GARDskin Dose-Response assay for PoD determination of fragrance materials and its application in conducting Quantitative Risk Assessment (QRA)

Abstract
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Abstract

The global fragrance industry applies Quantitative risk assessment (QRA) to develop risk management practices (IFRA Standards) for ingredients that are identified as potential dermal sensitizers. An important step in QRA is determination of a "No Expected Sensitization Induction Level" (NESIL), which has historically been determined using human data with the support of animal data (e.g., murine local lymph node assay (LLNA). The EC3 value determined in the LLNA is used as the guidance for selection of the dose level in HRIPTs (Human Repeated Insult Patch Test) to confirm a NESIL value. The fragrance industry has adopted new approach methodologies (NAM) to address skin sensitization. Although several NAMs for identifying skin sensitizers have been accepted as Test Guidelines by OECD, these methods have thus far been validated only for hazard identification. Since a NESIL value is a key requirement to evaluate sensitizing potency for conducting QRA evaluations, development of a NAM-based strategy capable of providing potency data in the form of NESIL remains a high priority for the fragrance industry.

The *in vitro* GARDskin assay was recently adopted by the OECD (TG 442E) for the hazard identification of skin sensitizers. Continuous potency predictions are derived using a modified protocol that incorporates dose-response measurements. Linear regression models have further been developed to predict LLNA EC3 and human NESIL values.

The aim of the study was to evaluate the precision and reproducibility of the continuous potency predictions from the GARDskin Dose-Response assay. A total of 17 test materials were evaluated, 11 of which were tested in three blinded studies separated in time. Preliminary results indicated that the GARDskin Dose-Response model predicted LLNA EC3 values and human NESIL values with geometric mean fold-misprediction factors of 3.8 and 2.5, respectively. For comparative reasons, the LLNA EC3 predicted the human NESIL values with a fold-misprediction factor of 3.7 in the same dataset. Results from the repeated assessment of the test materials were reproducible, with an estimated geometric mean range of fold-changes between replicates of 2.9.

Using isocyclocitral (CAS 1335-66-6) as an example, a QRA was conducted to determine its safe use levels in different consumer product types.

The results demonstrate that the LLNA EC3 values and the human NESIL values predicted from the GARDskin Dose-Response assay are reproducible between experiments and show good concordance with the published NESIL and EC3 values. Together with the reported performance data, this represents a major step towards the establishment of the assay as a relevant source of information to derive NESIL values for conducting QRA evaluations for fragrance materials to ensure product safety while avoiding the generation of new animal data.

Methods

The GARDskin dose response protocol

The GARDskin DR protocol is based on the validated protocols of GARDskin as outlined in OECD TG 442E[1]. In short, for each test item, cellular stimulations were performed in an extended range of concentrations (≥6), to investigate the dose-response relationship between GARDskin classifications (Decision Values, DVs) and test item concentrations. From the resulting dose-response curve, a cDV_0 value was identified, corresponding to the lowest concentration required to exceed the binary classification threshold in GARDskin (DV ≥ 0). Resulting cDV_0 concentrations were used to predict LLNA EC3, and human NESIL values, using regression models developed to exploit the significant linear relationship between cDV_0 and above-mentioned potency metrics[2].

Results

Predicted NESIL and EC3 vs Experimental NESIL and EC3

Test Item	Name	Predicted Human NOEL (µg/cm²) I	Reference Human NOEL (µg/cm²) I	Predicted LLNA EC3%	Reference LLNA EC3%I
TS-a758	Limoxal	361 (129, 1010)	5510	1.18 (0.605, 2.31)	22.7
TS-a759	Cedryl acetate	3300 (1180, 9220)	NA (6400 calculated value)	7.95 (3.95, 16)	26.05
TS-a760	Hexen-2-al	53 (11.2, 250)	18	0.222 (0.0872, 0.563)	3.56
TS-a761	Methyl-2-nonynoate	30.8 (3.89, 244)	24	0.156 (0.0463, 0.525)	3.33
TS-a762	Methyl Heptine Carbonate	111 (26.5, 461)	118	0.44 (0.185, 1.05)	0.65
TS-a763	Anisyl alcohol	-	1771	-	5.9
TS-a764	p-t-Butyl-dihydrocinnamaldehyde (Bourgenol)	575 (221, 1500)	1181	1.78 (0.938, 3.37)	4.3
TS-a765	Isocyclocitral	9970 (1880, 53000)	7087	17.9 (6.24, 51.5)	7.3
TS-a766	Ylang Ylang oil (UVCB)	5100 (1390, 18700)	1771	11.5 (4.8, 27.6)	6.8
TS-a767	Herbac (contains 2 contituents)	Non-sensitizor	NA	-	NA
TS-a768	Ylanganate	Non-sensitizor	NA	-	NA
TS-a769	Ethyl Vanillin	19200 (2750, 134000)	NA	31.2 (9.33, 104)	NA

I)95 % confidence interval for cDV_0, EC3 and NOEL within brackets.

Figure 1. GARDskin DR predicted LLNA EC3 & Human NESIL values compared to reference benchmark data. Geometric mean fold-misprediction factors of 2.37 and 4.88 respectively**.**

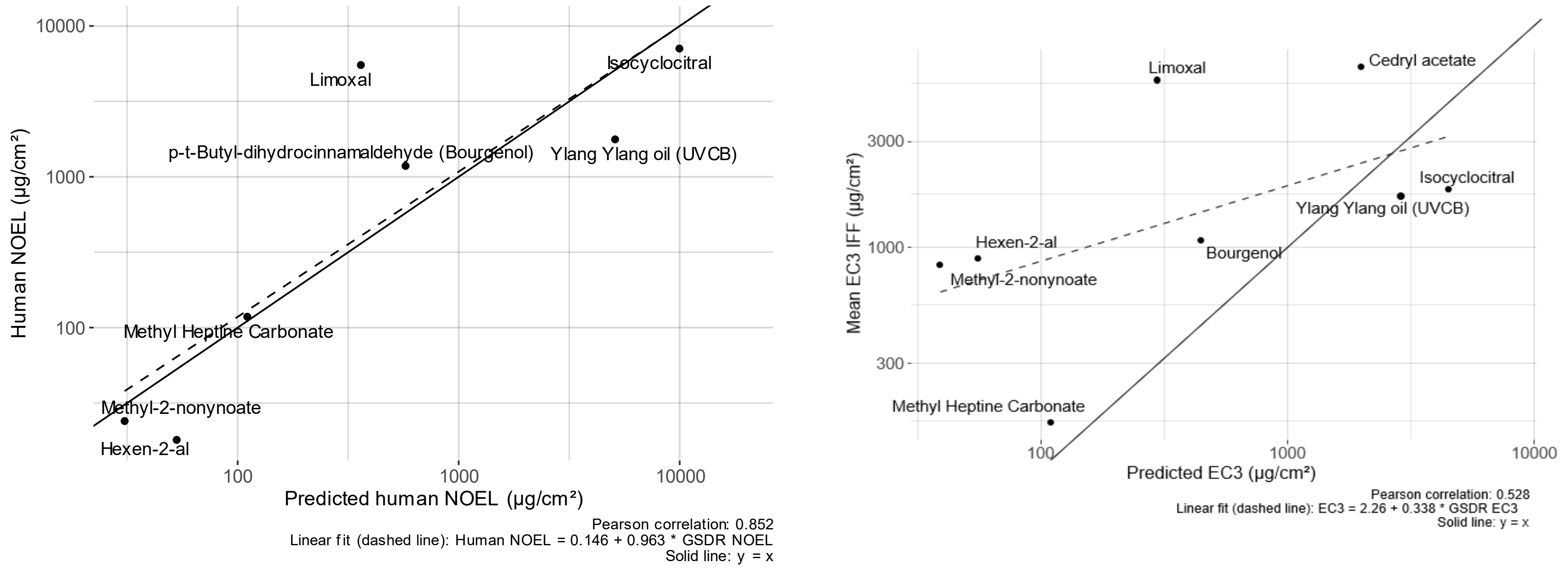


Figure 2. GARDskin DR predicted Human NESIL vs benchmark reference NESIL and GARDskin DR predicted EC3 vs benchmark reference EC3. GARDskin DR predicted human NESIL values correlated well with reference NESIL values, with a Pearson correlation of 0.85. GARDskin DR predicted EC3 values correlated moderately well with reference EC3 values, with a Pearson correlation of 0.53.

Reproducibility data

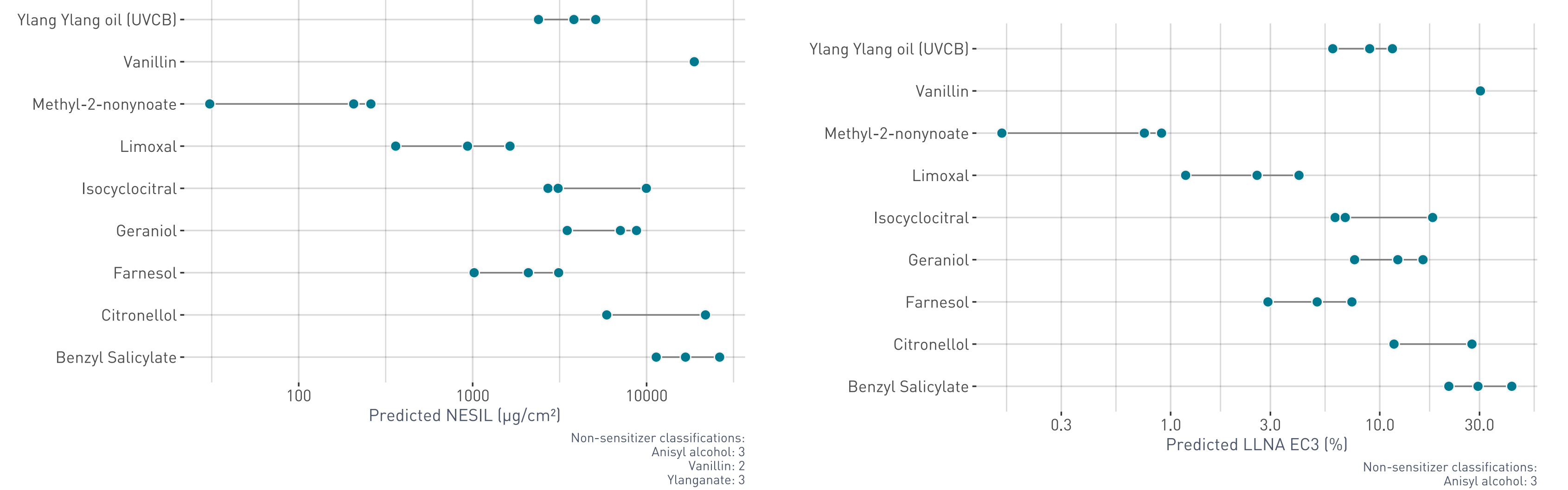
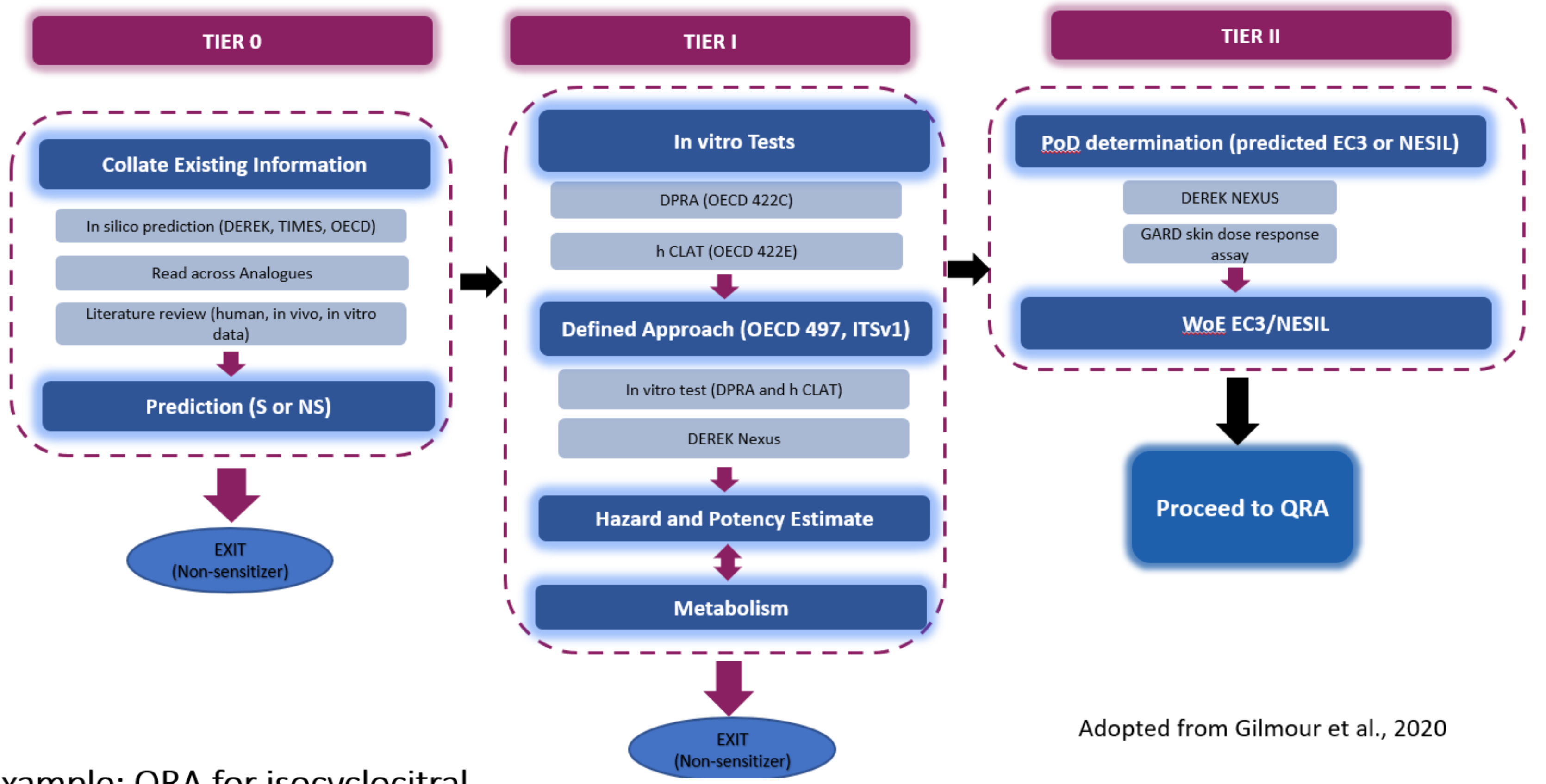


Figure 3. GARDskin DR reproducibility of predictions from repeated experiments (n=3). Predicted NESIL and LLNA EC3 values from replicate measurements were highly reproducible, with a median range of fold-changes between replicate of 2.5.

NGRA framework for Quantitative Risk Assessment (QRA) for Determining Maximum Use concentration of Fragrance materials in cosmetic products



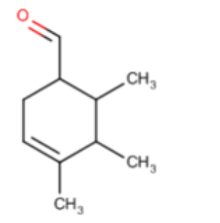
TIER 0
- Collate Existing Information
  - In silico prediction (DEREK, TIMES, OECD)
  - Read across Analogues
  - Literature review (human, in vivo, in vitro data)
- Prediction (S or NS)
- EXIT (Non-sensitizer)

TIER I
- In vitro Tests
  - DPRA (OECD 422C)
  - h CLAT (OECD 422E)
- Defined Approach (OECD 497, ITSv1)
  - In vitro test (DPRA and h CLAT)
  - DEREK Nexus
- Hazard and Potency Estimate
- Metabolism
- EXIT (Non-sensitizer)

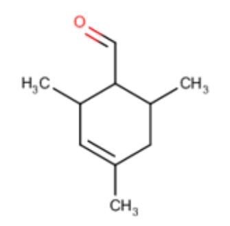
TIER II
- PoD determination (predicted EC3 or NESIL)
  - DEREK NEXUS
  - GARD skin dose response assay
- WoE EC3/NESIL
- Proceed to QRA

Adopted from Gilmour et al., 2020

Example: QRA for isocyclocitral



- **Tier 0**
  - Exposure estimation
    - ❑ Not applicable: QRA will determine the maximum use concentration
  - Existing data
    - ❑ In silico predictions
      - ❖ TIMES SS: Parent is a sensitizer (Cat 1B), All metabolites predicted to be non-sensitizers
      - ❖ DEREK: Plausable Sensitizer (Predicted EC3: 4.9% or 1225 ug/cm2)
      - ❖ OECD toolbox: Sensitizer, reacts with skin proteins
    - ❑ in vitro and in vivo data
      - ❖ No data available
    - ❑ Read across chemicals
      - ❖ 1423-46-7
      - ❖ Tanimoto score: 98%
      - ❖ In silico approaches predict to be a sensitizer
      - ❖ No in vivo or in vitro data available



Tier 0 Conclusion: Chemical is predicted to be a sensitizer

Tier I

❑Invitro tests
- ❖ DPRA: mean lysine and cysteine depletion ~30%
- ❖ hCLAT: MIT is 120 ug/ml

❑Defined approach
- ❖ Apply Integrated Testing Strategy ITSv1[(4)] for hazard classification and potency sub-categorization according to UN GHS
- ❖ Conducting assays addressing KE1 (DPRA) and KE3 (h-CLAT)
- ❖ In silico prediction – Derek Nexus
- ❖ Simple score-based system to determine potency

Score	h-CLAT MIT* ug/mL	DPRA Mean Cysteine and Lysine% depletion	Derek Nexus prediction
3	<=10	>=42.47	
2	>10 - 150	22.62 - <42.47	
1	>150 - 5000	6,38 - <22.62	Positive
0	Not calculated	<6.38	Negative

*Minimal Induction Threshold
MIT = min(EC150 CD86, EC200 CD54)

Score	h-CLAT MIT ug/mL	DPRA Mean Cysteine and Lysine% depletion	Derek Nexus prediction
Result	120	30%	Positive
Score	*2*	*2*	*1*
Total Score	5		
Potency prediction	1B		

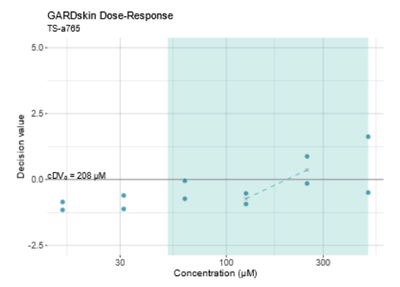
Potency	Total Battery Score
1A	*6-7*
1B	*2-5*
Not Classified	*0-1*

Reference:
(4) OECD Guideline No.497 (2021) Guideline on Defined Approach for Skin Sensitisation

Tier 1 Conclusion: Chemical is a skin sensitizer (1B)

Tier II

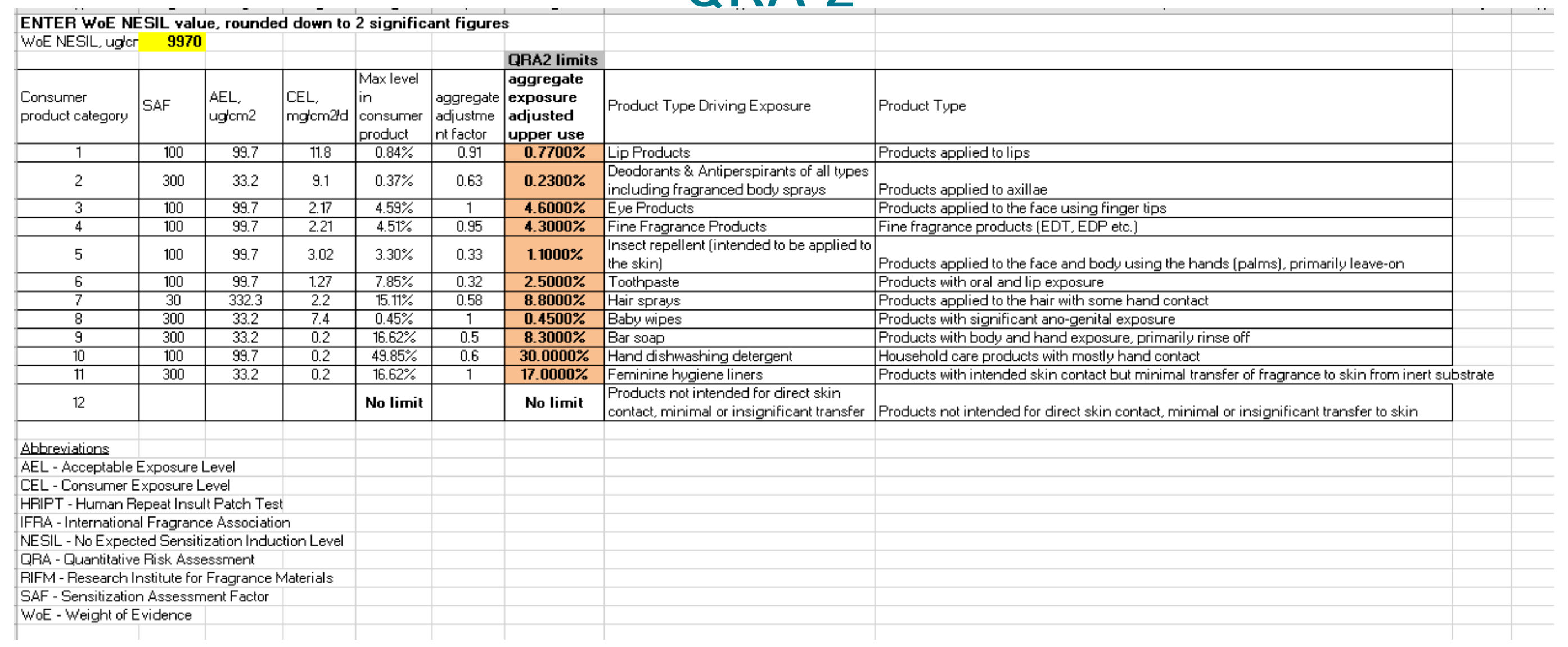
- PoD determination (predicted EC3/NESIL)
  - ❑GARD Potency Assay
    - ❖208 (51.5, >500)*, DV≥ 0, Sensitizer
    - ❖Predicted EC3: 17.9 ug/cm2 (6.24, 51.5)*
    - ❖Predicted NESIL: 9970 ug/cm2 (1880, 53000)*
    - * 95 % confidence interval for cDV_0, EC3 and NOEL within brackets.



- WoE NESIL
  - ❑9970 ug/cm2

Tier 3 Conclusion: A NESIL of 9970 ug/cm2 will be used for QRA 2

QRA 2



ENTER WoE NESIL value, rounded down to 2 significant figures
WoE NESIL, ug/cr **9970**

Consumer product category	SAF	AEL, ug/cm2	CEL, mg/cm2/d	Max level in consumer product	aggregate adjustment factor	QRA2 limits **aggregate exposure adjusted upper use**	Product Type Driving Exposure	Product Type
1	100	99.7	11.8	0.84%	0.91	**0.7700%**	Lip Products	Products applied to lips
2	300	33.2	9.1	0.37%	0.63	**0.2300%**	Deodorants & Antiperspirants of all types including fragranced body sprays	Products applied to axillae
3	100	99.7	2.17	4.59%	1	**4.6000%**	Eye Products	Products applied to the face using finger tips
4	100	99.7	2.21	4.51%	0.95	**4.3000%**	Fine Fragrance Products	Fine fragrance products (EDT, EDP etc.)
5	100	99.7	3.02	3.30%	0.33	**1.1000%**	Insect repellent (intended to be applied to the skin)	Products applied to the face and body using the hands (palms), primarily leave-on
6	100	99.7	1.27	7.85%	0.32	**2.5000%**	Toothpaste	Products with oral and lip exposure
7	30	332.3	2.2	15.11%	0.58	**8.8000%**	Hair sprays	Products applied to the hair with some hand contact
8	300	33.2	7.4	0.45%	1	**0.4500%**	Baby wipes	Products with significant ano-genital exposure
9	300	33.2	0.2	16.62%	0.5	**8.3000%**	Bar soap	Products with body and hand exposure, primarily rinse off
10	100	99.7	0.2	49.85%	0.6	**30.0000%**	Hand dishwashing detergent	Household care products with mostly hand contact
11	300	33.2	0.2	16.62%	1	**17.0000%**	Feminine hygiene liners	Products with intended skin contact but minimal transfer of fragrance to skin from inert substrate
12				**No limit**		**No limit**	Products not intended for direct skin contact, minimal or insignificant transfer	Products not intended for direct skin contact, minimal or insignificant transfer to skin

Abbreviations
AEL - Acceptable Exposure Level
CEL - Consumer Exposure Level
HRIPT - Human Repeat Insult Patch Test
IFRA - International Fragrance Association
NESIL - No Expected Sensitization Induction Level
QRA - Quantitative Risk Assessment
RIFM - Research Institute for Fragrance Materials
SAF - Sensitization Assessment Factor
WoE - Weight of Evidence

Conclusions

- The continous readout from the assay is reproducible and the assay predicts LLNA EC3 and human NESIL values with high correlation to reference benchmark data (geometric mean fold-misprediction factors of 3.8 and 2.5 respectively)
- The assay provides a nice tool for the fragrance industry to predict the NESIL value which can be used for conducting the quantitative risk assessment for generating the IFRA standard.