Update on the work program of the analytical task force, status, remaining activities, timelines and input to QRA2

13.12.2016, Andreas Natsch

IDEA Analytical HP task force: A multistage project

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Problem definition

- Hydroperoxides (HP) 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 added 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 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 HP)
  - Linalool: Up to now lowest elicitation level in humans: 560 ppm (based on one small published ROAT)

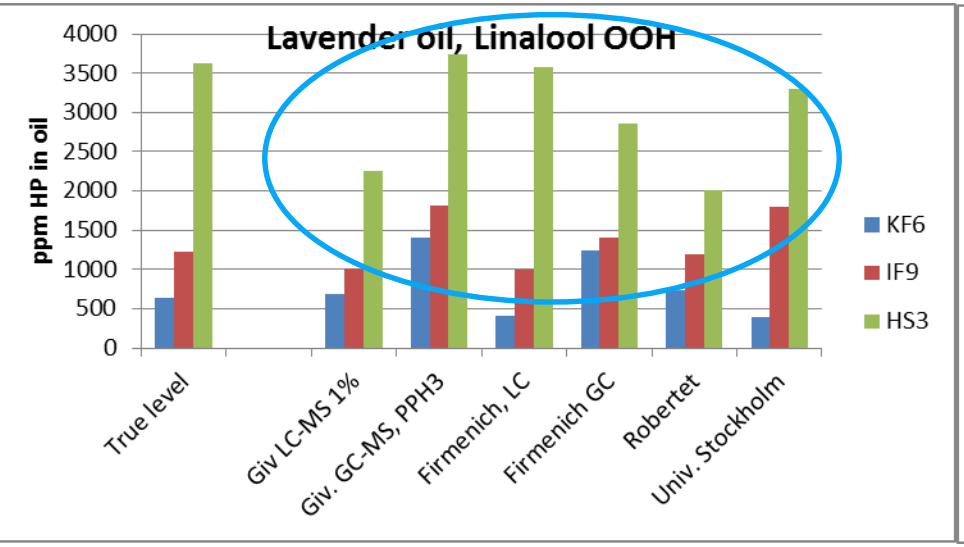
- **Revised analytical target** – based on improved analytical methods:
- **50 ppm in final consumer product (defined as 'reporting level')**
  - This is 100 fold below default induction level
  - 10-fold below reported elicitation level
  - Note: This lower level is set to have a full understanding and is based on analytical feasability: it does not mean that all levels above 50 ppm are of toxicological concern

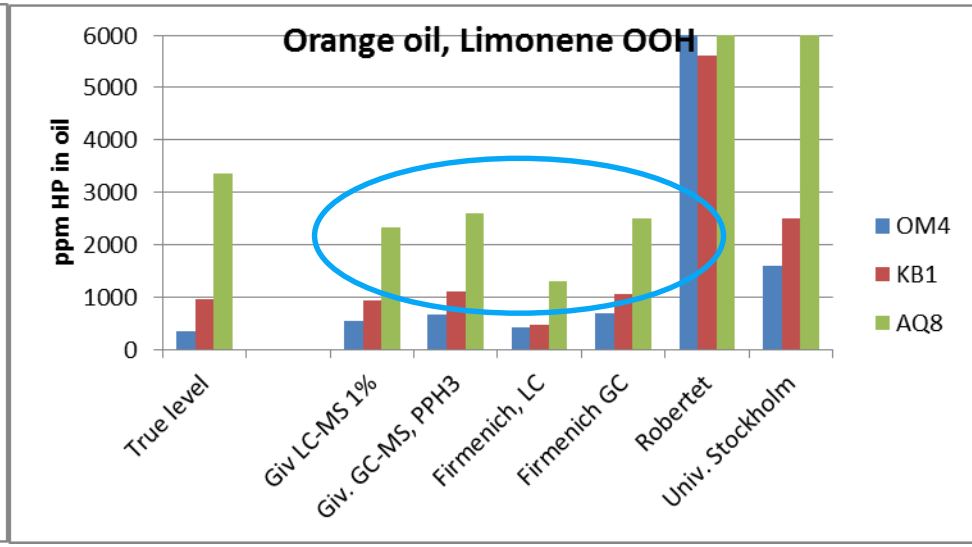
Study 1: Comparison of methods 1

- 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

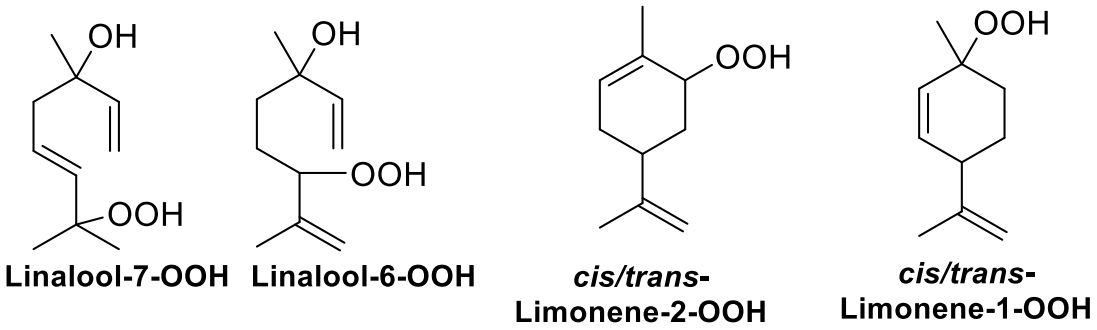
Tendency for underestimation in orange oil





An important step: Accurate analytical standards

- First study was run with analytical stdandards 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

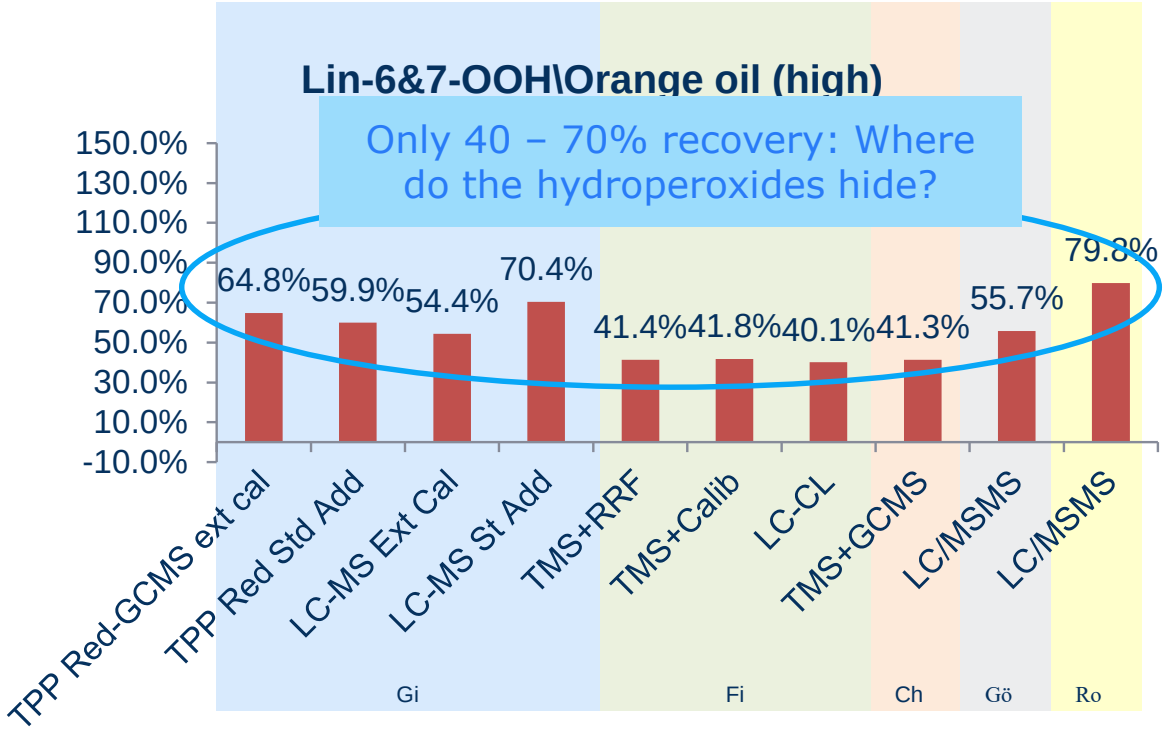


Linalool-7-OOH **Linalool-6-OOH** ***cis/trans*-Limonene-2-OOH** ***cis/trans*-Limonene-1-OOH**

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

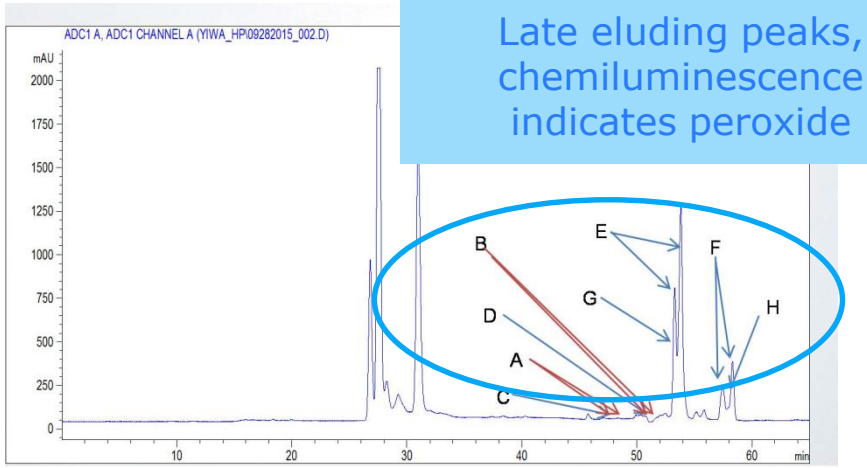
- Conclusion:
  - **General underestimation in orange oil and Lily fragrance with several methods**
  - Reduction / GC-MS method may be a robust method



An issue encountered: Loss of hydroperoxides in essential oils

- Only 40 – 70% recovery in some oils: **Where do the hydroperoxides hide?**

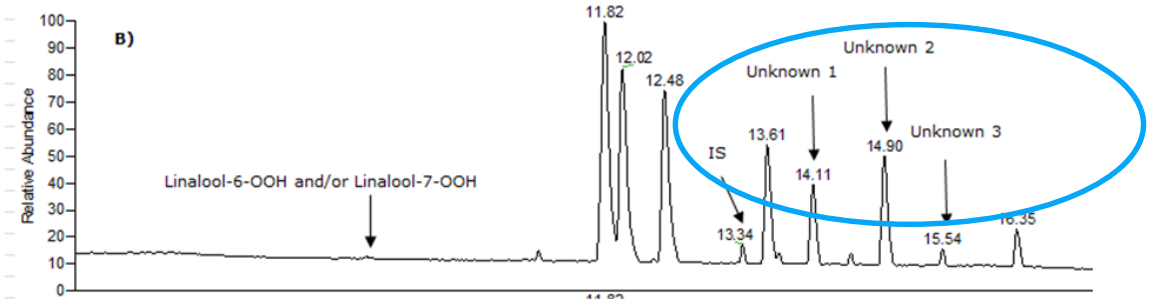
- HPLC-Chemiluminescence:



Late eluding peaks, chemiluminescence indicates peroxide

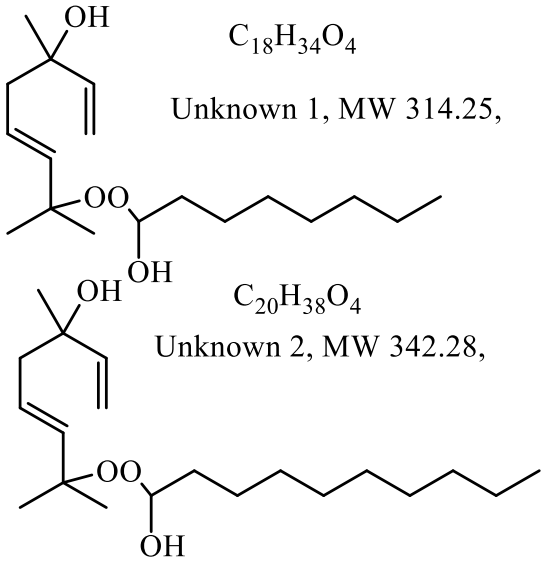
- LC-MS:

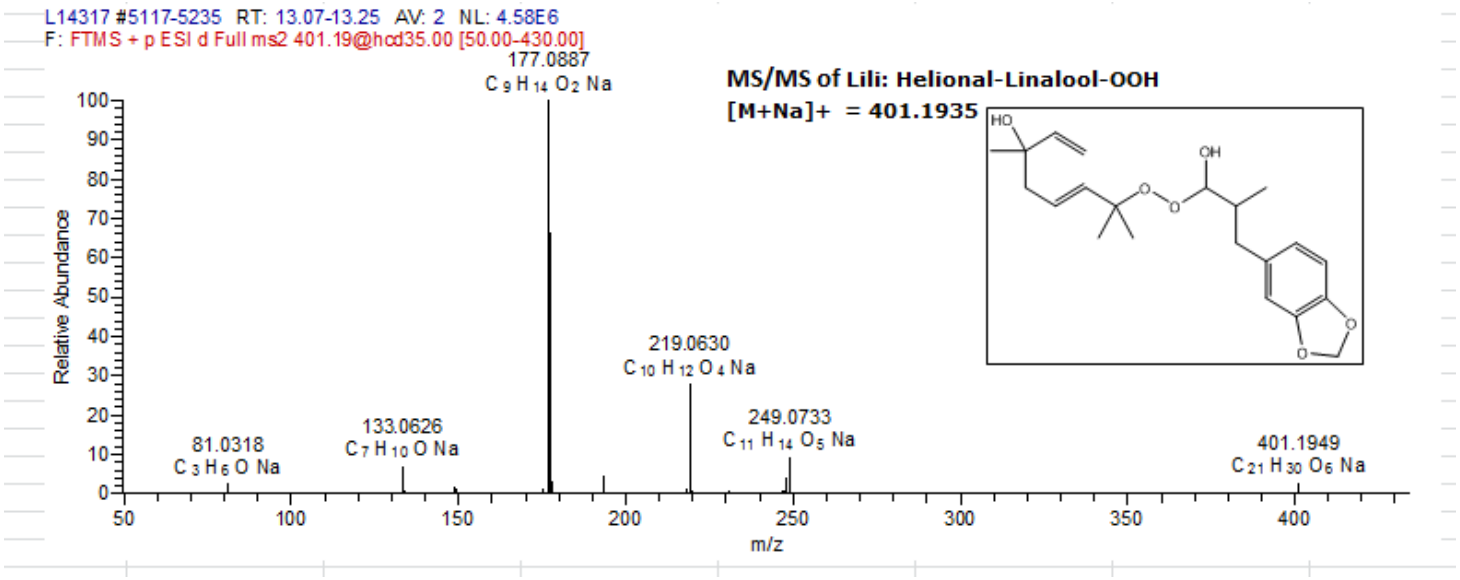
Late eluding peaks, MS indicates they contain Linalool-OOH substructure



Issue resolved: Formation of Peroxy-Hemiacetals

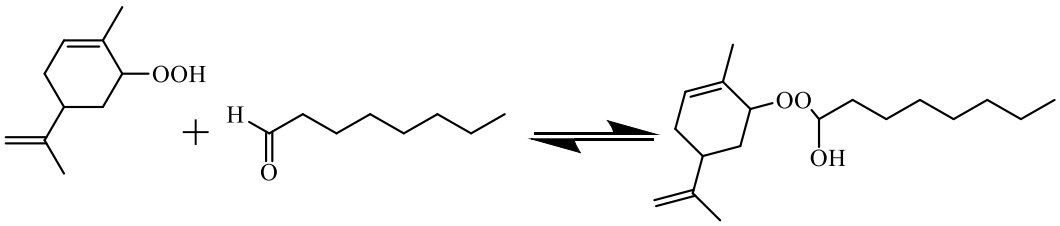
- Two laboratories could independently resolve the issue
- Hydroperoxides form hemiacetals with aldehydes in essential oils and fragrances
  - Adduct with decanal and octanal contained as trace impurities in orange oil
  - Adducts with synthetic aldehydes such as Helional in fragrance oils





Formation of Hemiacetals – a critical hurdle?

- This research is not finished – *the following conclusions are based on current understanding*
- For the time being it appears that:
- A) Hemiacetals formation is reversible



- B) Hemiacetals are mainly formed under aprotic conditions / in aprotic solvents: i.e. In neat raw materials and oils
  - May be an important interference in raw materials and ess. oils ⇒ **Quality control**
- C) Hemiacetals are present, but at low levels, in more complex mixtures with protic solvents (e.g. Fine fragrances)
  - Equilibrium is far on the left side
  - **May pose less problems in final products / when assessing consumer exposure**

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 detecte HP directly) **-> Method comparision**

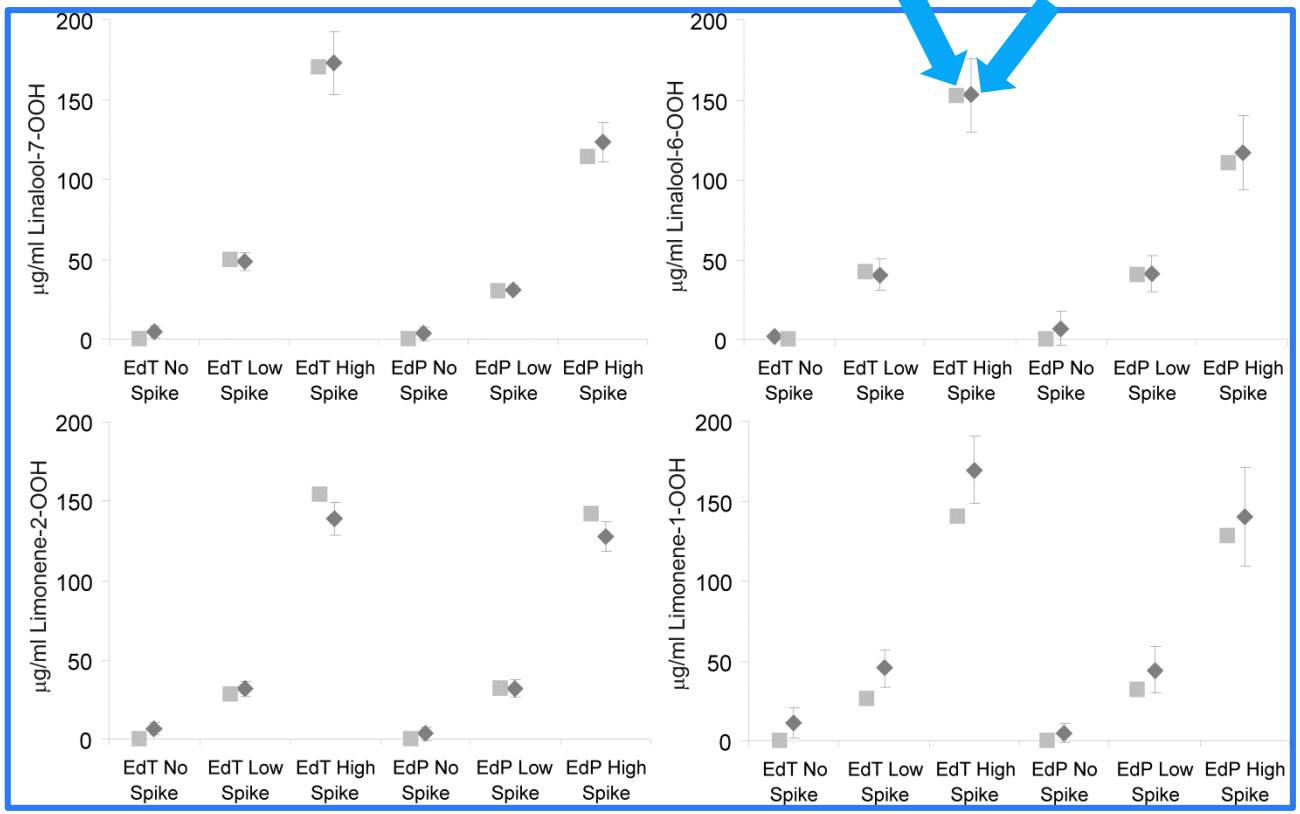
Eau de toilette, not spiked	Eau de toilette, low level Spiked with different levels of Limonenen-1-OOH, Limonenen-2-OOH, Linalool-6-OOH, Linalool-7-OOH in the range of **20 – 50 ppm**	Eau de toilette, high level Spiked with different levels of Limonenen-1-OOH, Limonenen-2-OOH, Linalool-6-OOH, Linalool-7-OOH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

Study 3: Method validation in real products – fine fragrances

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

Light grey squares: Spiked levels

Dark grey diamons: Found levels



Study 3: Method validation in real products – fine fragrances

- 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 body lotions
- 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 January 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: Quality control of raw materials

- Many raw materials (oils) contain substantial amount of peroxyhemiacetals
- Current IFRA method (POV, 1 min reaction time) does not fully detect these (slow reaction with potassium iodide)
  - POV value in aldehyde containing samples may be biased
- Adaptation of the POV method may be needed for accurate validation of raw materials
- Work is ongoing
- The new analytical methods and the analytical standards help to perform validation of this improved method

- **Deliverable: Updated POV method; may lead to adapted IFRA standard method (Q4 2017)**

Application: Market overview and patient's products

- Detection in final consumer products
  - Detection in general **market products**
  - Detection in **aged consumer samples**

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

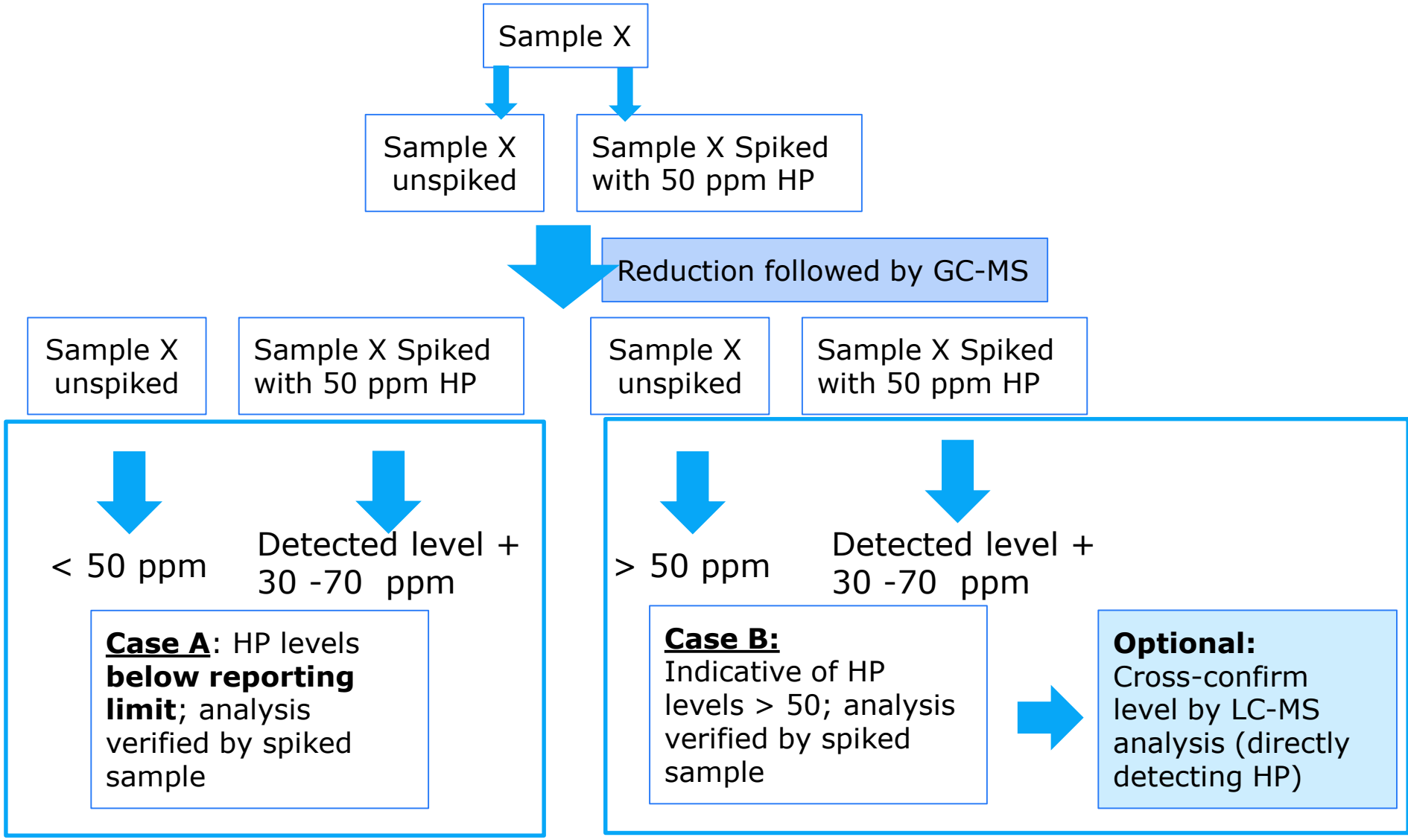
    ⇒Presence of potentially elicitating doses which may indicate relevance of reaction to actual disease?

- **How is such a study organized, and who will perform analysis?**
  - **Discussed 12.12.2016, analytical taskforce**
  - **Who:** ideally a CRO, e.g. Eurofins. Ideally CRO will already join final ring study to test their competency and validate the method with the lab applying it

Variables to be considered for study on for 'real products'

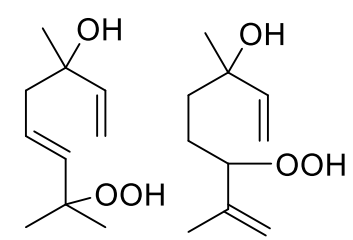
- Product batches, Nr. of replicates?
- Body regions where product is applied? (**link to cumulative exposure**)
  - This defines product types, e.g. **Body lotion, face or hand cream, deodorant**
- Number of brands in given product types?
- Geographic spread – number of countries?
  - Note: Differences in frequency of pos. patch test found between Sevilla and e.g. Gothenburg
  - E.g. Take Gothenbourg, ev. Leuven, Seville and London
    - Maybe select cities according to dermatological centers collaborating with patient samples
- Aged samples or freshly bought samples?
  - Aged samples? – Only take samples where we know product history.
  - Ask market research company to collect samples
  - Search fresh batches in shop to match samples of which we got an aged/used one –
    - **This answers question of effect of aging on HP formation in consumer products**

Proposal: How to test market samples, aged products and patient's samples



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

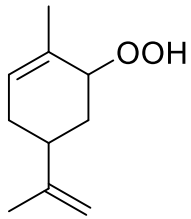
- We have good LLNA and guinea pig test data for hydroperoxides (or oxidized fractions with known hydroperoxide content)
- Based on these data we can derive NESIL values for individual HP
- Overall, potency in a similar range (EC 3 0.3 – 1.6 %)
- With a grouping / read-accross approach also potency / 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

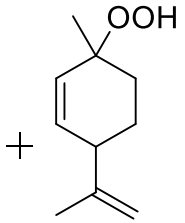
2 Linalool-6-OOH

EC3 of 5:3 mixture = 1.6%



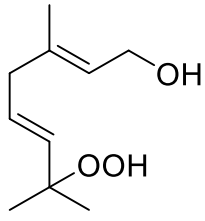
3 *cis/trans*-Limonene-2-OOH

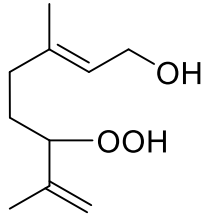
EC3 = 0.83%



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

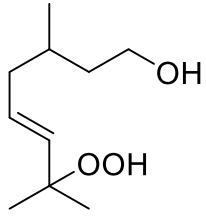
EC3 = 0.33%

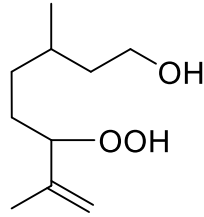




5 **Geraniol-OOH**

EC3 of mixture = 1.4%





6 **Citronellol-OOH**

EC3 of mixture = 2.3%

Interpretation - input to QRA2: Case study

- '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

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

Thank you

Contact