IDEA Annual Review

Risk Assessment of Pre- & Pro-Haptens

2014 Update

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Definitions of Pre- & Pro-haptens

- A **hapten** is a small low molecular weight molecule that can induce and elicit an immune response.

- **To be effective, a hapten needs to:**
  a. Gain access in sufficiently high concentrations to the target protein(s) responsible for triggering the induction of skin sensitization.
  b. React with these target protein(s) without also causing marked cellular damage.

The difference between pre- & pro-haptens

- **Pre- & pro- haptens** are hapten precursors and are often non-sensitizing themselves.

- A pre-hapten is a substance that needs to be chemically (**abiotically**) activated.

- A pro-hapten is a substrate for biotic activation (often involving the so-called drug-metabolising enzymes).

Key conclusions of 2013 WS

- There is clear qualitative indication that sensitizers can be formed in some formulations under realistic conditions as a result of abiotic hydrolysis of fragrance ingredients. The importance of biotic hydrolysis in the epidermis will require further investigation.

- Contact allergy (positive patch-tests) to oxidation products of some fragrance ingredients (like Linalool or Limonene) is common. There is presently insufficient data on exposure to these oxidation products to make a correlation to disease (allergic contact dermatitis).

Key conclusions of 2013 WS

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- On biotic and abiotic oxidation, the data available show the complexity with great challenges for predictability and analytical testing.

- The models do not sufficiently reflect exposure conditions or co-factors that interfere with sensitization.

- The development of new analytical methodologies such as HR MAS-NMR is a key requirement to clarify in situ phenomena.

Priorities identified during 2013 WS

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- Identify and characterize the actual consumer exposure from all sources to fragrance ingredients and closely related structures.
- Select and ensure the general availability of a suitable range of pure reference fragrance ingredients.
- Develop new analytical techniques for the detection and quantification of haptens derived from pre/pro-haptens (e.g. hydroperoxides) in fragranced consumer products and biological media (e.g. skin).
- From a clinical perspective, focus on pre- and pro-haptens that have been well characterized in laboratory studies.

High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance

HR-MAS NMR

HR-MAS NMR Project

Research project with University of Strasbourg (J-P Lepoittevin)

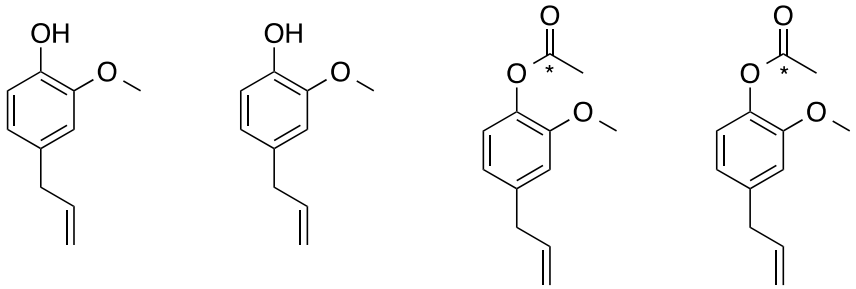
Pilot study to investigate if HR-MAS NMR (High Resolution-Magic Angle Spinning Nuclear Magnetic Resonance) spectroscopy in association with Reconstructed Human Epidermis (RHE) could be used to follow and characterize the metabolic transformation / activation of pro-haptens and their subsequent interactions with epidermal proteins (nucleophilic residues on amino-acid side chains)

HR-MAS NMR Project

- **Study materials**: Eugenyl acetate, Isoeugenyl acetate
- **Rationale**: Eugenol and Isoeugenol are used as pure materials but also in form of esters such as acetates. Clinical studies have shown that individuals sensitized to the parent material also reacted when patch tested with the ester – mechanism underlying this observation is still not clear, even so there is the hypothesis that the esters are hydrolized either enzymatically (epidermal esterases) or chemically (hydrolysis).
- **Aim**: initial phase to assess the potential of the HR-MAS NMR / RHE model to investigate and characterize the behavior of the study materials in a living tissue.

HR-MAS NMR Project

- Synthesis of ^{13}C-substituted eugenyl- and isoeugenyl-acetates



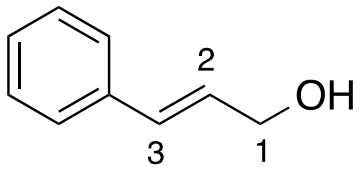
- Qualitative studies – RHE will be treated with the above mentioned materials and samples analyzed at 24h by HR-MAS NMR spectroscopy for identification of hydrolysis products. Experimental options for quantification of released acetate will be investigated.
- Time/response and dose response of the ^{13}C-labelled samples on RHE will be investigated to characterize the enzymatic / hydrolysis reaction.

HR-MAS NMR Project

- **Study material**: Cinnamyl alcohol
- **Rationale**: Cinnamyl alcohol is considered as the model of pro-hapten being activated by alcohol dehydrogenase to form cinnamaldehyde – however, about half of the patients sensitized to cinnamyl alcohol do not react when patch-tested with cinnamaldehyde – strongly suggesting that at least one alternative metabolic pathway is taking place in human epidermis activating cinnamyl alcohol into an unknown reactive hapten
- **Aim**: initial phase to assess the potential of the HR-MAS NMR / RHE model to investigate the metabolism of cinnamyl alcohol in living tissue

HR-MAS NMR Project

- Synthesis of ^{13}C-substituted cinnamyl alcohol



1-(^{13}C)
2-(^{13}C)
3-(^{13}C)

- Initial metabolic studies
  RHE will be treated with with the ^{13}C-substituted material and samples analysed at 24h by HR-MAS NMR to characterize the formation of cinnamic aldehyde in RHE and check if other metabolites are formed.

HR-MAS NMR Project

- Project started in July 2014 and mid term will be reached in December 2014.

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Develop suitable SAR tool(s) to identify likely pre- and pro-haptens

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- RIFM collaborates with Prof. Ovanes Mekenyan, Department of Physical Chemistry, Laboratory of Mathematical Chem., University of Bourgas, Bulgaria.
- A SAR for pre- and pro-haptens is part of a model called the TIMES SS (Tissue Metabolism Simulator Skin Sensitization), parts of which are incorporated in the OECD Toolbox.
- RIFM has a license for the TIMES SS model and agreed with Prof. Mekenyan to identify specific refinements to the SAR rules. It is not envisioned that a new tool needs to be added, but rather a review and potential refinement of the SARs that already exist in the model.
- Plan to be completed by the end of 2015.

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Scheduled: Study to better understand Pro-hapten Metabolism

Pro-hapten metabolism study

- Research Project with the Leibnitz Institute for Environmental-Medical Research, Düsseldorf, Germany.
- Most of the mechanisms proposed for the activation of pro-haptens in the skin are based on theoretical knowledge and have not been experimentally demonstrated.
- The fact that a chemical is a good substrate for a defined enzymatic system and is forming reactive intermediate(s) able to react with nucleophilic residues is not a demonstration that the same pathway is taking place in the skin.

Pro-hapten metabolism study

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- The study shall demonstrate a possible approach to assess the role of pro-hapten metabolism in the formation of a hapten in allergic reactions to small molecular weight compounds.
- A protocol has been provided beginning of December and is undergoing review.
- The project, which could become a model for studying further compounds, is planned to start in 2015.

Synthesis and quantification of hydroperoxides

IDEA Hydroperoxides TF

- First meeting March 24, 2014
- Team from the Academia and the Industry
  - Jean-Marie Aubry — University of Lille
  - Anna Börje — University of Gothenburg
  - Hugues Brevard — Robertet
  - Michael Calandra — Firmenich
  - Alain Chaintreau — Firmenich; chair (also of IFRA-AWG)
  - Elena Gimenez — University of Strasbourg
  - Ann-Therese Karlberg — University of Gothenburg
  - Clémentine Marteau — IFF
  - Andreas Natsch — Givaudan
  - Ulrika Nilsson — University of Stockholm
  - Neil Owen — Givaudan
  - Veronique Rataj — University of Lille
  - David W. Roberts — University of Liverpool
  - Matthias Vey — IDEA Management Team

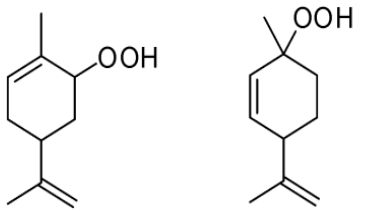
Objectives

- Ensure the general availability of a range of pure reference materials resulting from abiotic transformation.
- Because some products may be unstable, investigate for the half life as well as any other parameters related to their conservation.
- Use the reference materials to develop an analytical method for the reliable detection and quantification of chemically defined haptens resulting from abiotic transformation.
- Methods should be sensitive, specific, with target limits of quantification below the estimated induction levels and limits of detection below the estimated elicitation levels.
- This analytical method is critical for the subsequent execution of *in vitro* / biological assays and clinical investigations.

Targeted analytes

- Oxidation products from
  - Limonene, Linalool, Linalyl acetate and Citronellol
- Selection based on
  - Consumer exposure, oxidation potential
  - Existing knowledge and great difference of skin sensitization potency between parent compound and hapten
- Priority (strong skin sensitization potency)

  Linalool and Limonene hydroperoxides



***cis/trans*-Limonene-2-OOH**
EC3 = 0.83%

cis/trans-Limonene-1-OOH
EC3 = 0.33%

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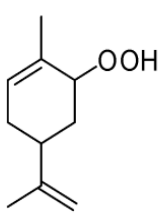

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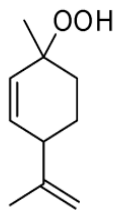

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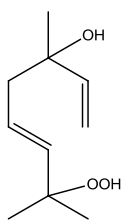
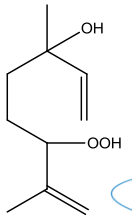


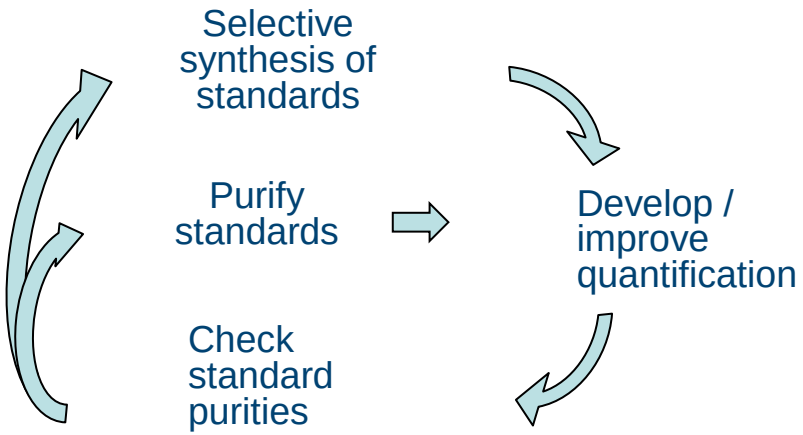
Linalool-7-OOH
EC3 of 5:3 mixture = 1.6%



Linalool-6-OOH

Synthesis

- Two potential manufacturers
  - Contract signed with Greenpharma S.A.S., Orléans (F)
- Preparing pure standards → an iterative process



- Feasibility study in progress
  - 4 isomers to be prepared separately
    - Limonene-1- and -2-OOH, and linalool-6- and -7-OOH
    - Expected purity: > 95%

Expected method performances

- Selectivity towards the hydroperoxides, or convenient means to locate hydroperoxides in a chromatogram.
- (Once the calibration of hydroperoxides has been achieved) use of recorded (relative) responses to avoid the further use of standards.
- Alternatively, if these two criteria cannot be met by a single method, several methods would be developed if each of them meets one of these criteria.

»

- Methods based on a spectrometric detection should comply with the state-of-the-art practices. Notably, the identity of quantified peaks should be checked to avoid analyte confusion and detect coelutions.

Level of detection needed

Target set: "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:
  - Linalool-OOH 16 000 ppm in LLNA [1]
  - Limonene-2-OOH 8 300 ppm in LLNA / 15 000 ppm in guinea pigs [2,3]
  - 5000 ppm maybe taken as a limit of quantification below the induction level

Note: Above levels are final levels in a preparation. If a fragrance oil or raw material is considered, dilution factor in product must be taken into account

[1] M. Sköld, A. Börje, E. Harambasic, A. T. Karlberg,*Chemical Research in Toxicology***2004,** *17*, 1697-1705.
[2] S. Johansson, E. Gimenez-Arnau, M. Grotli, A. T. Karlberg, A. Borje,*Chem Res Toxicol***2008,** *21*, 1536-47.
[3] A. T. Karlberg, L. P. Shao, U. Nilsson, E. Gafvert, J. L. Nilsson,*Arch Dermatol Res***1994,** *286*, 97-103.

Level of detection needed

Target set: "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 elicitation level:
  - Linalool hydroperoxide: Lowest elicitation level in humans = 560 ppm * [4]
  - No data on limonene hydroperoxide in humans, Lowest elicitation level in guinea pigs = 3000 – 10 000 ppm [3]

* Patients in elicitation study were exposed simultaneously over three weeks twice daily to a 5640 ppm dose on same arm, LOEL for elicitation may thus be clearly higher under realistic application of single low dose.

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[3] A. T. Karlberg, L. P. Shao, U. Nilsson, E. Gafvert, J. L. Nilsson, *Arch Dermatol Res* **1994,** *286*, 97-103.
[4] Y. Andersch Bjorkman, L. Hagvall, C. Siwmark, B. Niklasson, A.T. Karlberg, J. Brared Christensson, *Contact Dermatitis* **2014,** *70*, 129-38.

Quantification method comparison

- Objective: testing the performances of published and unpublished methods
- Five participants = five different methods
  - LC-TOFMS using a calibration curve
  - LC-MSMS using a standard addition
  - LC-Chemiluminescence
  - GC-MS (after reduction in the case of limonene-OOH)
  - GC-derivatization
- Identical samples were sent to all participants
  - Orange oil: 1 blank + 2 samples spiked Limonene-OOH
  - Lavender oil: 1 blank + 2 samples spiked Linalool-OOH
  - Coded samples (blind test)
- Results shared with the hydroperoxide-TF but not yet discussed
  - Next meeting: end of January 2015

Preliminary learnings

- A combination of methods could be required to quantify:
  - At all concentrations (induction and NOEL levels)
  - Different hydroperoxides
- Methods not yet satisfactory but one step has been achieved
  - Quantification feasible at known induction levels
  - With some methods also at lowest observed elicitation level
  - Determination of standard purity
  - Pure standards required to refine the methods at low levels

→ More work is required ←

Future steps

- Once fully developed, the quantification methods of pre- & pro-haptens will be used to monitor the market:
  - First to gather useful information on actual consumer exposure.
  - Provide methodology to ensure the level of haptens (e.g. hydroperoxides) in marketed products is acceptable.

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Thank you for your attention