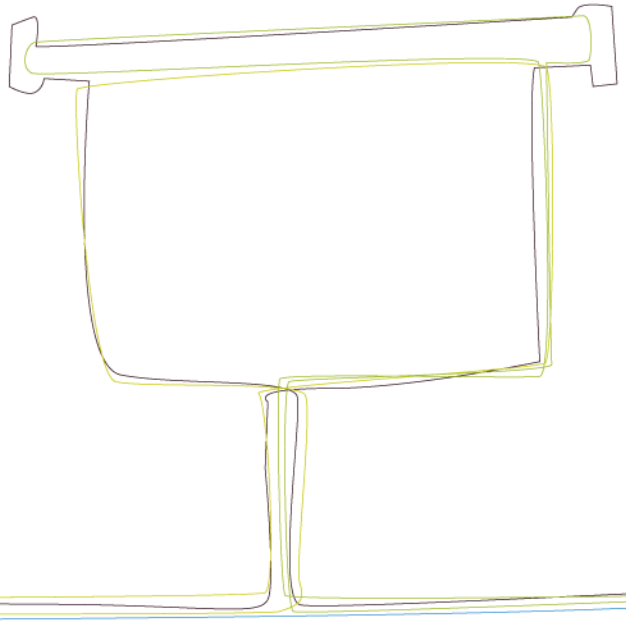
IDEA
International Dialogue for the Evaluation of Allergens

The exposure source for skin sensitizing hydroperoxides of limonene and linalool remains elusive

An analytical market survey



IDEA Hydroperoxides Task Force

Prepared for ESCD 2018
Oct 19th, 2018

IDEA Analytical Hydroperoxides (HP) task force: A multistage project

1. Problem definition
2. Analytical sensitivity: Targets set by the task force
3. Validation of analytical methods

Market overview and patient's products:

1. **Products analyzed**
2. **Results by the screening method and LC-MS confirmation**
3. **Validation by standard addition**
4. **Interpretation – Sensitivity and detected levels vs. toxicological / clinical data**

Problem definition

- Hydroperoxides (HP) of widely used terpenes (Limonene and Linalool) are skin sensitizers
- Positive patch test reactions to oxidized terpene fractions, containing these HP's, are frequently reported
- Hydroperoxides in these oxidized fractions presumed to be specific allergens
- **Limited evidence on occurrence of hydroperoxides in consumer products**
- Exposure source for induction of HP contact allergy is currently unknown
- What type of products?
- Status of products? Aged? Oxidized?

Problem definition: Analytical methods

- Analytical detection of HP is challenging
- HP are not intentionally added to products, but
  - They could be introduced as impurities from raw materials
  - They may form in products if sufficient oxygen is present or as a consequence of age
- There are very little exact data on HP levels in raw materials
- There are even less data on HP level in consumer products
- **Analytical data are needed to establish whether positive patch test reactions may come from use of fragranced consumer products**
- **Analytical methods able to detect HP in consumer products are required**

Scope:
What are analytical methods needed for

There are two different questions:

- **Quality control on raw materials**: Detection of HP in raw materials used in fragrance compounding
  - Complex essential oils from natural sources (e.g. lavender oil)
  - Synthetic raw materials (e.g. synthetic linalool)
- **Detection in final consumer products**
  - Detection in general market products and aged consumer samples

    ⇒ Presence of potentially **sensitizing doses** above levels considered safe by QRA?
  - Detection in products brought in by patch-test positive patients

    ⇒ Presence of potentially **elicitating doses** which may indicate relevance of reaction to actual disease?

Sensitivity:
Targets set for the task force

- **Initial analytical target agreed:**
  "Methods should be sensitive, specific, with target limits of quantification (LOQ) below the estimated induction levels and limits of detection (LOD) below the estimated elicitation levels"
  Estimated induction levels:
  - 5000 ppm taken as a default induction level (based on LLNA EC3 on multiple hydroperoxides)
  - Linalool: Up to now lowest elicitation level in humans: 560 ppm (based on one small published ROAT)

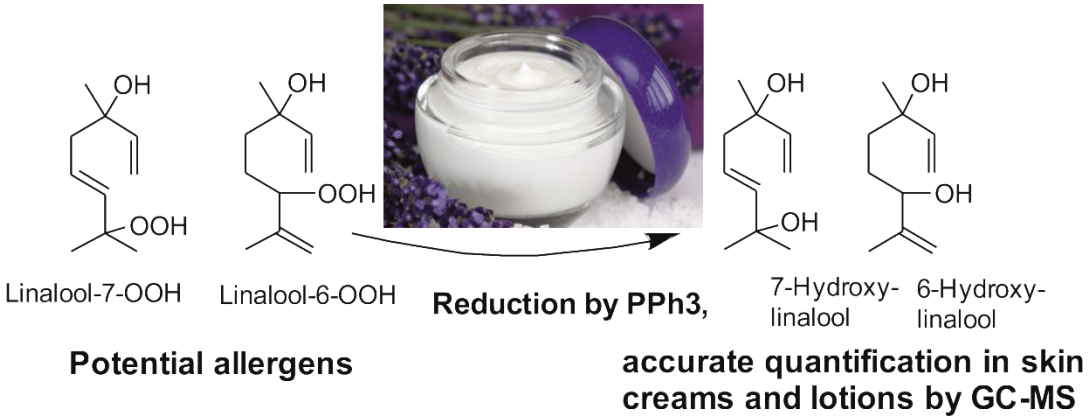
- **Revised analytical target** – based on improved analytical methods:

<u>50 ppm in final consumer product</u> (defined as 'reporting level')

- This is 100 fold below default induction level
- 10-fold below reported tentative elicitation level
- Note: This lower level is set to have a full understanding and is based on **analytical feasability**: it does not mean that all levels above 50 ppm are of toxicological concern!

Toolbox of methods: GC-MS-reduction method

- GC-MS-reduction method: HP are reduced to corresponding alcohols
- Alcohols are very stable analytes, which can be analyzed by conventional GC-MS methods



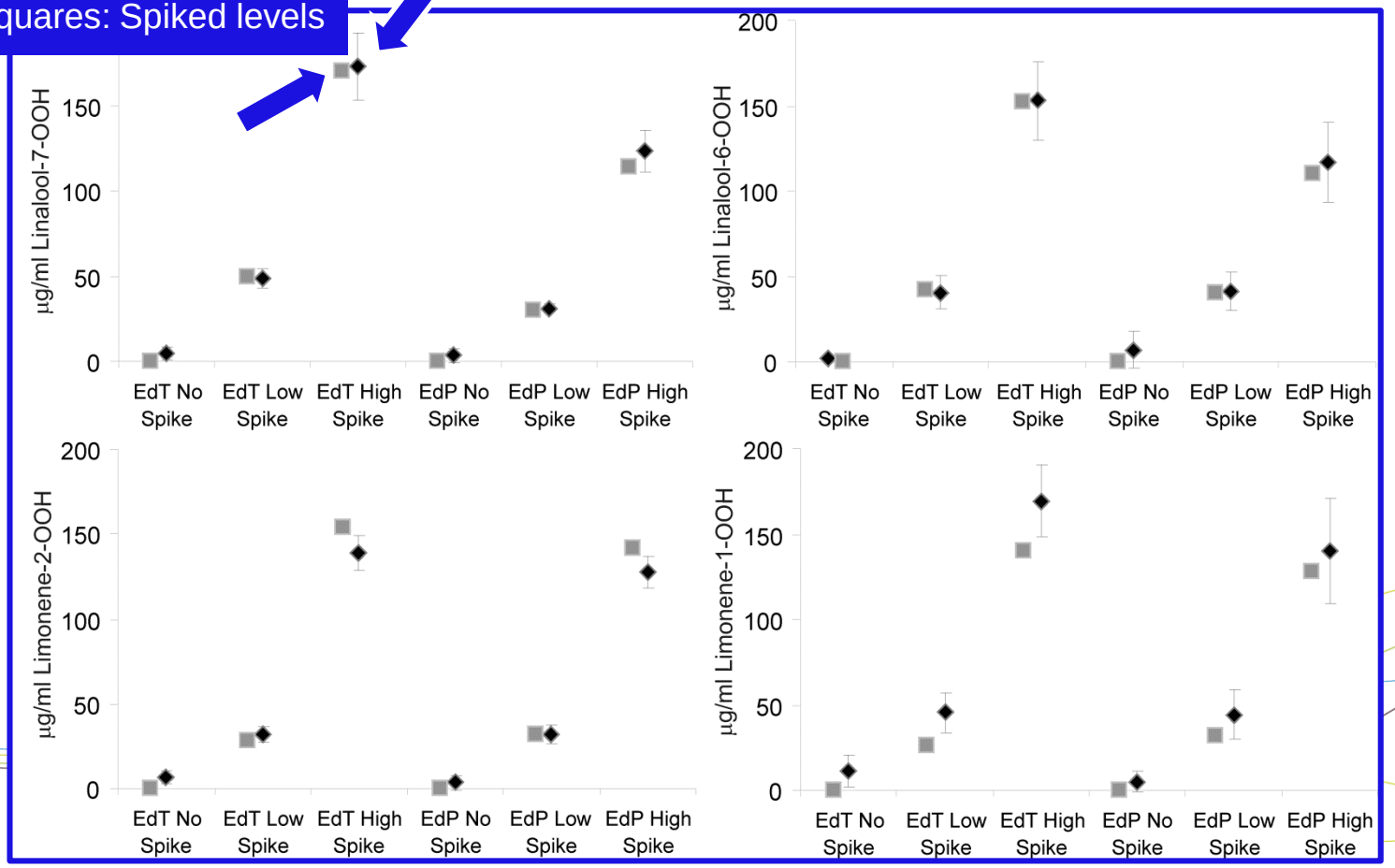
- This method is **very sensitive but** conservative, overestimation possible if alcohol is in product
- Method proven to be highly reproducible by **blind-coded** multilaboratory trials

Ring study: Method validation in fine fragrances

- Five labs tested **blind-coded** samples
- Eau de Toilette and Eau de parfum spiked with 4 HP at different levels
- Accurate detection with GC-MS reduction by all five labs
- **This method allows accurate quantification in commercial fragrances**

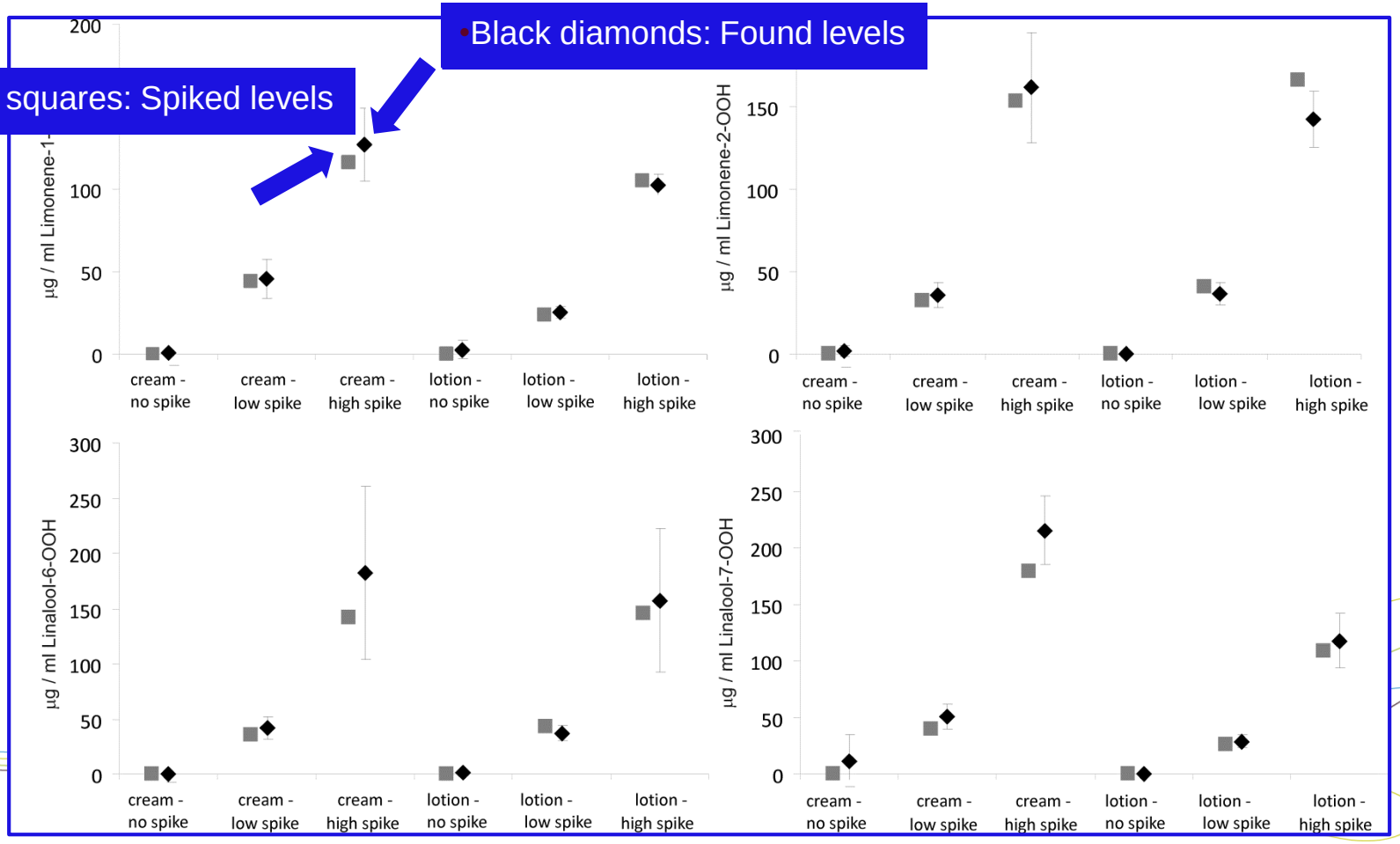
•Black diamonds: Found levels

•Grey squares: Spiked levels



Ring study II: Method validation in creams / lotions

- Five labs tested **blind-coded** samples
- Cream and lotion spiked with 4 HP at different levels
- Accurate detection with GC-MS reduction by all five labs
- **This method allows accurate quantification in complex cosmetic products**



LC-methods

- LC-method allow to directly detect parent HP
- LC-methods are **more specific** for the hydroperoxides
- More prone to matrix interaction
- Three LC-Methods were further validated as confirmatory methods
- Example of results:

	EdT No Spike	EdT Low Spike	EdT High Spike	EdP No Spike	EdP Low Spike	EdP High Spike
LC-Q-TOF MS	0.0	90.0	279.0	0.0	59.0	200.0
HPLC-CL	0.0	79.5	310.7	0.0	56.2	203.7
LC-orbitrap-MS	0.2	95.7	398.7	0.0	29.1	185.4
spike level added	0.0	92.0	322.0	0.0	70.0	224.0

Toolbox of methods: Analytical strategy

- Use versatile, **robust and sensitive** reduction method to screen samples
- Use LC-methods, which are **more specific** for the hydroperoxides, for confirmatory analysis
- Confirmatory analysis for **positive samples above reporting level** by reduction method, as method may be oversensitive
- Confirmatory analysis for **negative samples with high suspicion (patient samples)**

Application of the analytical methods: Market overview and patient's products

- Detection in final consumer products:
  - Detection in general **market products**
  - ⇒ Presence of potentially sensitizing doses above levels considered safe by QRA?
  - Detection in **aged consumer samples**
  - ⇒ Are products sufficiently protected against oxidation?
  - Detection in **products brought in by patch-test positive patients**

    ⇒Presence of potentially elicitating doses which may indicate relevance of reaction to actual disease?

Market overview – setup

- Samples from consumer homes, which are partly used
- Products should have declared linalool and limonene content and batch number /production code / date (to ensure traceability)
- For each aged product we searched for a matched fresh product
  - 31 different products (31 fresh and 31 aged, partly used)
  - Fine fragrances, deodorants, creams, lotions
- Samples from patients, collected by Spanish dermatological network

  - Mainly form patch test positive patients

  - If possible, samples also matched with fresh products

  - 28 samples; 11 samples from patients patch test positive to oxidized Linalool and / or oxidizedLimonene

- Specific products with controlled aging
- 'Aromatherapy' products
- A specific sample with rel. high level reported in previous study

Market overview – Results aged vs. new samples

- 31 products which could be matched with fresh products (62 samples, analyzed for 4 different hydroperoxides)
- Only one sample above reporting limit:
  91 μg/ml of Limonene-1-OH by GC-MS reduction method
- Presence of Limonene-1-OOH verified in this sample by three LC-based methods
- No evidence for HP accumulation in aged samples
- 33% of the analyzed samples contained > 1000 ppm of parent Linalool or limonene
- Compared to the significant level of parent linalool and limonene, HP are either very minor constituents or are not detectable at all in these products
- Aged samples are not more problematic than fresh samples

Results aged vs. new samples: Two products with controlled aging

- 2 products from manufacturer with controlled aging history
- No HP above reporting level
- Trace levels detected, no indication for increased HP level with aging
- No indication for degradation of parent HP

Two commercial fine fragrance samples with defined storage history analysed by the GC-MS-reduction method

Condition	Limonene -1-OOH	Limonene -2-OOH	Linalool-7-OOH	Linalool-6-OOH	Limonene	Linalool
Perfume 1, fresh	**16**	**33**	**18**	<16	4100	2200
Perfume 1, 3 years at RT	<16	<16	<16	<16	4200	2300
Perfume 1, 3 months, 45°C	<16	**18**	<16	<16	4300	2300
Perfume 2, fresh	**18**	**18**	**36**	<16	>5000	4200
Perfume 2, 6 years at RT	**19**	<16	**32**	<16	>5000	4100
Perfume 2, 3 months, 45°C	**24**	<16	**30**	<16	>5000	3900

•Indicated are ppm in final product as determined by the GC-MS reduction method

Market overview – Results products from patients

- 28 products obtained from patients over spanish dermatological network, suspected for being causative of reactions
- 11 of these samples were from patients which were positively tested to oxidized linalool or limonene
- None of these samples contained above 50 μg/ml by GC-MS method
- Three LC-MS methods could confirm this result: Absence of significant HP levels in all these products
- Neither induction nor clinical symptoms in these patients can be explained by HP level in the sampled, suspected products

Example of a patient product

Sample and history of donating patient	Analytical methods	Limonene-1-OOH	Limonene-2-OOH	Linalool-7-OOH	Linalool-6-OOH
O12, Body cream, Positive some fragrances, Positive Limonene ox	GC-MS red. (μg/ml)	<22	<22	<22	<22
	GC-MS red. (% recovery)	69%	70%	59%	84%
	LC-Orbitrap-MS (μg/ml)	NF	nr	NF	NF
	LC-Q-ToF-MS (μg/ml)	<5	<5	<5	11
	LC-CL (μg/ml)	NF	NF	NF	NF

⇐ Reduction method
⇐ Spike recovery
⇐ LC-MS method 1
⇐ LC-MS method 2
⇐ Chemilum. method

NF: Not found

Market overview – results essential oil containing products

- Limited number (five products) which contain essential oils according to declaration
- GC-MS reduction method could detect low amount of target alcohols in these samples
- LC-methods could NOT confirm these results
- The alcohols from HP reduction can be contained at low levels in natural essential oils (oversensitivity of the reduction method)
- See as an example next slide

Aromatherapy product with highest level according reduction method

- Shower oil preparation, from a company specialized on ess. oil containing product
- Claiming '***contains 12 essential oils***', limonene most abundant next to water
  - 21.5% Limonene in final product
  - 4.4% Linalool

Content of alcohols formed by reduction method

Limonene-1-OH	Limonene-2-OH	Linalool-7-OH	Linalool-6-OH
262	141	99	24

Content of parent

	Limonene	Linalool
Solvias	>7000 ppm	>7000 ppm
Diluted re-analysis (Giv)	**215'177 ppm** (21.5%)	**43'788 ppm** (4.4%)

Content of hydroperoxides (LC-MS methods)

	Limonene-1-OOH	Limonene-2-OOH	Linalool-7-OOH	Linalool-6-OOH
LC-Q-ToF-MS	**n.f.**	**n.a.**	**n.f.**	**n.f.**
LC-Orbitrap-MS	**<5**	**<5**	**<5**	**15**
LC-Chemiluminescence	**4.5**	**2.7**	**3.9**	**5.2**

All data in ppm

N.f.: Not found; n.a. not applicable

DECLARATION: Sulfated castor oil, Aqua (water), **Limonene**, Citrus aurantium dulcis (**orange**) peel oil, Lavandula angustifolia (**lavender**) oil, **Linalool**, **Cinnamomum camphora linalooliferum leaf oil**, Citrus aurantium amara (**bitter orange**) leaf/twig oil, Citrus nobilis (**mandarin orange**) peel oil, Cymbopogon martini oil, Origanum majorana flower oil, Cupressus sempervirens oil, Amyris balsamifera bark oil, Anthemis nobilis flower oil, Citrus aurantium amara (**bitter orange) flower oil**, **Lavandula** hybrida grosso herb oil, Geraniol, Citral, Farnesol

Market overview – Results: Re-analysis of a sample analyzed before

- One aftershave sample was recently found to contain 420 Linalool-6-OOH and ca. 20 ppm Linalool-7-OOH by a novel method [1)]
- This is a very unusal isomer ratio not occuring normally during oxidation
- We thus re-analyzed the same sample by all four methods
- Our three LC-methods could not verify the content of this hydroperoxide, much lower levels found by the reduction method

Aftershave analyzed before

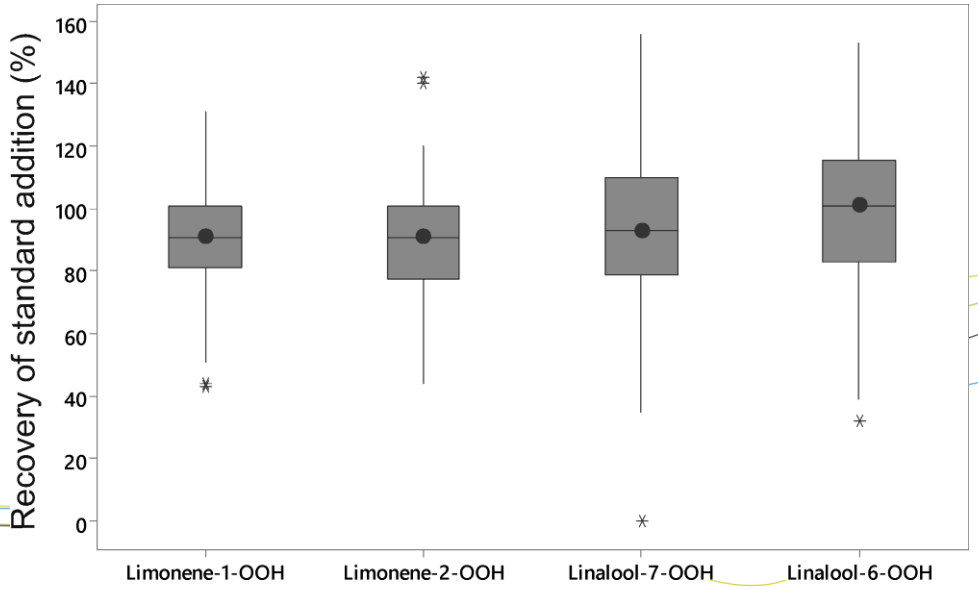
		Limonene-1-OOH	Limonene-2-OOH	Linalool-7-OOH	Linalool-6-OOH
Sample E, after shave	GC-MS red. (µg/ml)	18	20	**72**	**81**
	GC-MS red. (% recovery)	92%	97%	114%	102%
	LC-Orbitrap-MS (µg/ml)	7	n.r.	ca. 5-10	< 25
	LC-Q-ToF-MS (µg/ml)	17	<5	8	7
	LC-CL (µg/ml)	1.6	1	2.8	4.6

[1)] Ramzi A, Ahmadi H, Sadiktsis I, Nilsson U. A two-dimensional non-comprehensive reversed/normal phase high-performance liquid chromatography/tandem mass spectrometry system for determination of limonene and linalool hydroperoxides. *J Chromatogr A.* 2018.

Validation by standard addition

- In this work we report many negative results: The vast majority of samples does not contain hydroperoxides
- It is very important to validate these results – can we be confident that we can analyze the HP in these very different products?
- Thus each sample was analyzed in duplicate – once spiked with all four synthetic hydroperoxides at the reporting level (50 µg / ml)
- Spike could always be positively detected (one exception in 416 single determination)
- Spike recovery in general > 70%, and close to 100% on average

Recovery of standard addition (50µg/g) of four HP added to 104 products analysed by the GC-MS-reduction method.



Interpretation – Sensitivity and detected levels vs. toxicological / clinical data

- In general we could not detect and confirm hydroperoxides above reporting limit in great majority of the samples analyzed
- These negative results were validated by standard addition
- The first question is: Can the rare occurence of HP explain the high frequency of positive reactions in terms of ***frequency of occurrence?***

- But what do the figures mean in terms of ***quantity?***
- In one sample we could positively detect 90 μg/g of Limonene-1-OOH
- We can calculate what this means in terms of dose-per area and compare it to toxicological and clinical data….

Interpretation – Sensitivity and detected levels vs. toxicological / clinical data

Dose per area calculations for limonene-1-OOH

	Dose of hydroperoxide in test preparation	Dose per area
LLNA Dose inducing sensitisation (EC3)	3300 µg/g (0.33%)	82.5 µg/cm^2
Patch test limonene-HP *, **routine diagnostic level**	3300 µg/g (0.33%)	156 µg/cm^2
Patch test limonene-1-OOH **, diagnostic level	5000 µg/g (0.5%)	228 µg/cm^2
Defined reporting limit	50 µg/g	0.1 – 0.5*** µg/cm^2
Analytical data market survey: (Max. value of n = 104)	90 µg/g (0.009%)	0.2 µg/cm^2 ****

* Mixture of isomers, not specifically 1-OOH-isomer
** Dose used in study on specific Limonene-1-OOH isomer by Christensson, Contact Dermatitis 2015
*** Different dose depending on product type (Cream 10 mg/cm^2 higher than fine fragrance, 2.2 mg/cm^2)
**** Based on the typical application dose of fine fragrance per area

- Even the single positive sample leads to a dose per area exposure which is 400-fold below the inducing level in the LLNA
- Level is 1000-fold below the patch test dose when calculated as dose per area
- Reporting limit is also clearly below induction doses: the puzzle is not about analytical sensitivity

Conclusion

- This Study has significantly extended our knowledge on HP occurrence in Consumer Products
- This is the first study analyzing multiple products from patients

- HP of linalool and limonene are not widespread in consumer products
- Aging of Products has little to no impact on the HP levels found

- *Frequency of occurrence* and *quantity* (as exposure conc.) of HP cannot yet explain widespread induction / frequent patch test reactions
- An exposure source explaining frequent positive patch test reactions remains elusive

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Thank you for your attention