Implementation of *in vitro* methods in the safety evaluation of the skin sensitization potential of chemicals under REACH regulation

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REACH and SKIN SENSITISATION

REACH, Registration Evaluation Authorisation and Restriction of Chemicals

REACH Regulation (EC 1907/2006) asks for a complete registration dossier for all substances marketed in the EU in quantity above 1 t/y. This registration dossier contains toxicological information with increasing complexity in relation to the yearly tonnage band of the registered substance. Since 2007, EU Companies have registered more than 20,000 substances[1] to ECHA, the European Chemical Agency.

SKIN SENSITISATION

Skin sensitization endpoint is considered fundamental to guarantee safety of both workers and consumers and the assessment is mandatory for all substances, independently on the tonnage band excluding only intermediates used in low quantities and under strictly controlled conditions.

Point 8.3 in Annex VII in REACH, original text (2007)

COLUMN 1 STANDARD INFORMATION REQUIRED	COLUMN 2 SPECIFIC RULES FOR ADAPTATION FROM COLUMN 1
8.3. Skin sensitisation The assessment of this endpoint shall comprise the following consecutive steps: (1) an assessment of the available human, animal and alternative data, (2) *In vivo* testing.	8.3. Step 2 does not need to be conducted if: – the available information indicates that the substance should be classified for skin sensitisation or corrosivity; or – the substance is a strong acid (pH < 2,0) or base (pH > 11,5); or – the substance is flammable in air at room temperature. The Murine Local Lymph Node Assay (LLNA) is the first-choice method for *in vivo* testing. Only in exceptional circumstances should another test be used. Justification for the use of another test shall be provided.

Point 8.3 in Annex VII in REACH, Amended by Regulation 2016/1688

'8.3. Skin sensitisation Information allowing: — a conclusion whether the substance is a skin sensitiser and whether it can be presumed to have the potential to produce significant sensitisation in humans (Cat. 1A), and — risk assessment, where required.	The study(ies) under point 8.3.1 and 8.3.2 do not need to be conducted if: — the substance is classified as skin corrosion (Category 1), or — the substance is a strong acid (pH ≤ 2,0) or base (pH ≥ 11,5), or — the substance is spontaneously flammable in air or in contact with water or moisture at room temperature.
8.3.1. Skin sensitisation, *in vitro/in chemico* Information from *in vitro/in chemico* test method(s) recognised according to Article 13(3), addressing each of the following key events of skin sensitisation: (a) molecular interaction with skin proteins; (b) inflammatory response in keratinocytes; (c) activation of dendritic cells.	The(se) test(s) do not need to be conducted if — an *in vivo* study according to point 8.3.2 is available, or — the available *in vitro/in chemico* test methods are not applicable for the substance or are not adequate for classification and risk assessment according to point 8.3. If information from test method(s) addressing one or two of the key events in column 1 already allows classification and risk assessment according to point 8.3, studies addressing the other key event(s) need not be conducted.
8.3.2. Skin sensitisation, *in vivo*	An *in vivo* study shall be conducted only if *in vitro/in chemico* test methods described under point 8.3.1 are not applicable, or the results obtained from those studies are not adequate for classification and risk assessment according to point 8.3. The murine local lymph node assay (LLNA) is the first-choice method for *in vivo* testing. Only in exceptional circumstances should another test be used. Justification for the use of another *in vivo* test shall be provided. *In vivo* skin sensitisation studies that were carried out or initiated before 11 October 2016, and that meet the requirements set out in Article 13(3), first subparagraph, and Article 13(4) shall be considered appropriate to address this standard information requirement.'

VALIDATED *in vivo* METHODS

OECD 429 - Skin Sensitization: Local Lymph Node Assay (first adoption year 2002)
OECD 442A - Skin Sensitization: Local Lymph Node Assay: DA (first adoption year 2010)
OECD 442B - Skin Sensitization: Local Lymph Node Assay: BrdU-ELISA (first adoption year 2010)

VALIDATED *in vitro* METHODS

OECD 442C - In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA) (first adoption year 2015)
OECD 442D - In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method (first adoption year 2015)
OECD 442E - In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome pathway for Skin Sensitisation (first adoption year 2017)- Human Cell Line Activation test (h-CLAT) -U937 cell line activation Test (U-SENS™)

OECD Series on Testing and Assessment

No.168: The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins
Part 1: Scientific Evidence
Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing Approaches

ECHA Guidance on Information Requirements and Chemical Safety Assessment

Chapter R.7a: Endpoint specific guidance, Section R.7.3 Skin and Respiratory sensitisation
First publication in 2008, including only the LLNA
Main revision in 2016, including validated in vitro methods
Latest revision in 2017, including provisions of the Regulation 2016/1688

ANALYSIS OF THE SITUATION

The approach to assess skin sensitisation potential in the EU has been dramatically changed in the latest years. In spite of the many available guidance documents, REACH registrants have to cope with the uncertainty of using a new approach and CROs (Contract Research Laboratories) are overloaded of requests on methods for which they still have little experience. Most of the companies involved in the REACH registration deadline of the 31st May 2018 related to substances up to 100 t/y are SMEs (Small Medium Enterprise) with limited resources and minor toxicology expertise.

The aim of this work is to acknowledge the hurdles in applying new approach methodologies and to propose a strategy to improve confidence and acceptability of *in vitro* methods.

MATERIALS AND METHODS

TEAM mastery is a consultant company that has been involved in the preparation of hundreds of lead registration dossiers since the very beginning. Due to a precise commitment in avoiding *in vivo* methods as much as possible, it is now a leader in the use of *in vitro* approach according to the provisions of REACH with the important support of CAAT-Europe and the University of Milan. This favourable position has provided the opportunity to accumulate many examples of the applicability of the new approach for skin sensitisation assessment. The decision on the best method for testing, the selection of the CROs, the management of the sample deliveries, the monitoring of each study, the collection of the results and the final decision on the hazard classification are amongst the activity of TEAM mastery.

Hardly ever all three *in vitro* methods were performed due to the high cost and the difficulty in finding a CROs offering the complete set of *in vitro* tests. The table below reports few examples of recently registered substances. The substance name is not disclosed for confidentiality reasons.

In many cases, the registered substance was a UVCB (Unknown or Variable Composition, Complex Reaction Products and Biological Materials), adding uncertainty in the possibility of applying the in vitro method, in particular the DPRA. Non validated methods were considered to overcome the difficulties.

	Substance characteristics	DPRA	ARE-Nrf2 Luciferase	hCLAT	Other data	Conclusion	CRO Cost	Discussion
SUBSTANCE A	Mono constituent substance in the middle of a category: lower MW components are sensitisers, higher MW components are not sensitisers	Negative	Negative	Negative	Report from the doctor responsible for medical surveillance that no sensitisation has ever recorded in spite of 20 years of manufacturing	Not sensitiser	hCLAT 7,120 € (2012) DPRA + ARE-Nrf2 Luciferase: 5800 € (2018)	hCLAT was performed in 2012 before the publication of the OECD TG. ECHA asked for its rejection and requested ARE-Nrf2 Luciferase and DPRA to confirm the negative results
SUBSTANCE B	Sodium salt of a mono constituent substance	Positive	Not performed	Positive	The LLNA performed in 2013 on the acid form of the same substance is weakly positive	Sensitiser	7,100€ Cost of the LLNA: 4,300€	Possibility to classify as category 1B. This is not a big issue because the generic classification is equivalent to category 1B
SUBSTANCE C	UVCB, organometallic complex QSAR: out of the applicability domain	Positive	Negative	Positive	LLNA: Negative	Not sensitiser, but with great uncertainty	Information not available	There is a mismatch between in vitro and the in vivo. Very difficult to test UVCB *in vitro*, but organometallics may return false negative results with LLNA
SUBSTANCE D	High MW UVCB No water soluble, very difficult to test in vitro QSAR: Negative	Read Across with similar substances: Negative Tested with Sens-is[2] (validation on going) which measures the expression of sensitisation biomarkers in a 3D reconstructed epidermis model: Negative				Not sensitiser	5,500€	Uncertainty about the acceptability of a non-validated method, even if fully supported by QSAR and read across

DISCUSSION

There are some objective complications in applying the *in vitro* approach

- **Applicability** domain: very difficult to test low water soluble substances and UVCBs. In some cases, very high cytotoxicity or interference with the reagents prevent the performing of the cell assays
- Interpretation of the results can be challenging
- Very few CROs (Contract Research Laboratories) may offer the complete set of in vitro tests
- The general cost is higher compared to LLNA

However, LLNA has also some drawbacks that are often ignored[3]

- False negative findings with certain metals and false positive with skin irritant surfactants
- Impact of the vehicle on the final result
- Lack of reproducibility

CONCLUSION

There is a general suspicion in accepting the *in vitro* results, in spite of the validation efforts and the scientific evidence of the limitations of *in vivo* methods, that may also have restrictions in the applicability domain and experimental uncertainties, as any other scientific study. A cultural change is necessary.

Proposals for improving the situation:

1. Improve the culture of *in vitro* methods with the necessity of integrating information from different sources
2. Validate more *in vitro* methods, to enlarge the applicability domain
3. Make more use of QSAR and Read Across (RA) to support the interpretation of the results in an objective way
4. Fiscal incentive in the use of alternative method with more CROs offering the full service at a cheaper price

REFERENCES

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