IDEA
International Dialogue for the Evaluation of Allergens

Rapporteur's Progress Report on the IDEA Workshop on

Risk Assessment of Pre-& Pro-Haptens

May 28-29th 2013

Dolce La Hulpe Brussels
Chaussée de Bruxelles, 135
B-1310 La Hulpe, Belgium

1. Background information regarding the International Dialogue for the Evaluation of Allergens (IDEA):

Fragrance Allergy is a topic of high interest for the fragrance industry, its customers and the Authorities as expressed through the 2012 SCCS Opinion on Fragrance Allergens. The fragrance industry is determined to address this issue and provide solutions supported by a broad, multi-stakeholder approach.

To fulfil this objective, a work plan (att.01) was developed in the course of 2012 and submitted to DG Sanco Risk Assessment Unit for scrutiny. All comments and suggestions were taken into consideration and the final document, having received the Commission's support, is a clear roadmap intended to deliver positive outcomes for the consumers, the Authorities and the industry. This work plan has now moved into its execution phase and the International Dialogue for the Evaluation of Allergens (IDEA) represents its transposition into concrete actions and investments. Through the organization of experts' workshops and the planning of scientific studies, IDEA aims at providing an agreed and transparent framework for assessing fragrance sensitizers in a prospective way and, ultimately, to find optimal solutions to the issue of fragrance induced skin allergies.

The objective of this workshop was to review the current understanding of biological and chemical mechanisms involved in pre- and pro-haptens activity, and consider the potential implications for risk assessment. The main focus was on the processes of oxidation and hydrolysis. The workshop was aimed to enable the experts to bring all available inputs together to jointly identify, if needed, priorities for further research and agree on their design.

IFRA Operations
Avenue des Arts, 6
1210 Brussels, Belgium
Tel: +32-2 214 20 60
Fax: +32-2 214 20 69

www.ifraorg.org

2. Summary agreed at the workshop

The moderator identified a number of key conclusions on the work to date and identified a range of specific action steps: These were accepted at the workshop by the participants.

- 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 is common. There is presently insufficient data on exposure to these oxidation products to make a correlation to disease (allergic contact dermatitis).

- On biotic and abiotic oxidation, the data 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.

    - There is a need for more rigorous protocols (including ROAT) for clinical studies.

    - Different concepts of relevance (individual, group-related and epidemiologic data) need to be refined.

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

- The workshop produced a range of recommendations to identify and characterize pre- & pro-haptens, ranging from chemical characterization to confirmation through clinical studies.

- Future work should be conducted in transparency and with participation from stakeholders with relevant expertise.

3. Rapporteur's progress report

The workshop covered the whole area of pre- and pro-haptens from theoretical chemistry considerations to clinical practice observations. There was very active participation of workshop participants. This summary does not address all the technical details presented but rather focusses on the main conclusions and areas for further work.

A. Current scientific understanding of Pre- & Pro-Haptens

Pre- and pro-haptens are hapten precursors. To be an effective hapten a chemical needs to be:

- Able to gain access in sufficient concentrations to the target protein(s) in the skin that are responsible for the initiation of sensitization. A balance between hydrophilicity and lipophilicity has been identified as an important property for a hapten or pre- or pro-hapten to penetrate skin epidermal cells,
- Sufficiently reactive with the critical target sites of the protein(s),

However, to be an effective hapten, a chemical does not need to be markedly cytotoxic (or cause other substantial cell damage). If cytotoxic, then this chemical must only be so at higher doses than needed for sensitization to occur.

In order to act as a hapten some chemicals need to undergo conversion to more chemically reactive forms.
A pre-hapten is a chemical that needs to be chemically (abiotically) activated. Such activation can in principle arise:

i) During processing or storage of a raw material/formulation
ii) On the surface of the skin as a result of application
iii) Following skin penetration

Only in situation i) is there generally certainty about the mode of activation.

A pro-hapten is a chemical that is a substrate for the so called drug metabolising enzymes (biotic activation). To access these enzymes a chemical must penetrate the surface of the skin. In practice it may be difficult to identify whether a chemical is a pro-hapten.
On current evidence the most common pathways for hapten formation from precursors are oxidation and hydrolysis. It would appear that if a chemical is a pro-hapten it has the potential to be a pre-hapten too. However the converse does not necessarily apply. Thus pre-haptens are likely to be more common than pro-haptens. The number of fragrance ingredients in general use that can act as pre- or pro-haptens is unknown, there might be many hundreds. The LLNA data bases indicate that of those allergens that have been identified approximately two thirds direct acting haptens. It needs to be recognised that the formation of the hapten and its availability to the receptor protein will need to exceed a critical concentration (threshold amount) for sensitization to be initiated. It was pointed out that fragrance manufacturers are likely to avoid using fragrance materials that are unstable in the final cosmetic product.

A.1. Oxidation reactions that may lead to hapten formation

Activation of a pre- or pro-hapten can arise due to oxidation of:

- a double bond to form an epoxide
- a catechol or hydroquinone to form a quinone
- an allylic or benzylic C-H group to form a hydroperoxide
- an alcohol group to a carbonyl group.

In addition, reactions such as oxidative dehalogenation and photo-oxidation can occur.

A.2. Hydrolysis reactions that may lead to hapten formation

Some pre- and pro-haptens require conversion to form an alcohol or aldehyde in order to be activated. This can occur due to hydrolysis of:

- Schiff bases
- Esters (of particular importance are likely to be formates and acetates)
- Acetals

For some chemicals a two-step process involving both hydrolysis and oxidation may need to occur for the formation of an effective hapten (e.g. hydrolysis to an alcohol followed by oxidation to an aldehyde).

Data presented at the workshop provided good qualitative evidence that sensitizers can be formed in some formulations of fragrances (fragrance compounds or finished products) under realistic conditions..

Pre-haptens. It was evident that for a variety of reasons in investigations of fragrances the focus has been on pre-haptens. Studies on specific model and real fragrance products show that hydroperoxides are indeed formed although at concentrations that are lower than those generated under experimental conditions. Clinical studies on hydroperoxide-sensitive subjects using repeated open application tests showed that their elicitation thresholds may be quite low but may still be higher than levels of hydroperoxides measured in model and aged fragrance cosmetics.

Pro-haptens With regard to metabolic activation, it was pointed out that in the wider area of modes of toxicity of chemicals (metabolism of chemicals to reactive species that initiate toxicity), there is a very rich literature. This literature is concerned primarily with toxicity arising in organs other than the skin, in particular the liver, kidneys and lungs and with genotoxicity and cytotoxicity endpoints. Nonetheless many of the enzymes responsible for the formation of reactive species are also present in the skin. It may also be relevant that metabolic activation of a number of chemicals involves reactions other than oxidation and hydrolysis e.g. reduction and conjugation.

A.3. Factors affecting hapten formation

i) Oxidation reactions

The following factors were identified as having a potentially important influence on hapten formation from pre-haptens:

- Oxygen availability: This may be reduced if there are other components in the medium/formulation that compete for the oxygen.
- Temperature: As in all chemical reactions, the temperature plays an important role in hapten formation according to the Arrhenius equation.
- Alternative pathways for the chemical that do not result in hapten formation. It was noted that individual pre- and pro-haptens may be converted to a number of metabolites/products. This may result in the formation of haptens with differing potencies and/or the inactivation of the haptens formed and/or products that are not haptens.

Rate limiting factors for pro-hapten oxidation to haptens are the same as for pre-haptens but in addition include:

- Availability of an appropriate enzyme in the skin (e.g. a suitable isoform of Cytochrome P450).
- Ability of the pro-hapten to reach the enzyme. Availability of any relevant cofactors e.g. NADPH.

ii) Hydrolysis / solvolysis reactions

For hydrolysis of pre- and pro-haptens the requirements are basically the same as for oxidative reactions with the exception that:

- Hydrolysis can occur under both aerobic and anaerobic conditions.
- Abiotic hydrolysis and solvolysis are more dependent on the pH of the medium.

For the above reasons the sensitizing effects of pre- and pro- haptens that form haptens in the skin are likely to be less reproducible than the effects of substances that are direct sensitizing agents.

A.4. Understanding of the causes of human variability in the effects of pre- and pro- haptens

The principal reasons for inter-individual differences in the initiation of sensitization are not well characterised. In regard to pro-haptens it is probably relevant from studies in organs other than the skin, that major inter-individual differences due to genetic factors occur and that these may result in individual differences in toxicity. It is likely that genetic differences in skin drug metabolising enzymes also occur. For pre-haptens it is not clear whether greater inter-individual variation occurs than for haptens and if so what the critical factors are.

A.5. Known fragrance pre and/or pro-haptens

Particular attention was given at the workshop to the terpenes alpha-terpene, limonene, linalool and Geraniol. Other examples of initiation that were considered in some depth were cinnamic alcohol, cinnamic aldehyde, isoeugenol and eugenol.

A.6. Prediction of pre-and pro-haptens properties

The development of a structure activity (SAR) model for predicting pre- and pro- haptens would be valuable. The prediction of which fragrance ingredients will act as pre- and/or pro-haptens is limited at present for a number of reasons. Particularly:

- The restricted range of chemical classes that have been investigated in depth.
- The complexity of factors that influence the rate of formation and lifetime of the resulting haptens (see above and figure 1).
- Lack of knowledge of the critical sites on the proteins whose modification results in initiation of sensitization.

Key considerations in developing reliable prediction models that were discussed include:

- Determination of bond energy at vulnerable sites in a chemical.
- Determination of parameters influencing the kinetic rate of reactions (pH, oxygen availability, temperature).
- Improvement in the understanding of the ability of human skin to generate soft electrophiles/nucleophiles/free radicals.
- Assessment of lipophilicity and hydrophilicity of the pre- and pro-haptens and the resultant haptens.

- Understanding of the stability of the resultant haptens in the skin (the skin is a potential reservoir for both the absorbed pre- and pro- haptens and for the resultant haptens).

A.7. Prevention of the impacts of pre-and pro-haptens

The use of antioxidants to limit oxidative pre-hapten conversion to haptens was discussed. The conclusion that emerged was that prediction was not straightforward. In some circumstances the addition of antioxidants appeared to be beneficial but in others not.

Key factors in the generation of haptens are summarised in the following figure.

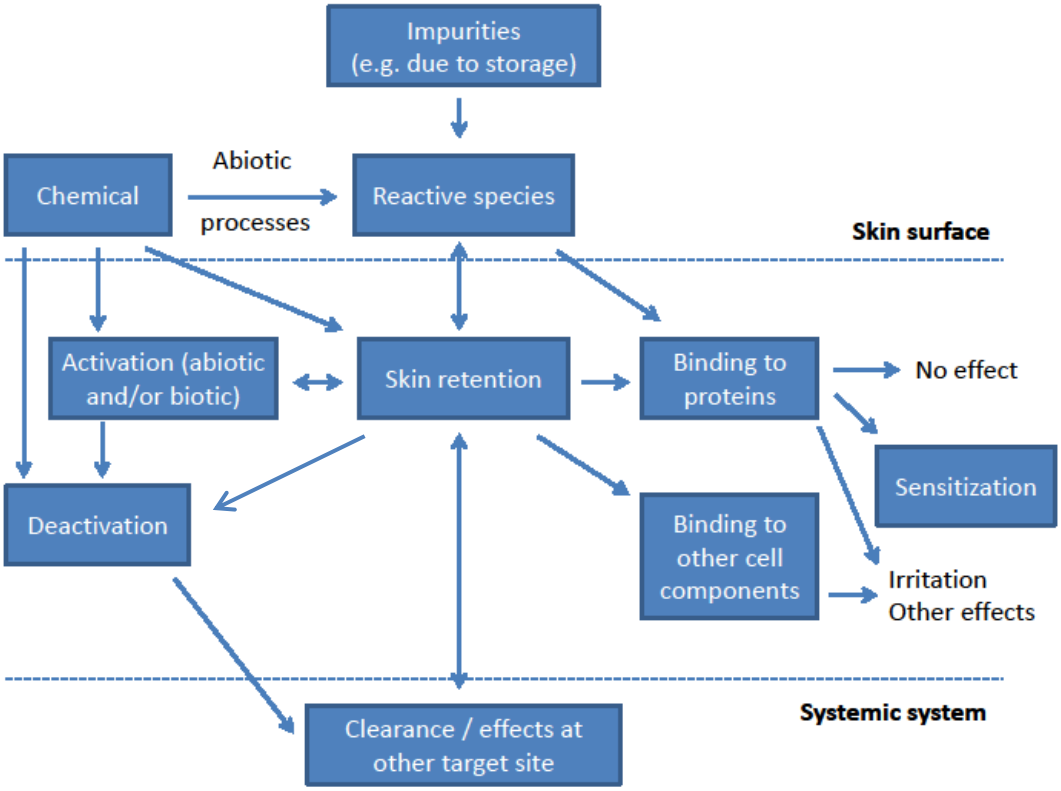

Figure 1: Distribution, transformation and effects of pre- and pro-haptens

The above approach should be integrated with the development of the Dermal Sensitization QRA (topic of the first IDEA workshop).

B. Methodological considerations

Progress in the identification, characterization and understanding of the modes of action of pre- and pro-haptens depends on the general availability of suitable methodologies.

Several stages can be identified in the process of establishing whether a fragrance is a pre- or pro-hapten (see also figure 2):

- Characterisation of physicochemical properties (informed by SAR / bond energy considerations)
- Obtaining evidence that it can form reactive products (under relevant abiotic / biotic conditions)

- Found to be a skin sensitizer in animal test(s)
- Found to cause a positive skin reaction in patch test(s) of allergic patients
- Estimates / measurements indicate that sufficient concentrations of the hapten can be generated to exceed the threshold for initiation under real world exposure conditions.

Suitable methodologies are required at each stage. A general requirement is also a reliable supply of well characterized high purity reference compounds (e.g. for analytical purposes).

B.1 Laboratory methods

Methods to measure / estimate hapten formation:

The chemically reactive nature of the products generated and consequently their instability makes this a crucial area. Several methods were mentioned (but not discussed in any detail) such as:

- Oxidation studies using a PetroOxy apparatus.
- Chemical methods for separating and characterizing the haptens generated e.g. use of chromatography, colorimetry, specific trapping agents, mass spectrometry.
- Magic angle spinning NMR: this technique appears to be of particular value in detecting the interactions of the generated hapten with cellular proteins.
- Chemical and biological models for studying hapten formation and their interactions.

Models for biotic transformation.

For studies on biotic and abiotic transformation by the skin, animal or human skin biopsy samples have been used by a number of researchers. Often homogenates or cell fractions have been used. These can provide useful information on conversion pathways and relative rates of formation of reactive products for different pre- and pro- haptens.

Lack of availability and relative poor reproducibility of such preparations in the past has resulted in the development of some skin culture models (e.g. the reconstructed human skin epidermis model). This preparation has good reproducibility but its relationship to normal human skin requires further examination. It is noted that the stratum corneum is not well developed and the source of keratinocytes is uncertain. More comparisons are also needed with skin biopsy samples on the drug metabolising capability.

Models for sensitization:

The favoured method is the local lymph node assay (LLNA) although non-animal testing alternatives are currently being investigated. The correlation between LLNA and patch test results is good (around 85% consistencies) but further studies are needed to determine its reliability in identifying different pro- and pre-haptens.

B.2 Clinical methods

B.2.1 Exposure assessment methods

- Information access:

  A major barrier to progress is the problem of estimating total real life exposure of consumers to individual fragrances and to other closely structurally related chemicals. A further issue is that there is often inadequate knowledge of the cosmetic matrix formulation(s) of consumer products. This is relevant because components in a formulation can have a substantive influence on the ability of pre- and pro- haptens to be converted to a hapten and/or on the half-life of the hapten.

- Measurements and modelling:

  Methods are available to determine the abiotic or biotic transformation of fragrances on the skin albeit it is particularly challenging to detect and quantify the generation of reactive products. As yet there is no viable methodology to determine hapten formation in the skin of patients.

B.2.2 Methods to identify and characterize pre- and pro-hapten sensitizers

Two types of study were identified for patch testing:

- Studies in individual patients
- Studies in randomized groups of volunteers

Some studies were described in outline that involved solvent extraction to remove a fragrance and its transformation products from the skin and measure them.

There was also some important discussion on the interpretation of patch testing results (a particular concern being consistency in categorization of fragrances as mild irritants and doubtful sensitizers).

Furthermore, the hypothesis was mentioned that low levels of weak sensitizers while not producing strong reactions may nonetheless lead to a subclinical state that can only be triggered via very high exposure doses. It was therefore recommended to reconcile clinical and exposure data under this hypothesis (this will be an important topic of the workshop on identification of sensitizers).

A framework was developed at the workshop for the identification of important pre- and prohaptens. It is a staged approach to facilitate prioritisation of fragrances for in depth study. To enable simplicity the various feed-back loops that might be important for individual investigations have been omitted.

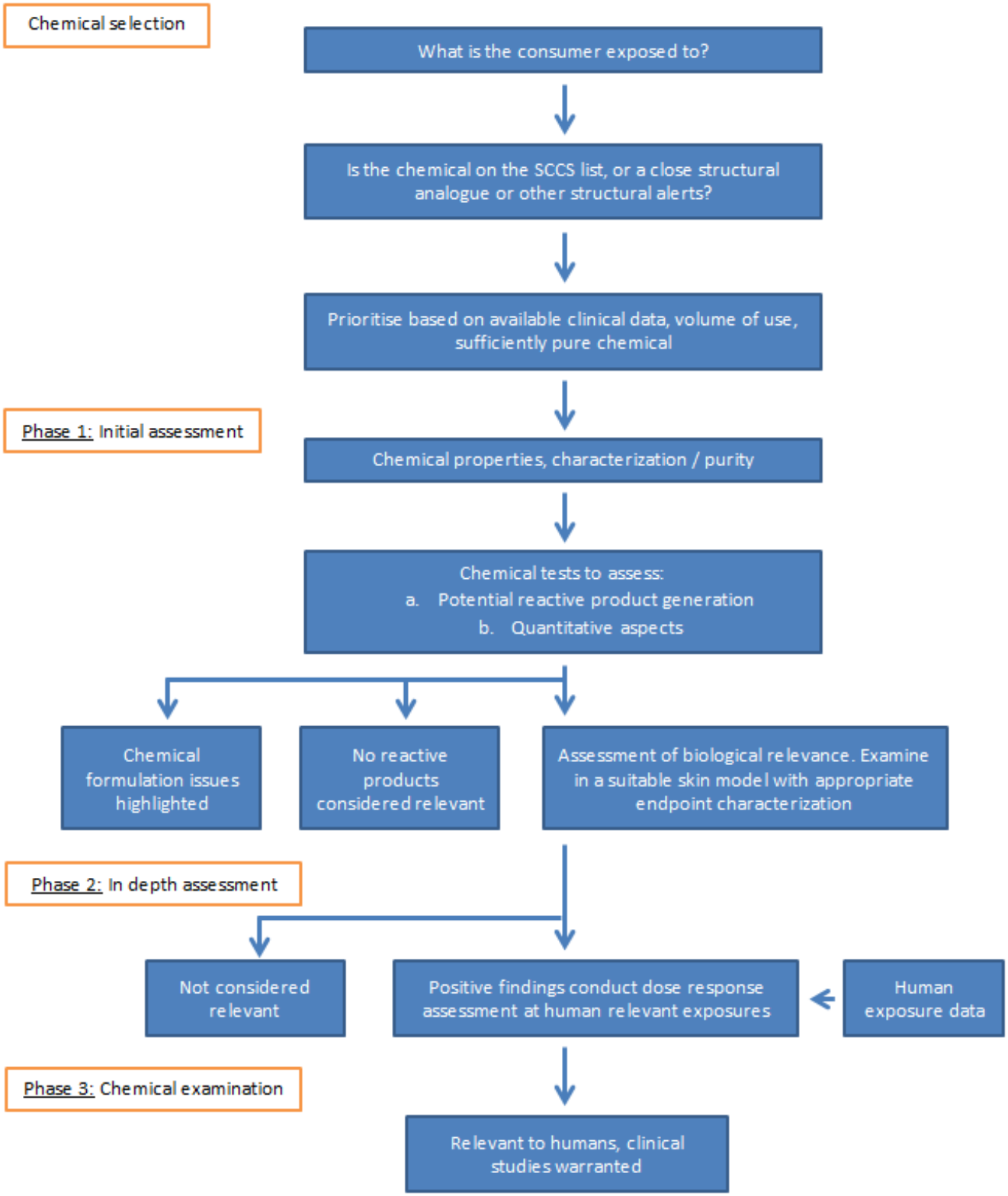

Figure 2: A staged approach for pre- and pro- hapten identification and characterization

C. General conclusions

- There are many fragrances with the potential to act as pre- and/or pro- haptens. Very few have been studied; the majority of these are terpenoids.
- There is a serious lack of reliable information on real life exposures to fragrances and closely related chemicals.
- Many of the tools required to investigate the significance of individual pre and pro haptens in the initiation of allergic contact dermatitis are already available. In addition there are some promising new developments.
- Even for the well-studied pre- and pro- haptens, the linkage between initial exposure, initiation and subsequent elicitation of allergic contact dermatitis is not fully established.

D. Priorities for further work

1. Identify and characterize the actual consumer exposure from all sources to fragrances and closely related structures.
2. Select and ensure the general availability of a suitable range of pure reference fragrance ingredients.
3. Develop new analytical techniques for the detection and quantification of haptens (e.g. hydroperoxides) in fragranced products and biological media (e.g. skin).
4. Develop a suitable SAR tool(s) to identify likely pre- and pro-haptens.
5. Characterize and develop skin models, particularly human skin models for studying hapten formation, protein interactions and subsequent fate.
6. Include pre- and pro-haptens in the development of Dermal Sensitization QRA (to be linked to the action items of the first IDEA workshop).
7. Examine how pre-hapten conversion to haptens can be minimized by improved formulation and storage.
8. From a clinical perspective, focus on pre- and pro-haptens that have been well characterized in laboratory studies.
9. Develop strategies for bridging the knowledge gap between induction and elicitation.

The participants agreed that in order to accomplish these important objectives, improved transparency and close collaboration between all active stakeholders in this field is a key to successfully addressing the issue. The subsequent follow-up from this workshop will provide the framework for achieving the expected outcomes. Further discussions are needed to identify and implement an effective and sustainable forum

APPENDIX 1: Programme

Tuesday, May 28th:

- First session – Abiotic and biotic transformations

  9:10 – 10:00 Toxicokinetic insights into the formation and the inactivation of haptens.
  Speaker: Prof. David Roberts

  10:00 – 10:50 State of knowledge on abiotic hapten formation (autoxidation, hydrolysis, …) using examples of fragrance ingredients and state of the art on the technical management of those transformations.
  Speakers: Dr. Andreas Natsch and Dr. Chris Powell

  11:10 – 12:00 State of knowledge on biotic transformations linked to fragrance ingredients.
  Speaker: Prof. Glenn Sipes

  12:00 – 12:50 First conclusions on the topic of abiotic and biotic transformations.

- Second session – Clinical considerations

  1:30 – 2:20 Clinical studies, dermatologic observations and toxicological considerations on haptens occurring from abiotic transformation of fragrance ingredients.
  Speaker: Prof. Ann Therese Karlberg

  2:20 – 2:40 Recent clinical studies on oxidized fragrance terpenes.
  Speaker: Dr. Johanna Bråred Christensson (discussion at 3:50 p.m.)

  2:40 - 3:30 Clinical studies, dermatologic observations and toxicological considerations on haptens occurring from the biotic transformation of fragrance allergens.
  Speaker: Prof. Jean-Pierre Lepoittevin

  3:50 - 5.30 Discussion and prioritisation of areas requiring additional research for a better comprehension of biotic and abiotic transformation as basis for adequate risk assessment.

Wednesday, May 29th:

9:00 - 9:30 a.m. Adoption of the agenda and wrap-up of Day 1

9:30 - 12:30 The participants will be subdivided into three working groups:

- Working group on abiotic transformations – Discussion on current tools and need of identification, prediction, control and areas for additional research.
- Working group on biotic transformation – Discussion on current tools and need of identification, prediction, control and areas for additional research.

- Working group on clinical studies – Discussion on current tools of identification of biotic and abiotic transformations (autoxidation, hydrolysis, etc.) of fragrance ingredients, clinical relevance of findings and areas for additional research.

1:10 - 3:00 Presentation of the conclusions and recommendations of the three working groups and establishment of priorities.

3:20 - 4:30 Conclusions of the workshop and next steps

APPENDIX 2: List of participants (30)

Prof.	Donald	Belsito	Columbia University Medical Center, USA and RIFM Expert Panel Member
Dr.	Hans-J.	Bender	Consultant *(Moderator of the Workshop)*
Prof.	Brunhilde	Blömeke	University of Trier, Germany
Dr.	Johanna	Brared-Christenson	University of Gothenburg, Sweden
Dr.	Halyna	Breslawec	PCPC, USA
Prof.	James	Bridges	University of Surrey, UK *(Rapporteur of the Workshop)*
Prof.	Magnus	Bruze	Lunds Universiteit, Sweden and RIFM Expert Panel Member
Dr.	Peter	Cadby	Chanel, France
Dr.	Michael	Calandra	Firmenich, USA
Dr.	Alain	Chaintreau	Firmenich, Switzerland
Dr.	Dominique	Favier	IFF, France
Prof.	Ellen	Fritsche	University of Düsseldorf, Germany (Day 1 only)
Dr.	Federica	de Gaetano	EU Commission - DG SANCO – Unit B2
Prof.	David	Gawkrodger	Royal Hallamshire Hospital, UK and Vice-chairman of the SCCS
Dr.	Carsten	Goebel	Procter & Gamble, Germany
Prof.	An	Goossens	Katholieke Universiteit Leuven (Day 1 only)
Prof.	Ann-Therese	Karlberg	University of Gothenburg, Sweden
Dr.	Jon	Lalko	RIFM, USA
Dr.	Fred	Lebreux	International Fragrance Association, Belgium
Prof.	Jean-Pierre	Lepoittevin	University of Strasbourg, France
Dr.	Andreas	Natsch	Givaudan, Switzerland
Dr.	Camilla	Pease	Environ (Day 1 only)
Dr.	Chris	Powell	Unilever, UK
Prof.	David	Roberts	Liverpool John Moores University
Dr.	Florian	Schellauf	Cosmetics Europe, Belgium
Prof.	Axel	Schnuch	IVDK / University of Göttingen, Germany
Dr.	Theodor	Schumacher	Smartpractice, Germany
Prof.	Glenn	Sipes	University of Arizona, USA
Dr.	Matthias	Vey	International Fragrance Association, Belgium
Dr.	Jonathan	Warr	Takasago, USA

APPENDIX 3: Additional reading

- Cinnamyl alcohol oxidizes rapidly upon air exposure / I. B. Niklasson, T. Delaine, M. N. Islam, R. Karlsson, K. Luthman and A.-T. Karlberg / Contact Dermatitis 2013.
- Air-oxidized linalool–a frequent cause of fragrance contact allergy / J. Brared-Christensson, K. E. Andersen, M. Bruze, J.-D. Johansen, B. Garcia-Bravo, A. Gimenez Arnau, C.-L. Goh, R. Nixon and I. R. White / Contact Dermatitis 2012.
- Stability of Essential Oils: A Review / C. Turek and F. C. Stintzing / Comprehensive Review in Food Science and Food Safety 2013.
- Haptens, pro-haptens and pre-haptens, or electrophiles and proelectrophiles / A. O. Aptula, D. W. Roberts and C. K. Pease / Contact Dermatitis 2007.
- Ann-Therese Karlberg, Moa Andresen Bergström, Anna Börje, Kristina Luthman and J. Lars G. Nilsson. Allergic Contact Dermatitis–Formation, Structural Requirements, and Reactivity of Skin Sensitizers. Chem. Res. Toxicol. 2008, 21, 53–69
- Determinants of skin sensitization potential / D. Roberts, A. Aptula / Journal of applied toxicology 2008.
- Oxidative degradation of fragrant aldehydes. Autoxidation by molecular oxygen / C. Marteau, F. Ruyffelaere, J.-M. Aubry, C. Penverne, D. Favier, V. Nardello-Rataj / Tetrahedron 2013.
- Not only oxidized R-(þ)- but also S-(-)-limonene is a common cause of contact allergy in dermatitis patients in Europe / M. Matura, M. Sköld, A. Börje, K. Andersen, M. Bruze, P. Frotsch, A. Goossens, J. D. Johansen, C. Svedman, I. WHITE, A.-T. Karlberg / Contact Dermatitis 2006.
- A sensitive method for determination of allergenic fragrance terpene hydroperoxides using liquid chromatography coupled with tandem mass spectrometry / J. Rudbäck, N. Islam, U. Nilsson, A.-T. Karlberg / J. Sep. Sci. 2013.
- HR-MAS NMR Spectroscopy of Reconstructed Human Epidermis: Potential for the in Situ Investigation of the Chemical Interactions between Skin Allergens and Nucleophilic Amino Acids / K. Elbayed, V. Berl, C. Debeuckelaere, F.-M. Moussallieh, M. Piotto, I.-J. Namer, J.-P. Lepoittevin / Chem. res. toxicol. 2013.
- Synthesis of Allylic Hydroperoxides and EPR Spin-Trapping Studies on the Formation of Radicals in Iron Systems as Potential Initiators of the Sensitizing Pathway / D. Kao, A. Chaintreau, J.-P. Lepoittevin, E. Gimenez-Arnau / J. Org. Chem. 2011.
- Mechanistic Proposal for the Formation of Specific Immunogenic Complexes via a Radical Pathway: A Key Step in Allergic Contact Dermatitis to Olefinic Hydroperoxides / S. Johansson, T. Redeby, T. Altamore, U. Nilsson, A. Börje / Chem. res. toxicol. 2009.
- Specific Adducts Formed through a Radical Reaction between Peptides and Contact Allergenic Hydroperoxides / T. Redeby, U. Nilsson, T. Altamore, L. Ilag, A. Ambrosi, K. Broo, A. Börje, A.-T. Karlberg / Chem. res. toxicol. 2010.