Exposure and specificity: Different theoretical scenarios to explain high frequency of patch test reactions

An alternative hypothesis on the role of cross reactivity

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Preamble

- The content of this presentation is **not the agreed Industry view**
- Content are data from **Givaudan laboratories** and possible interpretations of internal *and* literature data by Givaudan scientists
- Givaudan is using Linalool and Limonene in perfumes, but is neither producing nor selling these products directly

- There will be a slightly higher focus on oxidized linalool, as there are recently more data and discussions on linalool

- We are of the firm opinion that different scenarios need to be openly discussed, and that several questions have **not yet been answered to draw final conclusions** on
  - Relevant exposure to hydroperoxides
  - Specificity of reactions recorded by the patch tests

Background: Well established facts

- Different hydroperoxides are moderate to strong sensitizers
- These hydroperoxides act as specific haptens following specific induction (nicely demonstrated in animal / guinea pig tests)
- They form specific adduct with peptides / amino acids through radical reactions (nicely demonstrated in several NMR studies)

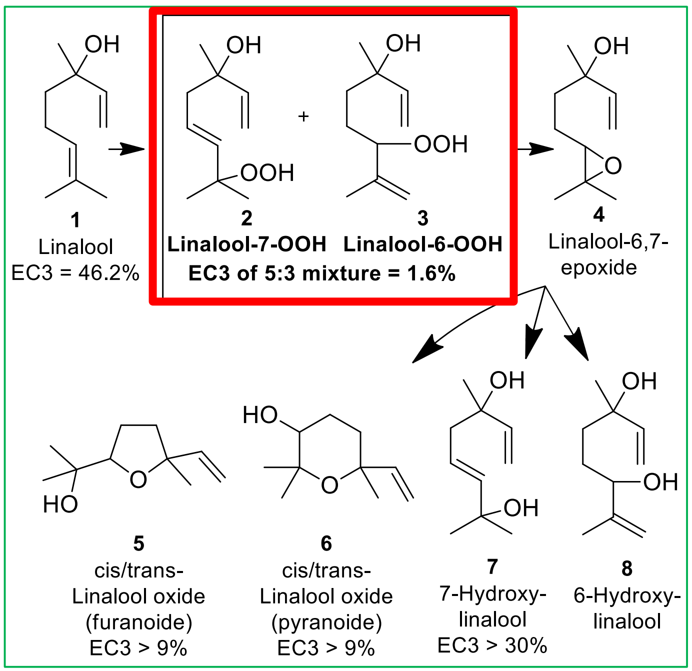


Figure 1. Main primary and secondary oxidation products of linalool with their reported LLNA EC3 values (in brackets)

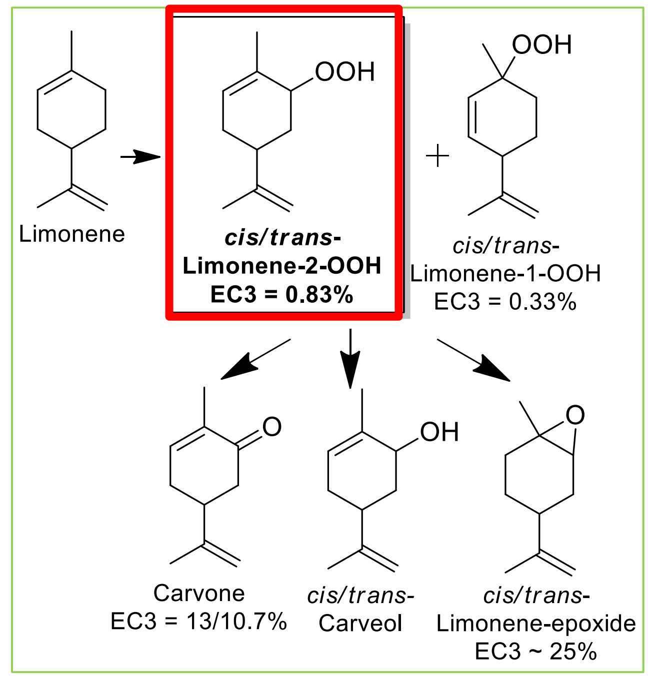
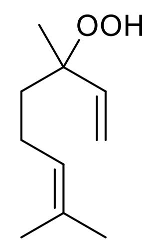


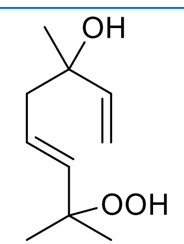
Figure 2. Main primary and secondary oxidation products of limonene with their reported LLNA EC3 values (in brackets)

History of oxidized linalool / linalool hydroperoxide

- Linalool is main ingredient in lavender oil and had been used for centuries in perfumes
  - There are no indications, that there are (new) problems specifically related to linalool containing products. Exposure to linalool has not changed.
  - But: 80 – 90% of perfumes contain linalool, any correlations to linalool content of products are difficult
- Work on oxidized linalool did not start from a clinical problem, but with chemical synthesis: Bezard et al. (1997) synthesized a hydroperoxide
  - Skin sensitizing in LLNA
  - This non-natural hydroperoxide was never found in essential oils/products
- Different hydroperoxide isolated from oxidized linalool
- Patch test developed with oxidized linalool



Linalool-hydroperoxide, non-natural hydroperoxide, never found from linalool oxidation
EC3 = 1.1% (*)

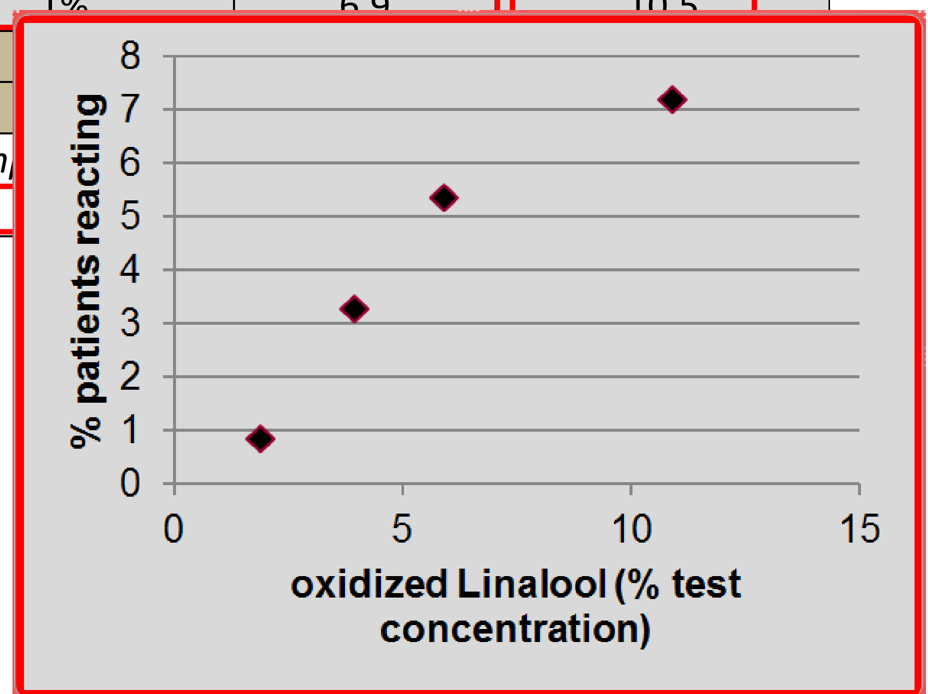


Linalool-7-OOH, main oxidation product in oxidized Linalool,
EC3 = 1.6%

Studies in dermatological patients

Table 1. Literature review of positive and doubtful reactions to the terpene hydroperoxides

Study reference	N patients	Target hydroperoxide	Hydroperoxide level in the patch test preparation	% of positive /allergy skin reactions	% of doubtful / irritants
[1]	1693	Linalool-OOH	0.38%	0.83	1.9
[1]	2075	Linalool-OOH	0.76%	3.2	5.1
[1]	1725	Linalool-OOH	1.14%	5.3	6.4
[1]	1004	Linalool-OOH	2.1%	7.2	7.3
[2]	4731	Linalool-OOH	1%	5.9	7.3 1)
[3]	2800	Linalool-OOH	1%	6.9	10.5
[4]	2800	Limonene-OOH			
[2]	4731	Limonene-OOH			
Data for com					
[6]	*37270*	Lyral			



⇒ **Similar frequencies in multiple studies**

⇒ **Test at relatively high concentrations**

⇒ **high test concentrations lead to high numbers of reactions**

⇒ **Even higher frequency of doubtfuls /irritants**

Why do we see these frequent reactions? Three possibilities:

- ***Scenario A*: The positive reactions are**
  - **Specific** to the specified terpene hydroperoxide
  - Induction stems from **oxidized terpenes in fragranced products**

A question of exposure to the hydroperoxides from fragranced products!

- ***Scenario B:* The positive reactions**
  - Come from oxidized terpenes present in **other exposures situations**

- ***Scenario C:* The positive reactions**
  - Are **not specific** to the specified terpene hydroperoxide
    - Induction stems from **other oxidized chemicals**

Exposure – oxidation under different scenarios

- We do have data on oxidation of linalool and limonene under different scenarios
- The following slides give an overview of these scenarios and what we know currently
- It is always important to look at the units – some data are on %, others are in mM, and analytical data are mostly in ppm / µg/g
- **What finally counts is exposure on the skin, usually expressed as $\mu g/cm^2$**

Experience from neat products and essential oils

- Under air saturation, complete degradation of Linalool
- formation of primary and secondary oxidation products
- Similar effects for citronellol, geraniol, linalyl acetate, lavender oil.
- Oxidation protocol developped in 1991 to '*mimic industrial handling of limonene (as a solvent)*', originally not related to fragrance industry

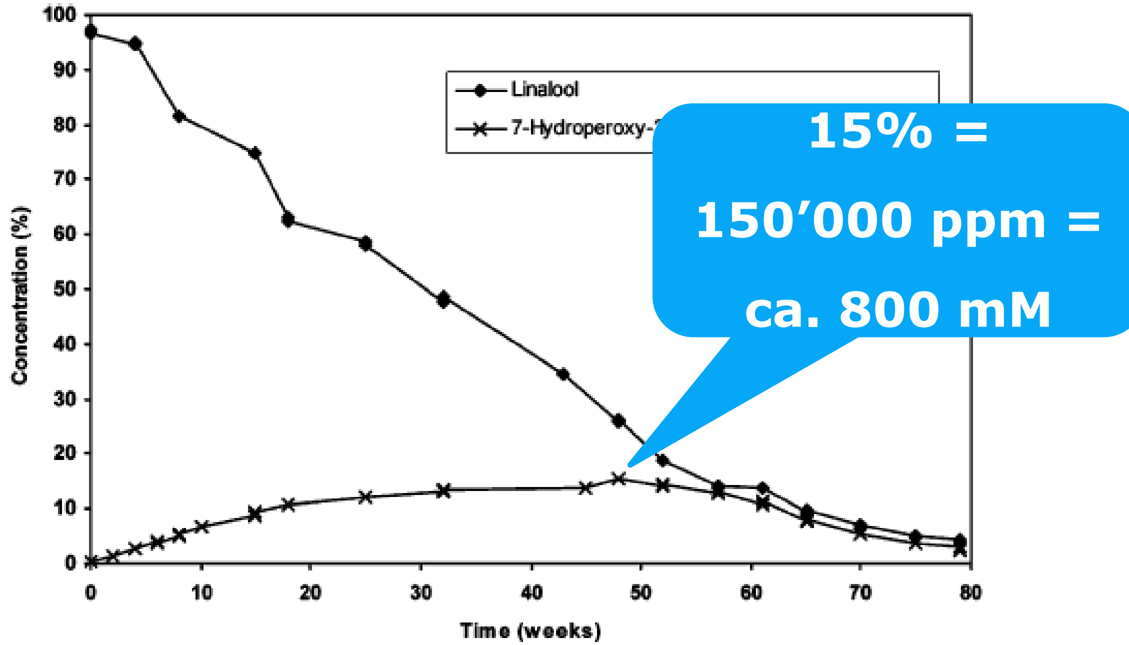
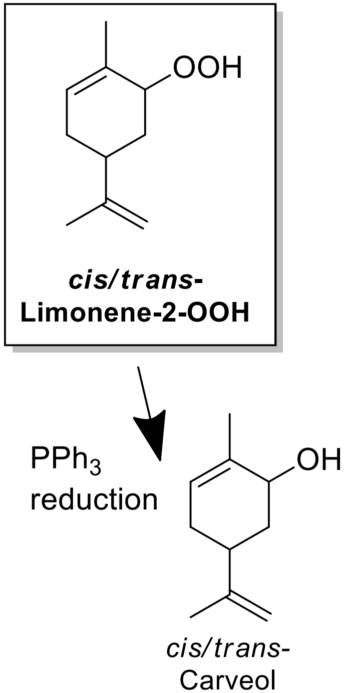


Figure 2. Concentrations of linalool and the major hydroperoxide 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol **4** in air-exposed linalool, over time. Quantification of linalool was performed with GC using the on-column technique. For the hydroperoxide, HPLC was used.

Air Exposure Procedure. Linalool (Lancaster) was air-exposed in an Erlenmeyer flask, covered with aluminum foil to prevent contamination. It was stirred for 1 h, four times a day for 80 weeks, as previously described (*13*). Samples were taken

Excurs – analysis of hydroperoxides

- **Iodometric titration**: IFRA quality control method –– measures oxidation of iodide.
  - Standard method used in fat oxidation studies
  - Sensitive – detects all peroxides.
  - Further validation is now possible based on synthetic references
  - Not selective – detects different (hydro)peroxides, and potentially other oxidants
- **HR-LC-MS of the hydroperoxide directly**
  - Selective and sensitive.
  - Feasabilty shown in complex products for linalool-OOH
  - Difficult / not useful for Limonene-OOH in complex products
- **Reduction to alcohol – followed by GC-MS**
  - Selective and sensitive
  - May give overestimation due to alcohol already in product
  - Also used in food chemistry



Question 1: Are the hydroperoxides added to products?

- Best addressed by quality control data
- Currently generated by iodometric titration

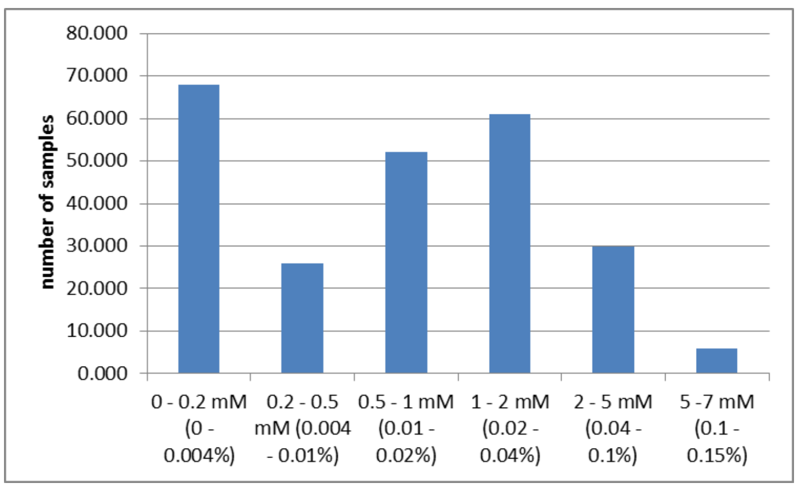
Iodometric titration: Peroxide levels in raw materials

- Raw materials are screened to comply with IFRA standards before added to fragrance compound / fragrance oil

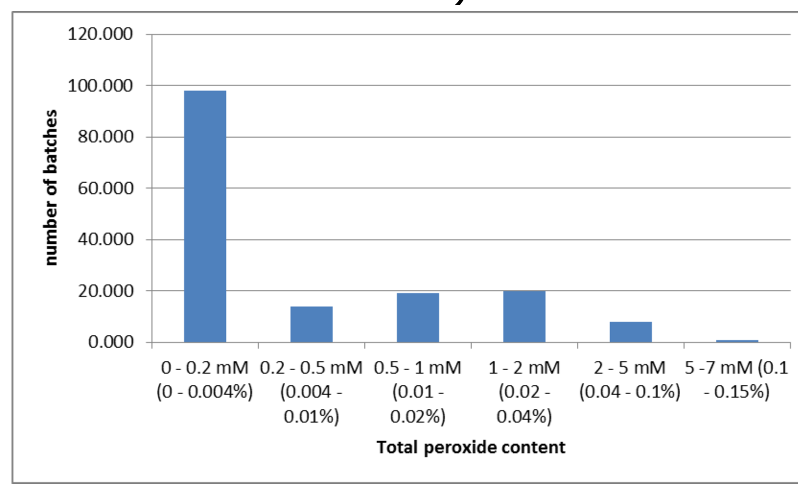
1.1 mM = 180 ppm
Top quartile: 0.6% limonene in EDT
Top quartile: 2.8 mM peroxide in raw material
⇒ **2.8 ppm in final eau de toilette**
⇒ **Level of Lim-OOH typically added to commercial product**

	Linalool Synth.	Orange terp.
average	**0.46 mM**	**1.10 mM**
median	**0.00 mM**	**0.80 mM**
n	160	243

commercial Limonene)



- Synthetic linalool (main source of commercial linalool)



Source: Givaudan raw material quality control, 2013

Question 2: Are the hydroperoxides formed in products?

- Studies performed with pure linalool in perfume formulation or linalool/ limonene containing perfumes

- Controlled stability studies in Eau de toilette and deodorant formulations

- Analysis by GC-MS and LC-MS

Effects of formulation parameters: controlled analytical study

- Synthetic linalool formulated as an eau de toilette (10% in ethanol), stored in small glass bottles
- Different parameters screened: Temperature, antioxidants, headspace air, bottle opening
- **No effect for these parameters – high stability of synthetic linalool in all conditions**

Table 2 Stability of pure linalool formulated as a hydroalcoholic fragrance in a 2-month standardized stability test

Linalool type	Storage temperature (°C)	Stabilizers	Half full	Half full/opened	Linalool (μg/g)[a]	Linalool7-hydroperoxide (μg/g)[b]	*cis/trans*-Linalool oxide (μg/g)	7-Hydroxylinalool (μg/g)
Synthetic	45	+			105,091±33	<LOD	3.6±0.2	<LOQ
Synthetic	45	+	+		105,978±7,708	<LOD	3.4±0.1	3.1±0.9
Synthetic	45	+	+	+	97,330±1,666	<LOD	3.7±0.2	3.6±0.4
Synthetic	5	+			100,003±1,405	<LOD	<LOD	<LOQ
Synthetic	45	-			100,008±2,032	<LOD	<LOQ	4.2±0.8
Synthetic	45	-	+		98,656±646	<LOD	<LOQ	3.7±0.8
Synthetic	45	-	+	+	104,931±2,552	<LOD	<LOQ	4.6±0.5
Synthetic	5	-			106,885±5,275	<LOD	<LOD	3.8±0.4

Formulation parameters – naturally derived linalool

- Naturally derived linalool contains higher hydroperoxide levels
- This quality also contains higher levels of secondary oxidation products
- This is a niche product, less than 1% of industrially used linalool
- **Again not affected by any of the studied formulation parameters**

Table 2 Stability of pure linalool formulated as a hydroalcoholic fragrance in a 2-month standardized stability test

Linalool type	Storage temperature (°C)	Stabilizers	Half full	Half full/opened	Linalool (µg/g)[a]	Linalool 7-hydroperoxide (µg/g)[b]	*cis*/*trans*-Linalool oxide (µg/g)	7-Hydroxylinalool (µg/g)
Natural grade	45	+			100,344±2,587	63±0	332±32	36±4
Natural grade	45	+	+		102,854±4,314	64±5	352±14	43±0.2
Natural grade	45	+	+	+	105,429±7,797	64±3	355±15	44±2
Natural grade	5	+			102,966±1,067	60±3	347±0.2	41±5
Natural grade	45	-			93,930±1,309	60±5	339±0.6	38±4
Natural grade	45	-	+		105,421±1,589	70±5	364±0.7	40±1
Natural grade	45	-	+	+	110,298±545	74±1	391±17	39±2
Natural grade	5	-			98,059±10,779	70±9	287±2	33±5

Kern, S., et al.. Anal. Bioanal. Chem., 2014. 406(25): p. 6165-78.

Prolonged storage of Linalool in EDT

- Samples with highest risk selcted – repeatedly opened
- Study prolonged to 9 months
- More sensitive LC-MS method for hydroperoxide detection developped
  - Hydroperoxide detected in synthetic linalool
- **No effect of storage temperature or antioxidants**

Table 3 Detailed analytical results after 9 months' storage for linalool formulated as a hydroalcoholic fragrance

	Storage temperature (°C)[b]	Linalool (μg/g)[a,c]	Linalool hydroperoxide (sum of isomers) (μg/g)[d]	*trans*-Linalool oxide (μg/g)[c]	*cis*-Linalool oxide (μg/g)[c]
Synthetic linalool plus stabilizers	45	110,553±2,499	18±0.4	10±1.3	<LOD
Synthetic linalool	45	113,100±5,102	15±0.2	<LOQ	<LOD
Synthetic linalool plus stabilizers	5	103,531±1,152	14±0.2	<LOD	<LOD
Synthetic linalool	5	117,980±664	14±0	<LOD	<LOD

Kern, S., et al.. Anal. Bioanal. Chem., 2014. 406(25): p. 6165-78.

Prolonged storage – natural linalool in EDT

- Again higher levels of the hydroperoxide in natural linalool
- No effect of temperature or antioxidants

Table 3 Detailed analytical results after 9 months' storage for linalool formulated as a hydroalcoholic fragrance

	Storage temperature (°C)[b]	Linalool (μg/g)[a,c]	Linalool hydroperoxide (sum of isomers) (μg/g)[d]	*trans*-Linalool oxide (μg/g)[c]	*cis*-Linalool oxide (μg/g)[c]
Natural linalool plus stabilizers	45	105,780±9,042	83±4	46±4	115±11
Natural linalool	45	107,732±5,033	83±4	49±4	29±5
Natural linalool plus stabilizers	5	108,424±2,403	97±0.1	20±2	75±0.1
Natural linalool	5	100,600±2,499	92±0.2	17±2	68±3

Limonene in 9 months fragrance stability study

- Partly filled, repeatedly opened bottles
- Parent limonene levels remains constant over 9 months stability study

	Storage Temp. [1)]	Theoretical limonene level (µg/g)	Detected limonene level (µg/g)
Fragrance B5	45°C	475 ± 47	428 ± 4
Fragrance B20	45°C	1900 ± 190	1976 ± 15
Fragrance B50	45°C	4750 ± 470	4935 ± 117
Commercial Fragrance D	45°C	990	840 ± 26
Fragrance B50	5°C	4750 ± 470	5037 ± 76
Commercial Fragrance D	5°C	990	922 ± 40

Kern, S., et al. Flavour Fragrance J., 2014. 29(5): p. 277-286.

Limonene-hydroperoxide in 9 months stability study

- No hydroperoxide found after 9 months stability study of limonene-containing fragrance
- No effect of storage parameters

Analyte	***trans*-carveol (µg/g)** [1]		
Reduction	**No** PPh_3	**With** PPh_3 **reduction**	
Spiking agent	**none**	**none**	**115 µg/g *trans*-limonene-2-OOH**
Fragrance B5, 45°C	<LOD	<LOD	122 ± 5 [2]
Fragrance B20, 45°C	<LOD	<LOD	125 ± 9
Fragrance B50, 45°C	<LOD	<LOD	122 ± 17
Commercial Fragrance D, 45°C	<LOD	<LOD	96 ± 1
Fragrance B50, 5°C	<LOD	<LOD	103 ± 11
Commercial Fragrance D, 5°C	<LOD	<LOD	112 ± 22

Carveol below limit of detection after PPh_3 reduction

Quantitative Carveol detection in spiked samples

⇒ **Method / negative result validated**

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Kern, S., et al. Flavour Fragrance J., 2014. 29(5): p. 277-286.

Question 2: Are the hydroperoxides formed in products?

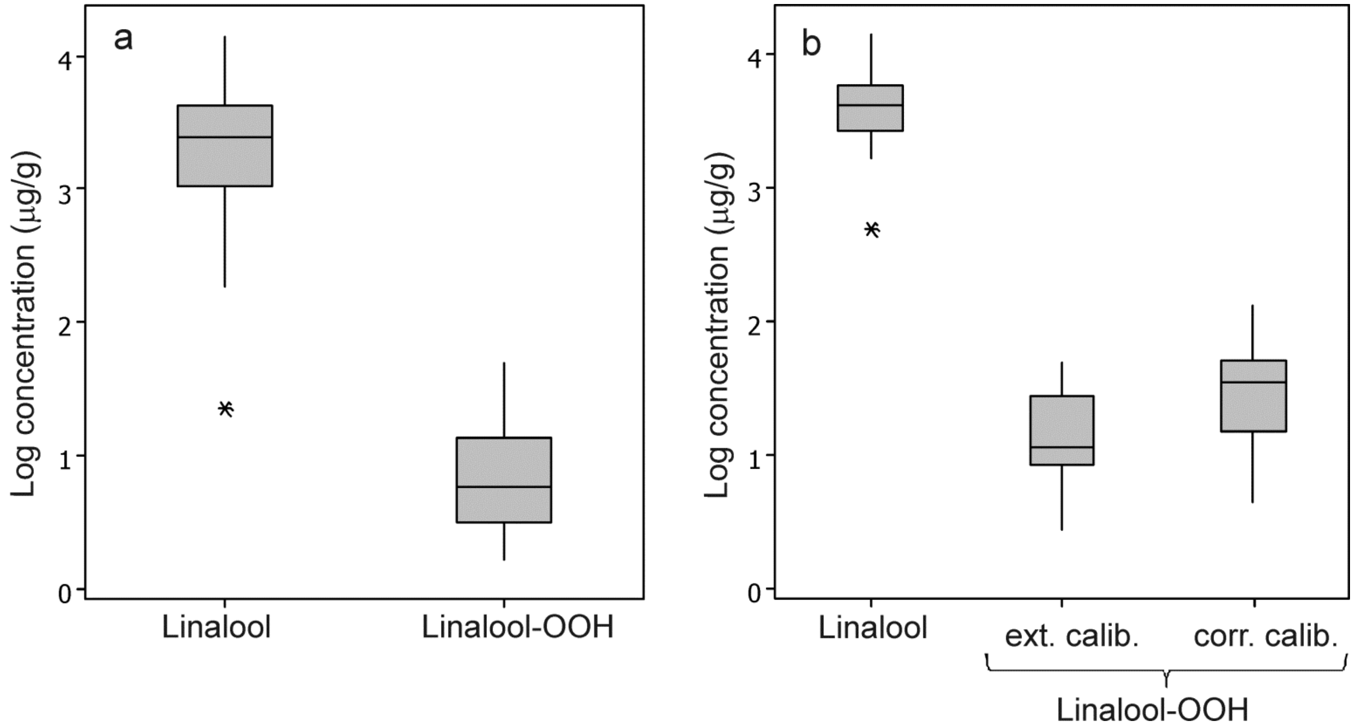
- Stability studies so far do not indicate that oxidation takes place in a final product
  - Conclusion for EDT and deodorants
  - No information on creams

Question 3: Are the hydroperoxides present in products used by the consumer?

- Studies performed with products recalled from consumers (EDT and deodorants)
- In depth analysis by GC-MS and LC-MS
- Validation with standard addition experiments

Linalool in fragrances aged 2 – 10 years in consumer homes

- Linalool hydroperoxide detectable in 33 of 39 fragrances
- Geometric mean **14 ppm** in 33 detectable samples, including matrix effect (= 0.66% of linalool content)
- Maximal level in one sample 130 µg/g
- We do not know how much is formed in product



A: all 30 samples

B: 18 samples reanalized with spiking experiments

Kern, S., et al.. Anal. Bioanal. Chem., 2014. 406(25): p. 6165-78.

Limonene in aged consumer fragrances

- 39 fragrances tested
- Limonene-OOH detected by reduction
- Only trace levels found (< 10 ppm)

Shown are the 10 samples with highest limonene content

Carveol detected in 9 of them

Successfull detection proven by spiking results

Analyte	Limonene (µg/g)	*trans*-carveol (µg/g)[2]		
Reduction		No PPh_3	With PPh_3 reduction	
Spiking agent		none	none	115 µg/g *trans*-limonene-2-OOH
Sample 31 (5)[1]	9343	2.8	4.9	123
Sample 26 (5)	8301	<LOD	<LOQ	130
Sample 24 (5)	7407	<LOD	<LOQ	124
Sample 7 (5)	6821	3.0	3.2	135
Sample 27 (5)	6748	1.7	3.9	112
Sample 37 (3)	6384	<LOQ	4.4	134
Sample 17 (2-3)	5941	<LOQ	2.8	134
Sample 30 (5)	5559	<LOD	<LOD	141
Sample 35 (7)	5152	1.9	1.7	138
Sample 33 (7)	5008	2.4	2.7	116

Exposure to linalool hydroperoxide:
Analytical and literature data calculated as dose-per area

- Levels in products 3 – 4 orders lower as compared to animal tests and patch tests

	Dose of hydroperoxide in test preparation	Dose per area
LLNA [a] Dose inducing sensitisation (EC3)	16‘000 µg/g (1.6%)	400 µg/cm^2
Patch test 2% oxidized linalool (**0.83% response**)	3’800 µg/g (0.38%)	152 µg/cm^2
Patch test 6% oxidized linalool**, diagnostic level**	10’000 µg/g (1%)	456 µg/cm^2
Patch test 11% oxidized linalool (7.2% response)	20’900 µg/g (2.09%)	836 µg/cm^2
Analytical data fine fragrance: median	14 µg/g (0.0014%)	0.031 µg/cm^2
Analytical data fine fragrance: (Max. value of n=39)	132 µg/g (0.0132%)	0.29 µg/cm^2

Do the present data establish **relevant** exposure

- It was claimed, that the maximal value in our exposure studies is not far away from the elicitation threshold in published ROAT

- **Based on maximal value:** Single extreme sample cannot explain population exposure
  - This claim is based on a single sample out of 39

- ROAT explains **elicitations not sensitization** – even if that unique sample would be at elicitation level, it could not explain widespread induction and high number of positives

- The published **ROAT may have two key limitations**
  - Simultaneous application of low and very high (3% ox. Linalool twice daily) doses
  - Potential of induction of enhanced sensitivity during ROAT

ROAT study – patient 1 (used to derive ellicitation level)

- Reaction prior to the study: + at 6%
- Reaction after the study: +++ at 0.7%

Reaction of patient 1 after the study

Concentration of oxidized linalool (% in pet.)	1
6.0	+++[a]
2.0	+++
0.70	+++
0.20	++
0.07	+

Response pattern during the study:

									Reading 1						Reading 2							Reading 3
		/day %	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Participant 1	Cream	3							-				i			16							
		1							-							-			6				17
		0.3							-							-				i, p			12
	Fine fragrance	1							-							-		r	13				17
		0.3							-							-				r			13
		0.1							-							-							2

- *Clinical picture might possibly be interpreted as active sensitization / enhancement of sensitization state and not elicitation of pre-existing sensitization*

⇒ Concomitant application of high dose appears not appropriate to determine elicitation level to pre-existing sensitization

Exposure sufficient for elicitation?

- Even with the comparison of:
  - the 'extreme' sample in the study of fragrances retrieved from consumers
  - and the 'extreme' patient 1 at the end of the ROAT – we still would get not to elicitation levels based on that aged fragrance

Table 6 Comparison of analytical results with doses in clinical and animal studies expressed as micrograms per square centimetre of a single-dose application

	Dose of hydroperoxide in test preparation	Application density	Dose per unit area
ROAT 0.3 % oxidized linalool: LOEL for elicitation[e–g]	564 µg/g (0.056 %)	10 mg/cm^2	5.64 µg/cm^2
ROAT 0.1 % oxidized linalool: NOEL for elicitation[e,f,h]	188 µg/g (0.019 %)	10 mg/cm^2	1.88 µg/cm^2
Fine fragrance: (median of positive samples; with median matrix correction factor)	14 µg/g (0.0014 %)	2.21 mg/cm^2[d]	0.031 µg/cm^2
Fine fragrance: (single sample of *n*=39 with highest content including matrix correction factor)	132 µg/g (0.0132 %)	2.21 mg/cm^2[d]	0.29 µg/cm^2

Terpene hydroperoxides in fragranced products – conclusion based on **the current state of the art**

- Conclusions below strongly affected by experience from hydroalcoholic products, and antiperspirants / deodorants (data not shown)
  - i.e. products giving highest local fragrance exposure
- Currently we have no indication that oxidation takes place in final product
- Oxidation mainly takes place in essential oils and neat products
  - Low /trace levels of hydroperoxides may then come into products by formulation
  - These levels are quite stable
  - Levels tend to be higher when natural ingredients are being used
- Storage / product parameters have surprisingly little effect
- BUT: Of course proper formulation with clean raw materials is needed
- So far we cannot derive any need for additional antioxidants, fixed shelf-life and expiry date, etc.
- Question is whether other product types show a different picture

Why do we see these frequent reactions? Three possibilities:

- ***Scenario A*: The positive reactions are**
  - **Specific** to the specified terpene hydroperoxide
  - Induction stems from **oxidized terpenes in fragranced products**
- ***Scenario B:* The positive reactions**
  - Come from oxidized terpenes present in **other exposures situations**

    A question of exposure to the hydroperoxides from other sources!
- ***Scenario C:* The positive reactions**
  - Are **not specific** to the specified terpene hydroperoxide
    - Induction stems from **other oxidized chemicals**

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Scenario B: Other exposure sources

- One may envisage that some aromatherapy application with concentrated essential oils may lead to significant exposure
  - Oxidation in neat oils proven to occur
  - Uncontrolled storage of oils for such applications may be an issue
- However: Use of such products rather a niche market – can it explain widespread induction leading to the high frequencies?

- The analytical methods have now been developed – this question can be studied
- We were considering such a study as methods are in our hands, but:
  - The fragrance industry should not act as a 'police' to judge what others do
  - This question must be eventually adressed by aromatherapy associations

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- ***Scenario A*: The positive reactions are**
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  - Induction stems from **oxidized terpenes in fragranced products**
- ***Scenario B:* The positive reactions**
  - Come from oxidized terpenes present in **other exposures situations**

- ***Scenario C:* The positive reactions**
  - Are **not specific** to the specified terpene hydroperoxide
    - Induction stems from **other oxidized chemicals**

A question of specificity of reaction

Specificity - Concomitant reactions
– cross-sensitization and tandem exposure

- **To interpret the numbers, several considerations are key:**

A) Interpretation of **doubtfuls**: Is doubtful reaction for one material indication for a possible concomitant reaction?

- Important as doubtful / irritants are more frequent as positives

B) Reproducibility of positives: How often will recorded upon re-testing?

Reproducibility of doubtfuls: How often wil retested and vice versa?

A) Dose: Hydroperoxide level in ox. linal limonene

- Higher positive rates for ox linalool
- By definition: More ox. Lin. positives among
- Frequency / concomitant reactions, if same

oxLin	**oxLim**	
+	+	Concomitant
?	+	Concomitant?
+	?	Concomitant?

1th test	**2nd test**	
+	+	reproducible positiv
?	+	Positiv?
+	?	Positiv?
?	?	Positiv?
?	-	negative?
-	?	negative?

Reproducibility of patch test reactions

- One study on reproducibility of patch test to oxidized materials: Matura *et al.* 2003
- 13 of 28 reactions to oxidized limonene were observed in both sessions
- 5 of 20 reactions to limonene hydroperoxides were observed in both sessions
- Retest reproducbility (test 1 to 2): 50% / 33%
  - 30% of 'not reproducibles' are doubtful in the other session

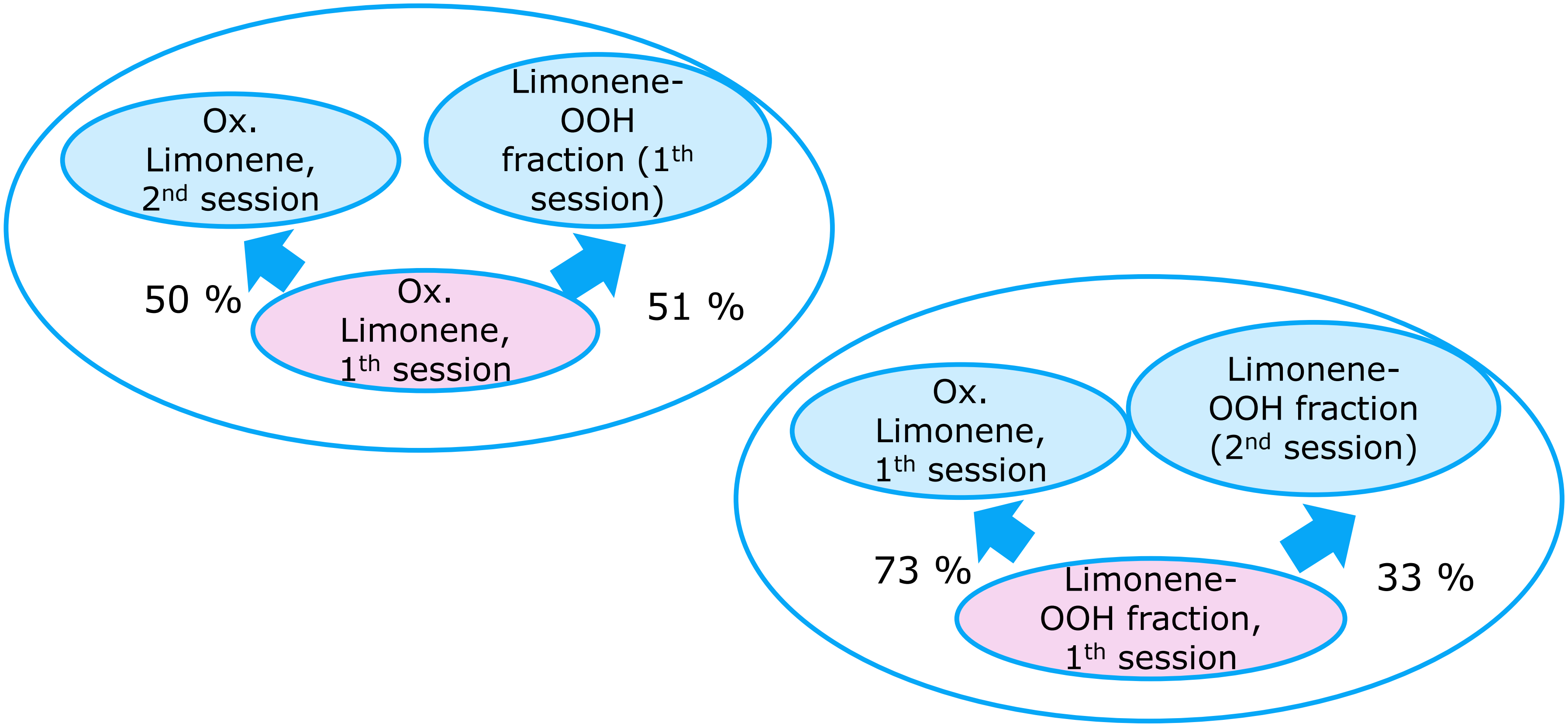
Table 4. Reproducibility of positive patch test results at retesting (test session II) in 30 subjects with contact allergy to oxidized *R*-(+)-limonene mixture and/or limonene hydroperoxide fraction at the screen testing (test session I)

				Differences in reactions between test session I and II			
				Non-positive test session I–positive test session II		Positive test session I–non-positive test session II	
	Positive reactions test session I	Positive reactions test session II	Positive reactions test sessions I and II	Negative test session I	Questionable test session I	Negative test session II	Questionable test session II
Ox. lim.[a]	26	15	13	2	0	7	6
Lim.-OOH[b]	15	10	5	3	2	7	3

[a]Ox.lim = oxidized *R*-(+)-limonene mixture, independent of the vehicle.
[b]Lim.-OOH = *R*-(+)-limonene hydroperoxide fraction, independent of the vehicle.

Reproducibility and concomitant reactions among Limonene markers in Matura, 2003

- Overall, the concomitant reactions between different oxidized limonene markers are in the same range as repeat testing with same markers.
- Only published data, but limited number of study subjects (n = 30)



Concomitant reactions in Christensson 2014

Limonene hydroperoxide analogues show specific patch test reactions

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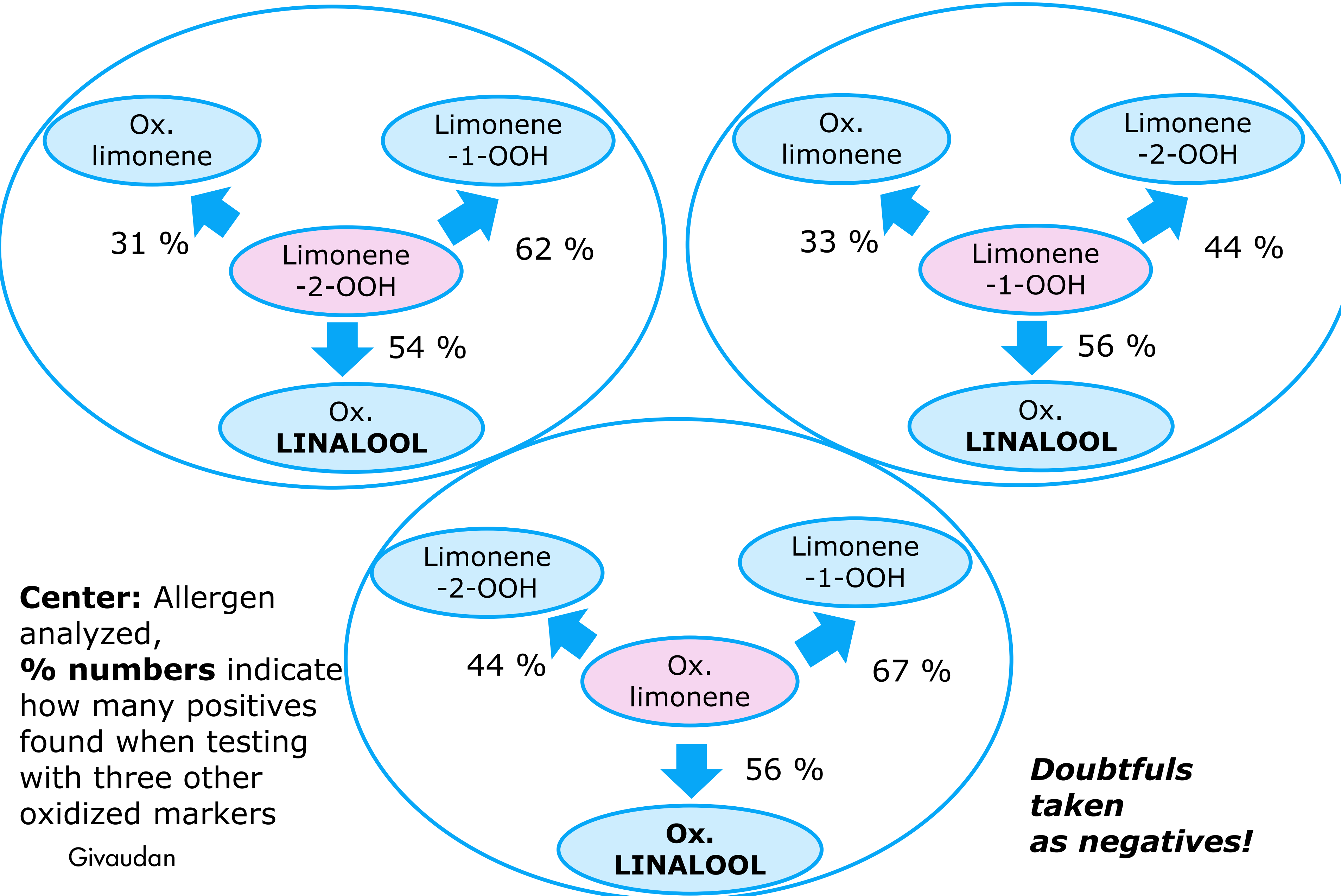
Table 3. Clinical data from individual patients showing maximum positive patch test readings to oxidized *R*-limonene, limonene-1-hydroperoxide, and limonene-2-hydroperoxide; the table also shows concomitant positive patch test reactions to fragrance markers and/or colophonium in the baseline series, as well as to oxidized linalool 6.0% pet.

						Other fragrance markers and oxidized linalool						
	Age (years)	Male (M)/ female (F)	Oxidized limonene 3.0%	Limonene-1-hydroperoxide 0.5%	Limonene-2-hydroperoxide 0.5%	Fragrance Mix I	Fragrance Mix II	*Myroxylon pereirae*	Colo-phonium	Oxidized linalool	Location of eczema	Relevant exposure [a]
2007	64	F	?	++	++	+	–	+	–	+	Hands	Cleaner
	49	M	–	+	+	–	–	–	–	+	Legs	–
	36	F	–	+	?	–	–	–	–	–	Around mouth	–
	36	M	++	++	++	+	–	+	–	++	Foot	–
	33	F	–	+	–	–	–	–	–	–	Axillae	Deodorant
	33	F	–	–	+	–	–	+	–	–	Dry skin, eczema on hands	Shower gel
2008	81	F	+++	+	+	++	?	+	+	++	Torso	–
	74	F	+	–	–	–	–	+	–	+	Axillae, torso	Positive reaction to deodorant
	66	F	+	+	–	–	–	–	–	–	Hands	Works in cookie factory
	72	F	–	++	+	–	–	–	–	–	Around ears	–
	55	F	?	+	+	?	–	++	++	+	Face	Many perfumed products
	47	F	–	–	+	–	–	–	–	–	Arms, hands	Works with paints
	33	F	+	+	+	++	–	++	++	+	Hands	–
	21	M	?	?	+	++	–	++	–	+	Around mouth	–
	49	F	–	+	–	–	–	–	–	–	Face	–
	21	F	–	+	–	–	–	–	–	–	Scalp	–
	14	F	–	+	–	–	–	–	–	–	Face, arms	Shampoo
2009	54	F	+	+	–	–	–	+	–	–	Face, around mouth	–
	32	M	+	+	?	+	–	?	–	–	Hands, feet, arms	Baker, pizza maker
	32	F	++	–	++	–	–	–	–	–	Genital area	–
	24	F	+	?	–	++	–	–	–	+	Hands	–
	57	F	–	+	+	–	–	++	–	–	Hands	–
	56	F	–	+	?	–	–	–	–	+	Hands, torso	Works in biscuit factory
	14	M	–	+	–	–	–	–	–	–	Face	–
	38	F	–	–	++	–	–	–	–	–	Hands	Sunscreen

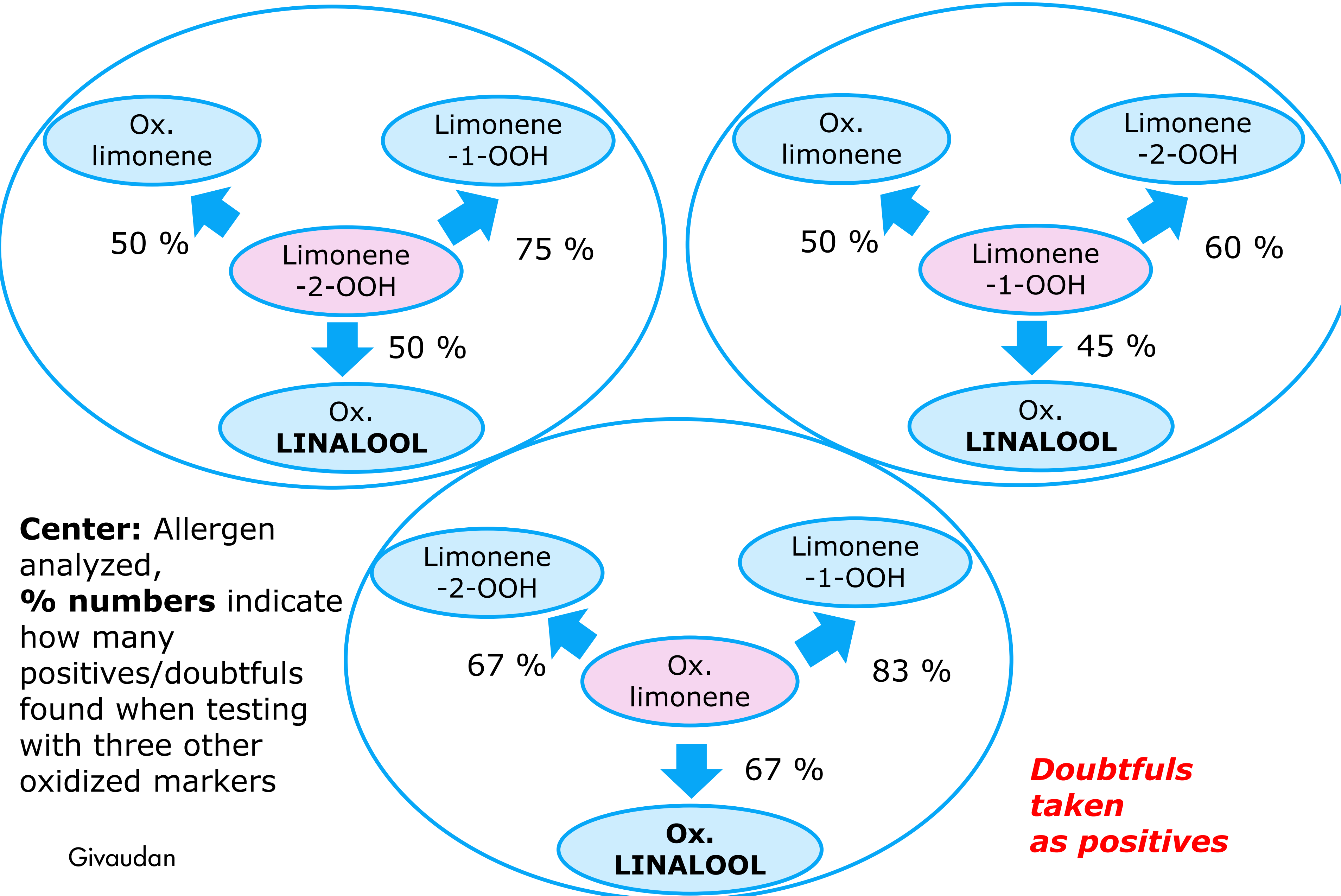
Analysis of concomitant reactions in Christensson 2014, Table 3



Center: Allergen analyzed,
% numbers indicate how many positives found when testing with three other oxidized markers

Doubtfuls taken as negatives!

Analysis of concomitant reactions in Christensson 2014, Table 3



Center: Allergen analyzed,
% numbers indicate how many positives/doubtfuls found when testing with three other oxidized markers

Doubtfuls taken as positives

Concomitant reactions in Christensson 2014

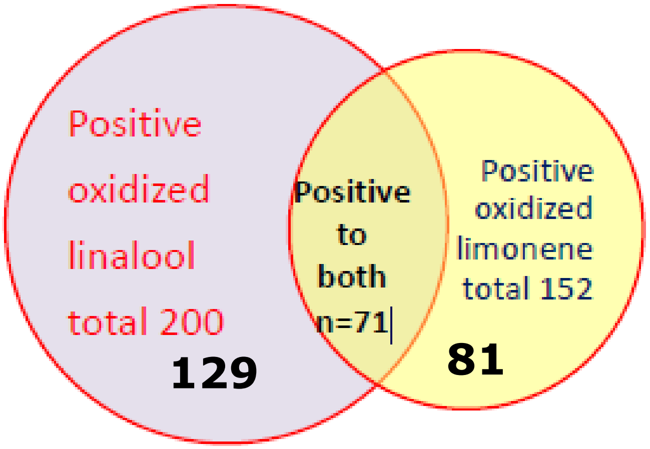
- Reaction to Limonene-2-OOH informs us better about probability of reaction to oxidized linalool than to oxidized limonene
  - Despite the fact that oxidized limonene, but not linalool contains Limonene-2-OOH

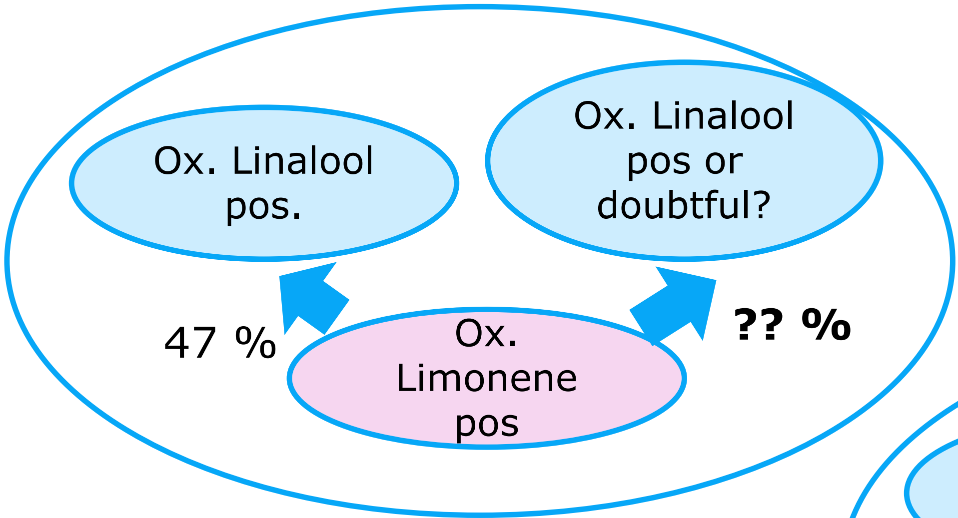
- Conclusion from these data would in my eyes not be that ox. linalool- and ox limonene sensitivity are unrelated, but that they **are** related

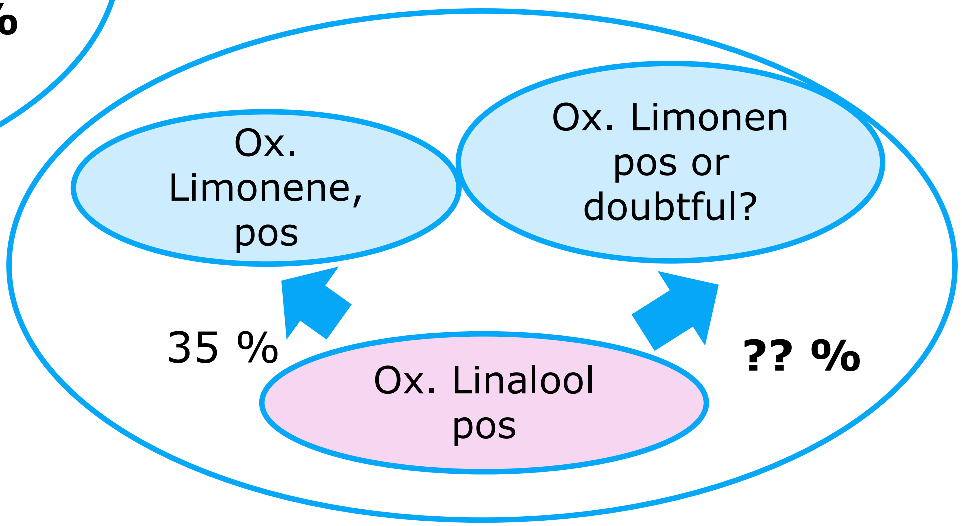
- However: These data need to be treated with caution:
  - Low patient numbers
  - Reproducibility of patch test reactions
  - Different test concentrations
  - **More data are needed for firm conclusions!**

Concomitant reactions between large studies (Christensson 2012, 2013)

- Lower figures than in Christensson 2014, but still in range of published retest reliability

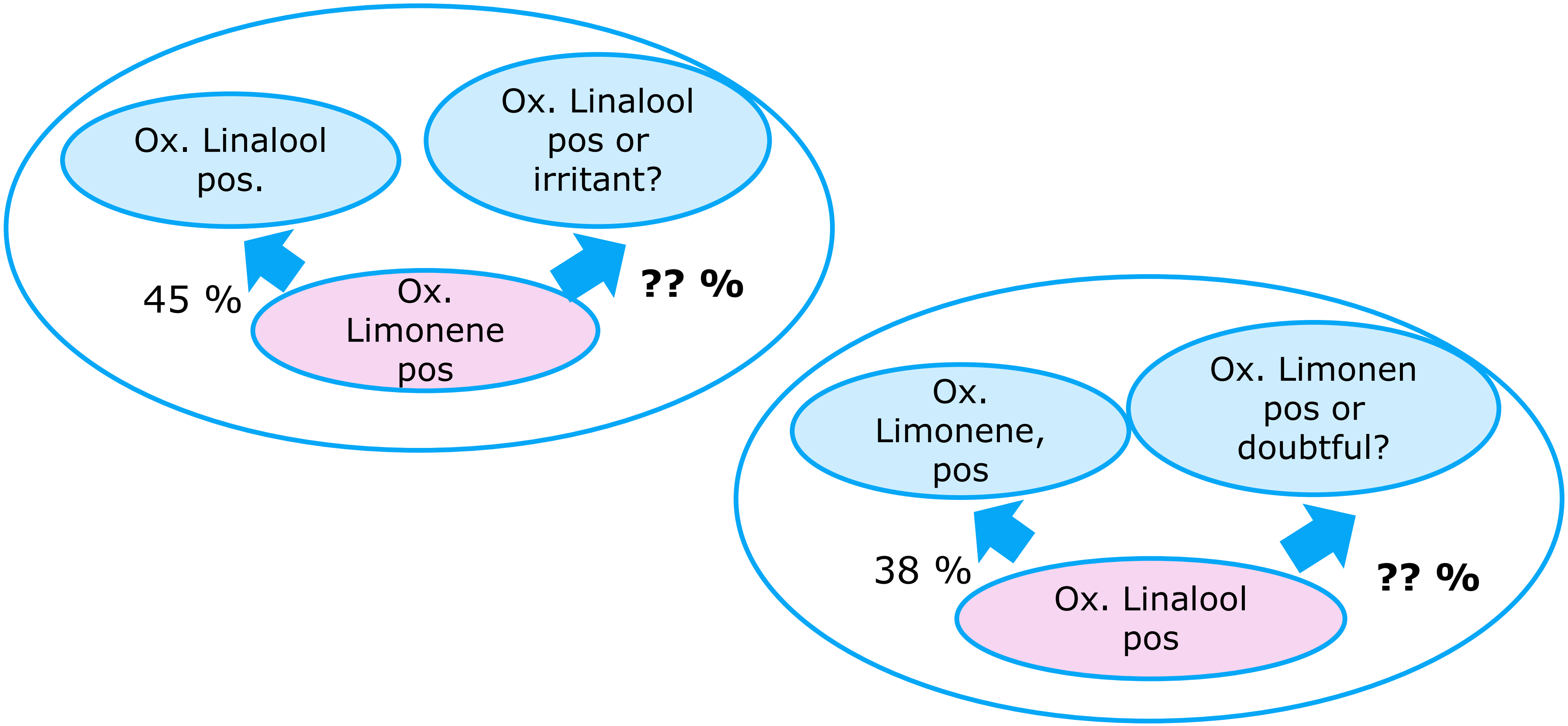






Concomitant reactions Audrain et al., 2014

- Very similar Figures in Audrain et al.



Concomitant reactions between large studies (Christensson 2012, 2013; Audrain; 2014)

- All studies found higher rates of doubtfuls than positives
  - There is clearly a gradient of reaction strength
- We would need detailed data also on the doubtfuls /irritants
- To further discuss we would need the below Table filled and we need reliable blind re-test reproducibility data
- We would need the data for both the Christensson and Audrain papers

	Patients Positive to Linalool-OOH	Patient Irritant/ doubtful linalool-OOH	Patients with no reaction at all to Linalool-OOH
Patient Positive to Limonen-OOH			
Patient Irritant/doubtful Limonen-OOH			
Patients with no reaction at all to Limonen-OOH			

What patch test evidence do we take to prove or disprove relationship among related allergens?

- Most cited case of clear cross-sensitization: Isoeugenol and its esters
- It is commonly agreed that patients sensitive to isoeugenol will also react to isoeugenol acetate, due to the extermly rapid hydrolysis in skin (See data JPL)
- Published patch test evidence: Only 32.5% of isoeugenol positives do react to the acetate:
  - We would never accept this (low) figure as a proof of **not existing** cross-sensitization

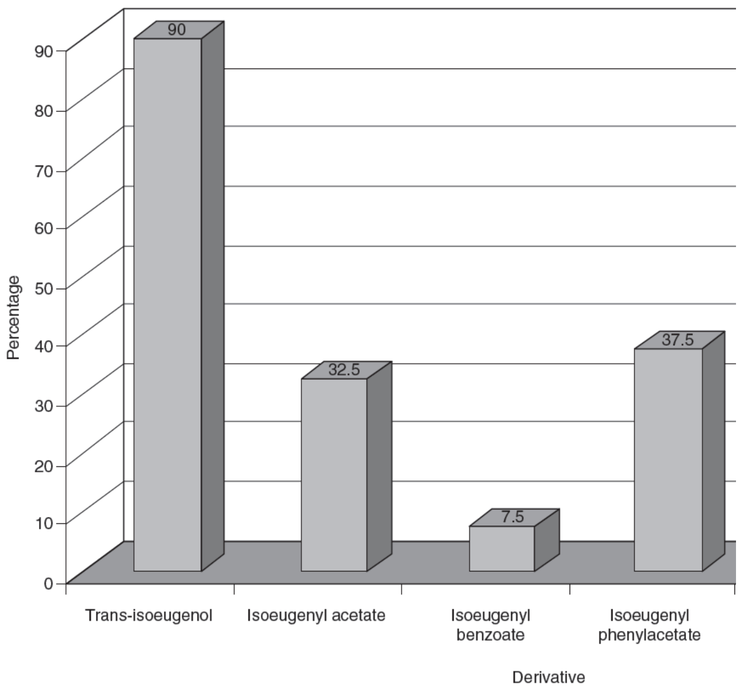


Fig. 3. Isoeugenol is positive: concomitant reactions to derivatives.

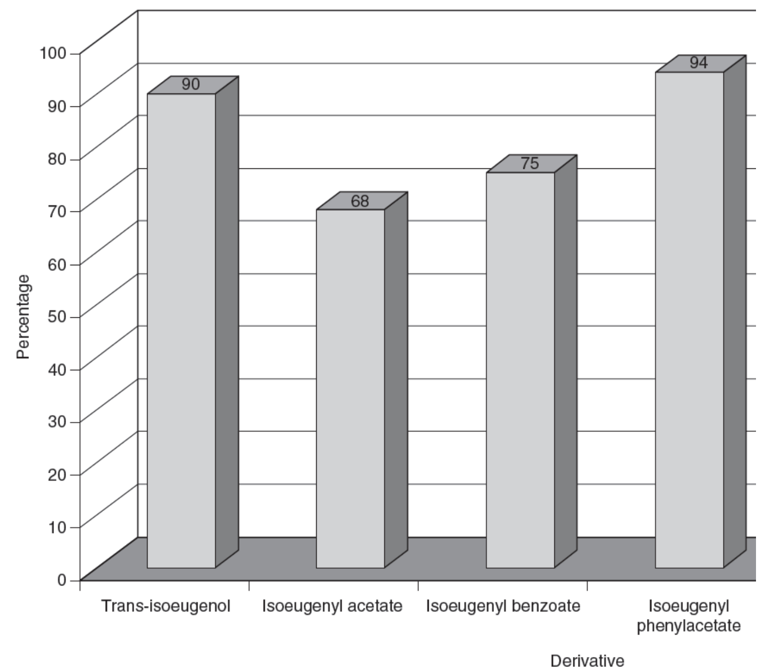


Fig. 2. Derivative is positive: concomitant reactions to isoeugenol.

Why do we see these frequent reactions? Three possibilities:

- ***Scenario A*: The positive reactions are**
  - **Specific** to the specified terpene hydroperoxide
  - Induction stems from **oxidized terpenes in fragranced products**
- ***Scenario B:* The positive reactions**
  - Come from oxidized terpenes present in **other exposures situations**

A question of exposure to the hydroperoxides!

- ***Scenario C:* The positive reactions**
  - Are **not specific** to the specified terpene hydroperoxide

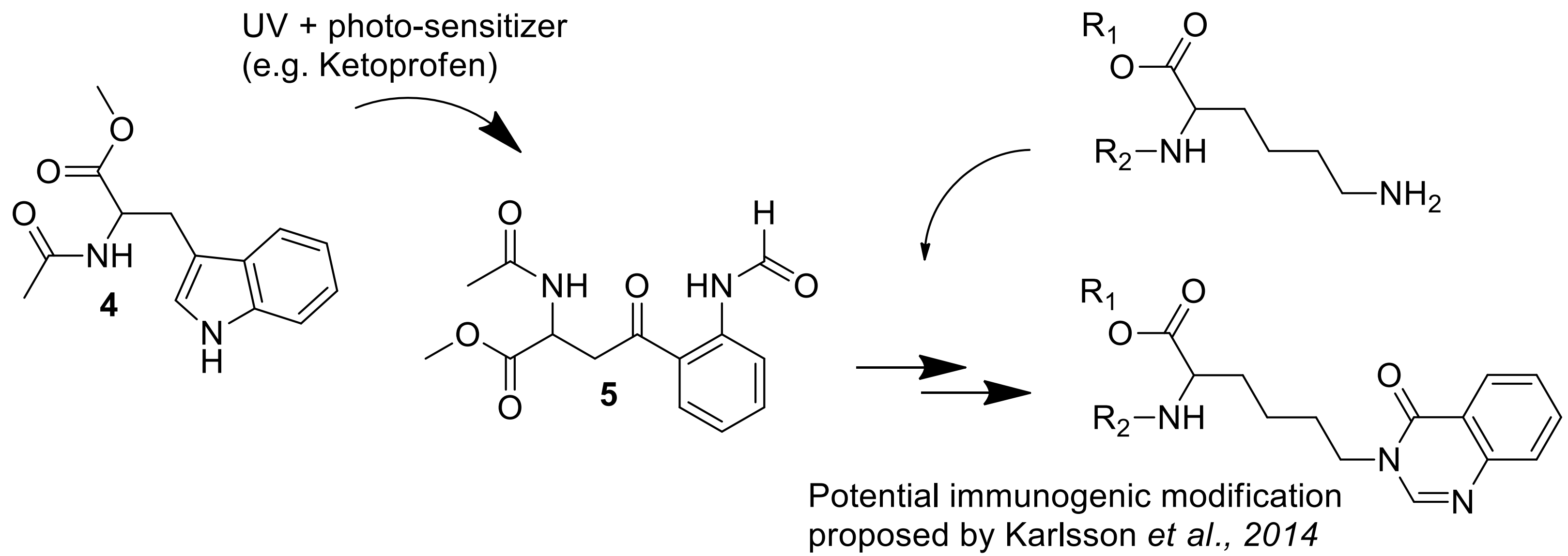
A question of specificty of the reaction!

  - Induction stems from **other oxidized chemicals**

Does oxidation play a role?

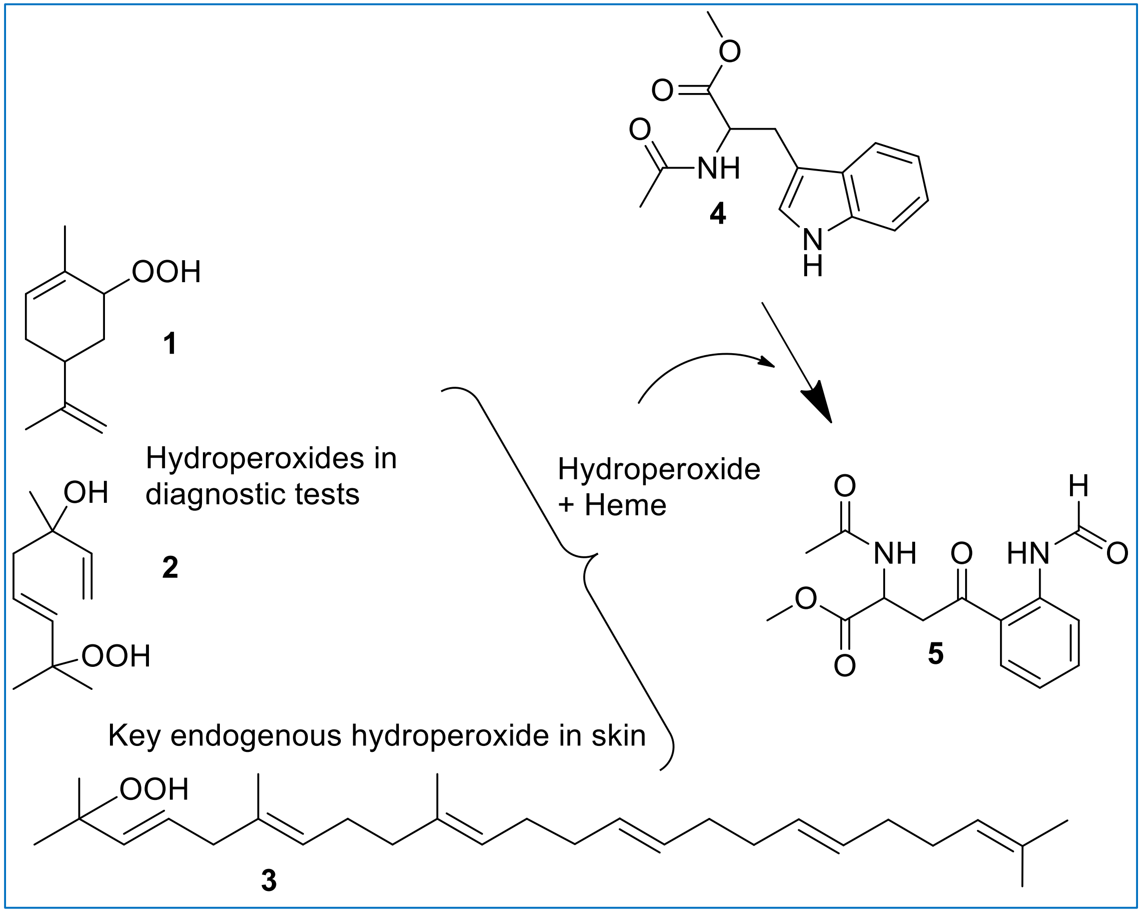
Oxidative events triggered by hydroperoxides

- Next to specific hapten formation, hydroperoxides trigger oxidation events
- Such oxidation events were proposed as possible explanation for cross-sensitation between different photosensitizers (Karlsson *et al.*, 2014)



Oxidative events triggered by hydroperoxides

- Tryptophan oxidation by hydroperoxides previously shown by Kao *et al.*, 2014
- We investigated this in more detail
- In presence of iron in the heme form (i.e. Physiologically predominant form), this process is particularly efficient
- Also works for key endogeneous hydroperoxide in the skin



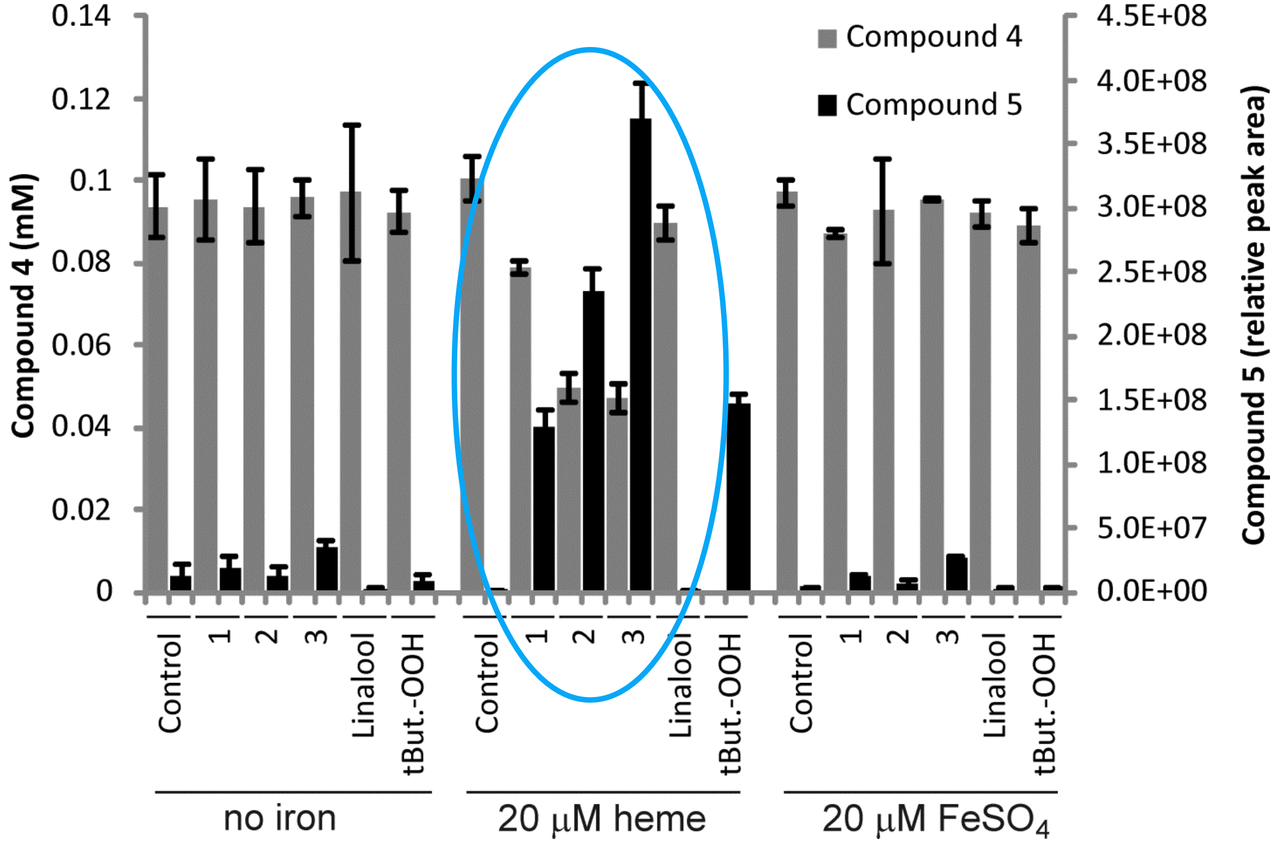
Oxidative events triggered by hydroperoxides

- Formation of formyl-kynurenine metabolite after 24 h incubation in the presence of different test chemicals and iron sources.

1: Limonene -OOH

2: Linalool-OOH

3: Squalene-OOH



Let’s not argue – let’s test….

Proposal

1. Further **exposure data needed**, focus on moisturizers, creams and lotions

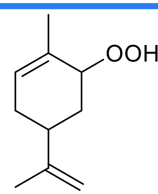
2. **Analytical data on products** used by positively tested patients

3. Enrol patients with a positive (and ev. doubtful) reaction to one or both of oxidized Linalool / Limonene in a **follow-up patch test** with different oxidized materials.

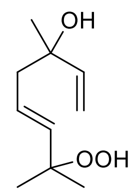
   Three aims:
   1. Repeated tests – **reproducibility**
   2. Blind tests – **method validation**
   3. Different pure hydroperoxides tested simultaneously– **Specificity of reaction**

Potential study set-up

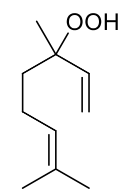
- Oxidized terpenes
  - retest reliability?
- In parallel reaction to natural and non-natural hydroperoxides
  - Specificity?
- Equimolar level of hydroperoxides in all samples
  - Two doses: e.g. 20 / 40 mM
- Blind testing and duplicate testing *
  - Method validation



1) Limonene-2-OOH, main oxidation product in oxidized limonene, EC3 = 0.83 %

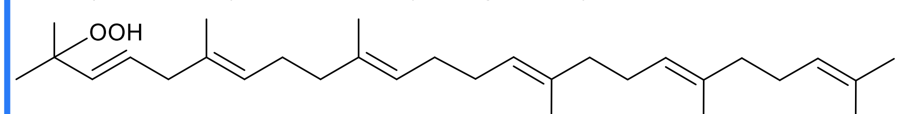


2) Linalool-7-OOH, main oxidation product in oxidized Linalool, EC3 = 1.6%



3) Linalool-hydroperoxide, **NON-NATURAL hydroperoxide, never found from linalool oxidation** EC3 = 1.1% (*)

4) Squalene-hydroperoxide, Key endogenous hydroperoxide in skin



5) Oxidized Linalool - containing ca. 16% Linalool-6/7-OOH

6) Oxidized Limonene - containing ca. 16% Limonene-1/2-OOH

} Commerical patch test preparations

* Both sides of the back as in Grollhausen et al., 1989

Possible outcomes

- *Current assumptions:*
  Hydroperoxides are present in fragranced consumer products at sensitizing doses, causing specific contact allergy

- **In this case we see reactions only to the hydroperoxides contained in the oxidized samples for which patients were previously positive**

- *Alternative proposal:*
  Some patients are sensitive to the oxidative nature of hydroperoxides and cross-react to different hydroperoxides.

- **In this case we see frequent co-sensitization**
- **we would even find positives to the non-natural hydroperoxide**
- **Association between oxidized terpenes and hydroperoxides contained therein is only loose**

Why do we see these frequent reactions? Three possibilities:

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- ***Scenario B:* The positive reactions**
  - Come from oxidized terpenes present in **other exposures situations**
- ***Scenario C:* The positive reactions**
  - Are **not specific** to the specified terpene hydroperoxide
    - Induction stems from **other oxidized chemicals**

In our view – we cannot currently conclude which scenario offers the true answer.

Thank you

Contact