

Analytical methodologies for common skin allergens

Organic thioureas, isothiocyanates and fragrance hydroperoxides in everyday life products

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Abstract

This thesis describes analytical methodologies for the determination of important skin-sensitizing chemicals in two types of commercial items: chloroprene rubber and fragrance products which are both well-known to be associated with contact allergy.

Chloroprene rubber (neoprene) is widely used in different applications and products, such as bags, gloves, wetsuits, braces, mouse pads et c. Exposure to chloroprene rubber materials has resulted in numerous cases of allergic contact dermatitis. Organic thioureas have been considered the main culprits, even though they at the same time have been classified as weak or non-sensitizers in the murine local lymph node assay (LLNA). Previous findings indicate that a possible reason for sensitization is that organic thioureas are being metabolized in the skin into more reactive electrophiles, such as isothiocyanates and isocyanates, after skin exposure. In this thesis, chemical analyses of a number of chloroprene products from the European open market showed the presence of diethylthiourea in all analyzed items, while other organic thioureas could not be detected. The levels of diethylthiourea varied, with the highest at $158 \mu\text{g cm}^{-2}$ in a used back support that had previously caused allergic contact dermatitis in a patient. Furthermore, it was discovered that all the examined items emitted ethyl isothiocyanate. LLNA showed that ethyl isothiocyanate is a strong skin sensitizer, as has been shown earlier for other tested isothiocyanates. Isothiocyanates were shown to be thermally formed from diethyl, diphenyl and dibutylthiourea at a temperature as low as $35 \text{ }^\circ\text{C}$, i. e. around skin temperature. Altogether, the results from these experiments, patch tests and chemical analyses revealed that isothiocyanates are important haptens in contact allergy to chloroprene rubber.

Fragrances constitute one of the main causes of contact allergy, next to nickel and preservatives. The most widespread fragrances in cosmetics and perfumes on the market are monoterpenes, such as linalool and limonene, which at air exposure easily oxidize to hydroperoxides, which are strongly skin sensitizing compounds and the main haptens. Despite this, there is so far no EU regulation concerning fragrance hydroperoxides in products, which may be due to lack of analytical methods which can reliably measure them in fragrances. Presented in this thesis is a toolbox of different analytical methods, applied on essential oils, shampoo, patch test preparations and different types of perfumes. Furthermore, one of our studies elucidated the first case (to my knowledge) of allergic contact dermatitis as being correlated to a product that contains a fragrance hydroperoxide.

Taken together, the thesis shows the importance of developing analytical methods for the identification, measurement and detection of important haptens in contact allergy.

Keywords: *Chemical analysis, contact allergy, chloroprene rubber, diethyl thiourea, ethyl isothiocyanate, fragrance hydroperoxides.*

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Cover image: Skin of my daughter, Leen Ramzi. Photo was taken by her mom, Nour Alshaar.

To my beloved family

List of Papers

- I. **Ramzy AG**, Hagvall L, Pei MN, Samuelsson K, Nilsson U (2015) Investigation of diethylthiourea and ethyl isothiocyanate as potent skin allergens in Chloroprene rubber. *Contact Dermatitis* 72 (3):139-146.

The author was responsible for generating ideas regarding all method development for chemical analysis and the major part of data evaluation and writing.

- II. **Ramzy AG**, Lammintausta K, Matura M, Brared Christensson J, Nilsson U, Hagvall L (2017) Isothiocyanates are important as haptens in contact allergy to chloroprene rubber. *British Journal of Dermatology* 177 (2):522-530.

The author was responsible for generating ideas regarding analytical method development, evaluation of data from chemical analyses and the majority of the writing.

- III. Rudbäck J, **Ramzy A**, Karlberg A-T, Nilsson U (2014) Determination of allergenic hydroperoxides in essential oils using gas chromatography with electron ionization mass spectrometry. *Journal of Separation Science* 37 (8):982-989.

The author contributed with method development and significantly to the chemical analyses, as well as to data evaluation.

- IV. Elliott JF, **Ramzy A**, Nilsson U, Moffat W, Suzuki K (2017) Severe intractable eyelid dermatitis probably caused by exposure to hydroperoxides of linalool in a heavily fragranced shampoo. *Contact Dermatitis* 76 (2):114-115.

The author developed the major part of the analytical method, performed the chemical analyses, evaluated data from the chemical analyses, and contributed to a significant part of the writing.

- V. **Ramzi A**, Ahmadi H, Sadiktsis, I, Nilsson U (2018) A two-dimensional reversed/normal phase high-performance liquid chromatography/tandem mass spectrometry system for determination of limonene and linalool hydroperoxides. *Manuscript*.

The author was responsible for generating ideas regarding analytical method development, all chemical analyses, data evaluation and writing.

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Populärvetenskaplig sammanfattning

Absorption genom huden är ett av flera sätt på vilka människan kan exponeras för kemiska ämnen i omgivningen. Lyckligtvis har huden ett immunförsvar och en metabolism som bidrar till ett skydd mot reaktiva skadliga ämnen. Immunsystemet aktiveras när det utsätts för nya kemikalier så att det "minns" och kan oskadliggöra dem vid nästa exponering. I en del fall uppstår en oönskad försvarsmekanism mot en substans som egentligen är ofarlig, så att en hudsensibilisering och kontaktallergi (hudallergi) utvecklas. Det är ett livslångt tillstånd; det finns ännu inget botemedel och minsta hudkontakt med ämnet kan orsaka hudreaktioner och eksem. Ofta är det dessutom svårt att härleda en uppkommen kontaktallergi till ett visst ämne eller kommersiell produkt. Det kräver tillräckligt bra och specifika analysmetoder, eftersom allergena ämnen kan vara svåra att mäta i blandningar med andra ämnen och halterna kan vara låga.

Huvudsyftet med detta arbete har varit att ta fram analysmetoder som kan användas för att mäta och kartlägga oxiderade parfymämnen och neoprengummikemikalier, båda vanligt förekommande i kommersiella produkter.

Det har varit känt sedan länge att parfymämnen, som linalool, geraniol och limonen, orsakar många fall av kontaktallergi. Det har emellertid visat sig att parfymämnen inte är speciellt allergena i sig, men lätt oxiderar vid kontakt med luftens syre och då bildar starkt hudsensibiliserande ämnen, hydroperoxider. Parfymhydroperoxider är värmeinstabila ämnen som hittills varit svåra att bestämma. En av metoderna som presenteras i avhandlingen kan användas för att mäta riktigt låga halter i parfym och bygger på en kombination av tvådimensionell vätskekromatografi och tandem-masspektrometri. En annan metod som presenteras utnyttjar gaskromatografi kopplad till masspektrometri

och är lämplig för exempelvis koncentrerade eteriska oljor. Därmed, med hjälp av en “verktygslåda” med metoder, kan parfymhydroperoxider identifieras och mätas i exempelvis aromaterapioljor, rakvatten, deodorant, schampo och eau de toilette.

Hudallergi mot neoprengummikemikalier har tidigare ansetts bero på en reaktion mot de rester av vulkaniseringsmedel som används vid tillverkningen, trots att dessa ämnen, organiska tioureor, inte är särskilt allergena i sig. I avhandlingen presenteras resultat från en undersökning av flera kommersiella neoprenprodukter. Dietyltiourea kunde detekteras i alla undersökta produkter med hjälp av vätskekromatografi kopplad till masspektrometri. Det mest intressanta resultatet från studien visar att neoprenmaterialen lätt avger ett gasformigt ämne, etylisotiocyanat. Redan vid 35°C, dvs ungefärlig hudtemperatur, bryts dietyltiourea ned till mätbara halter av extremt allergiframkallande etylisotiocyanat. Det innebär att kontaktallergi kan uppstå vid tillräckligt lång och upprepad hudkontakt med materialen. Mätningen av gasformigt etylisotiocyanat gjordes med hjälp av fastfas-mikroextraktion i kombination med gaskromatografi och masspektrometri.

Projektet som helhet har lett till ökade möjligheter för detektion och kartläggning av viktiga allergiframkallande ämnen i vanliga kommersiella produkter. På sikt kan det förhoppningsvis bidra till lagändringar och en effektivare prevention.

ملخص

حساسية التلامس مرض مزمن أخذ في التزايد حول العالم. السبب الرئيسي لانتشار هذا المرض هو الاستمرار في تقديم مُنتجات جديدة في الأسواق قد تحتوي على مواد كيميائية يمكن أن تسبب هذا المرض. يحدث هذا المرض عندما تتعرض البشرة لواحدة أو أكثر من المواد الكيميائية النشطة الموجودة في المُنتجات التي تلامس البشرة، مثل القلادات المعدنية والملابس والعطور ومستحضرات التجميل، لفترة معينة من الزمن، ونتيجة لهذا التعرض قد تخترق تلك المواد سطح البشرة وتتفاعل كيميائياً مع أحد مكونات طبقات البشرة العليا.

قد يتفاعل الجهاز المناعي ضد هذا التفاعل مسبباً واحداً أو أكثر من أعراض عديدة، كاحمرار في الجلد عند منطقة التلامس، أو حكة، أو خروج سائل من تلك المنطقة مع تشققات وتغير في لون البشرة. هذه الأعراض تُعرف طبيّاً بـ"إكزيما التلامس". التَّعرُفُ علي تلك المواد هو عمل شديد الصعوبة، إذ يتطلب إجراء عديد من التجارب في عيادات الجلدية، وكثيراً ما يتفاعل المرضى مع مُنتجات تجارية معروف عنها أنها لا تسبب حساسية التلامس، والسبب في هذا التفاعل أن المادة الكيميائية المسببة للحساسية قد تكون موجودة بتركيز منخفض جداً بحيث لا يمكن تعينه بطرق التحليل العادية أو لسبب آخر هو أن تكون تلك المادة بحاجة إلى تنشيط، سواء خارج الجسم أو من داخله، لكي تتفاعل مع أحد مكونات البشرة.

في هذه الأطروحة وُظف التحليل الكيميائي للتَّعرُفُ علي المواد الكيميائية المسببة لحساسية التلامس في المُنتجات المصنوعة من مطاط النيوبرين والزيوت العطرية ومستحضرات النظافة المعطرة والعطور، إذ استُخلصت وفُصِّلَت المواد المسببة للحساسية باستخدام طرق الفصل الكروماتوجرافي، وكُشف عنها باستخدام أجهزة كشف دقيقة مثل "مطياف الكتلة".

في حالة المواد المصنوعة من مطاط النيوبرين كُشف عن وجود مادة ثنائي إيثيل الثيوبوريا التي كان يُعتقد أنها مسؤولة عن الإصابة بحساسية التلامس في هذا النوع من المُنتجات، ولكن التجارب على الحيوانات بيّنت أن هذه المادة ضعيفة النشاط، وقد تكون غير نشطة لإحداث تفاعل مع البشرة. باستخدام التحليل الكيميائي كُشف عن التحلل الحراري لتلك المادة عند درجة حرارة مماثلة لدرجة حرارة البشرة، فتتحلل إلى مادة إيثيل الأيزوثيويانات ذات القدرة الفاتقة على التفاعل مع البشرة، والإصابة بحساسية التلامس طبّقاً، لنتائج التجارب على الحيوانات.

الحالة الثانية هي المواد المسببة لحساسية التلامس في الزيوت العطرية والمُنتجات المعطرة. تُستخدم التربينات الأحادية استخداماً موسّعاً في صناعة العطور والمُنتجات العطرية، وذلك لرائحتها الطيبة. مثالاً على ذلك مادة الليمونين التي تُعتبر مسؤولة عن الرائحة الطيبة في الزيوت العطرية المستخلصة من الليمون والموايح، ومادة اللينالول الموجودة في الزيوت العطرية المستخرجة من بعض النباتات مثل الخزامى. عندما تتلامس التربينات الأحادية مع الهواء الجوي فإنها تتأكسد مكونةً هيدروبيروكسيدات التربينات الأحادية التي تُعرف بأنها شديدة القدرة على الإصابة بحساسية التلامس. في هذا العمل صُممت وطُوّرت مجموعة من طرق التحليل الكيميائي لاستخلاص وتعيين مستويات هيدروبيروكسيدات التربينات الأحادية في الزيوت العطرية والمُنتجات المعطرة والعطور. أحد هذه المُنتجات هو نوع من الشامبو تسبب في إصابة طفلة في السابعة من العمر بحساسية التلامس، وشُخصت الحالة بأنها ناتجة عن التفاعل مع هيدروبيروكسيدات اللينالول التي كُشف عن وجودها في الشامبو، باستخدام التحليل الكيميائي .

في النهاية، نجد أن التحليل الكيميائي مهم جداً في هذا المجال البحثي، لمعرفة السبب الرئيسي للحساسية الناتجة عن التلامس مع كثير من المواد التي قد نتعامل معها يومياً.

Abbreviations

2D-HPLC	Two-dimensional high-performance liquid chromatography
ACD	Allergic contact dermatitis
BIC	Butyl isocyanate
BITC	Butyl isothiocyanate
C ₃	Propylsilane
C ₈	Octylsilane
C ₁₈	Octadecylsilane
Cit-OOH	Citronellol hydroperoxide
Cum-OOH	Cumene hydroperoxide
CYP450	Cytochrome P450
Da	Dalton
DBTU	Dibutylthiourea
DCs	Dendritic cells
DETU	Diethylthiourea
DPRA	Direct peptide reactivity assay
DPTU	Diphenylthiourea
EC3	Estimated concentration to induce a stimulation index of 3
EI	Electron ionization
EIC	Ethyl isocyanate
EITC	Ethyl isothiocyanate
ESI	Electrospray ionization
F ₅	Pentafluorophenyl propyl
FID	Flame ionization detector
GC	Gas chromatography
Ger-OOH	Geraniol hydroperoxide
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography

HS	Headspace
IL-1 β	Interleukin 1 beta
ICs	Isocyanates
ITCs	Isothiocyanates
<i>k</i>	Retention factor
LC	Liquid chromatography
Lim-OOH	Limonene hydroperoxide
Lin-OOH	Linalool hydroperoxide
Lin-OAc-OOH	Linalyl acetate hydroperoxide
LLNA	Murine local lymph node assay
LOD	Limit of detection
LogP	The partition coefficient of an analyte between two phases, usually octanol and water.
Monoterpene	A C ₁₀ hydrocarbon
Monoterpenoid	Includes both monoterpenes and modified monoterpenes, for instance linalool
MRM	Multiple-reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTBE	Methyl <i>tert</i> -butyl ether
<i>m/z</i>	Mass-to-charge ratio
NMR	Nuclear magnetic resonance (spectroscopy)
NP	Normal phase
OTUs	Organic thioureas
PDMS	Polydimethylsiloxane
PGC	Porous graphitized carbon
PIC	Phenyl isocyanate
PITC	Phenyl isothiocyanate
<i>R_S</i>	Chromatographic resolution
RIC	Reconstructed ion current

ROAT	Repeated open application test
RP	Reversed phase
SI	Stimulation index
SIM	Selected-ion monitoring
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TIC	Total ion current
TMS	Trimethylsilyl
TNF- α	Tumour necrosis factor alpha
UV	Ultraviolet (detection)

Introduction

The human skin, our largest organ, serves as a barrier from different environmental hazards. It protects from harmful solar UV-irradiation and from pathogenic microorganisms, as well as preventing chemicals from reaching the blood circulation and the inner organs that may cause a variety of adverse systemic effects. In addition, the skin functions as a regulator of body temperature, a centre of sensation and a site of vitamin D synthesis [1-3].

Due to all types of possible interactions with different environmental factors, the skin itself is also susceptible to numerous health problems. Exposure to excess sunlight is considered as one of the main causes of skin cancer [4]. The human skin is continuously exposed to numerous chemicals in our surroundings, such as substances originating from clothes, skin care products, cosmetics, air pollution etc. Skin proteins may react with compounds able to penetrate the skin barrier, resulting in an immunogenic complex formation that can give rise to one of the major skin health problems in the Western world, namely contact (skin) allergy [5,6]. This is a lifelong disease, which so far has no cure and causes much suffering and problems among those affected.

This thesis comprises studies on some of the most commonly occurring causative substances of skin allergy in daily life, e.g. organic thioureas (OTUs) and isothiocyanates (ITCs) in chloroprene rubber (**Paper I and II**) and monoterpene/monoterpenoid hydroperoxides formed in oxidized fragrances (**Paper III, IV, and V**). The focus has been on the development of analytical tools, necessary to be able to identify and measure these skin sensitizing chemicals (haptens) and study how they are formed in commercial products. The identification procedure is a challenging task, since the chemical composition of the matrix is often very complex, while the level of hapten is low. As in the case of hydroperoxides, the hapten is sometimes very reactive, making the analysis

difficult to perform. On the other hand, in some cases, the identification might be difficult since the chemical of interest has no skin sensitizing potency *per se* (prohaptens), but are metabolized in the skin to a protein-reactive and skin-sensitizing compound (haptens). In clinics, patients are seen who react with skin inflammation (allergic contact dermatitis, ACD) caused by a seemingly non-allergenic material or a product having only harmless compounds listed on the label. In some cases, a skin-reactive compound is formed unintentionally by exposure of the product to e.g. heat or air. Chemical analysis is then the only option to reveal the actual haptens. The identity is, of course, crucial knowledge for an affected individual to be able to avoid further exposure.

The focus of this thesis is mainly on the development of analytical tools, but the underlying research has been largely interdisciplinary and collaboration with researchers in dermatochemistry as well as dermatology has been important. **Paper I, II, and III** involved collaboration with Sahlgrenska Academy, University of Gothenburg (GU), Gothenburg, Sweden, Turku University Hospital, Turku, Finland, and Karolinska Institutet, Stockholm, Sweden while **Paper IV** was initiated by dermatologists from University of Alberta, Edmonton, Canada.

Contact allergy

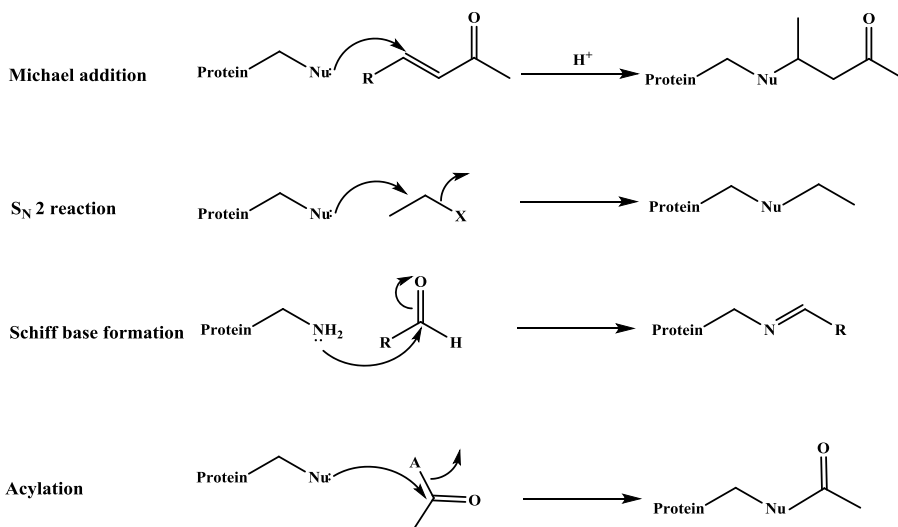
Contact allergy is chronic and implies a lifelong change in the immune system specificity that has developed after repeated skin contact with a sufficient amount of an allergenic compound [6]. Statistically, about 20% of the Western populations react to at least one sensitizing compound when tested at hospitals [6,7]. Metals, such as nickel and chromium, as well as fragrances, rubber chemicals, and dyes, are commonly occurring contact allergens on the market [7]. Despite the development of many alternative products, with less or no allergenic potential, the prevalence of contact allergy is still rising [7] due to a frequent and continuous introduction of new products and materials with hitherto unknown contents of allergenic compounds. The clinical manifestation of contact allergy is ACD, observed as eczematous skin inflammation, which is the most common immunotoxic reaction in humans [8]. Contact allergy is a delayed type of hypersensitivity and the symptoms in an already sensitized individual usually do not develop until after 24-48 h of re-exposure to the sensitizing compound [5]. For an affected individual, ACD often has a significant impact on the quality of daily life [9]. In order to recover from the symptoms, temporary or permanent sick leave may be necessary, which in turn often leads to psychological and economical problems [10]. For society, this widespread disease represents a huge economic burden.

Chemical properties of haptens

Haptens are protein-reactive chemical compounds, usually electrophiles, and are able to elicit an immunological response only if they bind to a carrier macromolecule in the *epidermis*, most likely proteins. The immune system then can process and recognize the hapten-protein adducts formed as xenobiotic [11]. Chemicals can be haptens *per se*, or they can be formed either outside the skin from prehaptens, *i.e.* chemicals that are activated by for instance autoxidation, or

within the skin from prohaptens, which refers to chemicals that are activated by skin metabolism [12,13]. To penetrate the *stratum corneum* into viable *epidermis*, the compound must be small enough, usually $< \sim 1000$ Da and possess a suitable lipophilicity ($\log P \sim 2$) [13].

Hapten-protein adduct formation can occur by covalent binding between a usually electrophilic hapten and a nucleophilic moiety in a protein, such as primary amines ($-\text{NH}_2$) and thiols ($-\text{SH}$) in the side chains of lysine and cysteine, respectively [11]. The reaction can go *via* different mechanisms, such as Michael addition or nucleophilic substitution. Some of the most common reactions are shown in **Scheme 1**.



Scheme 1: Common reaction mechanisms of hapten-protein adduct formation. Nu: nucleophile; R: alkyl, aryl or hydrogen; A: good leaving group; X: electron-withdrawing and a good leaving group, e.g. halogens.

However, some of the most important allergens studied in this thesis, fragrance hydroperoxides, are non-electrophiles and possibly form immunogenic

complexes *via* radical reactions. This is further discussed in fragrance hydroperoxides part.

Mechanism of the development of contact allergy and ACD

Development of ACD takes place in two steps, sensitization followed by elicitation. A hapten-skin protein complex is formed from a reactive compound (a hapten, such as an electrophilic compound) and a reactive site on a protein. The formed immunogenic complex is taken up by dendritic cells (DCs) where it is processed. The DCs mature and migrate to the regional lymph node *via* the afferent lymphatic vessels. The maturation and migration of DCs to lymph nodes are determined by the pro-inflammatory cytokines, such as IL-1 β and TNF- α , which are significantly increased when the immunogens are detected by the DCs [14]. In the lymph nodes, mature DCs activate naïve T-cells, causing them to proliferate and differentiate into memory T-cells, which then start to circulate in the blood and lymphatic system. Now, the exposed person has become sensitized towards the chemical (hapten). In the secondary step when the individual is re-exposed to the same hapten elicitation occurs leading to an inflammatory response, ACD [15]. In this phase, the formed immunogen follows the same pathway as in the sensitization step, but after being taken up by the DCs, processed and presented to memory T-cells, those T-cells that recognize the immunogen will be activated, resulting in the release of pro-inflammatory mediators, such as cytokines and chemokines [16]. This leads to further infiltration of T cells and the arrival of other cells, such as macrophages [17] to the site of exposure, initiating an eczematous inflammatory reaction, ACD.

Diagnosis of contact allergy

Patch testing

Patch testing is a standard *in vivo* method for diagnosis of contact allergy [18], carried out at dermatology clinics. The objective is to stimulate elicitation (but not sensitization) to a suspected contact allergen by applying the diluted chemical(s) in a vehicle, most often petrolatum, under occlusion on the skin (usually the upper back) for 48 h. The conditions are standardized as recommended by the European Society of Contact Dermatitis (ESCD) [18].

Recommendations require two readings of each patch test result and should be performed on day 2, 3 or 4 plus day 7. The reading times may differ owing to the allergens, organizational conditions, and/or geographic conditions.

The allergic reaction is evaluated based on the morphological characteristics, such as the presence of erythema, infiltration, papules, and vesicles, according to the globally acknowledged guidelines of the International Contact Dermatitis Research Group (ICDRG), see **Table 1**.

Repeated open application test (ROAT)

A ROAT is recommended when the results from patch testing are questionable and the clinical relevance of the suspected allergen is unclear [18]. The main goal of ROAT is to mimic everyday exposure of the suspected chemical. This test, nowadays often used by dermatologists, was developed by Hannuksela and Salo and published in 1986 [19]. Test solutions of the suspected hapten or sometimes products are applied twice a day for two weeks on for instance the forearm. If the patient does not show any allergic reaction using the ICDRG reading criteria (**Table 1**) during this time, the results are considered negative, but in some cases of highly suspected products, the ROAT might be extended for up to four weeks. In the study by Hannuksela and Salo, 44% of the

questionable results at patch testing (+?) were shown to be positive in ROAT [19].

Table 1: The ICDRG reading criteria [18], for assessment of skin reactions.

	Erythema	Infiltration	Papules	Vesicles	Various morphologies
- Negative	-	-	-	-	
+? Doubtful	✓	-	-	-	
+ Weak positive	✓	✓	✓ Possible	-	
++ Strong positive	✓	✓	✓	✓	
+++ Extreme positive	✓ Intense	✓	✓	✓ Coalescing	
IR Irritant reaction					✓ Soap effect & necrosis.

Predictive methods for assessment of skin sensitization potency

Murine local lymph node assay (LLNA)

The LLNA is an *in vivo* test to assess potency of potential skin sensitizers [20-22]. Besides improved animal welfare compared to other previously used animal methods, such as the guinea pig maximization test [23], it provides quantitative information with regards to sensitizing potencies. The principle of LLNA is to subject a set of mice to the proposed sensitizer by topical application on the *dorsum* of the ears at different concentrations in a suitable vehicle for three consecutive days. Mice are rested for two days and are then injected

intravenously with radioactive thymidine. The response of the mouse immune system to the hapten is correlated with the lymphocyte proliferation in the local lymph nodes. The lymph nodes are excised, pooled and prepared to single cell suspensions and the increased level of radioactive thymidine is measured by a β -scintillation counter. The rate of disintegration per minute (dpm) is then divided by the number of lymph nodes. The results are compared to a group of control mice in order to estimate the stimulation index (SI). An EC3 value is used, which is defined as the lowest concentration of a chemical required to induce an SI of 3, referring to three times the response of the controls. The sensitization potencies as EC3 values are classified as follows: <0.1% = extreme, 0.1-1% = strong, 1- 10% = moderate and 10-100% = weak [20]. One drawback of LLNA is that the method is limited to measuring the level needed for the induction only. In the guinea pig maximization test, however, the animals are challenged after sensitization in order to measure the levels of elicitation. Also, possible cross-reactions between compounds can be investigated [23].

Since 2013, European industries are forbidden to test cosmetics and substances for use in cosmetics for allergenic potential on animals [24]. The European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) has been working for several years on strategies for the replacement of animal testing, for skin sensitizers [25]. To my knowledge, there is so far no stand-alone alternative method that can fully replace the performance of LLNA. The Organization for Economic Co-operation and Development (OECD) has considered new non-animal methods that aim to determine the impact of a chemical on one or more key events in the adverse outcome pathway of skin sensitization, such as depleted peptide reactivity assay (DPRA), KeratinoSens™, human cell line activation test (h-CLAT) and human T cell proliferation assay [26]. As an example of a non-animal assay, the DPRA test is described below. Results from quantitative structure–activity relationship models (QSAR)

combined with results from animal and non-animal methods can provide a good prediction for skin sensitization potency [27].

Direct peptide reactivity assay (DPRA)

The DPRA is an *in chemico* method that was introduced by Gerberick *et al* in 2004 and predicts the allergenic potency of the test chemical by evaluating its ability to form a stable covalent bond with peptides via nucleophilic-electrophilic interactions [28]. There is a well-known correlation between the peptide reactivity of electrophilic skin sensitizers and their potencies [29].

The procedure of DPRA is simply to react the supposed hapten with synthetic peptides containing cysteine, lysine or histidine in solution for 24 h, where subsequent depletion of the peptides is monitored by HPLC/UV or HPLC/MS/MS [30]. However, when the latter analytical technique is used in the DPRA, it can provide more information regarding the peptide nucleophilic site binding to the electrophile [31] and molecular transformations that occur to the peptide, such as dimerization [32], that otherwise could lead to false positive results. The stability of peptide-hapten product can also be monitored by using MS/MS. For instance, Karlsson and coworkers [33] could monitor the transformation of initially formed cysteine-ITC adducts to stable lysine adducts and suggested the latter to be responsible for the strong allergenicity of isothiocyanates.

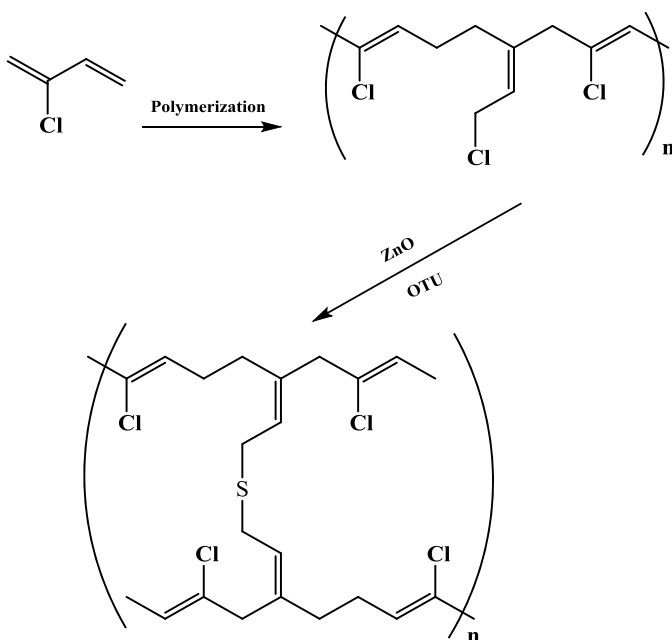
The test compound is classified as non-reactive if the peptide depletion is less or equal to 6.38%, low-reactive between 6.38 and 22.62%, moderate between 22.63 and 42.47% and highly reactive when higher than 42.47% [30]. DPRA has been considered scientifically valid by EURL ECVAM as an animal-free method for prediction and classification of haptens, however, not as a stand-alone method. Furthermore, DPRA does not work as a method for prediction of the allergenic potency of prehapten and prohaptens. However, efforts have been

made to circumvent this limitation either by modification of the DPRA procedure, for instance by addition of abiotic or metabolic conditions to the assay to activate the pre/prohaptens [31,34], or by statistic evaluation of a combination of results from non-animal methods (e.g. Weight of Evidence approach (WoE)) [31].

Contact allergy to chloroprene rubber

Background and aim of the study

Chloroprene rubber (with the commercial name Neoprene[®]) is one of the most common synthetic rubbers, with suitable chemical and physical properties for use across a wide range of applications in daily life and it is also produced at a reasonable cost. Chloroprene rubber was introduced by DuPont in 1933 as an alternative to naturally derived rubber [35] and is produced by polymerization of 2-chloro-1,3-butadiene, chloroprene monomers. In order to improve the physical properties of the rubber, a chemical reaction called vulcanization is carried out, in which bridges (cross-links) between the polymer chains are formed by the addition of a sulfur atom (**Scheme 2**).



Scheme 2: Polymerization of 2-chloro-1,3-butadiene and vulcanization of chloroprene rubber by addition of trace amounts of ZnO and organic thiourea (OTU).

There are many reports of ACD cases caused by commercial products made from chloroprene rubber, such as running shoes, gloves, wetsuits, elbow splints, orthopedic supports, and knee braces [36-41]. During the last 50 years, several patch test studies have been performed to diagnose contact allergy to these types of products [42-45]. From those studies, it was shown that organic thioureas (OTUs) have a clear clinical relevance for contact allergy towards chloroprene rubber. These compounds are used as vulcanization accelerators in rubber processing and traces may remain in the finished products. In 2009, mixed dialkyl thiourea (MDTU) consisting of diethylthiourea (DETU) and dibutyl thiourea (DBTU) was announced to be the allergen of the year by the American Contact Dermatitis Society (ACDS) at its 20th annual meeting in San Francisco [46], after a study was presented by the North American Contact Dermatitis Group (NACDG). This report presented a retrospective analysis of data from thousands of patients patch-tested with MDTU between 1994 and 2004 [47]. Among 21,898 patients tested with MDTU, 1.0% showed positive skin reactions. Also in Europe, many positive reactions to OTUs have been recorded in patients previously diagnosed with contact allergy towards chloroprene rubber. Dall and coworkers, from Odense University Hospital, Denmark, have evaluated patient data from patch testing performed in-house and at some private clinics during a period of 19 years to assess the prevalence of skin allergy towards DETU [48]. Of 239 patients allergic to chloroprene rubber, more than 15% (37/239) showed positive patch test reactions to DETU. The members of NACDG have included MDTU in their patch test series since 1991, but OTUs are still not a part of the European base line series of patch tests [49]. Despite this, three different OTUs are often used in so-called rubber series for patch testing, namely DETU, DBTU and diphenylthiourea (DPTU) [50] (see structures in **Figure 1**).

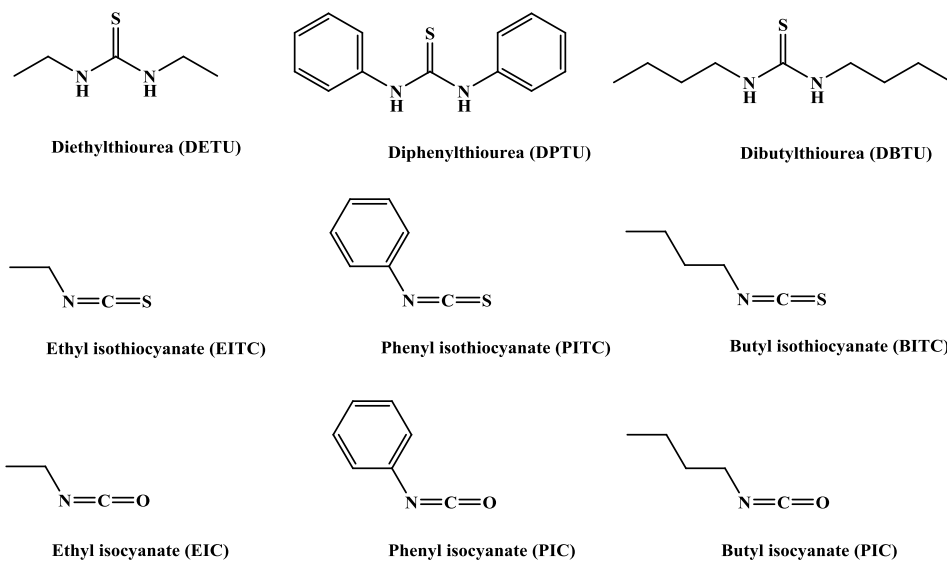


Figure 1: OTUs investigated in this study and their corresponding isothiocyanates and isocyanates. In an *in vitro* metabolism experiment, using skin-like enzymes, both isothiocyanate and isocyanate were formed from DPTU [51].

Despite the many reactions among chloroprene rubber allergic patients to OTUs, these compounds have been shown to act as weak sensitizers or non-sensitizers in the LLNA [51,52]. In an *in vitro* study, DPTU has been shown to be bioactivated when mixed with a group of skin-like enzymes (CYP450) resulting in a set of compounds, including the strong sensitizers phenyl isothiocyanate (PITC) and phenyl isocyanate (PIC) [53]. In the same study, PITC was detected even in the absence of the enzymes at 37 °C as a degradation product of DPTU. In a case of contact allergy to PVC tape, both DPTU and PITC were identified in the tape, the latter most likely a degradation product from DPTU [54]. From these studies and our results in **Papers I and II**, it is reasonable to suggest that OTUs are the main reason for the widespread chloroprene rubber allergy. However, the actual skin sensitization is not due to OTUs *per se*. Sensitization is

rather caused by degradation products from OTUs, isothiocyanates and most likely isocyanates (**Figure 1**), which are much more potent haptens.

Recently, a DPRA study by Karlsson *et al.* was performed to evaluate the peptide reactivity of three different strongly/extremely sensitizing ITCs, among them ethyl isothiocyanate (EITC) [33]. In this case, HPLC/ESI-MS/MS was used to follow the adduct formation during 24 h, instead of using the standard protocol with UV detection. All isothiocyanates were shown to be highly reactive to lysine and cysteine residues in the involved peptides. However, over the time the ITCs were shown to be detached from the cysteine and instead formed more stable adducts with lysine. These findings were further verified by density function theory (DFT) calculations. Due to this, it was suggested that adducts to lysine in the skin proteins in the viable *epidermis* are responsible for skin allergy to isothiocyanates. However, it was also speculated that cysteine residues may still play an important role in isothiocyanate skin allergy [33]. At dermal exposure to isothiocyanates, these compounds probably form adducts immediately to cysteine-containing peptides, which are highly abundant in the *stratum corneum*, the outermost layer of the skin. Under the acidic conditions (pH 5) in the *stratum corneum*, the cysteine adducts may hinder the isothiocyanates from hydrolysis and thus allow penetration further into the viable *epidermis*. This could explain why EITC can be classified as an extreme sensitizer, despite its high volatility (which otherwise would make it evaporate quickly from the skin) and its instability at acidic and physiological pH [33].

The work associated with this part of my thesis was aimed at studying the relevance of OTUs and ITCs in contact allergy to chloroprene rubber. To this end, it was necessary to first develop analytical methods to be able to measure the occurrence of the analytes of interest in chloroprene rubber products. This is described in **Paper I**.

Levels were measured in specific chloroprene rubber-containing products, which were brought in by patients diagnosed with a skin allergy to one or several of the OTUs, using the developed methods. The quantitative results were correlated with patient retesting, **Paper II**. From these studies, it was concluded that ITCs are relevant haptens.

Methods to measure OTUs and ITCs in chloroprene rubber products

Across a large number of chloroprene rubber samples, DETU, DBTU, and DPTU were first screened for qualitatively using ultrasonic solvent extraction followed by HPLC/ESI-MS analysis. Headspace solid-phase microextraction (HS-SPME) followed by GC/EI-MS was used for ITCs as these were difficult to extract with ultrasonication due to their high volatility.

The screening results indicated that DETU is most likely the overall predominant OTU in chloroprene rubber products in the European market today. It was also shown to decompose into the extreme sensitizer EITC already at room temperature, with an increasing decomposition rate at skin-like temperatures, of about 35 °C. The subsequent focus was then on quantification of DETU and EITC.

Chloroprene rubber samples

A collection of 16 newly-bought products made of chloroprene rubber (Neoprene[®]) was analysed in **Paper I**. These products, representing three different categories, namely diving gear, sports support products, and medical devices, were chosen since they are often used in prolonged and repeated contact with the skin. In **Paper II**, a set of five samples was investigated in correlation to skin reactions in four patients. These samples included a back support, two sailing gloves, and two knee braces.

Extraction of OTUs from chloroprene rubber samples

The sample materials were very different in terms of thickness and density, which most likely affect the extraction efficiency and the time needed for an exhaustive extraction. Ultrasonic solvent extraction is a static type of extraction, which implies that repeated extraction with new solvent is needed, but altogether it was found efficient for all investigated samples.

Ultrasonic solvent extraction is often used as an alternative to the well-known Soxhlet extraction due to significantly reduced extraction time and in many cases similar extraction efficiencies [55]. The ultrasonic waves produce cavitation bubbles in the solvent that implode causing shock waves, with both a mechanical and a thermal impact on the sample matrix. The heat and mechanical work increase the solvent penetration into the sample, which improves the mass transfer of the analytes, explaining the high efficiency [56,57].

Pentane, dichloromethane, and acetone were evaluated for their efficiency as extraction solvents. Pentane gave no detectable recoveries at all, while dichloromethane gave the highest recovery of DETU as shown in **Figure 2**.

A problem was experienced with DETU as standard compound dissolved in pure dichloromethane. It was shown to adsorb extensively to the walls of ordinary glass tubes, a problem which could be solved by adding acetonitrile to the solution (20% v/v). A comparison of two solutions with the same initial concentration of DETU is illustrated in **Figure 3**. This adsorption phenomenon was not observable for DETU in the samples, indicating that sample matrix components hinder DETU from adsorbing on glassware.

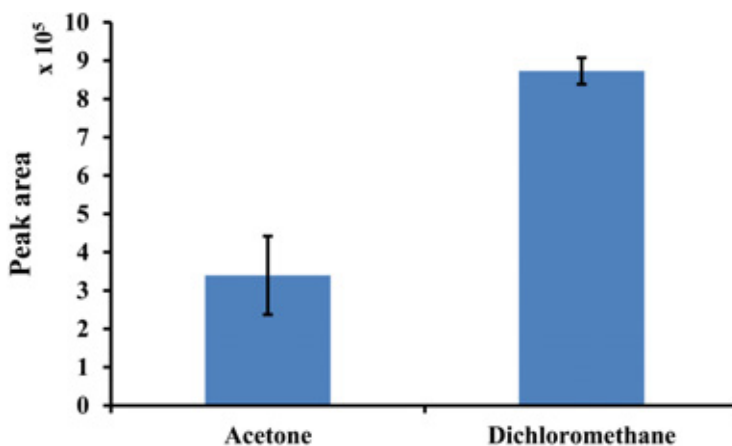


Figure 2: Comparison of ultrasonic extraction of OTUs from a chloroprene material in two different solvents, evaluated by the response in the HPLC/ESI-MS analysis. N=3.

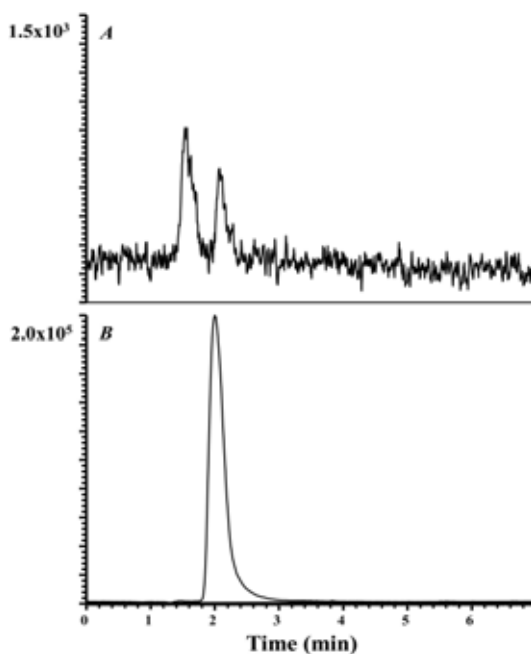


Figure 3: HPLC/ESI-MS chromatogram of DETU standard solution when dissolved in glass tubes in pure dichloromethane, showing complete depletion (**A**), and when dissolved in dichloromethane: acetonitrile (4:1) (**B**). The analyses were run in SIM mode and none of the peaks in (**A**) could be identified. The peak in (**B**) corresponds to DETU.

A mixture of dichloromethane: acetonitrile (4:1) was used for both standards and samples in order to keep the analytical consistency and avoid glass adsorption phenomena. An internal standard was added prior to the extraction and HPLC/ESI-MS analysis was performed after the extracts had been first carefully evaporated to dryness, the solvent changed to acetonitrile: MilliQ water and the samples subjected to syringe filtration.

HPLC/ESI-MS detection of DETU in chloroprene rubber samples

Reversed phase HPLC with UV detection has been used for determination of OTUs in an orthopedic brace made from chloroprene rubber by other investigators [39]. Since DETU is retained using a conventional C₁₈ column and the compound is well ionized by electrospray, HPLC/ESI-MS was applied in this work in order to improve the limit of detection. Selected-ion monitoring (SIM) gave, in this case, a more reproducible quantification of OTUs compared to multiple-reaction monitoring (MRM). To strengthen the identification, the samples were also analysed using columns in HILIC mode. In **Paper I**, aminopropyl silica was used as the HILIC column, while in **Paper II** a pentafluorophenyl propyl silica (F₅) column was used. MS/MS product ion scans were performed to verify that the fragments were the same as those formed from the standard compound.

Matrix effects are expected when samples of high complexity, such as chloroprene rubber materials, are analysed using an ESI interface. This was investigated by comparing calibration curves, *i.e.* spiking samples at several concentration levels and comparing these with the corresponding standard solutions. By using an isotope-labelled internal standard it is possible to compensate for suppressing or enhancing effects of the sample matrix on the ESI [58]. No suitable commercial internal standard was available and thus an internal standard compound was synthesized in house. In **Paper I**, the synthesis

of d₅-DETU is described and its identity, as well as purity, were verified with NMR. The mean accuracy for the quantification of DETU using this internal standard was 108%.

Extraction of ITCs from chloroprene

Generally, ITCs of low molecular weight are compounds with high volatility [59], such as allyl isothiocyanate which is the major component of natural volatile mustard oil. Volatile ITCs are also considered responsible for the taste and odor of fresh cabbage [60,61].

The investigated ITCs, EITC, butyl isothiocyanate (BITC) and PITC, showed very low responses in ESI. Also, ultrasonic-assisted solvent extraction was found unsuitable due to the high volatility of the analytes. This could be solved by derivatizing the ITCs with a dialkylamine to form derivatives which are easily ionized by ESI [62,63]. However, to avoid a derivatization step and instead take advantage of the ITC volatilities, HS-SPME followed by GC/EI-MS was the method of choice.

HS-SPME was efficient to sample the volatile ITCs from chloroprene materials. Pawliszyn and coworkers introduced this technique already in the 1990s [64]. It is a fast, simple and solvent-free extraction technique used for a variety of analytical applications, such as food and environmental analysis. It combines extraction and pre-concentration on a fused silica needle, which subsequently is directly thermally desorbed in the GC injector. The SPME sampler can be re-used up to a hundred times and it is a time- and cost-saving method when applicable. The needle tip of the SPME sampler is coated with a thin stationary phase, often based on silica and similar to GC stationary phases. The mechanism of SPME in headspace mode can be explained by the system's drive to keep an equilibrium between three different phases, the solid or liquid sample, the air above the sample (headspace) and the sampling SPME fibre [65]. The amount of

analyte that has partitioned into the stationary phase in HS-SPME at equilibrium can be expressed by this equation [66]:

$$n_f = C_0 \frac{K_{fh} K_{hs} V_f V_s}{K_{fh} K_{hs} V_f + K_{hs} V_h + V_s}$$

where n_f is the number of moles of analyte extracted by the SPME coating, C_0 is the initial concentration of analyte in the sample, K_{fh} is the fibre/headspace partition coefficient, K_{hs} is the headspace/sample partition coefficient, V_f is the volume of fibre coating; V_s is the volume of the sample and V_h is the headspace volume.

Sampling at equilibrium is the common way of using SPME, and in this case, the time needed for reaching equilibrium has to be determined. As an alternative, time-weighted average sampling can be performed, especially when equilibrium takes a long time to achieve. Several factors affect the sampling time needed for equilibrium, such as the headspace/sample partition coefficient and the SPME fibre coating/headspace partition coefficient, *i.e.* the type of fibre coating, as well as the SPME fibre coating thickness. An SPME sampling time of 20 min was found to be sufficient for all investigated ITCs to reach equilibrium. After sampling, the analytes were directly desorbed from the SPME fibre in a specially designed narrow liner in the GC injector by applying high temperature. This type of HS-SPME/GC analysis has earlier been used to determine methyl isothiocyanate from food and environmental samples [67-69].

In the screening studies presented in **Papers I** and **II** of products made of chloroprene rubber, only DETU and the corresponding EITC were detected among the targeted compounds. Neither DBTU, DPTU, nor the corresponding

ITCs were detected in any of the materials. This was surprising since cases of skin allergy towards both DPTU and DBTU have been reported [54,43,70]. Because of the screening results, the analytical work focused on EITC only. Two different stationary phases were evaluated for the SPME, a 100% polydimethylsiloxane (PDMS) and a carboxen-PDMS mixture. The highest recovery for the extraction/sampling of EITC was obtained with the latter coating as shown in **Figure 4**.

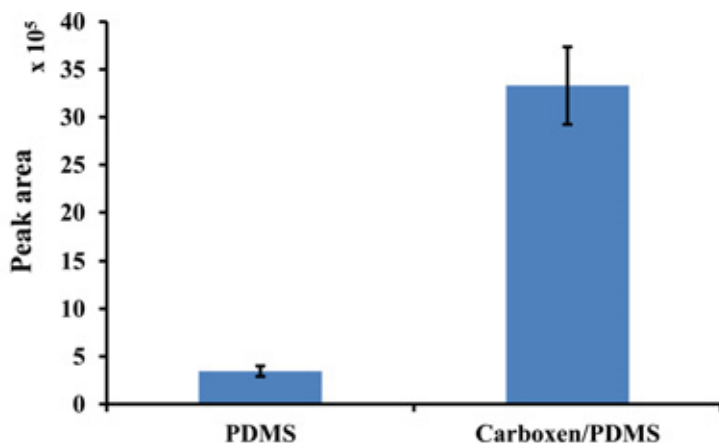


Figure 4: Sampled amounts of EITC at equilibrium conditions on SPME fibres made from PDMS, compared to the amounts sampled on Carboxen mixed with PDMS.

The mixed sorbent phases consist of PDMS, which is a highly viscous liquid with a silicon backbone, and carboxen, which is an adsorptive porous material with a carbon skeleton. Carboxen has a unique even distribution of micro, meso, and macro pores [71]. Pore size is an important parameter for optimal extraction in adsorptive SPME where the pore diameter should be at least two-fold in diameter compared to the analyte molecule. The relatively high content of micro pores ($\sim 10 \text{ \AA}$ in diameter) in carboxen makes it ideal for trapping small molecules such as EITC ($\sim 5 \text{ \AA}$ in diameter). In addition, the high porosity of

carboxen increases mass transfer and facilitates the adsorption as well as the desorption processes.

To decrease the time for HS-SPME extraction/sampling of EITC, the chloroprene rubber samples were cut into small pieces and spiked with the d₅-EITC internal standard. The equilibrium between EITC and the SPME fibre was achieved after 10 min in a sealed glass vial, at room temperature. This was determined by sampling a gaseous standard of EITC under identical conditions. For DETU-containing samples extracted at 35 °C (skin-like temperature), equilibrium for EITC was not achieved at any time during the experiments. The reason for this was found to be a continuous formation of gaseous EITC due to thermal degradation of DETU present in the sample. Instead of observing a constant level of the compound, the rate of EITC production at this temperature was measured. Two 20-min extractions were performed for each sample, after 2 and 6 h, respectively. Quantification by GC/EI-MS was performed by acquisition in full scan mode and integrating peaks in the reconstructed ion chromatogram (RIC).

LLNA

Since our results showed that DETU and EITCs were commonly detected in the investigated consumer products, it was of interest to examine the skin sensitizing potencies of these compounds in the LLNA. BITC and butyl isocyanate (BIC), which may be generated from DBTU were included in the assay, even though neither of these compounds was detected in the products. The sensitizing potencies of PITC and PIC in LLNA have been evaluated previously [53].

In the LLNA, three groups of mice were subjected to five different concentrations of the tested compound dissolved in acetone and olive oil as a vehicle. The control group was subjected only to the vehicle without the test

compound. The procedure of this test was similar to the one already described in the literature [72].

Patch testing

In order to study the clinical relevance of an experimentally identified skin sensitizer, patch testing should be performed. In the present study, eight patients with a positive reaction to DPTU were retested with in-house prepared patch test materials containing DPTU, PITC, and PIC in dilution series, in addition to a commercial DPTU patch test preparation. Patch testing was performed in three different Scandinavian dermatology clinics (Gothenburg - 4 patients, Turku - 3 patients and Stockholm - 1 patient). Reproducible patch tests could not be performed for EITC because of its high volatility. Instead, four patients (in Gothenburg) who previously were diagnosed with contact allergy to DETU were retested with dilution series of DETU, with concentrations between 0.00058% and 0.58%. The levels were chosen to be equimolar to the DPTU dilution series. Patch testing was performed according to ESCD guidelines. Readings were performed on days 3 and 7 and the strongest reaction obtained among them was recorded.

Analysis of OTUs patch test preparations

Due to the tendency of OTUs to be degraded at room temperature as well as skin-like temperatures, the stability of these compounds in the patch test preparations was questionable. Therefore, three commercially available patch test materials with specified levels of DETU, DPTU, and DBTU, respectively, were examined for the real concentrations using HPLC/ESI-MS. For the determination of corresponding ITCs, HS-SPME/GC/EI-MS was used. According to the label, the concentration of each OTU should be 1%.

Results and Discussion

Screening of 21 chloroprene rubber products resulted in detection of DETU only among the OTUs. The levels are presented per area, rather than sample amount, as this was deemed most relevant for skin exposure. Detected levels of DETU were in the range of 1.7 to 71 nmol cm⁻², except for a back support (B1) which had a much higher content, 1200 nmol cm⁻² (158 µg cm⁻²). A patient who had developed skin allergy and ACD to this product had brought the sample. Apart from B1, the levels of DETU were lower in the patient's samples compared to newly bought samples from corresponding products. This could be due to degradation of DETU in the older samples. Emission of EITC could be measured in all products shown to contain DETU. The EITC emission rate was significant for all samples, except for the S1 sample, as illustrated in **Figure 5**.

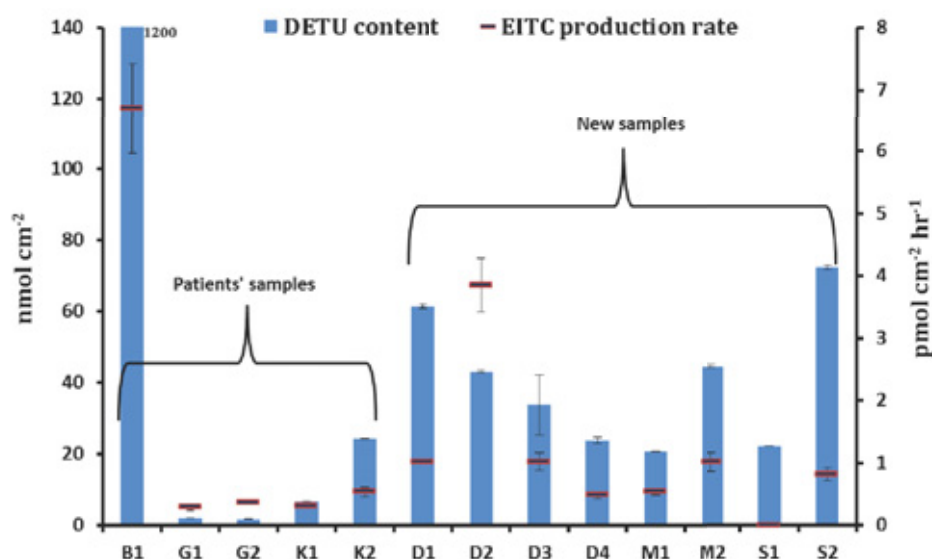


Figure 5: DETU content and EITC emission rate, respectively, for all analysed chloroprene rubber samples in *Paper I* and *Paper II*. Y-axis to the left refers to the DETU content, while the one to the right represents the EITC emission rates.

The emission rate of EITC did not directly follow the DETU level. Most likely, this is attributed to different material-specific properties, such as different diffusivities and densities.

The LLNA results showed that all corresponding ITCs and isocyanates (ICs) to DETU and DBTU are highly sensitizing. **Table 2** summarizes the results together with previously reported data for the degradation products from DPTU [53]. ICs were stronger sensitizers than the corresponding ITCs, except for EITC, which showed higher potency than the corresponding IC, EIC.

Table 2: Results from LLNA regarding skin sensitizing potencies of the ITCs and ICs included in this study.

Compound	EC3 (mM)	EC3 (%)	Classification
EITC	4.6	0.04	Extreme
EIC	56.3	0.40	Strong
BITC	39.1	0.45	Strong
BIC	6.1	0.06	Extreme
PITC	30	0.40	Strong
PIC	1.7	0.02	Extreme

All patients diagnosed as DETU-allergic reacted to a lower concentration at retest. Of four participants, three showed flare-up reactions at skin sites where they had been sensitized previously to the products. Seven of eight patients, previously shown to be DPTU-allergic, reacted also at the retest with DPTU. One of the seven skin reactions was doubtful (+?). All patients showed skin reactions to PITC and two reacted to PIC, a likely metabolite of DPTU. Results are summarized in **Table 3**.

Table 3: Summary of two different patch tests, one for DETU and one for DPTU and related compounds PITC and PIC.

	DETU	DPTU	PITC	PIC
Reacting/Investigated patients	4/4	7/8*	8/8	2/8
Lowest concentration for reaction	0.0058%	0.001%	0.0001%	0.001%
Classification of the reaction	+	+	++	+

* One doubtful reaction

Stability tests of patch-test preparations of OTUs showed that they emitted the corresponding ITC already at room temperature (25 °C) and at a significantly higher rate at a skin-like temperature (35 °C). EITC was released at the fastest rate among the measured ITCs.

The results from the studies presented here indicate that DETU seems to be a commonly occurring OTU in chloroprene products on the European market. It was also the only OTU detected in the analysed products. Furthermore, the materials containing DETU were shown to continuously emit the extreme sensitizer EITC. Since the investigated products mostly are worn close to the skin, it is possible that their content of EITC can cause skin sensitization and skin allergy. Due to the high volatility of EITC, it was, however, difficult to perform patch testing with this compound. The reactivity of EITC could be estimated when the results from patch testing were correlated with the LLNA assays and combined with chemical analysis of both the patients' products and the patch test preparations. Since a larger percentage of patients reacted to DETU than to DPTU [50, **Paper II**], and EITC exhibits a stronger potency than PITC in the LLNA, it is expected that EITC would show higher reactivity than PITC if possible to use in patch tests. Considering the emission rates of EITC from the products in comparison with the lowest dose for a positive reaction to PITC, roughly 100 h of exposure to EITC would be required for elicitation of a

skin reaction. However, a shorter time is expected when considering the faster emission rate for EITC compared to PITC from the patch test preparations. In addition, regarding levels needed for elicitation, patch tests concentrations are a bit misleading since much higher doses are used compared to amounts patients are usually exposed to [73,74]. Furthermore, a cutaneous metabolism of DETU to EITC could contribute to the total dose in real life situations. The two positive reactions to the DPTU metabolite, PIC, support the theory that cutaneous metabolism of OTU occurs and plays a role in contact allergy to chloroprene rubber.

Conclusions

In this work, the developed analytical methods were shown to be crucial in order to understand the cause of contact allergy towards chloroprene rubber. The results from chemical analyses were combined with those from predictive *in vivo* and *in vitro* skin sensitization tests (LLNA and DPRA, respectively) and clinical patch test results. A conclusion that can be drawn is that DETU acts as a prehapten since it decomposes to the strong hapten EITC in the materials at skin-like temperatures. DETU might also act as a prohapten *via* skin metabolism to EITC and EIC, although this has not been investigated to the best of my knowledge. The clinical relevance of ITCs, possibly together with ICs, was shown in **Paper II**. It was also shown that patch test preparations in petrolatum with OTUs were not stable since significant emissions of corresponding ITC could be measured over the investigated period of time. This shows the importance of controlling the content in patch test preparations using appropriate analytical methods. In my opinion, patch tests with OTUs for diagnosis of chloroprene rubber allergy should still be recommended, despite their tendency to degrade, since they simulate the conditions when patients are exposed to chloroprene rubber products.

Fragrance contact allergy

Background and aim of the study

Monoterpenoid compounds (C_{10} with two isoprene units), either oxidized or non-oxidized forms, constitute a major part of the class of organic compounds that is referred to as fragrances, which due to their pleasant odors are widely used in the perfume and cosmetic industry [75]. The story of fragrances started already in ancient times and they were used in the social and religious rituals of many ancient civilizations in China, India, Egypt, and Mesopotamia [76]. Nowadays, fragrances are ubiquitous on the market, in everyday items, such as cosmetics and household products. Their widespread use has unfortunately resulted in the increasing trend of contact allergy. Fragrance contact allergy today has a prevalence of 1-3% in the European population [77-80], only nickel and preservatives causing more cases of contact allergy.

To reduce the risk of skin sensitization and ACD from fragrances in the population, the 1999 European Cosmetics Directive declared that cosmetics containing any of 26 different fragrance chemicals have to be labelled; leave-on products with levels exceeding 0.001% and rinse-off products with levels above 0.01% [81].

Fragrances constitute thousands of compounds, often added to the products as mixtures, for instance as naturally derived essential oils from plants [82]. The chemical structures of some of the monoterpenoid fragrance compounds, included in the EU directive and discussed in this thesis, are shown in **Figure 6**. Linalool is a monoterpenoid that is one of the main fragrances in lavender oil together with linalyl acetate [83] and is found in more than 200 different essential oils [84]. The cyclic monoterpene limonene is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit) [85]. Geraniol

is another naturally occurring monoterpenoid found for instance in rose, palmarosa and citronella oils [86]. Citronellol is found mainly in rose and geranium oils, but also in at least 70 other oils [87,88].

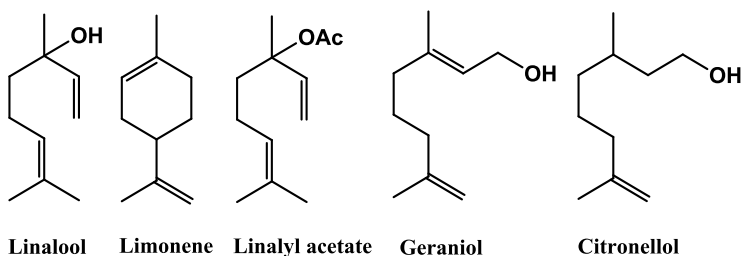
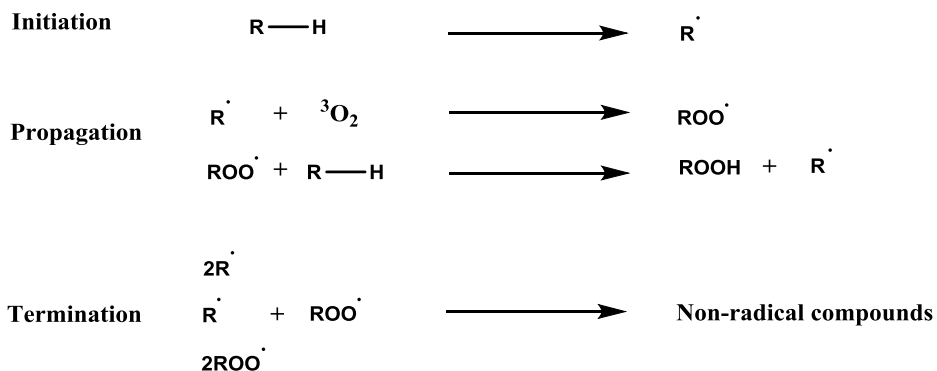


Figure 6: Structures of the monoterpenoids discussed in this thesis.

It is now well known that oxidized forms of the fragrance monoterpenes/monoterpenoids are the main culprits behind fragrance contact allergy [13]. Oxidation can occur either outside the skin, for instance in the product or on the skin, but also by metabolism within the skin. The unsaturated carbons facilitate a fast autoxidation in the presence of air [89]. Autoxidation is a spontaneous chain reaction, following a free radical mechanism that is difficult to stop. Antioxidants can prevent it for a while but are consumed with time, after which the reaction immediately starts as there is more oxygen available [90]. Primary autoxidation products are the hydroperoxides, which have been shown to be very important with regards to contact allergy [13].

The free radical mechanism of autoxidation involves three stages, initiation, propagation and termination, **Scheme 3**. The initiation step starts upon exposure to heat, visible/UV light, or a catalyst forming an alkyl radical by abstraction of an allylic hydrogen atom [13]. The alkyl radical then reacts with atmospheric triplet oxygen in the propagation step to form a peroxy radical. This, in turn, abstracts a hydrogen atom, resulting in the formation of a hydroperoxide (ROOH) together with a new alkyl radical, which propagates the reaction. The reaction is terminated when two radicals bind together resulting in non-radical

products. In unsaturated molecules such as monoterpenes, the radicals are mostly formed by abstraction of the allylic hydrogen atom because the radical is stabilized by resonance [13].



Scheme 3: The general mechanism of the autoxidation process.

Several clinical studies have shown that patch tests of fragrance-allergic patients with oxidized forms of limonene, linalool, linalyl acetate and geraniol have generated many more positive results than with the pure compounds [91-107]. Also in animal studies, such as LLNA, these parent fragrances have expressed low or non-sensitization potencies, while a significant rise in the sensitizing potency was observed after autoxidation [108-111].

Besides the hydroperoxides, autoxidation also leads to a complex mixture of secondary oxidation products, such as aldehydes, alcohols, diols, epoxides, ketones, and peroxides. These oxidation products have different sensitizing potencies depending on their overall structure, but generally, the secondary products have lower potencies than the hydroperoxides, which are considered the strongest sensitizers, confirmed by several animal tests [108-111] and clinical studies [91,92,112]. The studied fragrance hydroperoxides, shown in

Figure 7, are small molecules (168-228 Da) with log P (1.88-2.45), indicating that they have the possibility to penetrate the skin easily.

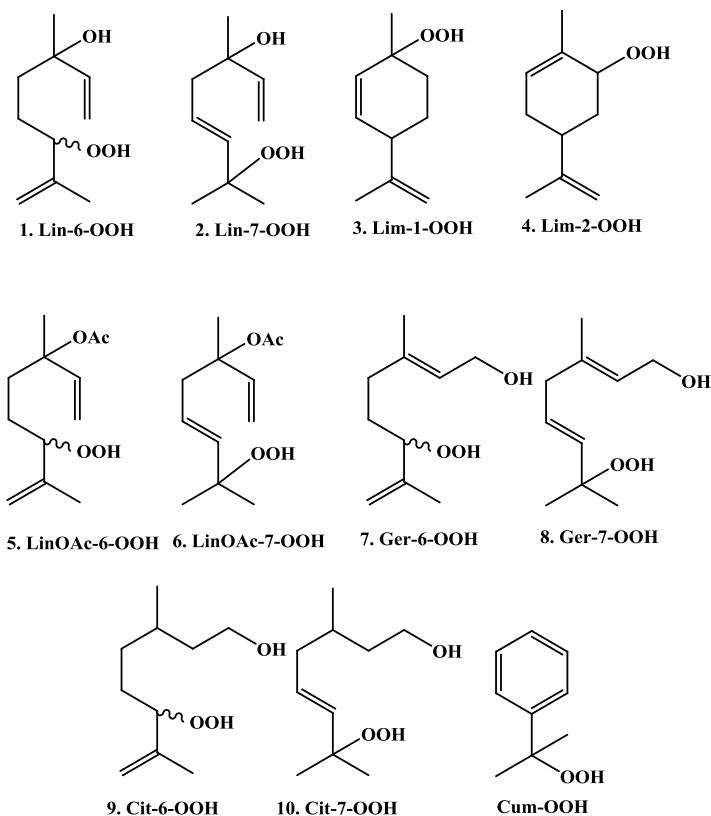


Figure 7: Hydroperoxides studied in this thesis, all of which were shown to be strong skin sensitizers. Cumene hydroperoxide (Cum-OOH) is not considered a fragrance hydroperoxide but was used as internal standard for quantification in **Paper V**.

Other factors may improve the skin penetration of hydroperoxides, for instance, their chemical stability or volatility. Preliminary results in this thesis show that the volatility of hydroperoxides is much lower when compared to the parent monoterpenoids. A commercial non-fragranced cream was spiked with oxidized citronellol containing 2% of Cit-OOHs, spread as a thin film on a glass plate and the evaporation of both the parent compound and the hydroperoxides were

investigated by HPLC/MS after extraction with a PDMS tape. The decreasing HPLC/MS responses were monitored over a period of five days. The results are shown in **Figure 8**. This experiment aimed at reflecting the situation with leave-on products, in which the hydroperoxide content has a longer time to penetrate the skin before evaporation and the relative levels of sensitizing hydroperoxides compared to low-sensitizing parent compounds will increase.

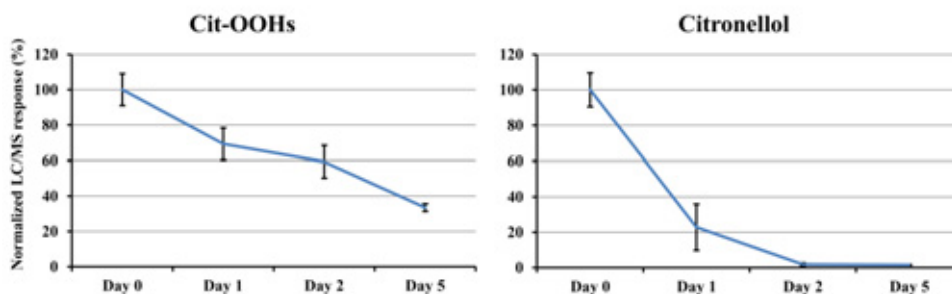


Figure 8: Results from a cream spread on a glass plate and spiked with citronellol containing 2% Cit-OOHs. The volatilities of parent compound and hydroperoxides were compared by measuring the remaining content after different timepoints.

Hydroperoxides may form immunogenic protein complexes *via* a radical mechanism, or an electrophilic-nucleophilic reaction after transformation to an electrophile, *e.g.* an epoxide [13]. However, to my knowledge, neither of these possibilities have been investigated or shown *in vivo*. A radical reaction would need a homolytic cleavage of the oxygen-oxygen bond leading to a reactive alkoxy radical [113]. This radical can either react directly or rearrange to a carbon-centred radical that then reacts with for instance sulfur or aromatic rings in the protein [114-116]. Therefore, hydroperoxides most likely need some form of activation before they react with proteins to form the immunogenic complex. For instance, CYP-450 or heme in hemoglobin in the viable *epidermis* could act as such activators that induce radical formation from the hydroperoxides [117,118].

The main route of skin exposure, commonly resulting in sensitization to hydroperoxides, has not been shown yet. One main route could be skin contact due to their proven occurrence in fragranced products [119, **Papers IV and V**]. Whether they are formed mainly in the finished products or in the essential oils prior to addition to the products has not been shown. However, it has been indicated that hydroalcoholic solutions, like in fine fragrances, afters-have and eau de cologne, prevent or slow down autoxidation [119].

Representatives from the European fragrance and perfume manufacturers have communicated that they consider the reported levels of hydroperoxides in finished products too low to be sensitizing or even eliciting since the test concentrations used for patch testing of patients usually are much higher. However, there are currently different opinions about this. For instance, it has been shown with ROAT on an already sensitized patient that levels close to those found in products actually can elicit skin reactions [120]. It has also been suggested that repeated exposure to low levels can trigger the immune system to react to much lower levels [73,74]. Another possible main route of exposure is that the pure fragrance compounds autoxidize on the skin after application. There is also a possibility that hydroperoxides form in the skin after skin penetration of the pure fragrance compounds and subsequent metabolism by skin enzymes. To my knowledge, nothing has been published so far on the two latter possibilities of exposure.

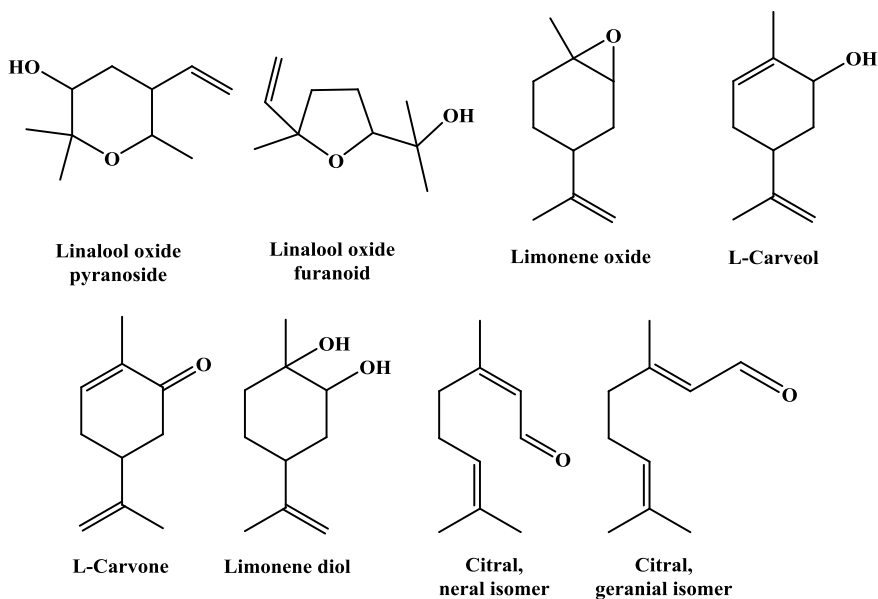


Figure 9: Some secondary autoxidation products formed via the primary oxidation products, hydroperoxides, from linalool and limonene. Citral is a naturally occurring monoterpenoid but is also a result of autoxidation of geraniol.

The composition of monoterpenes and their oxidized forms as constituents in both essential oils and fragranced products is usually highly complex, including various parent monoterpenes/monoterpenoids together with their oxidation products, such as those illustrated in **Figure 9**, while the actual skin sensitizers are only a part of the latter. Both chromatographic and mass spectrometric separations of hydroperoxides from the matrix are often highly demanding due to similar structures as well as often identical elemental compositions. Similarities in both LC retention behaviour and MS fragmentation pathways constitute a severe problem in the chemical analysis.

Legislation on levels of hydroperoxides in products is hitherto absent, despite their strong sensitization potencies. This can be explained partly by lack of analytical methods for commercial products, and partly by lack of commercially available reference compounds. Currently, work is ongoing on further

development and validation of analytical methods for Lin-OOHs and Lim-OOHs in fragranced products, coordinated by The International Dialogue for the Evaluation of Allergens (IDEA) project, involving both industry and academy partners, such as Stockholm University. This is one of the important results from the long-time research collaboration between researchers from the University of Gothenburg and Stockholm University, who initially started the analytical method development for these important skin allergens.

Part of the work underlying this thesis has been to develop GC/MS and HPLC/MS methods for fragrance hydroperoxides, in order to obtain a versatile toolbox of methods for different types of sample matrixes. So far, we have been able to determine accurately the levels in essential oils, hydroalcoholic products, and shampoo. The different methods and applications are discussed in the following sections.

Development of analytical methods for fragrance hydroperoxides

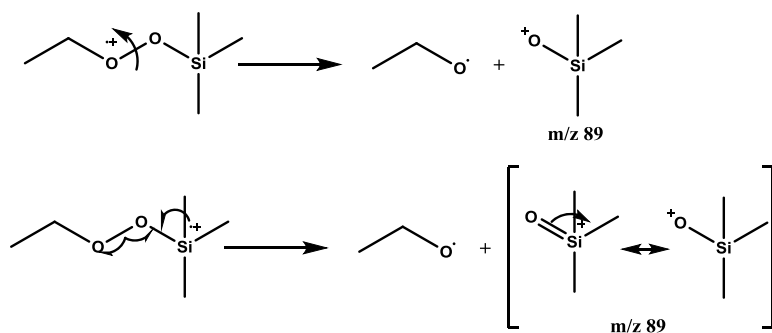
GC/MS

The content of fragrance compounds in essential oils is usually determined using GC/FID or GC/MS [121]. Direct determination of hydroperoxides using GC is generally not applicable due to the thermal instability of this type of compounds in addition to their higher vapor pressure. Limonene hydroperoxides (Lim-OOHs) are the only fragrance hydroperoxides that have previously been shown to be thermostable enough at on-column injection at a sufficiently low column temperature [122]. However, according to my experience, the stability is dependent also on the type of on-column injector. None of the hydroperoxides can stand the high temperature environment in a split-splitless injector.

Silylation creates more thermostable derivatives of hydroperoxides. This is an S_2N reaction, where the active hydrogen is replaced by a trimethylsilyl (TMS) group [123]. Silylation has previously been used for other organic hydroperoxides, such as cumene hydroperoxide (Cum-OOH), methoxyalkyl hydroperoxides and phenylethyl hydroperoxide, using N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as the derivatizing agent. After derivatization, they were stable enough to separate by GC [124,125]. In this work, two different essential oils (sweet orange oil and petitgrain oil, with limonene and linalool as main constituents, respectively) were analysed with GC/MS for their content of Lim-OOHs, hydroperoxides of linalool (Lin-OOHs), geraniol (Ger-OOHs) and linalyl acetate (LinOAC-OOHs) (**Paper III**). Each oil was autoxidized for two months by stirring in contact with air in order to simulate normal handling and usage.

The oil samples were diluted in dry toluene and derivatized to TMS derivatives using an excess of a mixture of N, O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) during 24 h prior to analysis.

The TMS derivatives of the hydroperoxides all showed a specific fragment at m/z 89 (**Scheme 4**), corresponding to $[\text{OSi}(\text{CH}_3)_3]^+$, although at a very low relative abundance of 1-5%, but still useful for identification. A big advantage with the developed GC/EI-MS method is the high separation capacity of GC compared to LC, which is advantageous for these highly complex oils. On the other hand, the detection limit is much higher. One reason is that SIM, which otherwise often lowers the detection limit significantly compared to fullscan mode, turns out to be less useful for the silylated hydroperoxides. The reason is the extensive fragmentation that yields many low-mass fragments, both in electron ionization (EI) and in positive ion chemical ionization (PICI) modes. For essential oils, for which the detection limit is not crucial, the GC/MS was very useful for quantification, and for identification of Cit-OOHs [126].



Scheme 4: Possible fragmentation pathways in EI of the TMS-derivative (upper by inductive cleavage and lower by homolytic), resulting in the characteristic fragment m/z 89.

Recently, another method for quantitative measurements of Lim-OOHs in hydroalcoholic products by GC/MS has been published [127]. Prior to analysis,

the hydroperoxides formed thermostable alcohols by a reduction reaction, for instance transferring Lim-2-OOH to carveol.

HPLC/ESI-MS/MS

Another way to circumvent the thermal instability of hydroperoxides is to use liquid chromatography as the method of separation. Together with MS, the detectability is significantly increased as shown in the paper by Rudbäck *et al.* 2008 [128]. Both reversed-phase (RP) HPLC [119,129,130] and normal-phase (NP) HPLC [122] have been used to separate hydroperoxides present in different matrices, such as essential oils and hydroalcoholic products. Different types of detection for LC have been used, such as UV [122], MS [129], and post-column chemiluminescence [130].

Part of the work included in this thesis involved further development of the HPLC/ESI-MS/MS method presented by Rudbäck *et al.* [129], in order to increase the selectivity for the fragrance hydroperoxides. In Rudbäck's work, a C₃-modified silica stationary phase had been used, so first other types of RP columns were tested. For the detection of Lin-OOHs in an allergenic linalool-fragranced shampoo (**Paper IV**), it was found that an F₅ stationary phase in RP mode was better suited and more selective than C₃ for separation, although with the drawback of long elution times. The retention mechanism is most likely composed of several types of interactions, apart from those due to London dispersion forces as in conventional RP mode. For instance, hydrogen bonding can occur between the fluorine substituents on the benzene ring and the hydroperoxide group [131]. Also, the fluorinated benzene ring is electron deficient, which may lead to interaction with the dipolar hydroperoxide function [132]. Furthermore, the planar fluorinated aromatic ring also most likely adds some shape selectivity to the separation [133].

In order to be able to shorten the retention time somewhat by using a bit higher flow rate, the mobile phase modifiers were changed from methanol and isopropanol to the less viscous acetonitrile [134].

Applications of the modified HPLC/ESI-MS/MS method

Analysis of a heavily fragranced shampoo (Paper IV)

In this work, a method using SPE in combination with analysis with HPLC/ESI-MS/MS was developed for quantification of Lin-OOHs and linalool oxide. The latter compound is a secondary oxidation product that can be used as a marker for oxidation as described in **Paper IV**. A seven-year-old girl had suffered from contact dermatitis symptoms for six months and no topical treatment helped her to recover. After patch testing and chemical analysis, it was realized that a specific shampoo was most likely the culprit, and her eczema disappeared as soon as she stopped using the shampoo.

The patient was tested with 60 common patch test preparations, including Lin-OOHs and Lim-OOHs. Only the preparation with linalool hydroperoxides gave a positive patch test reaction. Among the other tested compounds were preservatives, methylchloroisothiazolinone /methylisothiazolinone (MCI/MI), which both are strong sensitizers and common in hygiene products. Due to the clear result from the patient test, we subjected the shampoo to chemical analysis.

Before the shampoo sample was subject to analysis with respect to its content of hydroperoxides, it was analysed by HS-GC/MS, which revealed traces of linalool and linalool oxide. A multistep cleanup procedure enabled the extraction of Lin-OOHs and linalool oxide from the complex matrix (**Figure 10**). First, a liquid-liquid extraction of hydroperoxides from the shampoo was performed using dichloromethane. After centrifugation, a thin pellet was removed from the dichloromethane phase. The latter was loaded onto a silica

SPE cartridge on which Lin-OOHs are strongly retained. Pentane was used to wash the cartridge and was subsequently dried with nitrogen gas to facilitate solvent exchange. The analytes were then eluted with methanol and the internal standard (2,2'-biphenol) was added to the eluate. Despite the SPE cleanup, the sample started to turn opaque due to the residual matrix. The sample was then centrifuged once more, after which an aliquot of the clear supernatant was injected into the HPLC/ESI-MS/MS.

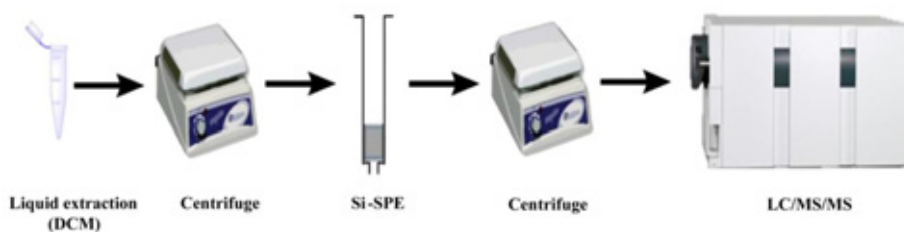


Figure 10: Schematic sketch of multistep cleanup procedure of the shampoo. DCM= dichloromethane

The extraction recoveries were evaluated using a spiked fragrance-free shampoo. The level of Lin-OOHs (including both the 6- and the 7-isomer) was found to be 0.2 ppm (RSD 9%). To my knowledge, this is the first time that a case of linalool hydroperoxide allergy has been correlated to a specific commercial product.

Analysis of patch test preparations for diagnosis of contact allergy to Lim-OOHs or Lin-OOHs (unpublished work)

Commercially available patch test preparations in petrolatum were obtained from two different suppliers. The preparations were labelled with 0.3% Lim-OOHs and 1% Lin-OOHs, respectively. The preparations were extracted using acetonitrile and ultrasonication. An aliquot of the extract was passed through a C₁₈ SPE cartridge to remove solid particles, and a volumetric internal standard

(2,2'-biphenol, IS_V) was added prior to the HPLC/ESI-MS/MS analysis using an F₅ silica column for the HPLC separation.

Two-dimensional HPLC/ESI-MS/MS method (Paper V).

Correct quantification of low levels of fragrance hydroperoxides in essential oils and commercial fragranced products of high complexity, in general, is demanding. As mentioned above, both the elemental compositions and fragmentation patterns are very similar or sometimes identical between hydroperoxides and a number of interfering compounds. One-dimensional HPLC usually does not yield sufficient separation selectivity, leading to matrix effects like ion suppression in the ESI-MS/MS analysis and low accuracy. For this reason, a two-dimensional liquid chromatography (2D-HPLC) coupled to ESI-MS/MS has been developed.

State-of-the-art method for 2D-LC separations is the comprehensive mode (LCxLC) at which narrow fractions from the entire 1st column dimension are sent to a 2nd column for very fast separation of the fractions one by one. This mode is frequently used for samples with a large number of analytes, such as proteomics, natural products, and polymers, where it is of interest to quantify or profile the entire content of analytes [135]. In the present work, accurate quantification of fragrance hydroperoxides Lim-2-OOH and Lin-OOHs was the aim, while other compounds were of little interest. Therefore, a 2D-HPLC system was used in which the hydroperoxides were fractionated on the 1st dimension column by heart-cutting and sent to the 2nd dimension column. Since a single heart-cut was used, the run time of the separation in the 2nd dimension is independent of the 1st, dimension. Due to this, a long separation time could be used in 2nd dimension, which gives the advantage of higher selectivity for the specific compound [135,136].

In the 2nd dimension, the separation was optimized for best possible separation from interfering compounds. The 1st dimension of chromatography was based on RP chromatography on C₈, while the 2nd was bare silica based on normal-phase retention. Orthogonality in the retention mechanisms is valuable in order to maximize the possibility to separate the analytes from interfering compounds [137]. This can be achieved by coupling, for instance, RP with size-exclusion, ion-exchange or hydrophilic interaction chromatography [138-140]. However, none of these three LC techniques is applicable to fragrance hydroperoxides, according to my experience. Different hydrophilic interaction chromatography (HILIC) columns were also tested (such as aminopropyl-, diol- and pentahydroxysilica and pure silica), but none of these showed sufficient retention of the hydroperoxides. HILIC usually works best at log P < 0, with ionizable or very polar compounds [141]. An RP/RP combination with ion pairing as one of the orthogonal separation mechanisms can be used for ionizable compounds. Also, NP with more lipophilic solvents and different retention mechanisms can be coupled to 2D-HPLC.

For our neutral fragrance hydroperoxide analytes, RP/NP was the only tested combination that was found to work satisfactorily. When designing a 2D-RP/NP HPLC system, the problem with the immiscibility and the incompatibility of the two different solvent systems have to be solved to obtain a working, automated system. One option to handle the problem with immiscible solvents is by evaporation [142] or use columns operating at low flow rates in the 1st dimension, *i.e.* narrow-bore columns for which flow rates can be reduced by 80% [143]. In our case, the problem was solved by introducing a short column, a “trap” column, between the two dimensions. As for packing material of the trap column, C₁₈, cyanopropyl (CN) and porous graphitized carbon (PGC) were evaluated for their ability to focus the target hydroperoxides. Due to the mobile phase composition used in the first column, the mechanism of retention on the

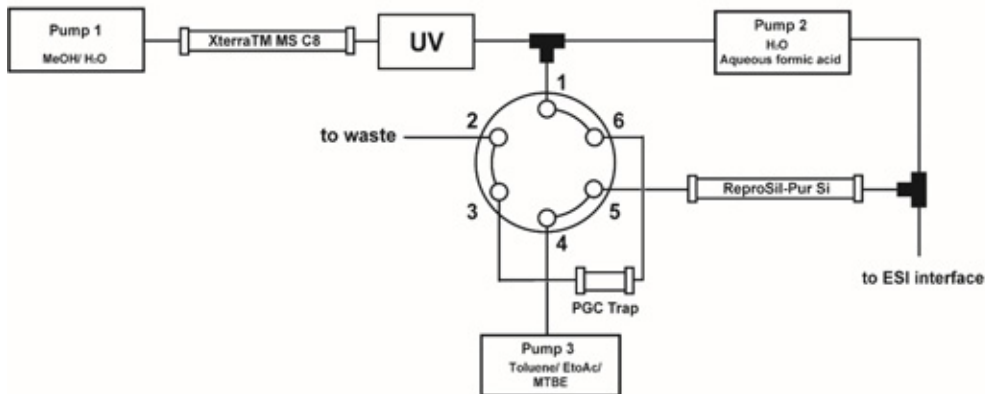
trap column was RP and highly aqueous conditions were used. Among the trap columns, PGC showed the best retention behaviour. The most polar of the hydroperoxides, Lin-OOHs, were retained only on PGC, while Lim-OOH showed strong retention on all three tested trap columns.

The PGC trap served two purposes; to focus the analytes from the 1st dimension, and at the same time reduce the transferred volume of polar mobile phase from the 1st to the 2nd second dimension. The physical dimensions of the 2nd column were important; a larger amount of stationary phase causes less water accumulation and less risk of unstable retention. A step with ethyl acetate to wash the 2nd dimension column between the runs gave good repeatability of the chromatographic separation. The mix of NP solvents used in the 2nd dimension, ethyl acetate/toluene/MTBE, is not ideal for coupling to the ESI interface of the MS. This was solved by using solvent-assisted ESI [144] by adding a flow of aqueous formic acid.

The final result was a repeatable 2D-RP/NP chromatographic system for the targeted Lim-2-OOH and Lin-OOHs fragrance hydroperoxides. The final setup is shown in **Figure 11**.

The hydroperoxide heart-cut fraction from the 1st dimension contains several interfering compounds, the number of which depend on the type of sample. Several commonly occurring compounds from autoxidation mixtures and essential oils were investigated for their retention. The inclusion of both Lim-2-OOH and Lin-OOHs in the same analysis would require a much wider heart-cut window, which would lead to a much larger number of interfering compounds. For that reason, separate methods were developed for the two different types of hydroperoxides.

Valve position 1→6



Valve position 1→2

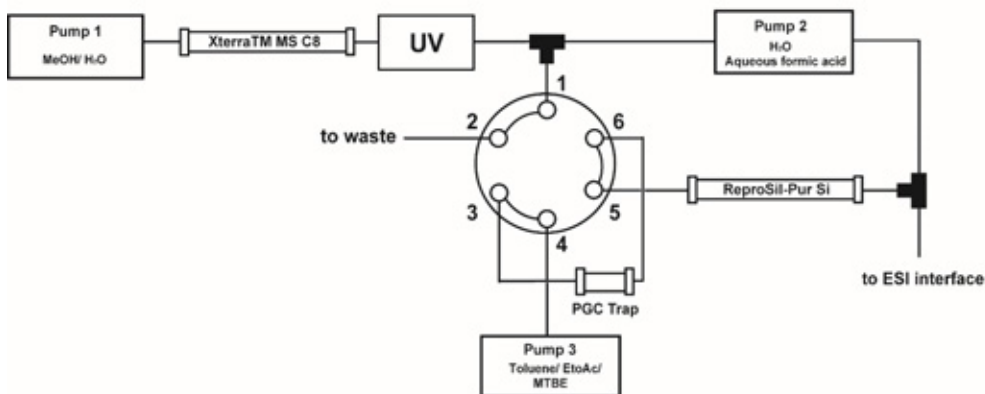


Figure 11: A schematic illustration of the 2D-HPLC/ESI-MS/MS system. The runs start with the 6-port divert valve in the 1→2 position. When the valve then is turned into the heart-cut position 1→6 (upper scheme), the target analytes are eluted to the PGC trap. Subsequently, when the 6-port valve is turned back into the waste position 1→2 (scheme below), the PGC trap is at the same time back-flushed and the heart-cut fraction trapped from the C₈ column transferred to the silica column for chromatographic separation followed by detection with ESI-MS/MS. MeOH= methanol, EtOAc= ethyl acetate, MTBE= methyl *tert*-butyl ether.

Cum-OOH was chosen as IS_V for the quantification, due to its similar retention time as Lin-OOHs on the C₈ column. They were trapped together from the C₈, and then completely separated in the silica column in the 2nd dimension. No suitable IS was found for Lim-2-OOH in spite of testing numerous compounds, so we decided to use Cum-OOH as IS_V also for Lim-2-OOH. In this case, Cum-OOH eluted into the UV detector connected to the first column, while Lim-2-OOH was trapped alone without IS and transferred to the 2nd column for detection by MS. The most suitable internal standards would be isotope-labelled analogues of Lim-2-OOH and Lin-OOHs, respectively, but none of these is yet commercially available.

Application of the 2D-HPLC/ESI-MS/MS method

Determination of monoterpene hydroperoxides in perfume

Perfumes (after-shave, fine fragrance, and eau de toilette) of different brands on the Swedish open market were analysed using the 2D-HPLC/ESI-MS/MS system for highly selective determination of Lin-OOHs and Lim-2-OOH. The perfume products had been stored and used during different periods of time after purchase, between 1-5 years.

The methods were evaluated for two different samples of the eau de toilette. Matrix effects were evaluated by using perfume samples spiked at different concentrations, which were compared with standard calibration curves made from analyte solutions in a pure solvent. The selectivity and degree of chemical interference were also investigated in terms of accuracy of the fragment ion ratios for the different hydroperoxides in both pure standard solutions, non-spiked and spiked samples.

Results and Discussion

GC/MS

GC/MS analyses of the derivatized hydroperoxides in autoxidized oils of sweet orange and petitgrain gave good separation and peak shape. This was due to the thermal stability of the TMS derivatives. The Lim-2-OOH level in sweet orange oil that had autoxidized for two months was found to be about 2% w/w as the sum of the *cis*- and *trans*-isomers. Four other isomers of Lim-OOH were separated and their identities verified by the fragment at m/z 89, as observed in the RIC chromatogram in **Figure 12a**.

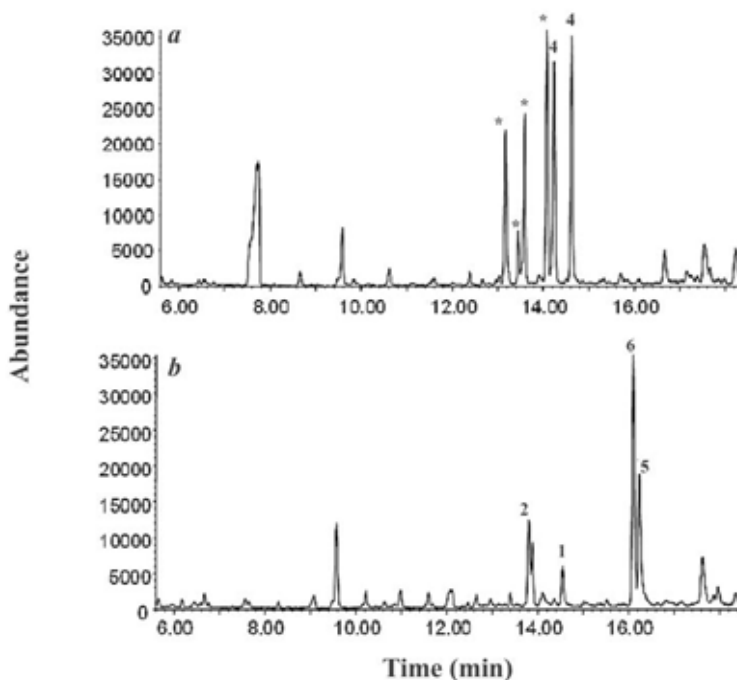


Figure 12: RIC chromatogram of m/z 89 for **a)** TMS derivatives of Lim-2-OOH isomers (4) in sweet orange oil after autoxidation for two months, **b)** TMS derivatives of 1) Lin-6-OOH, 2) Lin-7-OOH, 5) Lin-OAc-6-OOH, and 6) Lin-OAc-7-OOH in petitgrain oil after autoxidation for two months. The label * indicates other Lim-OOH isomers in the autoxidized sweet orange oil (Rudbäck *et al.*, 2014).

Among those were the corresponding isomer Lim-1-OOH, shown to be an even stronger skin sensitizer than Lim-2-OOH [110]. Due to lack of standards, except for Lim-2-OOH, the peaks could not be assigned to specific isomers. Assuming roughly similar response factors (which is a reasonable assumption in full scan EI-MS) the total content of Lim-OOHs in the oil can be estimated to be about three times the Lim-2-OOH content, approximately 5-6 %.

In petitgrain oil, the total concentration of Lin-OOHs (*cis*- and *trans*-isomers of both Lin-6-OOH and Lin-7-OOH) and corresponding Lin-OAc-OOHs was 0.5% and 1.2%, respectively, after two months of autoxidation, **Figure 12b**. Ger-OOHs were not detectable. The reasons for this are the initially low levels of geraniol in petitgrain oil and another type of autoxidation mechanism compared to limonene and linalool. Instead of geraniol hydroperoxides, hydrogen peroxide together with the aldehydes geranial and neral are formed *via* a hydroxyhydroperoxide [97].

HPLC/ESI-MS/MS

The F₅ column was shown to yield a better separation of fragrance hydroperoxides from interfering compounds in the investigated essential oils. Thereby, the risk of matrix effects on the ESI should be reduced and the accuracy of the quantification improved. To increase the flow rate without causing too high back-pressure, a mobile phase system with aqueous acetonitrile was tested and compared between the columns. Retention of isomers of Lin-OOHs, Ger-OOHs, Cit-OOHs, and Lim-2-OOH was compared in terms of retention factor *k*, as shown in **Table 4**. Also, the values of chromatographic resolution, *R_s*, between the isomers were calculated and compared.

Table 4: Comparison of retention and chromatographic resolution, R_s of hydroperoxides in three liquid chromatographic systems. R_s was calculated according to $2(t_{R2}-t_{R1})/(w_{b1}+w_{b2})$. “Isomer” refers to unknown isomeric forms.

Column/ Mobile phase	Analyte	Retention factor (k)	Chromatographic resolution (R_s)
F₅ Acetonitrile/water	Lin-6-OOH isomer 1	19.1	1.30
	Lin-6-OOH isomer 2	19.8	
	Lin-7-OOH	19.8	-
	Ger-OOH isomer 1	20.9	5.70
	Ger-OOH isomer 2	22.5	
	Cit-OOH isomer 1	24.6	2.68
	Cit-OOH isomer 2	25.8	
Lim-2-OOH	38.3	-	
C₃ Acetonitrile/water	Lin-6-OOH isomer 1	13.6	0.74
	Lin-6-OOH isomer 2	14.1	
	Lin-7-OOH	14.1	-
	Ger-OOH isomer 1	18.3	1.98
	Ger-OOH isomer 2	19.8	
	Cit-OOH isomer 1	22.9	1.13
	Cit-OOH isomer 2	23.7	
Lim-2-OOH	35.8	-	
C₃ Methanol/ Isopropanol/water (Rudbäck et al., 2013)	Lin-6-OOH isomer 1	4.7	0.60
	Lin-6-OOH isomer 2	5.0	
	Lin-7-OOH	5.0	-
	Ger-OOH isomer 1	5.8	0.00
	Ger-OOH isomer 2	5.8	
	Cit-OOH isomer 1	N.A.	N.A.
	Cit-OOH isomer 2	N.A.	
Lim-2-OOH	11.9	-	

As shown, the F₅ exhibited stronger retention with both mobile phase systems, and the chromatographic resolution was improved as well. The obtained method LODs with HPLC/ESI-MS/MS using the F₅ column were all below 200 ppm, about 20 times lower than those obtained with GC/MS (0.4% *i.e.* 4000 ppm).

The HPLC/ESI-MS/MS analysis of the fragranced shampoo that had been shown to cause ACD, **Paper IV**, showed a total concentration of 0.2 ppm of Lin-OOHs. The SPE method, applied to the HPLC/ESI-MS/MS analysis (using the F₅ column), was optimized for Lin-OOHs and the obtained extraction recoveries were 95%. Linalool and the secondary oxidation products linalool oxides were just estimated and since the aim was not to determine them, no method optimization was performed. The recoveries for linalool oxides were as low as 23%. The total linalool oxide and linalool level was 0.8 ppm and 75 ppm, respectively. Considering the ratio between oxidation products and the parent compound, this indicates that at least 1% of linalool had autoxidized. However, the history of the shampoo, *i.e.* the time of storage and usage, was not known, and the relative levels of oxidation products do change during the autoxidation process [108,109,145]. Also, it is not known if the autoxidation actually took place in the shampoo, or in the linalool oil prior to its addition to the product. Another question is if hydroperoxides at a level as low as 0.2 ppm, given their strong sensitization potencies, could lead to induction of contact allergy. The induction levels needed are still discussed within the community of fragrance allergy researchers, dermatologists and perfume industry representatives. Among the latter, the assumption is a limit of 5000 ppm for skin sensitization [146]. Nevertheless, it could be concluded that the shampoo was the culprit behind the girl's eyelid ACD and she reacted to no other tested haptens than Lin-OOHs. One possible explanation is that she previously had been sensitized to another product with higher levels and due to repeated use of the shampoo her immune system had been triggered to react also to very low levels.

Results from patch test materials showed deviations for all preparations with the largest discrepancies for the products from company B, **Figure 13**. The preparations had been stored at +4°C and had expiring dates labelled on the package. After reporting these results, company B immediately withdrew their

products from the market. It is important for the reliability of patch test results that the labelled content of the test preparation can be trusted. This is now possible to control with the analytical method presented here.

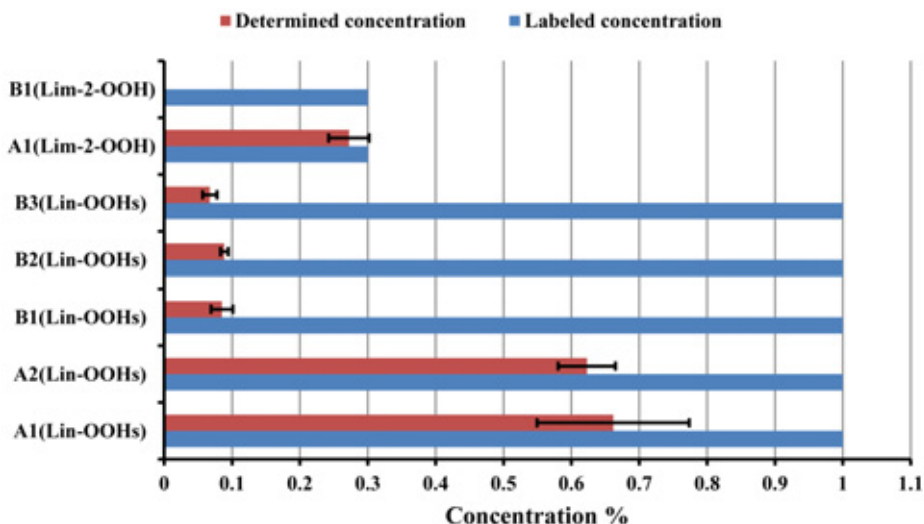


Figure 13: Comparison between the determined and labelled concentrations of hydroperoxides in patch test preparations. Lim-OOHs were not detected in **B1 (Lim-OOHs)**. **A1**, **A2**, and **B1 (Lin-OOHs)** had expired at analysis time.

Two-dimensional HPLC/ESI-MS/MS method (Paper V)

As mentioned above, a heart-cut technique was utilized to isolate the hydroperoxide fraction from the 1st dimension column in the 2D-HPLC system. Although some monoterpenoids co-eluted in the 1st dimension, the separation was achieved in the 2nd dimension (**Figure 14**). Limonene oxide was the only compound that interfered, partly co-eluting with Lim-2-OOH, but it could be fully separated by different MS/MS transitions and the quantification was not affected. The entire runtime for each hydroperoxide type was no longer than 60 min. The automation of the analysis is an advantage as well as the achieved reproducibility in retention. Furthermore, no sample preparation other than

dilution is needed for perfumes prior to injection. Screening of a much larger number of samples will, therefore, be more straightforward.

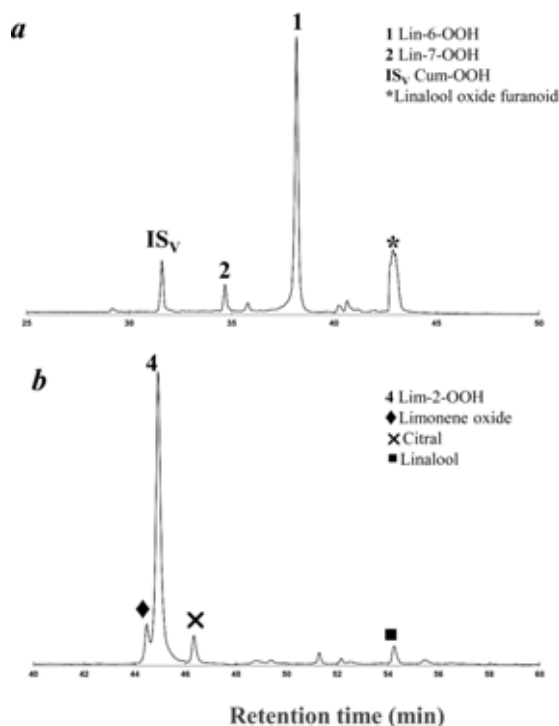


Figure 14: MRM chromatograms (TIC chromatograms) showing hydroperoxides and interfering monoterpenoids on the 2nd silica column after joint transfer from the first dimension via the PGC trap. **a)** Method for Lin-OOHs and **b)** method for Lim-2-OOH.

Lim-2-OOH was used as a marker for all hydroperoxide isomers of limonene that may occur in products. This was because 1) there is a lack of reference compounds of other isomers, 2) they have different retention behaviour between the isomers on the C₈ column, thus making it difficult to simultaneously transfer them into the 2nd column with the instrumental setup currently at hand. The Lim-2-OOH isomer has also been used as a marker for quantification by others [127,129]. The accuracy of quantification, as well as detection limits, were significantly improved with the 2D-HPLC/ESI-MS/MS system compared with the 1D-HPLC/ESI-MS/MS method. When samples were analysed some matrix

effects could still be observed (between +8% and -20%), but the ion ratios were shown to be reproducible and similar compared to standards (RSD < 9%) for all MS/MS ion transitions, **Figure 15**. Ten perfume samples were analysed and Lin-6-OOH and Lin-7-OOH were detected in all of them, while Lim-2-OOH could be identified in four of the samples. The highest level was found for Lin-6-OOH, which was detected in one sample at 436±24 ppm. This level is likely to be able to cause skin reactions in already sensitized individuals [120]. Whether it is able to cause skin sensitization, *i.e.* induce contact allergy, at this concentration level is not known. Kligman *et al.* and Friedmann *et al.* have suggested that repeated exposure during a long period of time could trigger the immune system so that much lower levels are able to induce skin allergy [73,74].

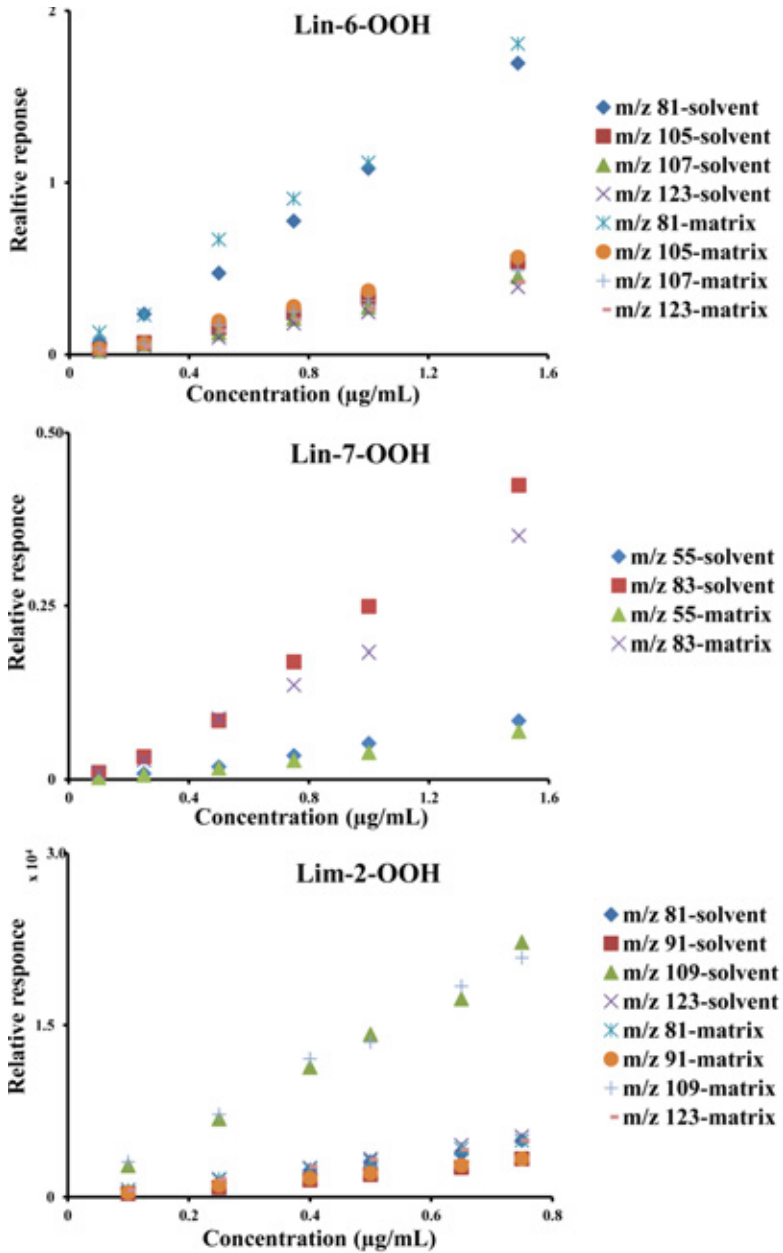


Figure 15: Comparisons between all mass transitions used for the determination of hydroperoxides when spiked in pure solvent and sample matrix, respectively.

Conclusions and future perspectives

This part of the work has been focused on developing and further improving analytical methodologies for the determination of mainly three different hydroperoxides, Lin-6-OOH, Lin-7-OOH, and Lim-2-OOH, which are all known as strong skin sensitizers. These compounds are primary oxidation products from two of the most commonly used fragrances used in cosmetics and household products. With the developed methods they could be quantified in complex and “difficult” sample matrices, such as essential oils, shampoos, patch testing preparations, perfumes, after-shaves and a deodorant (the latter unpublished).

A question that still remains to be answered is where the main exposure to fragrance hydroperoxides originates from. A toolbox of analytical methodologies is now available to screen the market for the occurrence of these compounds in many types of products.

It seems that hydroalcoholic solutions, like perfume, slow down the autoxidation. This has been suggested by Kern *et al.* [119] and is also supported by some of our experiments. This indicates, although not proves, that the hydroperoxides are added to rather than formed in such products, for instance as constituents of essential oils that have autoxidized already prior to their addition.

Another possible reason for exposure could be that fragrances like limonene and linalool form hydroperoxides by bioactivation, *i.e.* metabolism by enzymes in the skin. The latter has not been studied so far, most likely due to the lack of appropriate analytical methods. The low levels expected from skin metabolism are now possible to investigate by the 2D-HPLC/ESI-MS/MS method developed in this work, yielding detection limits as low as 1 ng/ml injected concentration.

Another scenario that could lead to exposure is autoxidation on the skin. Sunlight in combination with sunscreens (which are added to many skin care products) and fragrances might cause a fast oxidation into allergenic hydroperoxides directly on the skin. It has been shown previously by Karlsson *et al.* that the common sunscreen octocrylene can induce both contact allergy and photoallergy [147,148]. This compound is also known to produce singlet oxygen in the presence of UV light and air [149], which could lead to photooxidation of fragrance monoterpenoids. It would be of high interest to investigate whether cases of fragrance allergy are more prevalent in countries with more sunshine hours.

In my view, the development of the two-dimensional HPLC/ESI-MS/MS method is a significant contribution to the difficult field of hydroperoxide determination. The use of tandem mass spectrometry together with selective chromatographic separation improves the accuracy of quantification. The method comprises commonly available HPLC stationary phases and should be easily transferred to any lab housing a tandem mass spectrometer. An improvement still to be made is by having isotope-labelled hydroperoxides as internal standards. This would make it possible to set up this 2D-HPLC/ESI-MS/MS technique for strongly skin sensitizing hydroperoxides from other common fragrances, such as citronellol and geraniol.

Hopefully, fragrance hydroperoxides will soon be included in the EU regulatory framework, which is important for both prevention and to increase the common awareness of these compounds.

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