

# Pre & Pro Haptens in Fragrance:

## Part 1 – Abiotic oxidation

Givaudan<sup>®</sup>

 TAKASAGO

**IFF**

# Abiotic oxidation of fragrance raw materials

## 1. **Chemistry and theory – oxidation of neat raw materials**

1. Oxidation products as potential allergens
2. The rate of oxidation of fragrance materials as understood based on Bond Dissociation Energy (BDE) extensively used in the academic literature
3. Comparison of oxidation rates

## 2. **The scope of the problem in real products**

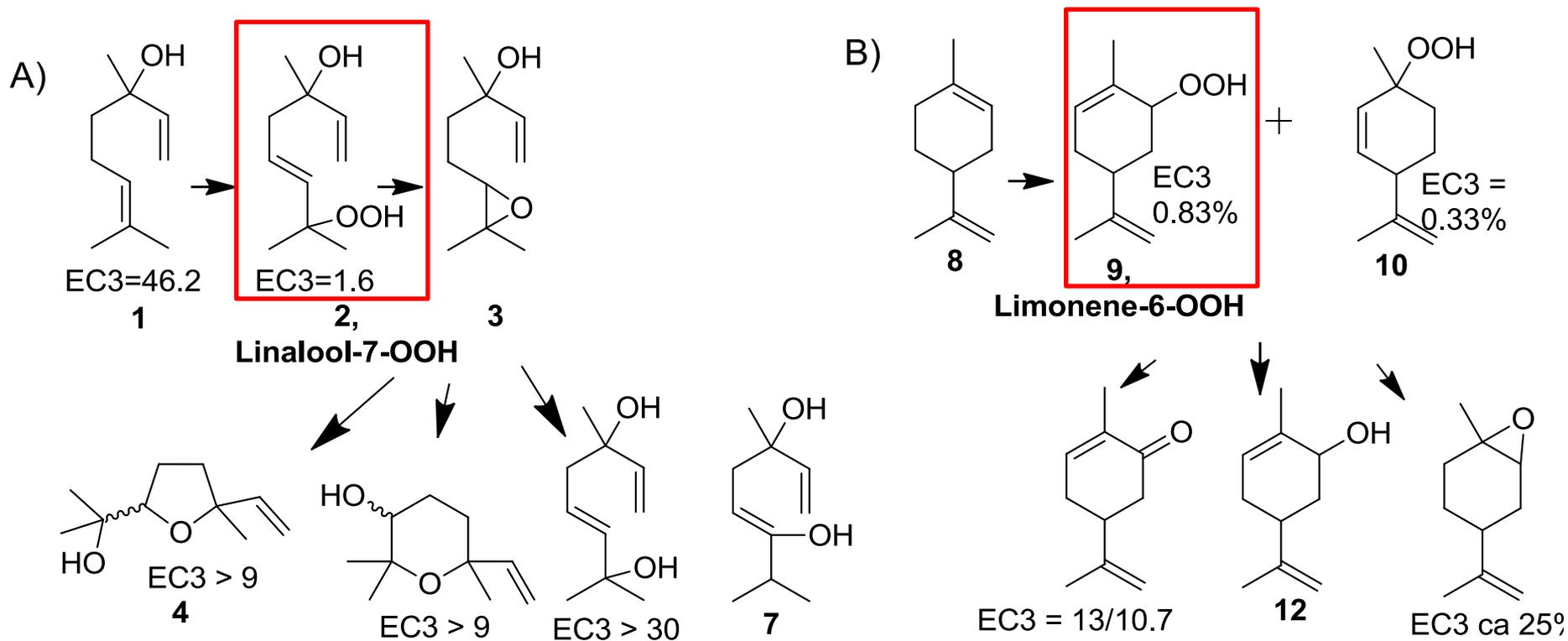
1. Study 1: GC-MS and LC-MS methods
  1. Fully controlled stability study on linalool and limonene
  2. Analytical determination of specific hydroperoxides in samples recalled from consumers
2. Study 2: Peroxide detection in fragrance samples: Titration method

## 3. **Conclusions**

## 4. **Gaps in our understanding and potential issues to address**

# 1) Chemistry and theory – oxidation of neat fragrance raw materials

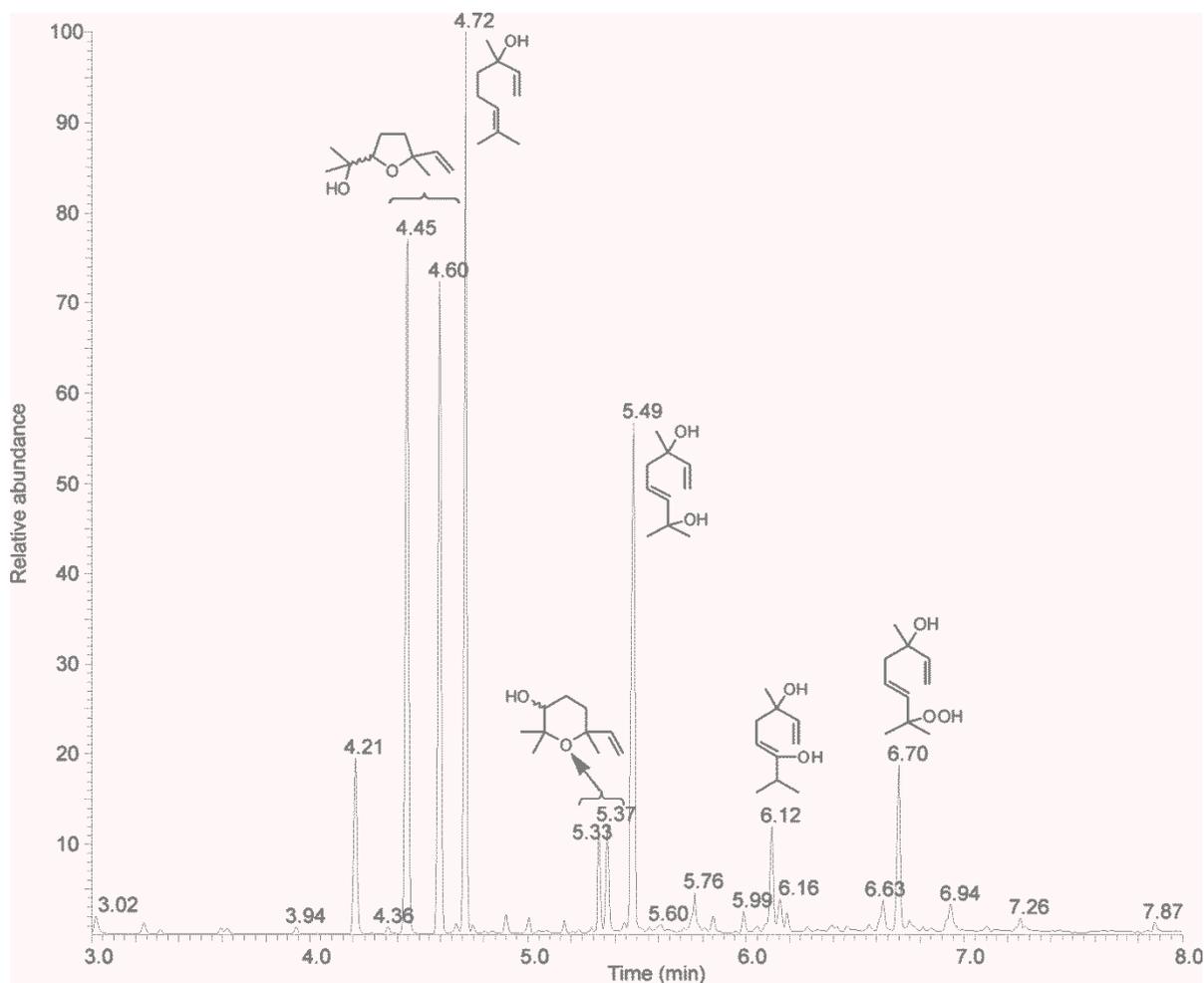
# The key results from the literature on autoxidation of linalool and limonene



The primary oxidation products (= the hydroperoxides) are the main sensitizers formed.  
 The secondary oxidation products are weak sensitizers / non-sensitizers

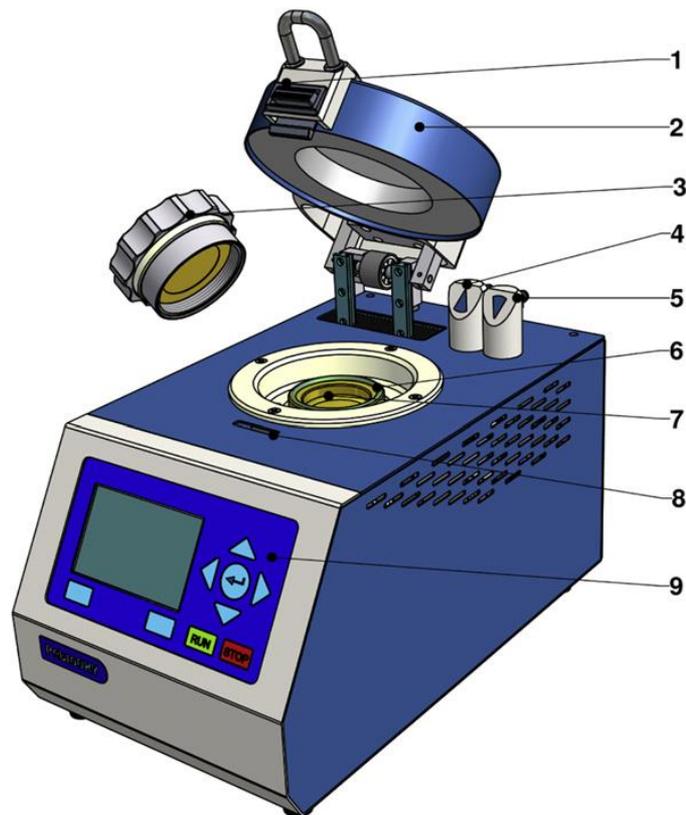
# Forced oxidation of Linalool under O<sub>2</sub> atmosphere

- We observe the same main primary and secondary oxidation products as reported by Prof. A.T. Karlbergs group in the experiments with repeated stirring in ambient air



Pure Linalool stirred under oxygen atmosphere, then stored for 1 year. GC-MS analysis

# Technique for the study of oxidation



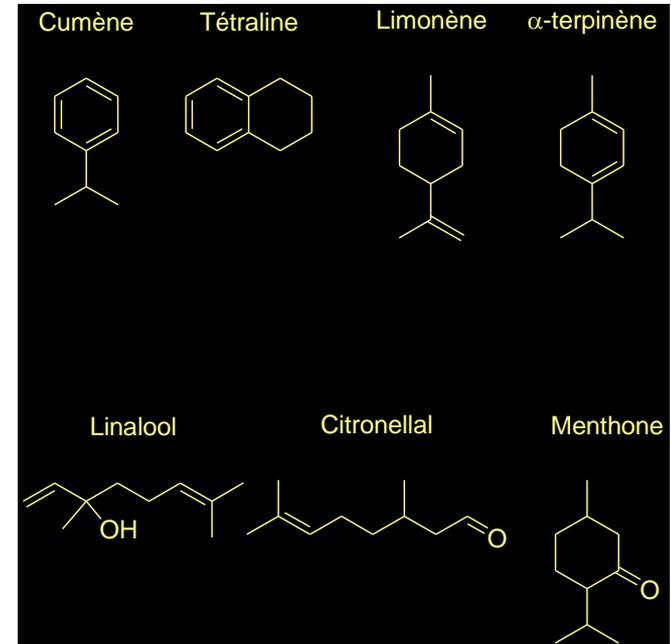
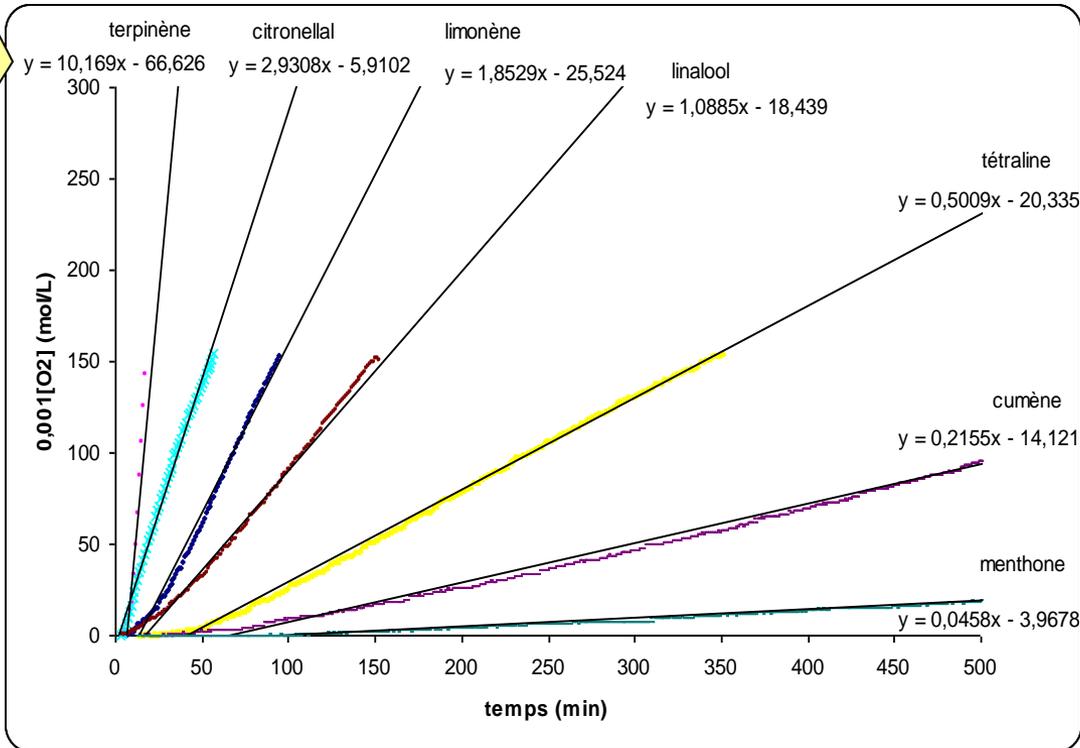
The PetroOxy apparatus was equipped with an inox cell (7) corresponding to a total volume of 25 mL in which the sample was introduced (the recommended volume is 5 mL) at ambient temperature. The cell was then closed by a screw cap (3) and a safety hood (2), which was locked by a latch (1 and 8). The gas was removed of the cell by the extraction gas connection (4) and replaced by only pure dioxygen, which was injected through the gas alimentation (5) at the pressure indicated on the interface screen (9) (300 kPa). The cell was then heated up to the temperature set (25 or 40 C) and the pressure was monitored every minute by the pressure sensor (6).

## Oxidative degradation of fragrant aldehydes. Autoxidation by molecular oxygen

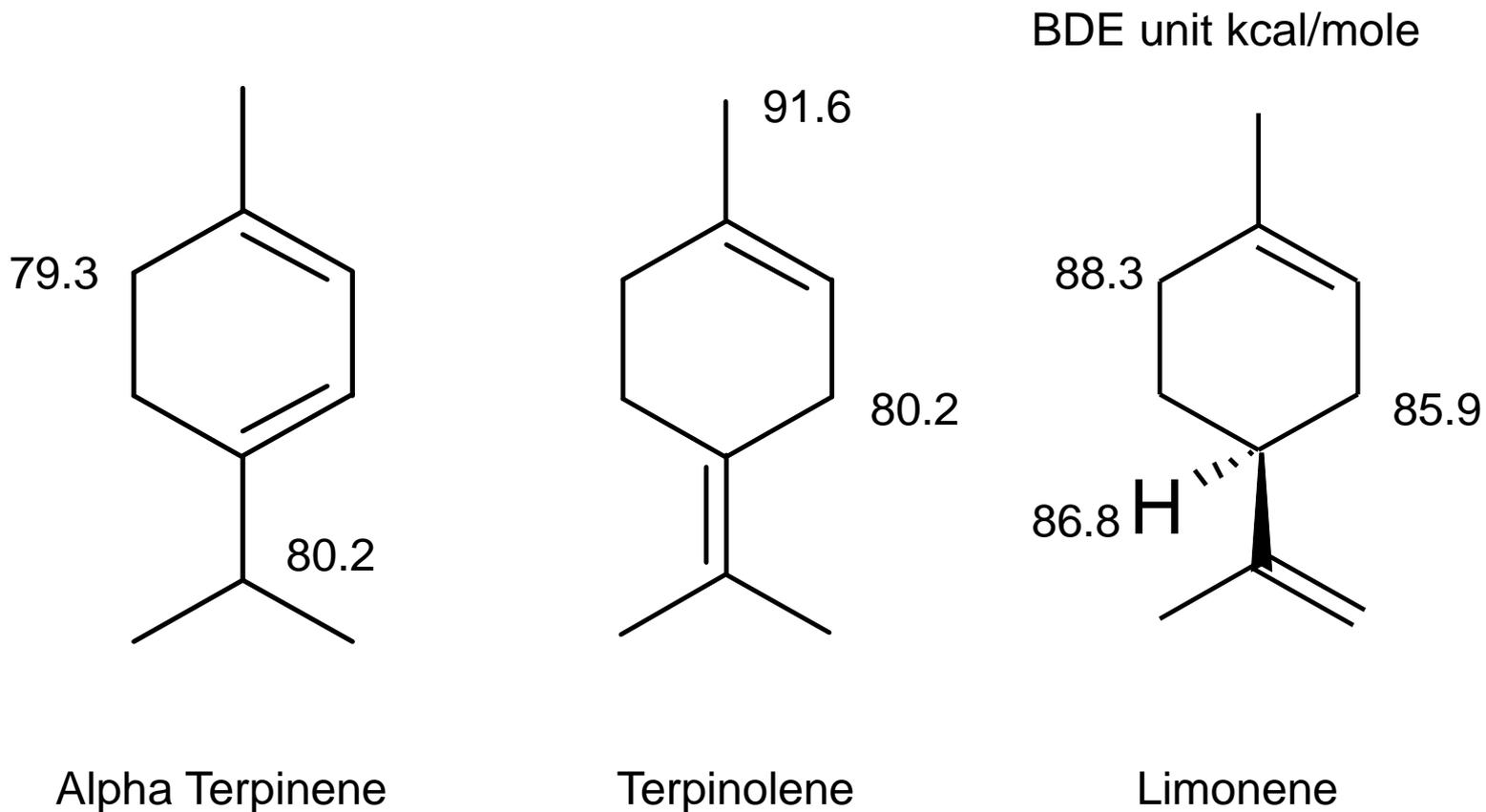
C. Marteau a,b, F. Ruyffelaere a,b, J.-M. Aubry a,b, C. Penverne a,b, D. Favier c, V. Nardello-Rataj a,b,\* *Tetrahedron* 69 (2013) 2268-2275

# Oxidability of fragrance materials

80°C 200kPa

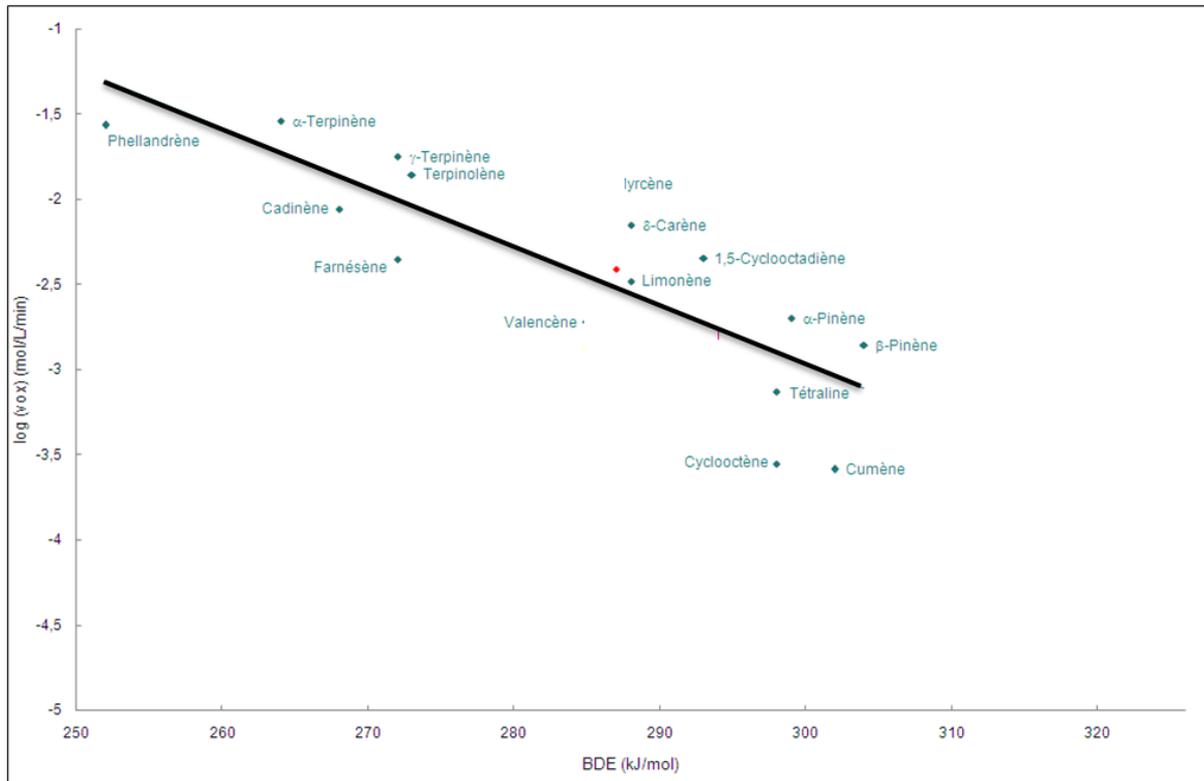


# Bond Dissociation Energy a key factor



# Calculated BDE/Oxidation rate relationship

- Bond Dissociation Energy of C-H bonds can help to estimate the sensitivity to oxidation of fragrance materials
- The oxidability of fragrance materials can be easily estimated by generating O<sub>2</sub> pressure vs time curves



## 2) The scope of the problem in real products

# Peroxides as skin sensitizing ingredients in consumer products – the missing link

- Linalool and limonene can autoxidize to form primary and secondary oxidation products
- The hydroperoxides as primary oxidation products are moderate to strong skin sensitizers as shown in animal tests
- The oxidation process as reported in the literature was performed on pure raw materials / concentrated essential oils and may not reflect the situation under real use and storage conditions
- However: Dermatitis patients react to patch test materials with high content of hydroperoxides (and other oxidation products)
  - *Is this sufficient proof that patients have become sensitized by fragranced products containing hydroperoxides?*
- We have so far no information, whether the hydroperoxides exist in relevant quantities in consumer products
  - ⇒ A quantitative method for hydroperoxide analysis is needed
  - ⇒ Stability studies and market surveys can help to understand fate of linalool and limonene in consumer products

# Study 1: Stability of linalool and limonene in consumer products

## ■ Three project stages:

### A) Detailed stability study, industry standard (45°C, 2 months).

- A) Linalool formulated in fine fragrance formulation
- B) Fully defined experimental fine fragrances with defined linalool and limonene content
- C) Market fragrances with typical linalool and limonene content
- D) Antiperspirants with defined linalool content

Analysis by GC-MS and GC-FID

### B) Prolonged stability study up to 9 months, detailed analytics of peroxide formation with high-resolution LC-MS

### C) Survey of aged fine fragrances retrieved from consumers, analytics of peroxide formation with high-resolution LC-MS

*Large study – only example results given below*

# Stability of synthetic linalool for 9 months in a hydroalcoholic formulation

- Linalool in a FF formulation (10% = 100'000 ppm linalool)
- Stored at 45°C for 9 months / in half-empty bottles / opened every 14 days
- contains a stable peroxide level of around 0.015%
- No significant degradation of linalool

Detailed analytical results after 9 month storage for linalool formulated as a hydroalcoholic fragrance					
	Storage Temp. <sup>2)</sup>	Linalool (µg/g) <sup>1)</sup>	Linalool-hydroperoxide (µg/g)	trans-linalool oxide (µg/g)	cis-linalool oxide (µg/g)
Linalool synthetic + stabilizers	45°C	110553 ± 2499	<b>18 ± 0.4</b>	10 ± 1.3	NF
Linalool synthetic	45°C	113100 ± 5102	<b>15 ± 0.2</b>	traces	NF
Linalool synthetic + stabilizers	5°C	103531 ± 1152	<b>14 ± 0.2</b>	NF	NF
Linalool synthetic	5°C	117980 ± 664	<b>14 ± 0</b>	NF	NF

- Theoretical initial content = 100'000 ppm (10%)
- All samples for the nine month study were half-empty and the bottles were opened every 14 days throughout the study duration to allow for contact with fresh air.

# Stability of natural linalool for 9 months in a hydroalcoholic formulation

- Natural linalool contains a 6x higher, but stable level of hydroperoxides
- No significant degradation of linalool

Detailed analytical results after 9 month storage for natural linalool formulated as a hydroalcoholic fragrance

	Storage Temp. <sup>2)</sup>	Linalool (µg/g) <sup>1)</sup>	Linalool-hydroperoxide (µg/g)	trans-linalool oxide (µg/g)
Linalool natural 2 months	45°C	110298 ± 545	<b>74 ± 1</b>	391 ± 17
Linalool natural 2 months	5°C	98059 ± 10779	<b>70 ± 9</b>	287 ± 2
Linalool natural 9 months	45°C	107732 ± 5033	<b>83 ± 4</b>	78 ± 4
Linalool natural 9 months	5°C	100600 ± 2499	<b>92 ± 0.2</b>	85 ± 2

1) Theoretical initial content = 100'000 ppm (10%)

2) All samples for the nine month study were half-empty and the bottles were opened every 14 days throughout the study duration to allow for contact with fresh air.

## Linalool in complex fragrances

- Linalool is stable in complex fragrances stored in half-empty bottles, opened every 14 days. Only traces of hydroperoxides detected
- Commercial fragrance C contains 60 ppm hydroperoxide if stored at 5°C, degrade to secondary products at 45°C

Analytical results after 9 month storage for linalool-containing complex hydroalcoholic fragrances							
	theoretical			<b>Linalool-</b>			
	linalool	level	Storage	Linalool	<b>hydroperoxide</b>	trans-linalool	cis-linalool
	( $\mu\text{g/g}$ )		Temp. <sup>1)</sup>	( $\mu\text{g/g}$ )	( $\mu\text{g/g}$ )	oxide ( $\mu\text{g/g}$ )	oxide ( $\mu\text{g/g}$ )
Fragrance A5	495 $\pm$ 49		45°C	591 $\pm$ 109	<b>1.5 <math>\pm</math> 0.1</b>	NF	NF
Fragrance A20	1980 $\pm$ 198		45°C	2099 $\pm$ 249	<b>2.4 <math>\pm</math> 0.2</b>	NF	NF
Fragrance A50	4950 $\pm$ 495		45°C	5048 $\pm$ 534	<b>2 <math>\pm</math> 0.1</b>	NF	NF
Commercial Fragrance C	2800		45°C	2550 $\pm$ 151	<b>2.1 <math>\pm</math> 0.1</b>	10 $\pm$ 0.4	22 $\pm$ 0.8
Fragrance A50	4950 $\pm$ 495		5°C	5308 $\pm$ 583	<b>1.8 <math>\pm</math> 0</b>	NF	NF
Commercial Fragrance C	2800		5°C	2938 $\pm$ 228	<b>59 <math>\pm</math> 6</b>	NF	Traces

## Stability of limonene in complex fragrances

- Limonene is stable in complex fragrances for 9 months, even if bottles are half-empty, and repeatedly opened
- No accumulation of hydroperoxides detected, but LC/MS-detection of limonene-hydroperoxide in complex fragrances is tricky, and limit of quantification is > 200 ppm.

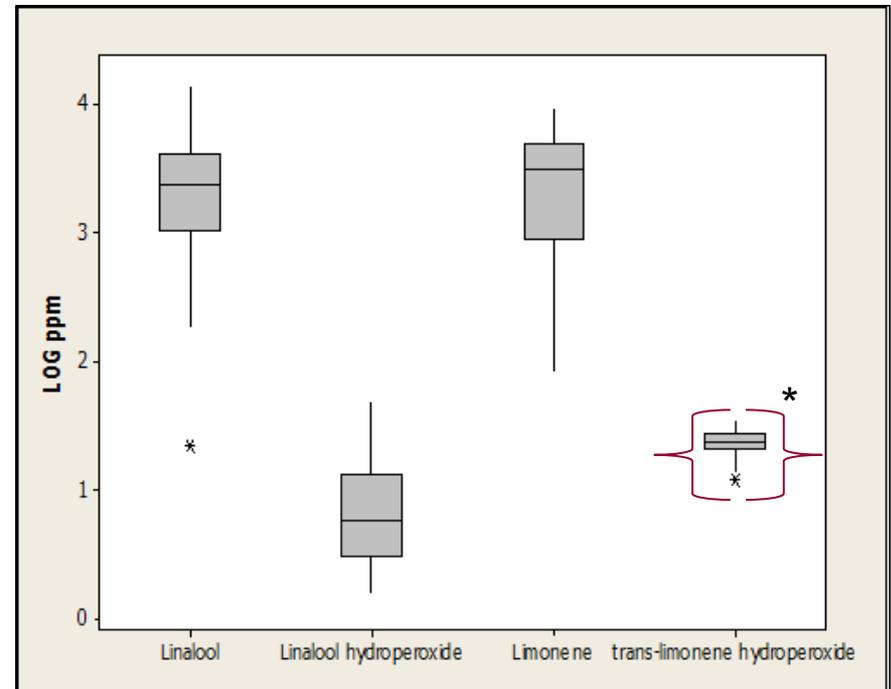
Recovery of limonene from complex hydroalcoholic fragrances over a nine month standardized stability test

Fragrance	Theoretical	1 month		2 month		9 month	
	initial limonene content	5°	45°	5°	45°	5°	45°
		½ full - opened		½ full - opened		½ full - opened	
B5	<b>475 ± 47</b>	483 ± 7	445 ± 9	452 ± 11	406 ± 11	n.d.	<b>428 ± 4</b>
B20	<b>1900 ± 190</b>	1871 ± 60	1797 ± 9	1792 ± 150	1624 ± 14	n.d.	<b>1976 ± 15</b>
B50	<b>4750 ± 47</b>	4775 ± 191	4624 ± 165	4324 ± 109	3810 ± 50	5037 ± 76	<b>4935 ± 117</b>
D <sup>1)</sup>	<b>990</b>	873 ± 13	829 ± 24	903 ± 9	863 ± 2	922 ± 40	<b>840 ± 26</b>

# An what happens in 'real' products, stored under 'real' conditions?

- 39 hydroalcoholic fragrances recalled from consumers
- Linalool and limonene content determined by GC/MS and hydroperoxide level determined by high resolution LC/MS

- linalool identified in 38 of 39 samples
- linalool hydroperoxide present in 33 out of 39 samples
- Median of hydroperoxide 600 fold below linalool content
- Maximal level is 50 ppm, below the generalized elicitation threshold of 100 ppm proposed by SCCS
- Similar result for limonene, but high analytical uncertainty
  - \* Spiking experiments indicate that limonene hydroperoxide cannot be detected in all product matrices



## Antiperspirant study

- Neat Linalool slightly degraded (18%)
- Degradation products are rearrangement products:  $\alpha$ -terpineol (6.2%), geraniol (1.5%) and a number of other terpenes
- Linalool stable in complex perfumes formulated into antiperspirant
- No hydroperoxides detected by GC/MS (LC/MS not available)

Fragrance	Theoretical initial linalool content	5° control	45° 1 month	45° 2 month
A5	<b>50 ± 5</b>	46 ± 5 <sup>1)</sup>	51 ± 5	<b>49 ± 4</b>
A20	<b>200 ± 20</b>	235 ± 10	202 ± 5	<b>217 ± 20</b>
A50	<b>500 ± 50</b>	508 ± 14	505 ± 39	<b>525 ± 34</b>
Linalool synth.	<b>10'000 ± 1000</b>	10514	n.d.	<b>7049 ± 11</b>

# Study 1: Conclusions

- High stability of linalool and limonene in complex fragrances in the studied products
- No evidence for accumulation of hydroperoxides over time during product storage
- Hydroperoxides may come from the raw materials
  - Can then be very stable in products or degrade to secondary oxidation products
- However, quantitative levels coming from quality –controlled raw materials are quite low and of no concern from risk assessment perspective

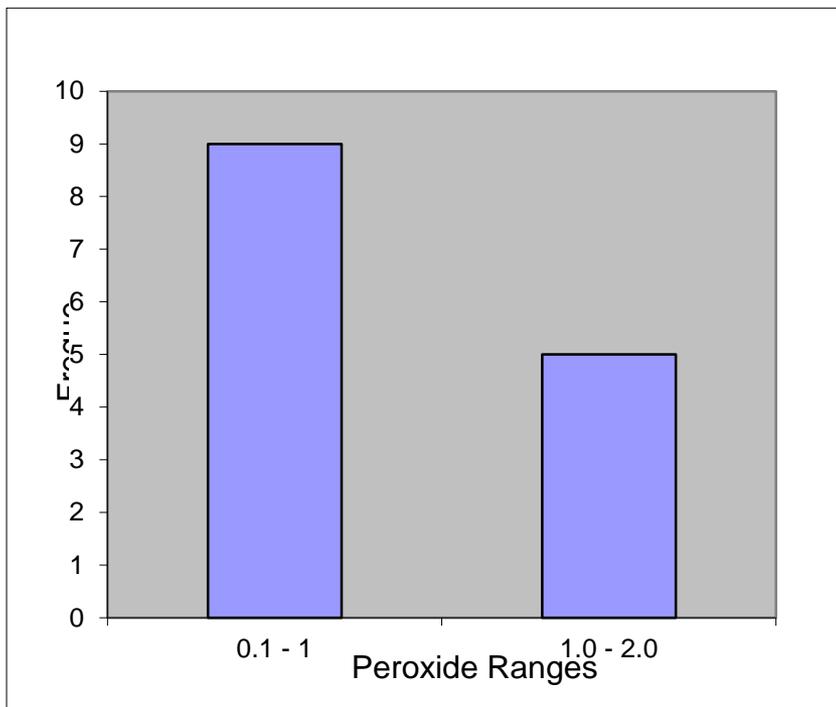
## Study 2: (hydro)peroxide detection in fine fragrances

- Three project stages:
  - A) Phase I: Production Batches of Fragrance Oils (data not shown)
  - B) Phase II: Unopened fine fragrance products purchased at retail
  - C) Phase III: Fine fragrance products that have been used by consumers under typical conditions

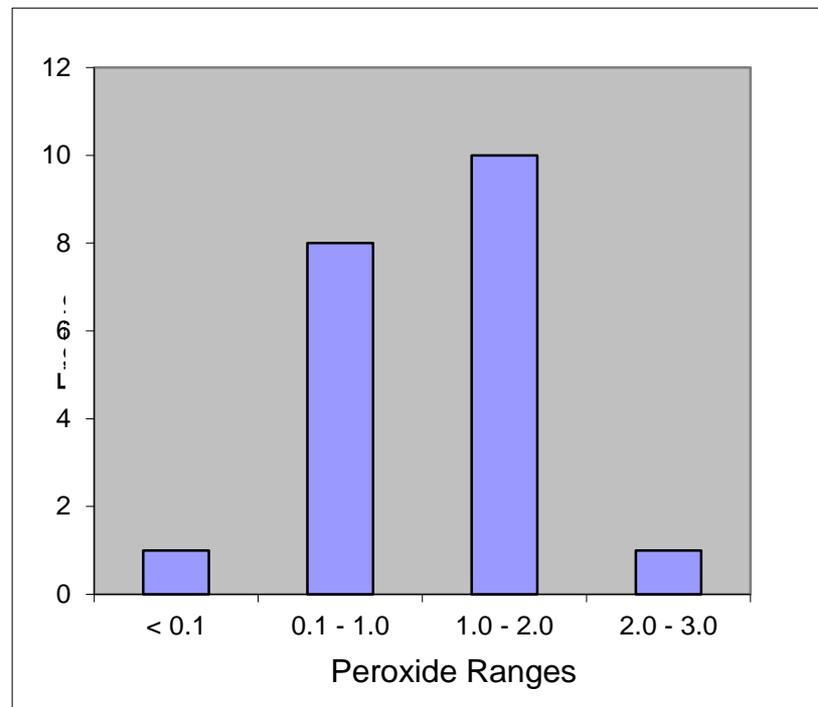
## Phase II Results

- Total peroxide values are below 2 mmol/L for 33 of 34 fragrances tested
- If this would correspond to a single terpene hydroperoxide with MW 170 -186, it would correspond to < 350 ppm
- Most likely the colorimetric signal comes from different peroxides and hydroperoxides in the multicomponent fragrances

Unopened Men's Fine Fragrances  
Purchased at Retail (n = 14)

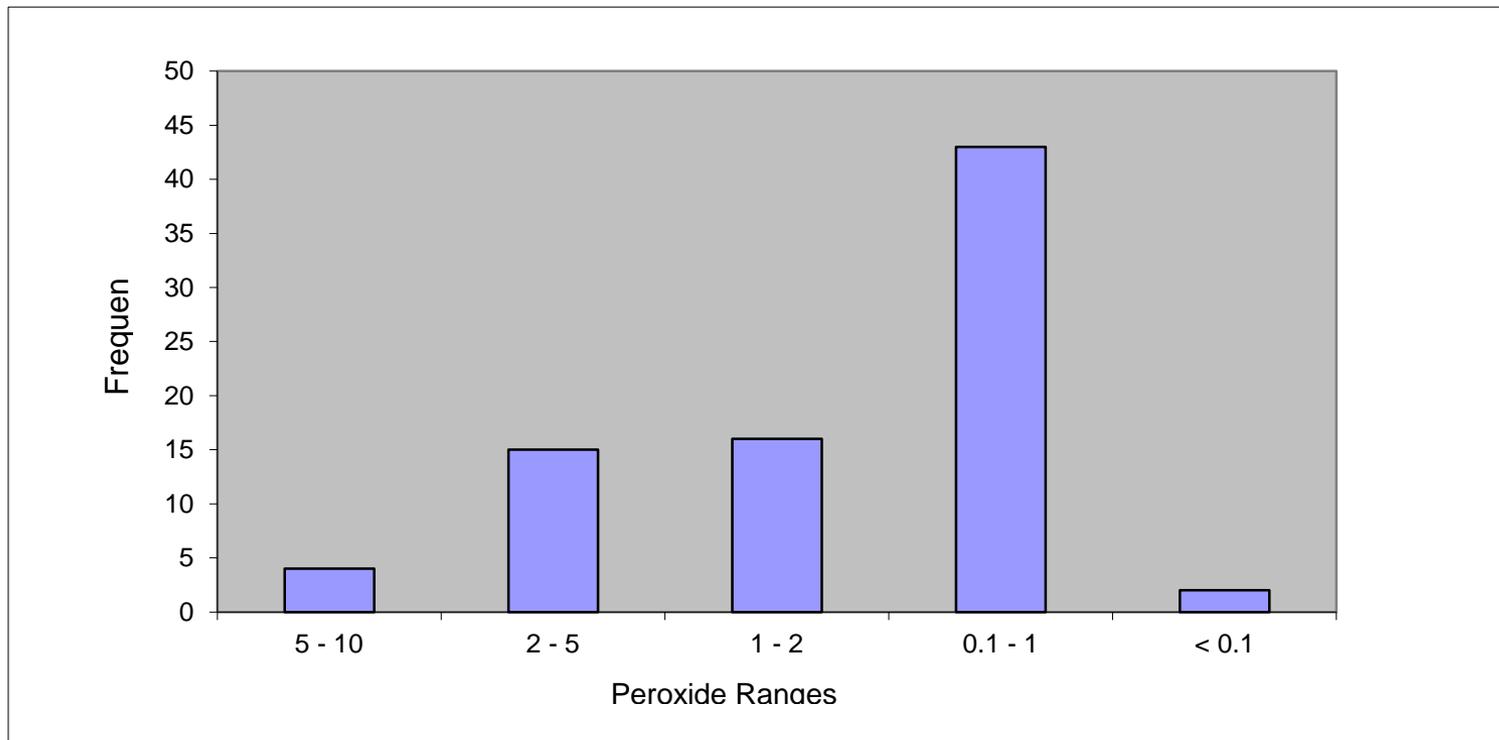


Unopened Women's Fine Fragrances  
Purchased at Retail (n = 20)



# Phase III Results – Fine Fragrance Products after typical storage/use (n = 80)

- Total peroxide values are below 2 mmol/L for most fragrances tested
- In general close to fresh fragrances
- 20% contain higher levels



## Study 2: Conclusions

- Fine fragrance products typically contain some of the highest fragrance levels of all consumer products and result in the greatest amount of dermal exposure to fragrance materials
- One product had a peroxide level of  $\leq 3$  mmol/l, while all other products tested contained  $\leq 2$  mmol/l peroxides
- Prior to opening, fine fragrance products contain extremely low levels of peroxides
- Peroxide levels were measured in fine fragrance products that had been used by consumers under typical conditions, which includes storage under less than ideal circumstances and repeated opening
- Except for 4 oils (5% of total), the peroxide levels were all  $< 5$  mmol/l
- A total of 75 / 80 fragrance oils (95%) exhibited peroxide levels  $\leq 5$  mmol/l and 76.25% had levels  $\leq 2$  mmol/l
- Peroxide levels do not appear to be a concern even after fine fragrance products have been opened and used for periods up to 5 years

# Overall Conclusions (I)

- Hydroperoxides present at low levels (< 100 ppm) in commercial perfumery ingredients, esp. of natural origin
- Hydroperoxides can be brought into final product from these raw materials at levels below the generalized elicitation threshold of 100 ppm proposed by SCCS
- But: No evidence for accumulation of hydroperoxides during product storage, even if air contact is ensured by repeated opening of bottles
- Similar conclusion for standardized stability test and 'random/real' storage at customers home

## Overall Conclusions (II)

- Colorimetric method gives total content of all peroxides and hydroperoxides
  - Gives therefore somewhat higher levels (but not compared in same products)
- Specific LC-MS method targeting single hydroperoxide gives a more accurate measure to calculate risk for exposure by a single ingredient
- LC-MS method works well in complex fragrance background for Linalool-hydroperoxide.
- For limonene-hydroperoxide method currently works well in essential oils and products containing only limonene
  - Method is not yet adequate for complex fragrances due to high load of equivalent ions
  - Reliable and accurate detection of low levels of limonene peroxide in complex products remains an analytical challenge.
- Also matrix effects in complex products affect accuracy of the measurements – however this does not affect the overall conclusions

## A gap remains

- EC3 level in mice for linalool hydroperoxide: 16'000 ppm (Sköld et al., 2004)
  - Significant reactions in guinea pigs at 26'000 ppm (rechallenge) and 51'000 ppm (first challenge), but only 1 of 15 animals reacting at 10'000 ppm (Sköld et al., 2002)
  - Patch test reactions in dermatitis patients (Christensson et al., 2010)
    - 0.83% of patients react to 3800 ppm hydroperoxide
    - 7.2% to 20'900 ppm
    - Standard concentrations in recent studies (Brared-Christensson et al., 2012) is 10'000 ppm
- ⇒ Therefore the concentrations reported both for induction and elicitation in man and in animals are >3 orders of magnitude higher as compared to the measured concentrations in consumer products
- Key questions therefore:
    - Are the reactions specific to oxidized linalool?
    - If they are: Have the consumers been induced by fragranced consumer products or by other sources?
    - What would explain induction by these extremely low concentrations?

# Gaps in our understanding and potential issues to address

- What is the inducing product in patients reacting to oxidized fragrance materials?
  - i.e. Where does induction come from
- Are oxidized materials present in products used by patch test positive patients?
  - i.e. Is there elicitation by currently used products
- Why do patients predominantly react to high levels of the hydroperoxides?
- How specific are reactions to high levels of hydroperoxides?
- Are other products besides fine fragrances and antiperspirants which should be investigated whether they contain critical hydroperoxide levels?
- Accurate detection method for limonene-hydroperoxide in complex products?

BACKup slides

## Autoxidation reaction – overall scheme

- **Initiation**       $\text{RH} \rightarrow \text{R}^\circ$   
The reaction rate is a function of the Bond  
Dissociation Energy **C-H**
  - Catalyzed by trace metals
- **Propagation**  
 $\text{R}^\circ + \text{O}_2 \rightarrow \text{ROO}^\circ \quad k \sim 10^9 \text{ l} \times \text{mol}^{-1} \text{s}^{-1}$   
 $\text{ROO}^\circ + \text{RH} \rightarrow \text{ROOH} + \text{R}^\circ$
- **Termination**
  - Stable products $\text{R}^\circ + \text{R}^\circ \rightarrow \text{R-R}$  $\text{ROO}^\circ + \text{R}^\circ \rightarrow \text{ROOR}$  $\text{ROO}^\circ + \text{ROO}^\circ \rightarrow$