
	0.4%	**0.8%**	**0%**	**0.4%**	**0.8%**	**0%**	**0.4%**	**0.8%**
Candida albicans	P[a]	P[a]	F	P[a]	P[a]	F	P[a]	P[a]

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; P[a] = passed level A criteria; P[b] = passed level B criteria; F = failed both A and B criteria of the European Pharmacopoeia [12].

Table VI Challenge test results with 0.125% diazolidinyl urea and different concentrations of MCI/MI and phenoxyethanol

	Diazolidinyl urea 0.125%							
	MCI/MI 0%		**MCI/MI 0.0001%**			**MCI/MI 0.0003%**		
	Phenoxyethanol		**Phenoxyethanol**			**Phenoxyethanol**		
	0.4%	**0.8%**	**0%**	**0.4%**	**0.8%**	**0%**	**0.4%**	**0.8%**
Staphylococcus aureus	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]
Pseudomonas aeruginosa	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]
Aspergillus niger	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]
Candida albicans	P[a]	P[a]	F	P[a]	P[a]	P[a]	P[a]	P[a]

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; P[a] = passed level A criteria; P[b] = passed level B criteria; F = failed both A and B criteria of the European Pharmacopoeia [12].

Table VII Challenge test results with 0.005% MI and different combinations of diazolidinyl urea and phenoxyethanol

	Diazolidinyl urea (0%)		**Diazolidinyl urea (0.05%)**			**Diazolidinyl urea (0.1%)**		
	Phenoxyethanol (%)		**Phenoxyethanol (%)**			**Phenoxyethanol (%)**		
	0.2	**0.4**	**0**	**0.2**	**0.4**	**0**	**0.2**	**0.4**
Staphylococcus aureus	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]
Pseudomonas aeruginosa	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]
Aspergillus niger	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]	P[a]
Candida albicans	P[a]	P[a]	F	P[a]	P[a]	F	P[a]	P[a]

MI, methylisothiazolinone; P[a] = passed level A criteria; P[b] = passed level B criteria; F = failed both A and B criteria of the European Pharmacopoeia [12].

Table VIII Challenge test result with 0.4% phenoxyethanol in combination with diazolidinyl urea, MCI/MI or MI

	Diazolidinyl urea (%)		MCI/MI (%)			MI (%)		
	0.05	0.1	0.00005	0.0001	0.0003	0.0005	0.0015	0.003
Staphylococcus aureus	P^a	P^a	P^b	P^b	P^b	P^b	P^b	P^b
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Aspergillus niger	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a
Candida albicans	P^a	P^a	P^a	P^a	P^a	P^a	P^a	P^a

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; MI, methylisothiazolinone; P^a = passed level A criteria, P^b = passed level B criteria, F = failed both A and B criteria of the European Pharmacopoeia [12].

Table IX Challenge test result with 0.2 phenoxyethanol in combination with diazolidinyl urea, MCI/MI or MI

	Diazolidinyl urea (%)		MCI/MI (%)			MI (%)		
	0.05	0.1	0.00005	0.0001	0.0003	0.0005	0.0015	0.003
Staphylococcus aureus	P^a	P^a	P^b	P^b	P^b	P^b	P^b	P^b
Pseudomonas aeruginosa	P^a	P^a	P^a	P^a	P^a	P^b	P^b	P^b
Aspergillus niger	F	P^a	P^a	P^a	P^a	P^b	P^b	P^a
Candida albicans	F	F	F	P^a	P^a	P^b	P^b	P^a

MCI/MI, methylchloroisothiazolinone/methylisothiazolinone; MI, methylisothiazolinone; P^a = passed level A criteria, P^b = passed level B criteria, F = failed both A and B criteria of the European Pharmacopoeia [12].

were found in the creams that were not inoculated.

Discussion

Using additive or synergistic combinations of preservatives has several advantages, for example a wider spectrum of activity, reduced concentrations of preservatives and potentially fewer cases of allergic contact dermatitis. This study showed that when combined with phenoxyethanol, the concentrations of the allergenic preservatives MCI/MI, diazolidinyl urea and MI could be lowered 8- to 16-fold compared with when the preservatives were used alone.

The MIC tests were preformed according to CLSI standards, with the exception of the temperature, which was set at 25°C for all microorganisms. This temperature was chosen because it is the same temperature that is used in the challenge tests, as described in the European Pharmacopoeia [12]. Furthermore, it is similar to room temperature, where most cosmetics are stored, and thereby mimics an authentic use pattern. The MIC results showed that only MCI/MI was effective against all four microorganisms (Table II). Phenoxyethanol against *S. aureus*, MI against *A. niger*, and diazolidinyl urea against *C. albicans* all resulted in MIC values at or above the maximum permitted concentrations in the European Union (Table II). In some cases, the determined MIC values differed from those found by the manufacture; MCI/MI, in particular, had lower MIC values in this study compared with those found by the manufacturer [15]. There are several potential reasons for the differences in the MIC values; for example, the inoculum size and age, temperature and incubation time can greatly influence the results [16].

Using the MIC values as a guideline for the concentration of preservatives in cosmetics is somewhat troublesome. Cosmetic products contain many ingredients that can have a synergistic or antagonistic effect on the preservatives' effect against microorganisms. This is one of the reasons

why all cosmetics have to be challenge tested before marketing to ensure that the demands are fulfilled. In general, concentrations below the MIC values are most likely ineffective, but other ingredients that are not listed as preservatives may have antimicrobial properties and thereby reduce the need for preservatives in the formulation or vice versa. Ingredients that are antimicrobial or enhance the effect of preservatives are, for instance, EDTA, pentylene glycol or some fragrance ingredients, such as citral or eugenol [17]. However, the use of fragrances as preservatives is not recommended because they are the other main class of cosmetic ingredients that can cause allergic contact dermatitis [18, 19]. In this study, all these ingredients were avoided to test the preservatives' activity alone.

The combinations of preservatives tested in this study were not antagonistic (FIC >4), and the majority of the mean FIC values were below 1 (Table III). No FIC value was below 0.5, which is the break point for synergy set by ASM [11]. However, FIC values around 1 indicate that the preservatives have an additive effect. An additive effect means that low concentrations of two or more preservatives combined are just as effective as a higher concentration of one preservative used alone. In cosmetic preservation, this is very important because the development of allergic contact dermatitis is dose dependent. The challenge tests showed that some concentrations above the MIC values did not adequately preserve the cream when the creams were preserved with a single preservative. Phenoxyethanol was the only preservative tested alone that was able to preserve the cream. However, 0.8% phenoxyethanol made the cream very thin and 0.4% just barely passed the B criteria in the European Pharmacopoeia (Table IV). MCI/MI used alone in a concentration 16 times higher than the MIC of *C. albicans* failed the challenge test (Tables II and IV). When combined with 0.2% phenoxyethanol, only 0.00005% MCI/MI was needed to preserve the cream (Table IX). This is 30 times less than the maximum permitted concentration in the EU (0.0015%) [20].

The maximum permitted diazolidinyl urea concentration in the European Union and the United States of 0.5% failed to preserve the cream; *C. albicans* was the only microorganism that was not eradicated in the challenge test with diazolidinyl urea alone. However, when 0.05% diazolidinyl urea was combined with 0.4% phenoxyethanol, it was sufficient to pass the challenge test (Table VIII). The 0.05% diazolidinyl urea corresponds to 1/10 of the ineffective and maximum permitted concentration tested previously (Table IV) [20].

MI (0.005%) in combination with 0.05% diazolidinyl urea could not preserve the cream. When MI was combined with 0.2% phenoxyethanol, only 0.0005% was needed (Table IX). This is 20 times below the maximum permitted concentration [20]. The manufacturers of MI suggest that cosmetics are preserved with 0.0045–0.0095% MI [21]. A combination of MI and phenoxyethanol is already available; however, the manufacturer recommends concentrations from 0.3% phenoxyethanol and 0.005% MI up to 0.6% phenoxyethanol and 0.01% MI [22], much higher than this study found to be necessary.

In this study, *C. albicans* was almost solely responsible for the failed challenge tests, even though some of the tested concentration were well above its MIC values. In other cases, challenge tests with preservative concentrations well below some of the microorganisms' MIC values had no difficulty in fulfilling the demands of the European Pharmacopoeia [12]. This illustrates the influence the formulation can have on the ability to preserve and why it is necessary to conduct a challenge test in every different formulation. A preservative system that is effective in one formulation might not be effective in another.

A study on the eliciting concentration of diazolidinyl urea showed that formaldehyde-allergic patients did not react to 0.05% diazolidinyl urea, but nine out of 10 diazolidinyl urea-allergic patients reacted to 0.15% [23]. This indicates that formaldehyde-allergic individuals can use the cream preserved with 0.05% diazolidinyl urea and 0.4% phenoxyethanol without an allergic reaction to the released formaldehyde. Even though the exact no-effect level for diazolidinyl urea is unknown, a reduced level of diazolidinyl urea will decrease the risk of an allergic reaction.

In a separate study, MCI/MI-allergic patients were exposed to three different concentrations of MCI/MI for 4 weeks. The study showed that the eliciting threshold for MCI/MI-allergic patients was in the proximity of 0.0002% [24]. In this study, the lowest effective concentration of MCI/MI was 0.0005% (Table VIII). Again, this indicates that MCI/MI-allergic patients might not react to this cream when it is preserved with MCI/MI in combination with phenoxyethanol.

Studies of the concentration of cosmetic preservatives show that in similar products the concentration of the same preservative can vary greatly. Diazolidinyl urea concentrations in 736 different products in the United States are between 0.0003% and 0.5% [25]. In Sweden and Denmark, MCI/MI is found in concentrations between 0.00008% and 0.0015% [26–28]. In Denmark, concentrations of phenoxyethanol are between 0.023% and 0.957% [27]. The concentration of MI has not been investigated. There is currently no information available on the distribution of high and low preservative concentrations in cosmetic products and whether they are used in combinations. However, with so many different products available, it could be speculated whether many of them could be preserved with either a lower concentration of preservative or a combination of preservatives. Currently, some of the most frequently used preservatives are also the most frequent allergens [2, 4]. Many different combinations of preservatives are available today, but it is not known precisely which combinations are most effective and in which concentrations. Furthermore, if preservation is achieved with concentrations in the proximity of the maximum permitted concentrations, it should be considered whether another preservative or preservative blend is more effective.

It is important to note that the combinations found effective in this study might not necessarily work in other formulations. Nevertheless, the general idea of investigating different combinations of preservatives to achieve effective preservation with the lowest amount of preservatives, especially the allergenic ones, should be considered by every cosmetic-manufacturing company. In the long term, this could reduce the cost of manufacturing, prevent withdrawal of the most effective preservatives from the market because of allergenic reactions, and potentially reduce the number of allergic reactions to preservatives.

In conclusion, this study verified that combinations of preservatives can be considerably more effective than individual preservatives used at higher concentrations.

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References

1. Alvarez-Lerma, F., Maull, E., Terradas, R. *et al.* Moisturizing body milk as a reservoir of *Burkholderia cepacia*: outbreak of nosocomial infection in a multidisciplinary intensive care unit. *Crit. Care* **12**, R10 (2008).
2. Steinberg, D.C. Voluntary registration of cosmetics and 2007 frequency of preservative use. *Cosmet. Toil.* **123**, 47–52 (2008).
3. The Scientific Committee on Consumer Product and Non-Food-Products intended for Consumers Opinion concerning Methylisothiazolinone (COLIPA n P94) (2004).
4. Thyssen, J.P., Engkilde, K., Lundov, M.D., Carlsen, B.C., Menné, T. and Johansen, J.D. Temporal trends of preservative allergy in Denmark (1985–2008). *Contact Derm.* **62**, 102–108 (2010).
5. Lundov, M.D., Thyssen, J.P., Zachariae, C. and Johansen, J.D. Prevalence and cause of methylisothiazolinone contact allergy. *Contact Derm.* **63**, 164–167.
6. Schnuch, A., Uter, W., Lessmann, H. and Geier, J. Contact allergy to preservatives. Results of the Information Network of Departments of Dermatology (IVDK) 1996–2007. *Interdiscip. J. All. Environ. Med.* **17**, 631–638 (2008).
7. Lundov, M.D., Moesby, L., Zachariae, C. and Johansen, J. Contamination versus preservation of cosmetics: a review on legislation, usage, infections, and contact allergy. *Contact Derm.* **60**, 70–78 (2009).
8. NCCLS. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standards, 6th edn. NCCLS Document M7-A6 (2003).
9. NCCLS. Reference method for broth dilution antifungal susceptibility testing of yeast; Approved Standards, 2nd edn. NCCLS document M27-A2 (2002).
10. NCCLS. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard. NCCLS document M38-A (2002).
11. Moody, J. Synergism testing: broth microdilution checkerboard and broth microdilution methods. In: *Clinical Microbiology Procedures Handbook* (Isenberg, H.D., ed.), p. 5.12.1–5.12.23. ASM Press, Washington DC (2004).
12. European Pharmacopoeia 6.0. *European Pharmacopoeia 6.0. General Texts; 5.1.3. Efficacy of Antimicrobial Preservation*. Directorate for the quality of Medicines and Health Care Council of Europe, Strasbourg, France. 528–529 (2007).
13. European Pharmacopoeia 6.0. *European Pharmacopoeia 6.0. Methods of analysis; 2.6.12. Microbiological Examination of Non-sterile Products: Total Viable Counts*. Directorate for the quality of Medicines and Health Care Council of Europe, Strasbourg, France. 4769–4773 (2007).
14. European Pharmacopoeia 6.5. 2.6.13. *European Pharmacopoeia 6.5. 2.6.13. Microbiological Examination of*

Non-sterile Products: Test for Specified Micro-organisms. Directorate for the quality of Medicines and Health Care Council of Europe, Strasbourg, France. 4773–4777 (2010).
15. ROHM and HAAS. *KATHON™ CG A Safe, Effective, Globally Approved Preservative for Rinse-Off Products*. DOW Chemical. Midland, MI (2006).
16. Schuurmans, J.M., Nuri Hayali, A.S., Koenders, B.B. and ter Kuile, B.H. Variations in MIC value caused by differences in experimental protocol. *J. Microbiol. Methods* **79**, 44–47 (2009).
17. Steinberg, D.C. *Preservatives for Cosmetics*. Allured Publishing Corporation, Carol Stream (2006).
18. Goossens, A., Beck, M.H., Haneke, E., McFadden, J.P., Nolting, S., Durupt, G. and Ries, G. Adverse cutaneous reactions to cosmetic allergens. *Contact Derm.* **40**, 112–113 (1999).
19. Warshaw, E.M., Buchholz, H.J., Belsito, D.V. *et al.* Allergic patch test reactions associated with cosmetics: retrospective analysis of cross-sectional data from the North American Contact Dermatitis Group, 2001–2004. *J. Am. Acad. Dermatol.* **60**, 23–38 (2009).
20. European Commission. Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (Consolidated version) (2008).
21. ROHM and HAAS. *NEOLONE™ 950 A safe, Broad Spectrum Bactericide for the Preservation of Leave-on Products*. DOW Chemical. Midland, MI (2006).
22. ROHM and HAAS. *NEOLONE™ PE A safe, Broad Spectrum Preservative for Leave-on Products Based on Methylisothiazolinone and Phenoxyethanol*. DOW Chemical. Midland, MI (2006).
23. Zachariae, C., Hall, B., Cottin, M., Cupferman, S., Andersen, K.E. and Menné, T. Experimental elicitation of contact allergy from a diazolidinyl urea-preserved cream in relation to anatomical region, exposure time and concentration. *Contact Derm.* **53**, 268–277 (2005).
24. Zachariae, C., Lerbaek, A., McNamee, P.M., Gray, J.E., Wooder, M. and Menné, T. An evaluation of dose/unit area and time as key factors influencing the elicitation capacity of methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) in MCI/MI-allergic patients. *Contact Derm.* **55**, 160–166 (2006).
25. Cosmetic Ingredient Review. Annual Safety Assessment Review (2007).
26. Rastogi, S.C. Kathon CG content in cosmetic products. *Contact Derm.* **19**, 263 (1988).
27. Rastogi, S.C. Analytical control of preservative labelling on skin creams. *Contact Derm.* **43**, 339–343 (2000).
28. Gruvberger, B., Bruze, M. and Tammela, M. Preservatives in moisturizers on the Swedish market. *Acta Derm. Venereol.* **78**, 52–56 (1998).

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CONTACT DERMATITIS

Prevalence and cause of methylisothiazolinone contact allergy

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Background: Methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) has been one of the most frequent sensitizers since the 1980s. In 2005, the use of MI alone was approved for the preservation of cosmetic and household products in the EU. Before that, MI was used in industrial products, and the first cases of isolated MI contact allergy were published.

Objectives: To present the prevalence and causes of MI contact allergy.

Materials and methods: Patch test results from 2536 dermatitis patients tested with MI at Gentofte University Hospital between May 2006 and February 2010 were analysed. A retrospective investigation of medical records from MI-allergic patients was performed to reveal the causes of their MI contact allergy.

Results: Of patch-tested patients, 1.5% had MI contact allergy. It was associated with occupational dermatitis, hand eczema and age above 40 years. Exposure to MI in cosmetic products was found in 12 (32%) cases, and exposure to MI in occupational products was found in 11 (30%) cases; 5 of the 11 were painters.

Conclusions: The prevalence of MI contact allergy is already at the same level as that of other sensitizing preservatives, which have been on the market for several years, but no rising trend was identified. MI contact allergy was associated with both occupational and consumer products.

Key words: contact allergy; cosmetics; methylisothiazolinone; occupation; prevalence.

Conflict of interests: The authors have declared no conflicts.

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Preservatives are necessary in a wide variety of industrial and consumer products to prevent spoilage by microorganisms. Some of the most common preservatives are also prevalent sensitizers (1). The combination of methylchloroisothiazolinone (MCI)/methylisothiazolinone (MI), in a 3:1 ratio, has been frequently used as a preservative since the beginning of the 1980s (1, 2). MCI/MI is used in a wide variety of industrial products, such as paints, lacquers, varnishes, polishing agents, toners and printing ink, as well as in household and cosmetic products (1–4). Both MCI and MI can cause contact allergy. MCI has been recognized as the more potent sensitizer of the two, although MI is also categorized as a strong sensitizer (5–7). In Denmark, the prevalence of contact allergy to MCI/MI has been stable at approximately 2% among patch-tested individuals since 1985 (8).

MI was approved for the preservation of cosmetics and household products in the EU in 2005, with a maximum permitted concentration of 100 ppm (0.01%) (9, 10). This corresponds to a more than a 25-fold increase in the maximum exposure to MI when compared to the maximum permitted concentration of MCI/MI (3.75 ppm MI in 15 ppm MCI/MI). Before approval for cosmetic and household products, MI was permitted in different industrial products such as paints, lacquers, varnishes, polishing agents, toners and printing inks (3). The first cases of isolated MI contact allergy were

published in 2004 and 2006, and were caused by occupational MI exposure (11, 12). In one of these studies, two patients had a positive patch test reaction to 30 ppm MI (12). In a recent review, it was found that contact allergy epidemics often begin with detection of occupational cases, and are then followed by the occurrence of allergic contact dermatitis among consumers (13). In this study, we present the prevalence and causes of MI contact allergy in dermatitis patients patch tested since 2006.

Materials and Methods

The European baseline series was supplemented with MI [2000 ppm aq., Chemotechnique Diagnostics (Vellinge, Sweden)] and used to patch test 2536 dermatitis patients from May 2006 to February 2010. Patch testing was performed using Finn Chambers® (Epitest, Oy, Finland) on Scanpor® tape (Norgesplaster A/S, Alpharma, Vennesla, Norway).

The patch tests were applied on the upper back and occluded for 2 days. Readings were performed on D2, on D3 or D4, and on D7, according to the recommendations from the International Contact Dermatitis Group (14). Reactions that were 1+, 2+ and 3+ were interpreted as positive reactions. Irritant responses, doubtful responses (?+) or negative readings were interpreted as negative.

The MOAHLFA (Male, occupational dermatitis, atopic dermatitis, hand eczema, leg dermatitis, facial dermatitis, age above 40 years) index was routinely registered throughout the study period by the treating physician. Medical records from patients with MI allergy in the period were examined retrospectively. This was done to obtain information about relevant exposures and the exact anatomical location of the dermatitis reaction (hands, face, scalp, arms, trunk, legs, feet, universal, other, missing). Comparisons between MI-allergic patients and patients without MI allergy were performed using the χ^2 test. A χ^2 trend test (linear by linear association) was used to test for possible significant trends of contact allergy across patch test years. A one-sample Kologorow–Smirnow test was used to test for normal distribution of the age of MI-allergic patients. Data analyses were performed using the SPSS package (SPSS Inc., Chicago, IL, USA) for Windows (release 17.0).

Results

The overall prevalence of MI contact allergy was 1.5% (37/2536) between 2006 and 2010, with a stable prevalence over the 5-year test period ($P_{trend} = 0.88$) (Table 1). MI-allergic patients were aged 18–81 years (mean 52.2 years, $P = 0.90$, one-sample Kologorov–Smirnov test). According to the MOAHLFA index, MI contact allergy was significantly more often associated with occupational exposure, hand eczema and age above 40 years (Table 2). When characteristics were compared between male and female MI-allergic patients, men had occupational dermatitis significantly more often ($P = 0.02$). Besides hand eczema (68%), the anatomical site of dermatitis included the face (30%, $n = 11$), the arms (30%, $n = 11$) and the legs (27%, $n = 10$).

Table 1. Prevalence of methylisothiazolinone (MI) contact allergy among 2536 dermatitis patients patch tested from May 2006 to February 2010

Year	Total tested	MI allergy		
		Total, % (*n*)	Men, % (*n*)	Women, % (*n*)
2006	416	1.7 (7)	2.1 (3)	1.5 (4)
2007	655	1.1 (7)	1.8 (4)	0.7 (3)
2008	623	1.7 (11)	2.0 (4)	1.7 (7)
2009	749	1.3 (10)	1.3 (3)	1.4 (7)
2010	93	2.2 (2)	4.2 (1)	1.4 (1)
Total	2536	1.5 (37)	1.8 (15)	1.3 (22)

Table 2. MOAHLFA index among 2536 patch-tested dermatitis patients stratified into methylisothiazolinone (MI) patch test positives and MI patch test negatives

	MI-positive ($n = 37$), % (*n*)	MI-negative ($n = 2499$), % (*n*)	*P*-value*
Male	41 (15)	33 (812)	0.3
Occupational dermatitis	38 (14)	23 (570)	0.03
Atopic dermatitis	8.1 (3)	17 (429)	0.15
Hand dermatitis	68 (25)	42 (1041)	0.002
Leg ulcers	8.1 (3)	3.7 (93)	0.17
Facial dermatitis	30 (11)	23 (590)	0.39
Age > 40 years	81 (30)	65 (1632)	0.05

*χ^2-test, except in 'Leg ulcers', where Fisher's exact test was used.

A total of 40 patients had positive patch test reactions to MCI/MI. Concomitant positive patch test reactions to MI and MCI/MI were found in 41% (15/37) of MI-allergic patients ($P < 0.001$). Comparison of the two groups revealed that MI-allergic patients had hand eczema significantly more often ($P = 0.03$).

Concomitant positive patch test reactions to three or more allergens, besides MI and MCI/MI, occurred significantly more often among MI-allergic patients ($P < 0.001$). The most prevalent concomitant contact allergy was methyldibromoglutaronitrile allergy (12/37), followed by nickel allergy (9/37).

Exposure caused by cosmetics was found in 32% (12/37) of patients. Seven of the nine wash-off products were hair care products (shampoo or conditioner), and two were liquid soaps. Leave-on products were creams (2), cleansing milk (1) and a

Table 3. Exposure to methylisothiazolinone (MI) among the 37 MI-allergic patients

	Source			Substance(s)*		
	Men	Women	Total	MI	MCI/MI	Both
Occupational	—	—	11	—	—	—
Cleaning	1	1	—	1	1	—
Painters	4	1		3	1	1
Others[†]	3	—	—	—	3	—
Unknown	1	—	—	—	—	—
Cosmetics[‡]	—	—	12	—	—	—
Leave-on	1	3	—	2	2	—
Rinse-off	4	5	—	5	3	1[$]
Unknown	—	1	—	—	—	—
Household products	—	1	1	1	—	—

MCI, methylchloroisothiazolinone.
*Based on products, ingredient labels or Material Safety Data Sheets brought in by the patients.
[†]Chemical factory, glue factory and cutting oils.
[‡]Two patients had both a stay-on and a wash-off product with either MI or MCI/MI in it.
[$]One patient used two different wash-off products, one with MI and one with MCI/MI.

suntan lotion (1). Occupational exposure was found in 30% (11/37) of patients; 5 of these were painters (Table 3). Exposure caused by household products was found in 1 of the 37 patients. Current exposure to MI alone was found in 12 patients, to MCI/MI alone in 10 patients, and to both MCI/MI and MI in 2 patients (Table 3).

Discussion

Our study showed that the overall prevalence of MI contact allergy (1.5%) is at the same level or just below the level of other preservatives that have been on the market for several years. for example formaldehyde releasers and MCI/MI (8).

MCI/MI and MI are used in both industrial and domestic products. MCI/MI is most frequently used in products such as paints, lacquers, printing inks and glues (3, 4). To date, there is no published information on the frequency of MI use in industrial products. However, in the Nordic database SPIN (Substances in Preparations in Nordic Countries), it is possible to determine the types of industrial products that MI is used in (3). On the basis of recent data from the SPIN database, MI is used in the same types of product as MCI/MI. Usage data for MI in cosmetics is only published in the USA, where manufacturers voluntarily report their use of preservatives in cosmetics (15). In 2007, MI alone was used in only a small percentage of cosmetic products, whereas MCI/MI was the 10th most frequent preservative, being found in approximately 5% of cosmetic products (15).

The first cases of MI contact allergy were published in 1986, and the patients were all exposed and allergic to both MCI and MI (16). The first cases of occupational MI contact allergy caused by MI alone were described in 2004 and 2006 (11, 12).

The legislation on cosmetic and household products is stricter than the legislation on industrial products, and new compounds are typically introduced earlier in industrial products than in cosmetics and household products. This is probably one of the main reasons why the first cases of allergic contact dermatitis caused by preservative may be occupational (13). Workers who handle allergenic compounds at industrial sites are often exposed to higher concentrations, and are thereby at risk of developing chemical burns and subsequent sensitization (11, 12). Furthermore, when the pattern of exposure is compared between occupational exposure to, for example, paints or cleaning agents, and consumer exposure to, for example, cosmetics or household products, it is clear that occupational exposure is greater than consumer exposure. We found that MI contact allergy was associated with occupational dermatitis especially in men, and that 30% of MI-allergic patients had a specific relevant exposure associated with their occupation; almost half of them were painters (Table 3).

The overall prevalence of preservative contact allergy increased significantly from 1985 to 2008 (8). This was primarily caused by the introduction of new preservatives in the European baseline series, for example methyldibromoglutaronitrile. However, each time a new allergenic preser vative is introduced to the market and subsequently patch tested, the overall burden of allergenic preservatives will increase, as well as the overall prevalence of preservative contact allergy (8).

MI is available at 2000 ppm from Chemotechnique as a standard preparation. This was the concentration used in the current study. In previous studies on occupational contact dermatitis caused by MI, the patients reacted to 1050 ppm in one study (11), and from 500 ppm and down to 30 ppm in another (12). This indicates that the 2000 ppm currently used for patch testing should be sufficient to detect most MI-allergic patients. A more detailed evaluation would have been possible if a serial dilution of MI had been employed. Such a study has been performed, and will be published (personal communication M. Bruze).

Concomitant positive reactions to MI and MCI/MI were seen in 40% of MI-allergic patients. Whether this was caused by exposure to the MI in MCI/MI or contact allergy to MCI with cross-reactivity to MI is unknown. A clinical study showed that some patients had a positive patch reaction to 30 ppm MI, just above the concentration of MI in the MCI/MI patch test, supporting the first option (12). However, an animal study showed that

cross-reactivity to MCI was indicated when MI was the primary sensitizer, giving some support to the latter explanation (7).

We also found that MI-allergic patients were often polysensitized and reacted to more than three different allergens (besides MCI/MI and MI), which could indicate that the MI-allergic patients had a higher degree of exposure and/or were more susceptible to other allergens.

In this study, we showed that MI contact allergy is frequent and occurs at the same level as that of other sensitizing preservatives (8). Relevant exposures to cosmetics or occupational products were found in two-thirds of the MI-positive patients. Hair care products were the most frequent cause of cosmetic exposure to MI, and painters seemed to constitute a specific subgroup of occupational exposure to MI.

At present, there are no available clinical experiments, for example repeated open application tests or dose–response tests. These are needed to establish the threshold levels of MI-allergic dermatitis, as a basis for safer use levels.

References

1. Lundov M D, Moesby L, Zachariae C, Johansen J. Contamination versus preservation of cosmetics: a review on legislation, usage, infections, and contact allergy. *Contact Dermatitis* 2009: 60: 70–78.
2. Fewings J, Menné T. An update of the risk assessment for methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) with focus on rinse-off products. *Contact Dermatitis* 1999: 41: 1–13.
3. Anon. SPIN, Substances in Preparations In Nordic Countries. 2010 April 9.
4. Flyvholm M A. Preservatives in registered chemical products. *Contact Dermatitis* 2005: 53: 27–32.
5. Kimber I, Basketter D A, Butler M et al. Classification of contact allergens according to potency: proposals. *Food Chem Toxicol* 2003: 41: 1799–1809.
6. Basketter D A, Gilmour N J, Wright Z, Walters T, Boman A, Lidén C. Biocides: characterization of the allergenic hazard of methylisothiazolinone. *Cutaneous Ocul Toxicol* 2003: 22: 187–199.
7. Bruze M, Fregert S, Gruvberger B, Persson K. Contact allergy to the active ingredients of Kathon CG in the guinea pig. *Acta Derm Venereol* 1987: 67: 315–320.
8. Thyssen J P, Engkilde K, Lundov M D, Carlsen B C, Menné T, Johansen J D. Temporal trends of preservative allergy in Denmark (1985–2008). *Contact Dermatitis* 2010: 62: 102–108.
9. European Commission. Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (Consolidated version). 2008.
10. European Commission. Regulation (EC) No 648/2004 of The European Parliament and of the Council of 31 March 2004 on detergents, 2004.
11. Thyssen J P, Sederberg-Olsen N, Thomsen J F, Menné T. Contact dermatitis from methylisothiazolinone in a paint factory. *Contact Dermatitis* 2006: 54: 322–324.
12. Isaksson M, Gruvberger B, Bruze M. Occupational contact allergy and dermatitis from methylisothiazolinone after contact with wallcovering glue and after a chemical burn from a biocide. *Dermatitis* 2004: 15: 201–205.
13. Thyssen J P, Johansen J D, Menné T. Contact allergy epidemics and their controls. *Contact Dermatitis* 2007: 56: 185–195.
14. Wilkinson D S, Fregert S, Magnusson B et al. Terminology of contact dermatitis. *Acta Derm Venereol* 1970: 50: 287–292.
15. Steinberg D C. Voluntary registration of cosmetics and 2007 frequency of preservative use. *Cosmet Toiletries Mag* 2008: 123: 47–52.
16. Pilger C, Nethercott J R, Weksberg F. Allergic contact dermatitis due to a biocide containing 5-chloro-2-methyl-4-isothiazolin-3-one. *Contact Dermatitis* 1986: 14: 201–204.

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Methylisothiazolinone contact allergy and dose-response relationships

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Abstract

Background Methylisothiazolinone (MI) used alone is a new preservative with a high prevalence of contact allergy. The eliciting threshold of MI is unknown. Combination of MI and phenoxyethanol enhanced the antimicrobial efficacy of MI.

Objectives The eliciting doses of MI contact allergy in a patch test and a ROAT were investigated. In the patch test it was tested if phenoxyethanol influenced the reactivity to MI.

Methods Eleven MI-allergic individuals were patch tested with two dilution series of 12 doses of MI and the same 12 doses with phenoxyethanol. The ROAT mimicked use of a cream preserved with 100, 50 and 5ppm MI (corresponding to 0.21, 0.105 and 0.0105 µg MI/cm^2).

Results Phenoxyethanol had no influence on the reactions to MI. The lowest eliciting dose in the patch test was 1.47 µg MI/cm^2. In the ROAT 7 patients (64%) reacted to 0.21 and 0.105 µg MI/cm^2 and 2 patients (18%) reacted to 0.0105 µg MI/cm^2, corresponding to a cream preserved with 5ppm MI.

Conclusion A maximum of 100ppm MI can be added to cosmetic products. 18% of MI-allergics reacted to a concentration 20 times lower in a ROAT. The development of MI contact allergy should be monitored closely.

Introduction

Methylisothiazolinone (MI) is a preservative used in cosmetic, household and industrial products. It has been on the market since the 1980's in a 1:3 combination with methylchloroisothiazolinone (MCI). This combination is one of the most frequent causes of contact allergy to preservatives (1). In 2005 MI alone was permitted in cosmetic products at a maximum concentration of 100ppm (2). Prior to approval for cosmetic products MI had already been used in industrial products, and in 2004 the first cases of occupational contact allergy caused by exposure to MI alone from a wallpaper glue and a chemical burn was published (3). In 2010 the first cases of cosmetic related contact allergy to MI was published (4). The prevalence of MI contact allergy among patch tested dermatitis patients in Denmark is 1.5% which makes it one of the most common contact allergen among preservatives (1;5).

The manufacture of MI recommends use concentrations from 50 to 100ppm MI either alone or in combination with phenoxyethanol (6;7). However, a recent study showed that 5ppm MI in combination with 0.4% phenoxyethanol was sufficient to preserve a standard cosmetic cream (8). Phenoxyethanol is a widely used preservative, which rarely causes allergic contact dermatitis (9;10).

Besides the few case reports with patients reacting to 10 and 30ppm MI at patch testing (3;4) there is no information available on the dose-response relationship in already sensitized MI patients. Fischer *et al* has developed a model for non-volatile compounds which converts dose-response patch test data to repeated open application test (ROAT) data (11).

In this study we investigated eliciting doses of MI with and without the addition of phenoxyethanol. Further it was tested if the previously developed conversion model between patch tests and ROAT also was valid for MI.

Material and Methods

Test subjects

MI in different concentrations (1000, 1050, 1500 and 2000ppm in aqua) corresponding to 30, 31.5, 45 and 60 µg MI/cm^2 respectively, has been part of a supplemental series used at Gentofte Hospital from 2005 and forth. In this period 52 patients has had a positive reaction (+, ++, +++) to MI to at least one of the different concentrations, which were the inclusion criteria in the study. Exclusion criteria were: age <18, eczema on the tested area, exposure to UV-light within the last three weeks, systemic immunosuppressive therapy, pregnancy, breast feeding and not being able to cooperate.
To include additional participants in the study we invited the patients with a positive reaction to MCI/MI (100ppm) from 2000 to 2005 into the study as well, as approximately 40% of MCI/MI allergic patients has a concomitant reaction to MI as well (5). Fifty MCI/MI allergic patients were invited and 5 accepted to participate. Before inclusion into the general study the MCI/MI patients were patch tested with MI (60 µg MI/cm^2). Inclusion and exclusion criteria were the same.
In total 11 test subjects with MI contact allergy were recruited into the study, two women and 9 men aged 37-68 years (mean 49.7).

Control subjects

Healthy volunteers were included in the study as a control group. Exclusion criteria were the same as for the test subjects. The control subjects were responders to a post on the website www.forsøgsperson.dk about the study. Six women and eight men aged 20-44 years (mean 27.5) were included as control.
All control and test subjects received written and oral information and signed a written consent prior to enrolment in the study. The study was performed according to the Helsinki II declaration and was approved by the local ethic committee (Capital region of Denmark, H-2-2010-015)

Patch test

The patch test series consisted of 12 decreasing doses of MI in Neolone 950™ 9.7% active ingredient (kindly supplied by Rohm & Hass, now DOW chemicals) in 10% ethanol and 90% water and the same 12 doses of MI combined with 9.26 µg phenoxyethanol/cm^2 (Sigma Aldrich, Schnelldorf, Germany) in the same vehicle. The dose of phenoxyethanol corresponded to a concentration of 0.4%, which together with MI were shown to be an effective preservative combination in a previous study (8). The blank was phenoxyethanol (9.26 µg/cm^2) in 10% ethanol and 90% water. The doses of MI in the patch test were: 60, 30, 15, 8.82, 4.41, 2.94, 1.47, 0.441, 0.21, 0.147, 0.105 and 0.0105 µg MI/cm^2 (Table 1). Control subjects were only patch tested with 60 µg MI/cm^2 and the blank. Fifteen µl of each dilution was applied on a filter disc in a Finn Chamber (Epitest, Oy, Finland) on Scanpor tape (Norgesplaster A/S, Alpharma, Vennesla, Norway). The patch test were applied on the upper back and occluded for 2 days. Readings were performed on D2, D3 or D4 and on D7, but only reactions from D3 or D4 were used for statistical calculations. As suggested by Fischer *et al* the following reading scale of reactions were used: 0 = no reaction; 1 = few papules with no erythema, no infiltration; 2 = faint erythema with no infiltration or papules; 3 = faint erythema with few papules and no homogenous infiltration; 4 = erythema, homogenous infiltration; 5 = erythema, infiltration and a few papules; 6 = erythema, infiltration and papules; 7 = erythema, infiltration, papules and a few vesicles; 8 = intensive erythema, infiltration and vesicles (12;13). Placement of the different doses were randomised and blinded for the investigator and subjects. The lowest dose (minimum score =1) in a continuous line from 60 µg MI/cm^2 and down wards was defined as the threshold dose.

ROAT

All participants were thoroughly instructed in applying 20 µl from four different bottles twice a day on four areas on the volar aspect of the forearm. Each area were 3 x 3cm. Solutions were applied with a fixed volume micropipette (Acura 815, 20 µl, Buch & Holm, Herlev, Denmark). Each bottle was numbered 1-4 which referred to one of the corresponding area on the fore arm also numbered 1-4. The solutions were spread out on the entire area with the tip of the pipette and allowed to dry by evaporation. The four bottles contained three different concentrations of MI combined with phenoxyethanol in 10% ethanol and 90% water and one blank without MI. The intention of the ROAT was to mimic the use of a cream preserved with three different concentrations of MI. One hundred ppm MI which is the maximum permitted concentration, 50ppm which is the lowest concentration recommended by the manufacture of MI and 5ppm which was the concentration sufficient to preserve a cosmetic cream when combined with 0.4% phenoxyethanol (8). Based on a usage of 4.2 mg cream/cm^2/day (14) the doses used in the ROAT was converted to an exposure of 0.21, 0.105 and 0.0105 µg MI/cm^2 per application (Table 1). Reactions were read on D2, D3 or D4, D7, D14 and D21 routinely and additionally if a reaction occurred between visits. If an area scored 5 or above in a system developed for ROAT, exposure to this area was terminated (15). If no reactions occurred or an area scored under 5 all exposures were terminated after 21 days. A typical reaction in the ROAT is shown in Figure 1. The threshold dose was the lowest dose with a score of 5 or above, or the lowest dose which gave a visible reaction that remained at D21 if the exposure had not been terminated. Readings in both the patch test and the ROAT were done by the same investigator together with one of the nurses from the allergy laboratory. As a control 5 sets of ROAT bottles were weighted before and after the 21 exposure days.

Comparison of patch test and ROAT

Repeated exposure is an important eliciting factor in allergic contact dermatitis. However, the ROAT is a very time consuming experiment. A model that converts patch test data to ROAT has been suggested (11):

$$ED_{xx}(ROAT) = 0.0296 \times ED_{xx}(patch\ test)$$

Where ED_{xx} is the eliciting dose in xx% sensitized individuals, and 0.0296 is the factor that converts patch test data into ROAT data. The model is based on results from nickel and the preservative methyldibromo glutaronitrile (MDBGN)(11).

Calculations and Statistics

We used standard logistic regression analysis to estimate the dose-response relationship in the patch tests. The eliciting doses (ED) which predicts a dose that will elicit a reaction in 5, 10, 25, 50, 75, 90 and 95% of sensitized patients were calculated and a fitted dose-response curve was drawn. Comparison between the patch test reaction with or without phenoxyethanol was performed using the Wilcoxons ranked sums test and correlations between the individual threshold doses were investigated by Spearman's ranked correlation. Difference in reactions to the same doses in the patch test and ROAT was investigated using the McNemar's test. If the model for converting patch test data to ROAT data is used two conditions has to be fulfilled. First, a positive correlation between the two test methods should be ascertained. Second, the dose-response curves have to be parallel. Spearman's ranked correlation was used to analyse the correlation between results from patch test and ROAT performed on the same patients.

Results

Patch test

A total of 11 test subjects and 14 control subjects participated in the study. Nine of the 11 were patients with a previous positive patch test to MI. Five MCI/MI allergic patients were patch tested with MI. Two developed a positive reaction and were included in the study.

All test subjects reacted to the maximum tested dose on D2, but 1 patient had no visible reaction on D4. The results of the patch test reactions are shown in Table 2. The lowest threshold dose was 1.47 µg MI/cm^2 of which more than half of the test subjects reacted to. No reactions to 60 µg MI/cm^2 or the blank were seen in the control subjects.

Figure 2 shows the fitted dose-response curve for all 11 test subjects with and without phenoxyethanol. The two curves are almost identical and statistically there are no differences between the results in the patch test with and without phenoxyethanol (Wilcoxon's ranked sum test P=0.27). Furthermore, there where no differences in the individual threshold doses with or without phenoxyethanol (Spearman's ranked correlation coefficient r_s=0.98 P=0.002). Based on the patient's reactions in the patch test it is possible to calculate the ED for already sensitized individuals. These results are shown in Table 3.

ROAT

Out of the 11 test subjects 9 followed the application scheme for all 21 days. One patient lost the pipette, tips and solutions and did not receive a new set until 4 days later. He did not develop any reactions to any of the doses. One patient applied the solutions for only 19 days due to travelling the last 2 days, this patient had reacted to the 2 highest doses in the ROAT within the first 10 days, and had no reaction to the lowest dose after 19 days. Seven test subjects (64%) reacted to the highest dose (0.21 µg MI/cm^2). The same 7 test subjects also reacted to the middle dose (0.105 µg MI/cm^2).

Two (18%) reacted to the lowest dose (0.0105 µg MI/cm^2). Three of the test subjects reacting to the middle dose (0.105 µg MI/cm^2) did not score 5 or above in the ROAT evaluation scale, but had a clear visible reaction. None of the test subjects reacted to the control. None of the control subjects developed any reactions in the ROAT. Weighing 5 sets of ROAT bottles before and after the experiment showed that the participants followed the application scheme and were exposed to the calculated values of MI and phenoxyethanol.

Comparison between the patch test and the ROAT

The frequency of reaction to the dose pr. application in the ROAT and the same dose in the patch test were compared (Table 4). The highest and middle dose in the ROAT (0.21 µg MI/cm^2 and 0.105 µg MI/cm^2) were statistically significant (McNemar's test P= 0.023). In Figure 3 the fitted dose-response curves for both the patch test and the ROAT is showed. The ROAT dose-response curve is displaced to the left meaning that repeated exposures caused the test subjects to react to lower doses compared to single exposures. The threshold doses found in the patch test and ROAT were correlated (Spearman's ranked correlations coefficient r_s=0.64 P=0.043). However, visually the two curves are not completely parallel, but when the patch test data is converted to ROAT data by the model (Figure 4) the two ROAT dose-response curves are not that different. The model for converting patch test data into ROAT based on the results in this study would be:

$$ED_{xx}(ROAT)= 0.0362xED_{xx}(patch\ test)$$

Discussion

MI used alone is a relatively new preservatives for cosmetics, but it is already the fourth most common preservative contact allergen (1;5). When MCI/MI contact allergy appeared in the 1980's it was followed by studies that showed that both MCI and MI where sensitizers, with MCI as the most potent (16-18).

Nine out of 52 MI allergic patients were eligible and interested in participating in the study. To include additional test subjects the 50 patients with a positive MCI/MI patch test from 2000 to 2005 were invited to the study. Concomitant reactions to MI in MCI/MI allergic patients is app. 40% (5). Five of the 50 patients with MCI/MI contact allergy chose to participate and 2 (40 %) reacted to MI, and were included in the study. The concentration of MI in the 100ppm MCI/MI patch test concentration is 25ppm (0.75 µg MI/cm^2). In this study the two MCI/MI allergic patients reacted to 1.47 µg MI/cm^2 but not to 0.441 µg MI/cm^2. These two patients could potentially have reacted to the MI part and not MCI in their previous MCI/MI patch test.

In two studies on MI contact allergy some of the patients were patch tested with different concentrations of MI. These studies showed that patients reacted to as low concentrations as 10 and 30 ppm (3;4). In this study the lowest dose the test subjects reacted to was 1.47 µg MI/cm^2 (corresponding to 49 ppm). The next dose tested was 0.441 µg MI/cm^2 (15 ppm) three times lower than the previous dose and none of the test subjects reacted to this dose.

Phenoxyethanol is the third most often used preservative in the US, but a very rare sensitizer (9;10). Phenoxyethanol is not a very effective antimicrobial so it is often necessary to combine it with other preservatives (8;19). We have shown that both 0.2% and 0.4% phenoxyethanol in combination with 5ppm MI were sufficient to preserve a cosmetic cream (8). Phenoxyethanol is a potential skin irritant so we chose to use 9.26 µg phenoxyethanol/cm^2 (corresponding to 0.4%) to see if this had

any effect on the reactions (20). Phenoxyethanol did not influence the reactions in the patch test (Figure 2) and we did not find any differences in the reactions to the same doses of MI.

MI has been used in four different patch test concentrations at Gentofte Hospital since 2005. Based on the results in this study it appears that the concentration used currently (60 µg MI/cm^2 or 2000ppm) is the best. All test subjects reacted to this dose, one patient had a weak reaction which disappeared by D4, and none of the 14 control subjects developed any irritant reactions. Furthermore all reactions except one in the diagnostic patch were either + or ++. A study from the IVDK network in Germany showed a prevalence of MI contact allergy at 1%, however, they only tested with 500ppm MI (10), this could indicate that the prevalence in their study might be underestimated.

In the ROAT setup we wanted to investigate if MI allergic patients could use a cream preserved with different concentrations of MI in combination with phenoxyethanol. The different concentrations of MI was chosen based on the maximum permitted concentration in cosmetic, the lowest concentration suggested by the manufacture of MI and the concentration proven effective in a cosmetic cream (2;6;8). These concentrations were 100ppm, 50ppm and 5ppm. Based on an exposure of 4.2 mg cream/cm/day as calculated by Colipa (14) this corresponded to an exposure per application of 0.21 µg MI/cm^2, 0.105 µg MI/cm^2 and 0.0105 µg MI/cm^2, respectively, in the ROAT.

Two test subjects (18 %) reacted to 0.0105 µg MI/cm^2 in the ROAT (Table 4). When exposed to the maximum permitted dose (0.21 µg MI/cm^2) and the lowest recommended dose (0.105 µg MI/cm^2) 64% of the test subjects reacted. There is no information available about the use concentration of MI in cosmetics, but assuming that cosmetic manufactures follows the advice given by the manufactures of MI use concentrations between 50ppm and 100ppm is most likely.

As already shown in animal studies MI is a potent sensitizer (16;18), and when MI alone was used in industrial products the first cases of MI contact allergy quickly appeared (3;21). A recent study showed that the majority of occupational MI allergic patients were painters (5). The use concentrations in industrial products are unknown but from a few manufactures of paints we know that use concentrations ranges from 113 to 270ppm MI.

The ROAT dose-response curve was not parallel to the patch test dose-response curve. Hence, it did not fulfil the conditions set for the model for converting patch test data to ROAT data (11). However, the threshold doses were still correlated so it is still possible to compare the two different ROAT dose-response curves, and visually they are not that different (Figure 3 and 4). The conversion model were developed based on experiments with nickel and MDBGN (12;13). If looked at independently there is a difference in the conversion factors found in both studies. For MDBGN it was 0.0265, while for nickel it was 0.0330 (11). In this study the conversion factor was 0.0362.

This is fairly close to the other factors despite a series of differences between the studies.

First, it is unknown if the model fits for all allergens. Second, the setup in this study differed from the setup in the nickel and MDBGN studies. Third, fewer test subjects participated in our study compared to the nickel and MDBGN studies (12;13). Still it is obvious that repeated exposure is an important factor in eliciting an allergic reaction.

In this study we showed that the concentration capable of eliciting an allergic reaction is 10 and 20 times lower than the lowest concentration recommended by the manufacture and the maximum permitted concentration in cosmetics.

When combined with MCI the maximum permitted concentration of MI in cosmetics is 3.75ppm. This low concentration has probably not sensitized many individuals to MI, but since 2005 MI alone has been permitted in cosmetics and it may also be used in detergents (2;22). The first cases

of cosmetic and household products related MI contact allergy has just been published (4;5). Based on the recent publications on MI contact allergy and the results found in this study we recommend that the permitted concentration of MI is reduced. If the prevalence of MI contact allergy increases without regulations in the permitted concentrations there is a considerable risk that MI will cause an epidemic of contact allergy and eventually be banned from cosmetic products.

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Reference List

(1) Thyssen JP, Engkilde K, Lundov MD, Carlsen BC, Menné T, Johansen JD. Temporal trends of preservative allergy in Denmark (1985-2008). Contact Dermatitis 2010: 62(2):102-8.

(2) Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (Consolidated version), European Commission, (2008).

(3) Isaksson M, Gruvberger B, Bruze M. Occupational contact allergy and dermatitis from methylisothiazolinone after contact with wallcovering glue and after a chemical burn from a biocide. Dermatitis 2004: 15(4):201-5.

(4) Garcia-Gavin J, Vansina S, Kerre S, Naert A, Goossens A. Methylisothiazolinone, an emerging allergen in cosmetics? Contact Dermatitis 2010: 63(2):96-101.

(5) Lundov MD, Thyssen JP, Zachariae C, Johansen JD. Prevalence and cause of methylisothiazolinone contact allergy . Contact Dermatitis 2010: 63(3):164-7.

(6) ROHM and HAAS. NEOLONE^{tm} 950 A safe, Broad Spectrum Bactericide for the Preservation of Leave-on Products. 2006.

(7) ROHM and HAAS. NEOLONE^{tm} PE A safe, Broad Spectrum Preservative for Leave-on Products Based on Methylisothiazolinone and Phenoxyethanol. 2006.

(8) Lundov MD, Zachariae C, Johansen JD, Moesby L. Low level efficacy of cosmetic preservatives. Int J Cosm Science 2009: Accepted.

(9) Steinberg DC. Voluntary Registration of Cosmetics and 2007 Frequency of Preservative Use. Cosmetics and Toiletries® magazine 2008: 123(10):47-52.

(10) Schnuch A, Uter W, Lessmann H, Geier J. Contact allergy to preservatives. Results of the Information Network of Departments of Dermatology (IVDK) 1996-2007. Interdisciplinary Journal of Allergy and Environmental Medicine 2008: 17:631-8.

(11) Fischer LA, Voelund A, Andersen KE, Menné T, Johansen JD. The dose-response relationship between the patch test and ROAT and the potential use for regulatory purposes. Contact Dermatitis 2009: 61(4):201-8.

(12) Fischer LA, Johansen JD, Menne T. Methyldibromoglutaronitrile allergy: relationship between patch test and repeated open application test thresholds. Br J Dermatol 2008: 159(5):1138-43.

(13) Fischer LA, Johansen JD, Menne T. Nickel allergy: relationship between patch test and repeated open application test thresholds. Br J Dermatol 2007: 157(4):723-9.

(14) QRA Expert Group. Dermal Sensitization Quantitative Risk Assesement (QRA) for fragrance ingredients. 2006.

(15) Johansen JD, Bruze M, Andersen KE, Frosch PJ, Dreier B, White IR, Rastogi S, Lepoittevin JP, Menne T. The repeated open application test: suggestions for a scale of evaluation. Contact Dermatitis 1998: 39(2):95-6.

(16) Basketter DA, Gilmour NJ, Wright Z, Walters T, Boman A, Lidén C. Biocides: Characterization of the Allergenic Hazard of Methylisothiazolinone. Cutaneous and Ocular Toxicology 2003: 22(4):187-99.

(17) Bruze M, Dahlquist I, Fregert S, Gruvberger B, Persson K. Contact allergy to the active ingredients of Kathon CG. Contact Dermatitis 1987: 16(4):183-8.

(18) Bruze M, Fregert S, Gruvberger B, Persson K. Contact allergy to the active ingredients of Kathon CG in the guinea pig. Acta Derm Venereol 1987: 67(4):315-20.

(19) Steinberg DC. Preservatives for Cosmetics. 2nd ed. Carol Stream, IL: Allured Publishing Corporation; 2006.

(20) MSDS Phenoxyethanol. 2003; http://msds.chem.ox.ac.uk/PH/2-phenoxyethanol.html

(21) Thyssen JP, Sederberg-Olsen N, Thomsen JF, Menné T. Contact dermatitis from methylisothiazolinone in a paint factory. Contact Dermatitis 2006: 54(6):322-4.

(22) Regulation (EC) No 648/2004 of The European Parliament and of the Council of 31 March 2004 on detergents, European Commission, (2004).

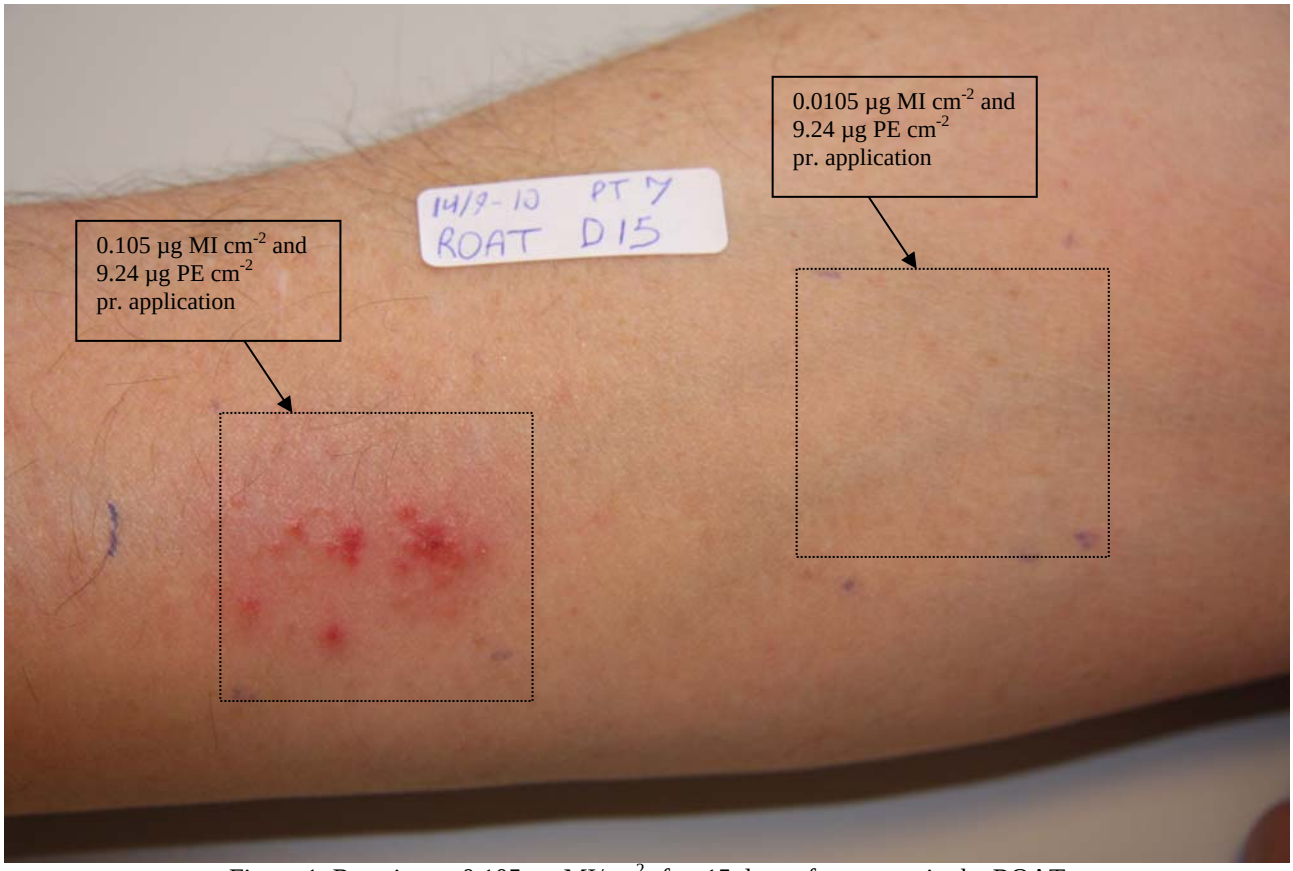

Figure 1: Reaction to 0.105 µg MI/cm^2 after 15 days of exposure in the ROAT

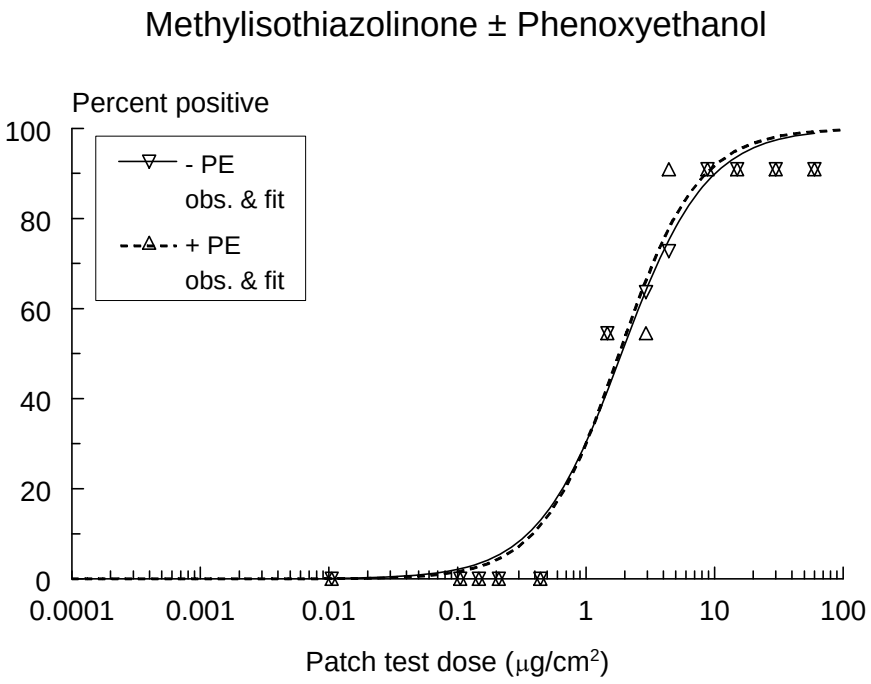

Figure 2: Fitted dose-response curves for MI ± phenoxyethanol.

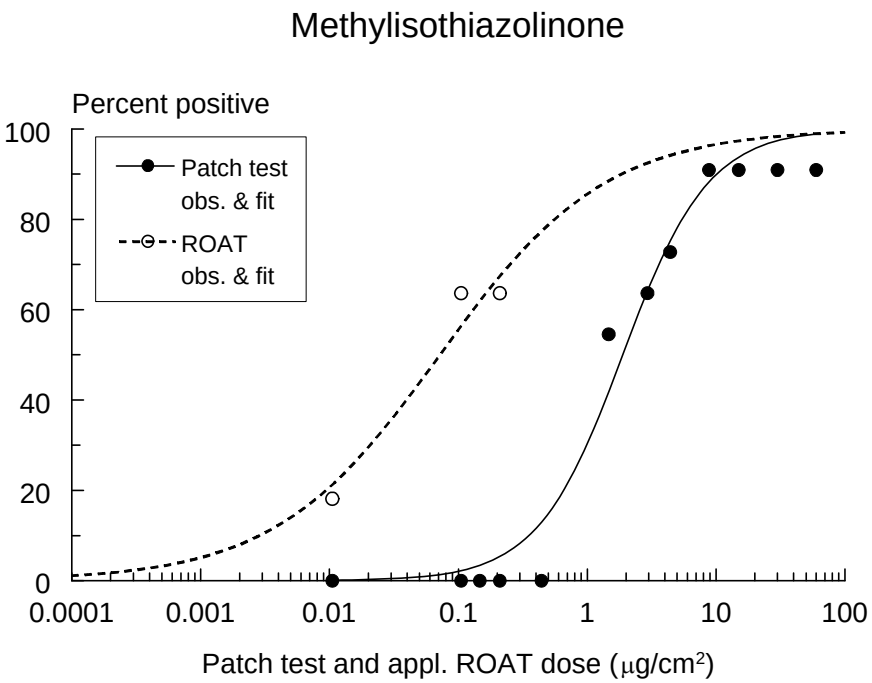

Figure 3: Fitted dose-response curves for patch test and ROAT.

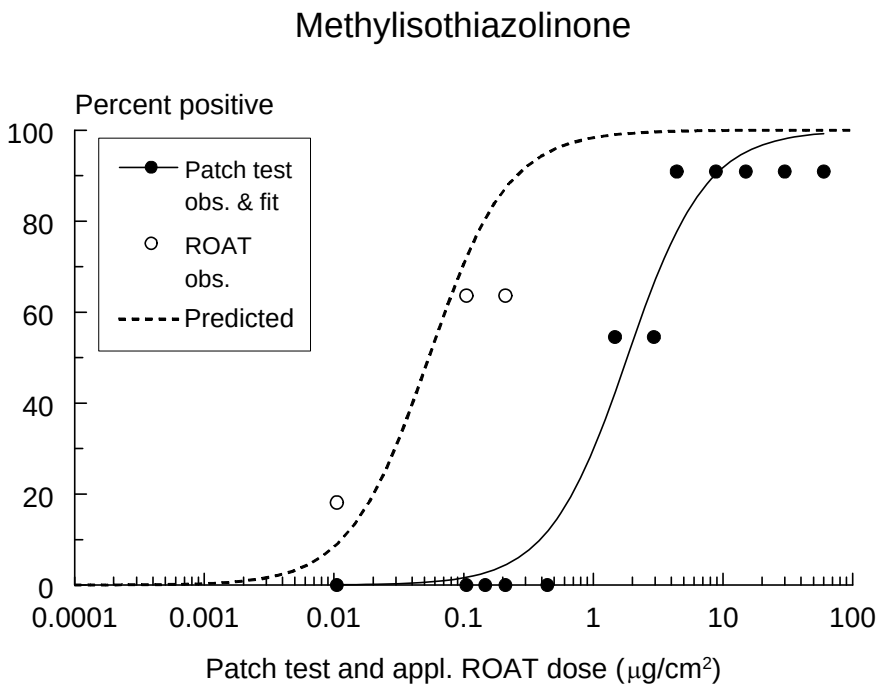

Figure 4: Fitted dose-response curve for patch test and calculated dose-response curve for ROAT based on the conversion model (11)

Table 1: Doses in the patch test and the ROAT

Patch test dilution series* (µg MI/cm^2)	Equivalent ROAT doses (µg MI/cm^2)
60	—
30	—
15	—
8.82	3 weeks accumulated dose (0.21) in the ROAT
4.41	3 weeks accumulated dose (0.105)
2.94	1 week accumulated dose (0.21) in the ROAT
1.47	1 week accumulated dose (0.105) in the ROAT
0.441	3 weeks accumulated dose (0.0105) in the ROAT
0.21	Highest dose per application in the ROAT
0.147	1 week accumulated dose (0.0105) in the ROAT
0.105	Middle dose per application in the ROAT
0.0105	Lowest dose per application in the ROAT

* The same 12 concentrations were also applied with 9.26 µg phenoxyethanol/cm^2.

Table 2: Number and % of reactions to the doses in the patch test

	Reactions n (%)	
Patch test dose (µg MI/cm^2)	Without phenoxyethanol	With phenoxyethanol (9.24 µg /cm2)
60	10 (91)	10 (91)
30	10 (91)	10 (91)
15	10 (91)	10 (91)
8.82	10 (91)	10 (91)
4.41	8 (73)	10 (91)
2.94	7 (64)	6 (55)
1.47	6 (55)	6 (55)
0.441	0	0
0.21	0	0
0.147	0	0
0.105	0	0
0.0105	0	0

Table 3: Calculated elicitation dose (ED) and 95% confidence interval (CI) in the patch test with and without phenoxyethanol

	Without phenoxyethanol		With phenoxyethanol	
	Dose (µg MI/cm2)	95% CI	Dose (µg MI/cm2)	95% CI
ED_5	0.20	0.012 – 0.54	0.23	0.016 – 0.58
ED_{10}	0.35	0.040 – 0.84	0.38	0.048 – 0.88
ED_{25}	0.82	0.20 – 1.8	0.84	0.22 – 1.7
ED_{50}	1.9	0.77 – 5.1	1.8	0.79 – 4.4
ED_{75}	4.4	2.0 – 21	4.0	1.9 – 16
ED_{90}	10	4.1 – 111	8.6	3.8 – 76
ED_{95}	18	6.3 – 362	15	5.6 – 227

Table 4: Comparison of the response frequencies in the doses identical in the patch test and the ROAT

Dose (µg MI/cm^2)	Patch test response n (%)	ROAT response n (%)	P-values (McNemar's test)
0.21	0 (0)	7 (64)	0.023
0.105	0 (0)	7 (64)	0.023
0.0105	0 (0)	2 (18)	0.48

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