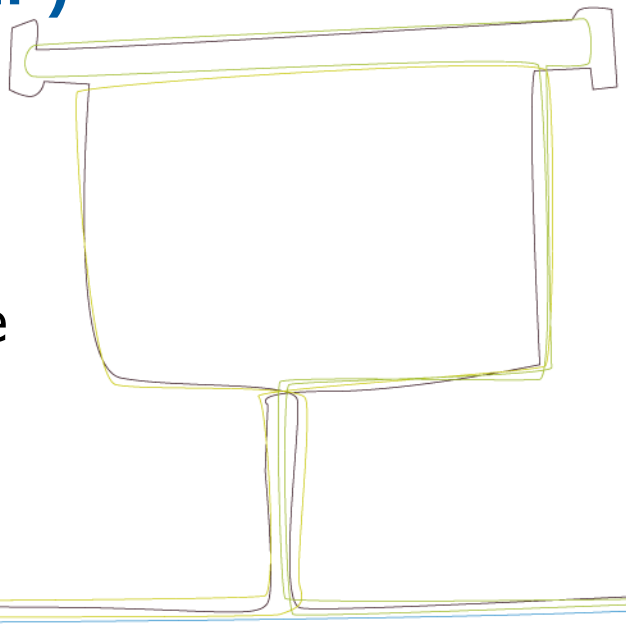
IDEA
International Dialogue for the Evaluation of Allergens

Risk assessment of pre- and pro- haptens
Development of an analytical method for detection and quantification of hydroperoxides (HP)



Dr. Andreas Natsch
Chair of the IDEA Hydroperoxides Task Force

IDEA Annual Review 2016
March 6th, 2017

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

1. The Problem
2. Scope: What are methods needed for?
3. Sensitivity: Targets set for the task force
4. Ring Study 1: Comparison of methods
5. Important step: Accurate analytical standards
6. Ring Study 2: Comparison of methods – continued
7. Ring Study 3: Method validation in real products – fine fragrances
8. Method development work – analysis in complex matrices (creams, lotions)
9. Ring Study 4: (Planned): Method validation in real products – Creams, lotions, and deodorants
10. Application: Market overview and patient's products
11. Interpretation – how will we judge results? – Input to QRA2

Problem definition

- Some slow oxidizing pre- haptens can form hydroperoxides (HP).
- Hydroperoxides are sensitizers
- Positive patch test reactions to oxidized products are reported
- Analytical detection of HP is challenging
- HP are not intentionally added to products, but
  - They could be present as impurities from raw materials
  - They may form in products if sufficient oxygen is present
- 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 find out 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 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. orange 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

- **Initially set analytical Target:**
  "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:

50 ppm in final consumer product (defined as 'reporting level')

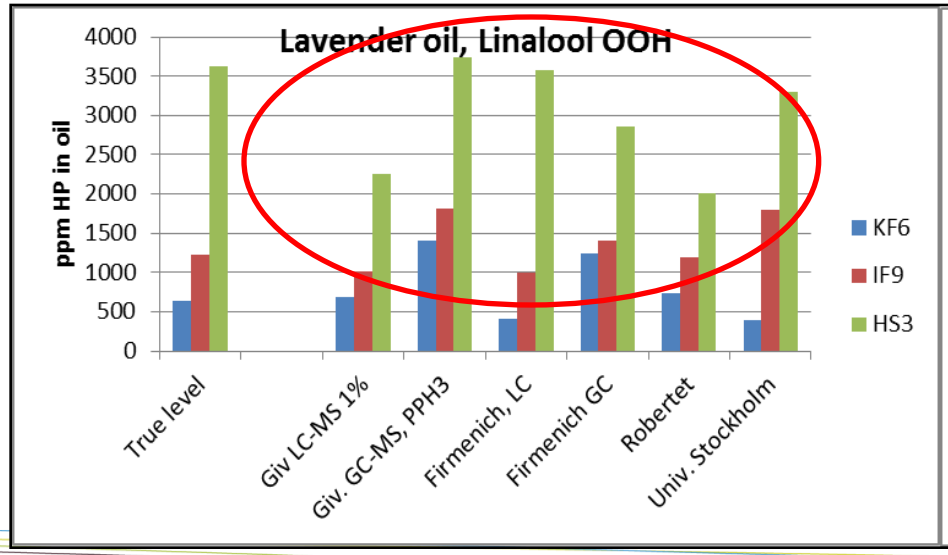
- This is 100 fold below default induction level
- 10-fold below reported elicitation level
- Note: This lower level is set to have a full understanding and is based on analytical feasability: it does not mean that all levels above 50 ppm are of toxicological concern

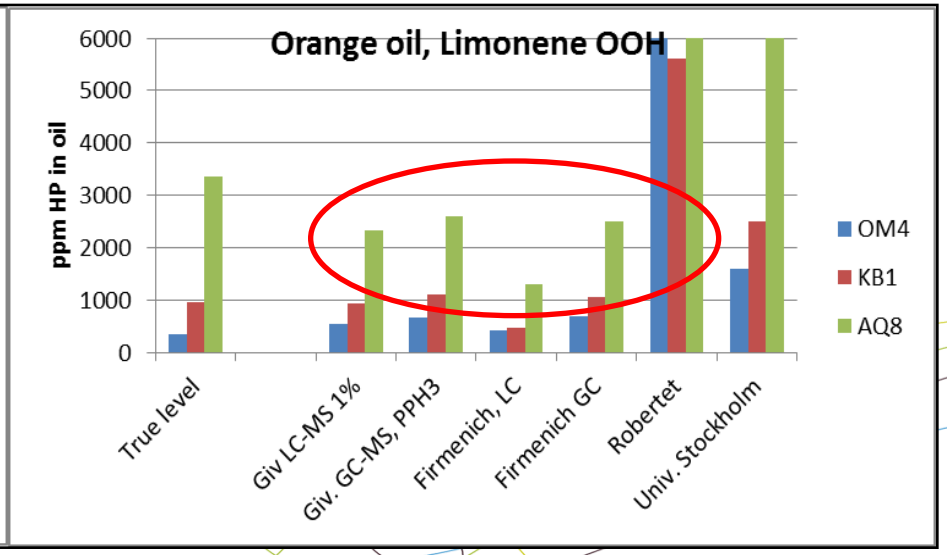
Ring study 1:
Comparison of methods

- Lavender oil spiked with Linalool-OOH and orange oil spiked with Limonenen-OOH
- Spike levels 500 (**red bars**) and 3000 ppm (**green bars**); blinded samples
  - Spike levels defined by initial target sensitivity
- 6 different methods by total 5 different laboratories

Methods can detect the HP, but significant variation from true level

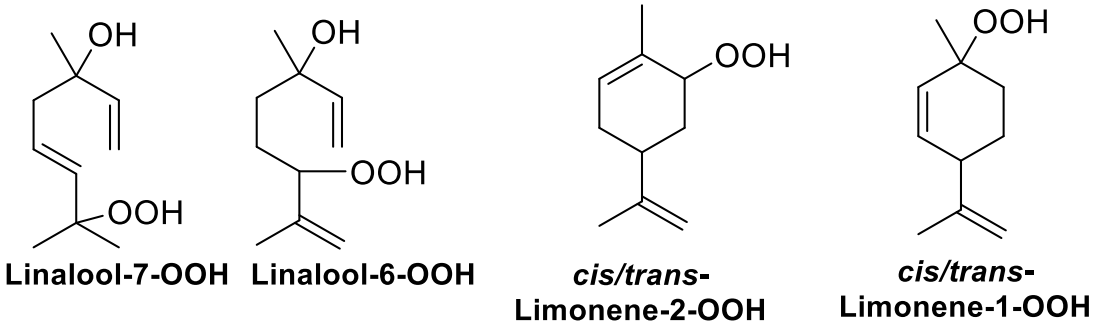
Tendency for underestimation in orange oil





An important step:
Accurate analytical standards

- First study was run with analytical standards containing **mixtures** of hydroperoxides, **not completely purified**
- Key to improve methods: **Highly pure reference standards**
- External company was asked to prepare 4 highly pure standards
- **These standards served to:**
  - Prepare exact spiked samples in subsequent ring tests
  - Calibrate analytical methods



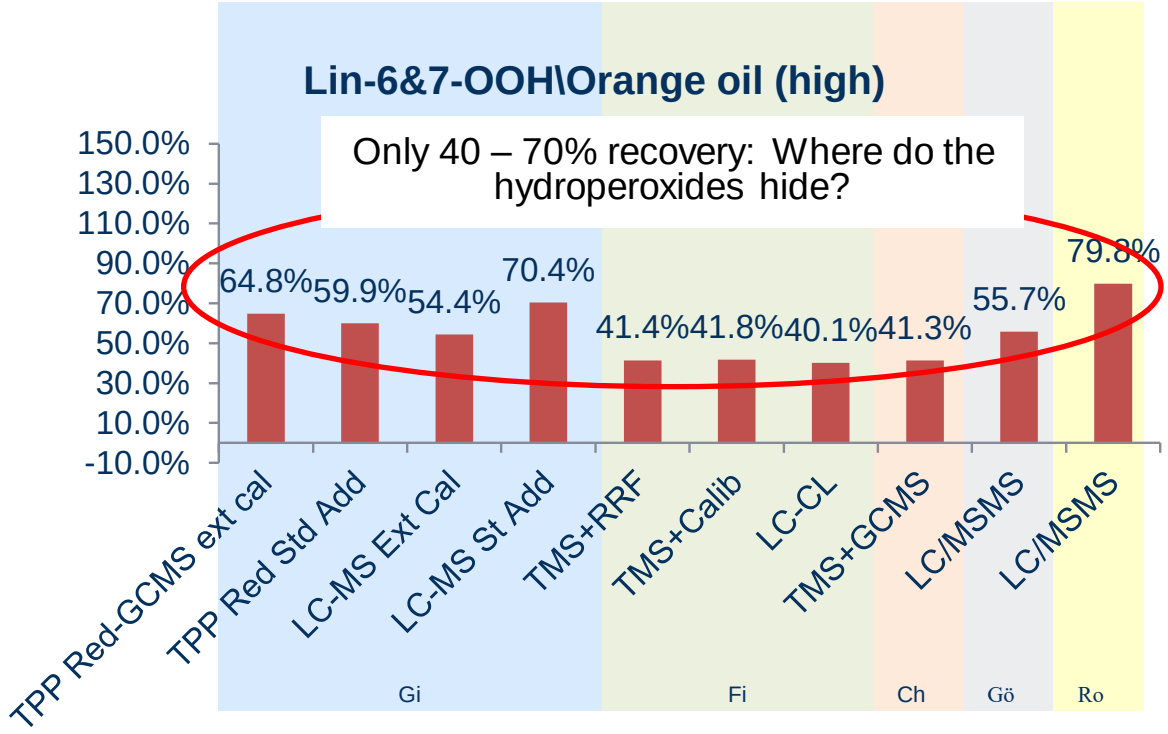
Linalool-7-OOH **Linalool-6-OOH** ***cis/trans*-Limonene-2-OOH** ***cis/trans*-Limonene-1-OOH**

Ring study 2:
Comparison of methods – continued

- Blind spiked samples with accurate analytical standards
- Three matrices of increasing complexity
  - Simple solvent
  - Orange oil
  - Model fragrance (Lily)
- 6 labs with a total of 10 different methods / quantification approaches

Conclusion:

- **General underestimation in orange oil and Lily fragrance with several methods**
- Reduction / GC-MS method may be a robust method



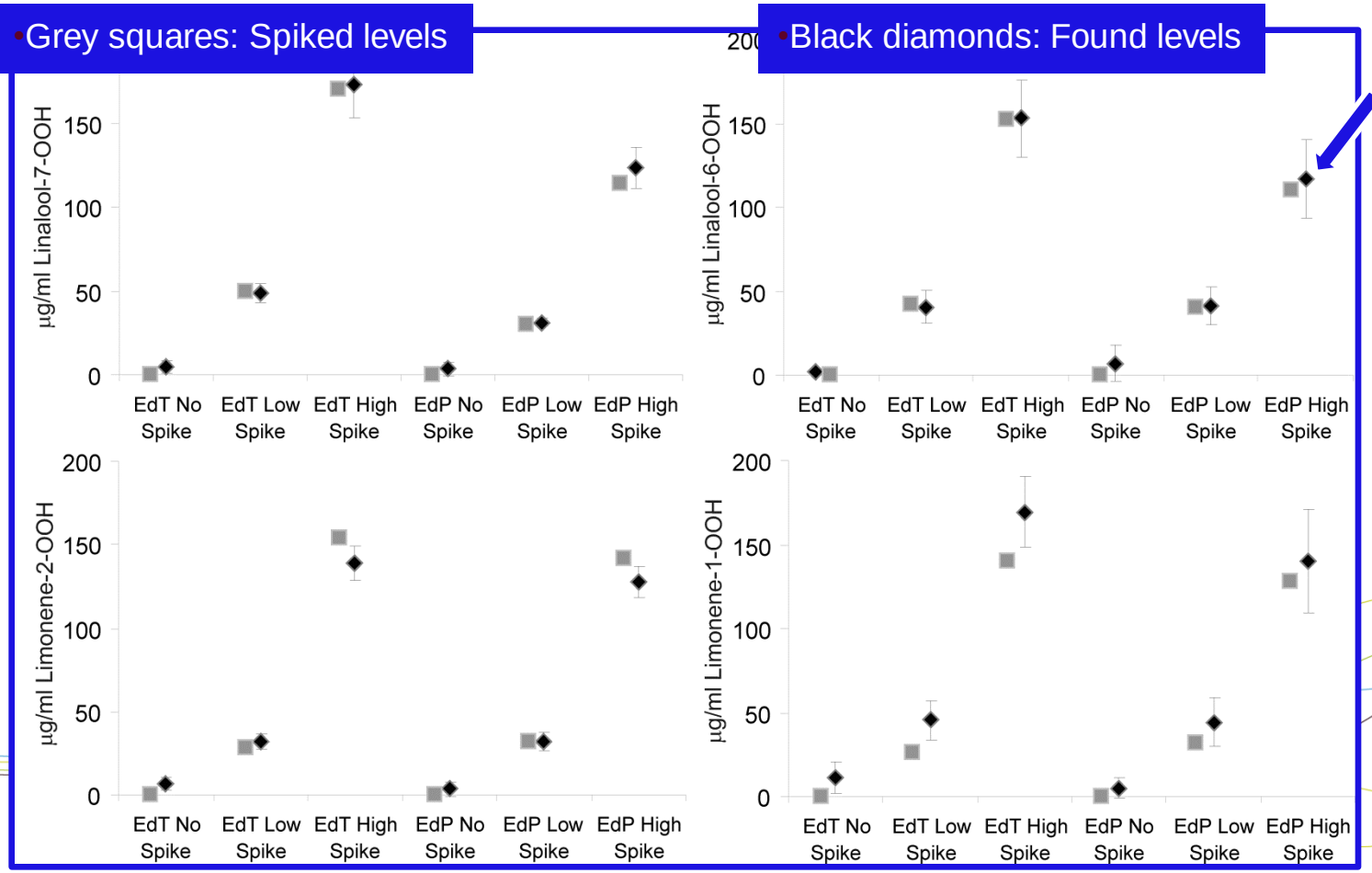
Ring study 3: Method validation in real products – fine fragrances (2016)

- Real market products, 2 samples with three spike levels of 4 different HP
- Blindly spiked with different levels
  - Lower analytical target levels taken
- Five labs compared same method (**GC-MS reduction method** to detect HP indirectly) **-> Method validation**
- Three labs tested additionally different methods (**LC-methods** to detect HP directly) **-> Method comparision**

Eau de toilette, not spiked	Eau de toilette, low level Spiked with different levels of Limonenen-1-OOH, Limonenen-2-OOH, Linalool-6-OOH, Linalool-7-OOH in the range of **20 – 50 ppm**	Eau de toilette, high level Spiked with different levels of Limonenen-1-OOH, Limonenen-2-OOH, Linalool-6-OOH, Linalool-7-OOH in the range of **100 – 200 ppm**
Eau de parfum, not spiked	Eau de parfum, low level Spiked with different levels of Limonenen-1-OOH, Limonenen-2-OOH, Linalool-6-OOH, Linalool-7-OOH in the range of **20 – 50 ppm**	Eau de parfum, high level Spiked with different levels of Limonenen-1-OOH, Limonenen-2-OOH, Linalool-6-OOH, Linalool-7-OOH in the range of **100 – 200 ppm**

Ring study 3: Method validation in real products – fine fragrances (2016)

- Accurate detection with GC-MS reduction by all five labs
- **This method allows accurate quantification in real products**



Ring study 3: Method validation in real products – fine fragrances (2016)

- Three different LC-methods
- Also allow good quantification without derivatisation in most samples

Detection of Linalool-OOH (sum of isomers) by different analytical methods (data in µg/ml)

	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

- **A Toolbox of methods is now available for analysis in fine fragrances**
- **What about more complex matrices such as creams and lotions?**

Method development work: analysis in Creams, lotions, complex matrices (2016)

- Two standard **creams and a standard deodorant**
- Each lab tried different methods
- Based on results promising method chosen
- Allows good recovery from different product matrices

Analysis is now also possible in complex consumer products

% recovery of 100 ppm spike	trans-Carveol ex Limonene-2-OOH	
	T=24 h	T=28 days
Woolwax Alcohol Creme	106.6	111.7
Deodorant Base	83.7	85.8
Bodylotion'	94.1	88.4
Anti ageing cream'	96.5	90.8
All natural deo	92.8	98.1
Lotion II	87.7	84.9
Average recovery	*93.6*	*93.3*

Ring Study 4 (Planned Q1 2017): Method validation in real products – Creams, lotions, and deodorants

- Last ring trial: Same setup as for fine fragrances
- Now with creams and deodorants
- 4-5 labs will again test reduction method
- 3 labs test different LC-methods
- Validation of the Method toolbox for more complex products
- Timeline: Sample preparation February 2017
- Data available End Q1 2017

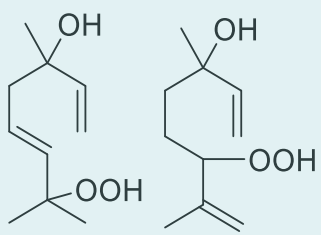
With this last step – toolbox of methods to extract HP and detect them with different methods ready for Roll-out

Application:
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?
  - Demonstrate relevance of patch test reactions by ROAT with the suspected product
- **How is such a study organized, and who will perform analysis?**
  - **Who:** ideally a CRO
    - Ideally CRO will already join final ring study to test their competency and validate the method with the lab applying it

Interpretation – how will we judge results? - input to QRA2

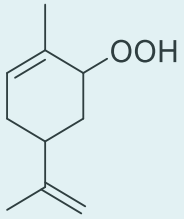
- We have good LLNA and guinea pig test data for HP (or oxidized fractions with known HP content)
- Based on these data we can derive NESIL values for individual HP
- Overall, potency in a similar range (EC3 = 0.3 – 1.6 %)
- With a grouping approach also potency (NESIL) of unknown HP can be predicted
- **Based on QRA2 we can then derive maximal levels in different product types which should not be surpassed**



1 Linalool-7-OOH

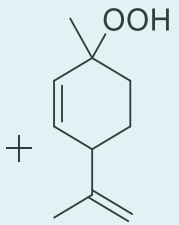
2 Linalool-6-OOH

EC3 of 5:3 mixture = 1.6%



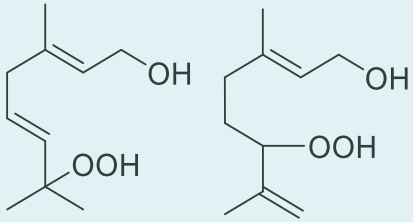
3 ***cis/trans-*** Limonene-2-OOH

EC3 = 0.83%



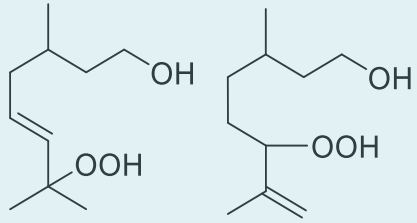
4 ***cis/trans-*** Limonene-1-OOH

EC3 = 0.33%



5 Geraniol-OOH

EC3 of mixture = 1.4%



6 Citronellol-OOH

EC3 of mixture = 2.3%

Interpretation - input to QRA2: Case study from market survey

- 'All natural' deodorant (made of natural products only) was analyzed
- Contains 28 ppm Linalool-6-OOH and 27 ppm Linalool-7-OOH: Total 56 ppm
- EC3 for Linalool 6/7-OOH Mixture: 1.6% = 400 µg /cm^2
- NESIL 400 µg /cm^2

	Linalool Hydroperoxides NESIL = 400 µg/cm^2		
Product Type	**Proposed SAF for QRA 2**	**Exposure (mg/cm2/day)**	**QRA2 product type upper use levels**
Deodorants and antiperspirants of all types including fragranced body sprays	300	9,1	**0.015% = 146 ppm**

- The **analytical result** is below QRA2 level, and indicates the product is fine according QRA2
- Also the **analytical level** is 10 fold-below lowest reported elicitation level.

Expected outcome

- The analytical toolbox will be applied to market samples
- Based on the results we will be able to calculate whether hydroperoxide levels are above QRA2 limits
- Results will indicate how frequent samples are, which contain hydroperoxides above QRA2 levels
- Further insight will be provided into the relevance of positive patch test reactions to oxidized materials.

- **Results should help to understand whether exposure to terpene hydroperoxides above QRA2 limits comes from IFRA regulated products**

IDEA

International Dialogue for the Evaluation of Allergens

Thank you for your attention