The Importance of Understanding Drivers of Irritation *In Vivo* for Selection of Chemicals Used in the Development and Evaluation of *In Vitro* Serious Eye Damage/Eye Irritation Assays: Cosmetics Europe Analysis

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Introduction

Cosmetics Europe's Task Force Eye Irritation (TFEI) is actively involved in the development and evaluation of alternative methods to assess serious eye damage/eye irritation potential of cosmetic ingredients using *in vitro* methods, based on optimizing current *in vitro* test methods, applied research projects and collaborative activities with external partners. Selecting chemicals for use in the development and evaluation of *in vitro* eye irritation assays based on a thorough understanding of what drives irritation in classification of ocular effects of chemicals in the *in vivo* rabbit Draize test is therefore a critical and essential element. This enables identification and evaluation of predictive capacity and applicability domain at an early stage of development. To facilitate understanding of the importance of such drivers of irritation (DroI), Cosmetics Europe TFEI has undertaken an in depth analysis of the publicly available external databases containing *in vivo* eye irritation data for 462 chemicals and solutions of chemicals tested in the Draize eye irritation test. This analysis is based on having good quality *in vivo* data that has allowed a clear understanding of the different ocular tissues effects that drive classification. These include corneal opacity (CO), iritis (IR), conjunctival redness (CR), conjunctival chemosis (CC), days to clear and/or persistence of effects. In addition, all 462 chemicals were screened for their commercial availability, assurance that they cover the whole range of irritation potential and represent relevant classes and physical states. Until today such an analysis is unprecedented and it will have important implications for *in vitro* methods development, evaluation and validation activities.

Strategy for Building a Master Chemicals List

1. Collection of chemicals with good quality *in vivo* data from existing databases (e.g. ECETOC, ZEBET and Laboratoire National de la Santé (Gautheron)) and generation of UN GHS classifications for eye irritation
2. Identification of the *in vivo* drivers of UN GHS classification, as depicted in Table 1 (priority order of criteria from left to right)
3. Differentiation of chemicals according to the drivers of classification as depicted in Table 2 (selected subset of chemicals) and Figure 1 (all 462 chemicals in the database), using the following prioritization scheme:
   a) Category 1:
      - Chemicals classified based on severity (mean scores of Days 1-3)
      - Chemicals classified based on persistence at Day 21, but not severity
      - Chemicals classified based on CO = 4, but not severity or persistence
      - Choice of endpoint driving classification dependent on the number of animals
   b) Category 2:
      - Choice of endpoint driving classification (2B: severity; 2A: severity and persistence at Day 7) dependent on the number of animals
   c) No Category (NC):
      - CO = 0 → chemicals showing CO scores equal to zero in all animals and all observed timepoints
      - CO > 0 → chemicals showing at least one CO score higher than 0 at any timepoint in any animal (when CO mean < 1)

Results: Tables

Table 1: List of the *in vivo* drivers of UN GHS classification

Category 1						Category 2			No Category (NC)	
Severity (Mean scores of Days 1-3)		Persistence at Day 21				Severity (Mean scores of Days 1-3)			CO = 0	CO > 0
CO mean ≥ 3 in ≥ 67% of the animals	IR mean > 1.5 in ≥ 67% of the animals	CO	CR and/or CC	IR	CO = 4 in at least 1 animal	CO mean ≥ 1 in ≥ 67% of the animals	CR mean and/or CC mean ≥ 2 in ≥ 67% of the animals	IR mean ≥ 1 in ≥ 67% of the animals	Chemicals with at least 1 animal with a mean score of days 1-3 for at least one endpoint above the classification cut-off marked with *	
						Persistence at Day 7 (2A)				
						CO	CR and/or CC	IR		

*: examples see Table 2

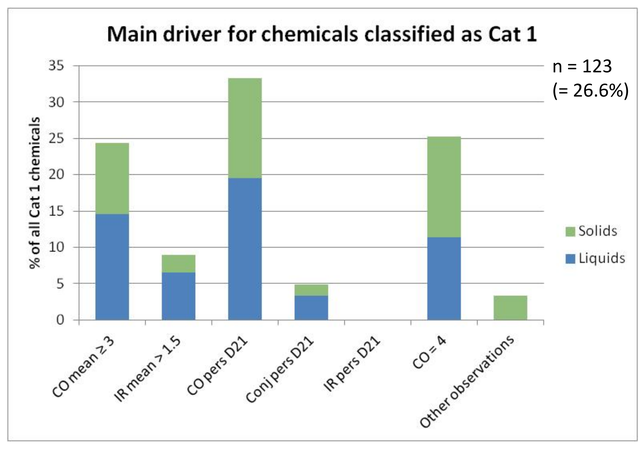
Table 2: Drivers of classification for several representative chemicals selected from 3 publically available databases: ECETOC, ZEBET and Gautheron

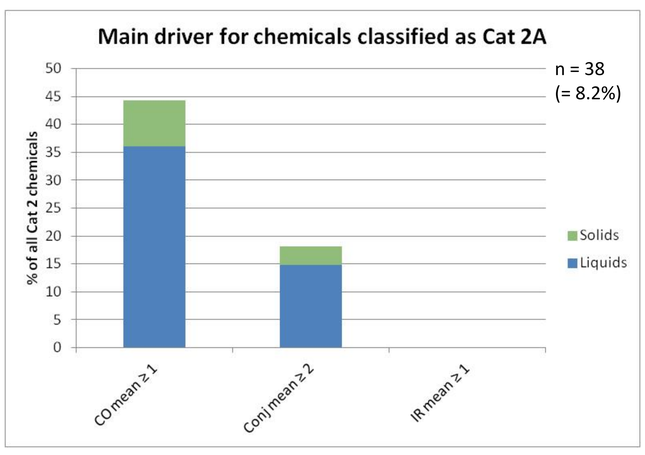
Chemicals	GHS Class	Drivers of Classification						Comments	CAS #	Physical Form (S = solid, L = liquid)	Data Source
		Severity		Specific observations		Persistence					
		Cut-off values	Number of animals	CO = 4 or other observations	Number of animals	Cut-off time	Number of animals				
Promethazine HCL	1	CO mean ≥ 3	2/3					Delayed effects	58-33-3	S	Gautheron
Quinacrine	1	IR mean > 1.5	2/3						69-05-6	S	ECETOC
3,6-Dimethyloctanol	1	CO mean ≥ 1	3/3			CO pers D21	1/3	Delayed effects	151-19-9	L	ZEBET
2,5-Dimethylhexanediol	1	CO mean ≥ 1	3/3			IR pers D21	1/3		110-03-2	S	ZEBET
Butyl cellosolve	1	CO mean ≥ 1	3/3			Conj pers D21	3/3		111-76-2	L	ECETOC
Methyl thioglycolate	1	CO mean ≥ 1	3/3	CO = 4	1/3			CO = 4 at D1 in 1/3 fully reversed by D10	2365-48-2	L	ECETOC
Tetra aminopyrimidine sulphate	2A	CO mean ≥ 1	2/3			CO pers D7	1/3		5392-28-9	S	Gautheron
iso-Butanal	2B	CO mean ≥ 1	2/3						78-84-2	L	ZEBET
Methyl cyanoacetate	2A	IR mean ≥ 1	3/3			IR pers D7	2/3		105-34-0	L	ECETOC
m-Dinitrobenzene	2B	IR mean ≥ 1	2/3						99-65-0	S	ZEBET
N-Lauroyl sarcosine Na salt (10%)	2A	Conj mean ≥ 2; CO mean ≥ 1	3/3; 2/3			Conj pers D7	1/3		7631-98-3	S (L as tested)	Gautheron
Sodium monochloroacetate	2B	Conj mean ≥ 2	3/3						3926-62-3	S	ZEBET
N,N-Dimethyl guanidine sulphate	NC	CO = 0	* Conj 1/3						598-65-2	S	ECETOC
4-Chloro-4-nitrodiphenylether	NC	CO = 0							1836-74-4	L	ZEBET
Tetra aminopyrimidine sulphate	NC	0 < CO < 1							5392-28-9	S	ECETOC
Methanol	NC	0 < CO < 1 *	* CO 1/3						67-56-1	L	Gautheron

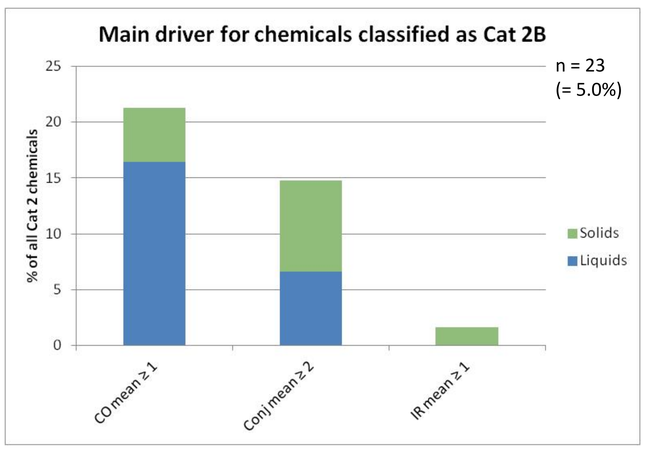
(Conj = conjunctival; pers = persistence)

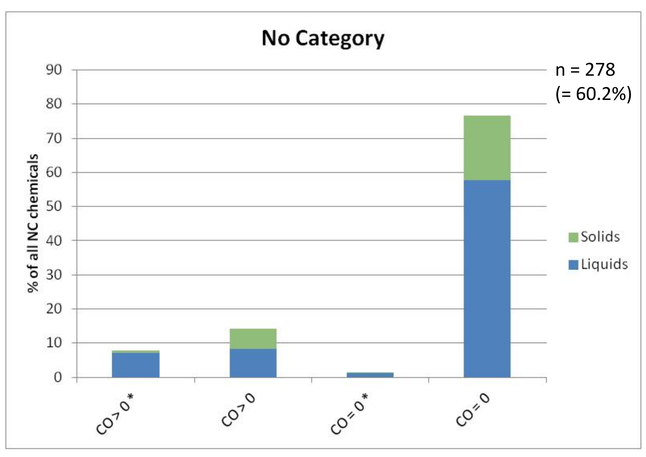
Results: Graphs

Figure 1: Distribution of chemicals according to drivers of classification for 462 chemicals (Cat 1: 123, Cat 2A: 38, Cat 2B: 23 and NC: 278) from the ECETOC, ZEBET and Gautheron and other databases (physical state: 319 liquids/solutions and 143 solids)









Conclusions

Our analysis clearly demonstrates the importance of understanding the *in vivo* tissue effects which drive eye irritation classification according to the UN GHS system. This builds on recent more general publications in this area (e.g Adriaens *et al*., 2013 [#]). The availability of such a comprehensive list of the drivers of irritation for identified chemicals is a critical and essential tool when selecting chemicals for development, evaluation and validation of new *in vitro* eye irritation tests. Valuable insights can be taken from this analysis of the DroI: 1) high involvement of corneal effects driving Cat 1, Cat 2A and Cat 2B classifications; 2) when persistence of effects at day 21 drives Cat 1 classification, this is primarily based on corneal effects; 3) low prevalence of iris effects driving classification.

Importantly, some inconsistencies within the *in vivo* data could be identified, e.g.: 1) tetra aminopyrimidine sulphate (CAS-No. 5392-28-9) which is classified differently (Cat 2A and NC) in two independent *in vivo* studies; 2) methyl thioglycolate (CAS No. 2365-48-2), which is identified as Cat 1 based on CO=4, even though corneal opacity fully reversed by day 10 of the study.

As such, provided here is a strategy for selecting reference chemicals based on understanding ocular effects that drive irritation in the *in vivo* rabbit Draize test in classification of chemicals. It is proposed that use of this approach would facilitate early and accurate assessment of the performance of a new method within tiered testing strategies, for example, that suggested from the EURL ECVAM 2005 expert meeting (Scott *et al*. , 2010 [##]).

As data become available from validation study analyses they will be integrated in this analysis.

[#]: *Adriaens et al.: Retrospective analysis of the Draize test for serious eye damage/eye irritation: importance of understanding the in vivo endpoints under UN GHS/EU CLP for the development and evaluation of in vitro test methods. Archives of Toxicology, 88, 701–723, 2014.*

[##]: *Scott et al.: A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom-Up and Top-Down approaches. Toxicology in Vitro 24: 1–9, 2010*