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The Importance of Understanding Drivers of Classification *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

A thorough understanding of which of the effects assessed in the *in vivo* Draize eye test are responsible for driving UN GHS classification is critical for an adequate selection of chemicals to be used in the development and/or evaluation of alternative methods and for properly assessing their predictive capacity and limitations. For this purpose, Cosmetics Europe undertook to compile an extensive database of chemicals - the Draize eye test Reference Database (DRD) - for which historical *in vivo* Draize eye test data are available. In the Draize rabbit eye test, the hazard potential of a test chemical is determined based on its effect on corneal opacity (CO), iritis (IR), conjunctival redness (CR), and conjunctival chemosis (CC) in combination with full reversibility or persistence of any effect on day 21 after instillation. In order to achieve full replacement of the *in vivo* Draize eye test, it is clear that alternative methods, alone or in combination, need to address the main ocular tissue effects that drive classification. An evaluation of the various *in vivo* drivers of classification compiled in the DRD was performed to establish which of these are most important from a regulatory point of view. This approach will facilitate an early and thorough assessment of the performance of a new alternative method and will help better identifying its limitations and applicability within testing strategies such as those suggested by Scott *et al*. (2010)*. Taken together, the key goals for compiling the DRD were to: i) enable a comprehensive analysis and understanding regarding *in vivo* drivers of classification based on the Draize eye test; ii) further evaluate the variability of the Draize eye test based on data obtained from repeat studies; iii) enable a critical review of the UN GHS/EU CLP classification criteria for eye damage/irritation; iv) make available an extensive database of chemicals with OECD Test Guideline 405 *in vivo* data, beyond those generally used historically, for further method development and validation and v) to provide guidance for selecting reference chemicals based on understanding ocular tissue effects that drive classification in the *in vivo* rabbit Draize eye test.

Strategy for Developing the DRD

The DRD was primarily compiled using different sources of historical *in vivo* Draize eye test data i.e. ECETOC, ZEBET, Laboratoire National de la Santé (Gautheron), NICEATM, EURL ECVAM, which were created to support past validation activities. These data were produced according to OECD Test Guideline 405 using proprietary and commercially available chemicals. The studies were classified according to the serious eye damage/eye irritation classification criteria defined by UN GHS and EU CLP which implemented UN GHS in the EU. According to these classification systems, there are several criteria derived from the four ocular tissue effects assessed in the Draize eye test, namely CO, IR, CR and CC (with CR and CC identified here as conjunctival effects or "Conj"), which can each independently drive the classification of a chemical. Therefore, a chemical can be classified based on a single or multiple drivers of classification. These drivers of classification are described in Table 1. A full description of all drivers of classification observed in each individual *in vivo* study are reported in the DRD. Selection of the main driver of classification in each study was performed according to the driver appearing in the largest number of animals and, in case of equal number of animals, according to the prioritisation scheme identified in Table 1 in moving from the left to right of the table for each UN GHS/EU CLP category. Overall, the DRD contains 681 independent *in vivo* studies on 634 individual chemicals representing a wide range of chemical classes and different physical states.

Results: Distribution According to Drivers of Classification

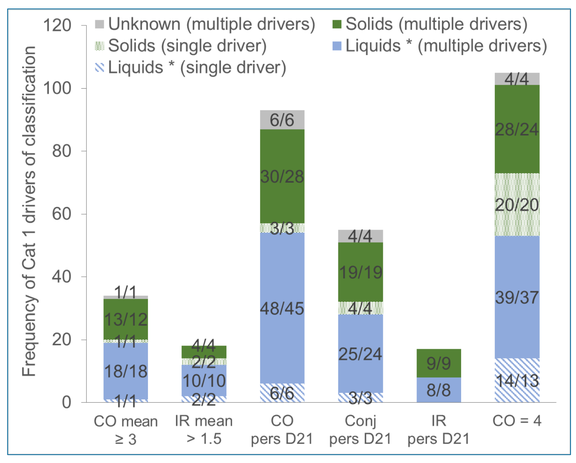
Table 1: List of the *in vivo* drivers of UN GHS classification for the chemicals requiring classification and subgroups for the chemicals not requiring classification. This table also contains the proportion (%) and number (n) of studies according to main driver of classification or according to the subgroups (No Cat).

Category 1 28.1% (n=165)						**Category 2** [a] 13.5% (n=79)			**No Category** 58.4% (n=343)			
Severity [b] *in ≥ 60% of the animals* 27.3% (n=45)		Persistence on Day 21 *in at least one animal* 46.7% (n=77)			Severe CO *in at least one animal* 20.6% (n=34)	Severity [b] *in ≥ 60% of the animals*			*in at least one observation time in at least one animal*		*in all observation times in all animals*	
CO mean ≥ 3	**IR mean > 1.5**	**CO**	**Conj**	**IR**	**CO=4**	**CO mean ≥ 1**	**Conj mean ≥ 2**	**IR mean ≥ 1**	**CO > 0 ****	**CO > 0**	**CO = 0 ****	**CO = 0**
73.3% (n=33)	26.7% (n=12)	80.5% (n=62)	19.5% (n=15)	0% (n=0)	100% (n=34)	60.8% (n=48)	38% (n=30)	1.3% (n=1)	8.7% (n=30)	13.1% (n=45)	1.7% (n=6)	76.4% (n=262)

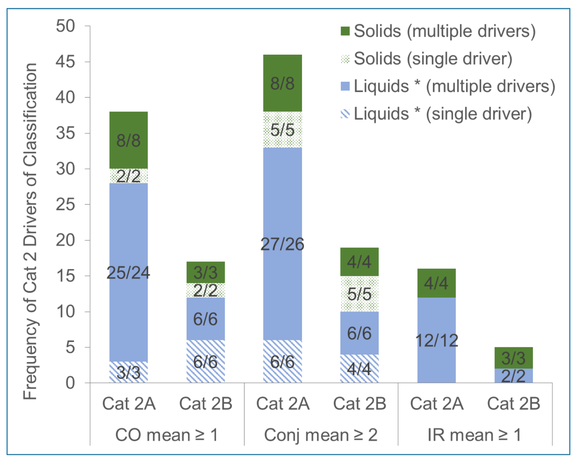
[a] sub-categorised in two categories: Category 2A (irritant to eyes) when any of the eye effects in any animal is not fully reversible within 7 days of observation (i.e. CO, IR, CR and/or CC > 0 at 7 ≤ day < 21) and 2B (mildly irritant to eyes) when all observed eye effects are fully reversible within 7 days of observation (i.e. CO, IR, CR and CC = 0 on day 7 and beyond); [b] Mean scores calculated from gradings at 24, 48, and 72 hours after instillation of the test chemical; ** at least one animal with a mean score of days 1-3 above the classification cut-off for at least one endpoint

Figure 1: Total frequency of Cat 1 drivers of classification observed in 156 UN GHS/EU CLP Cat 1 studies **(Fig. 1A)** and of Cat. 2 drivers of classification observed in 51 and 27 UN GHS/EU CLP Cat 2A/2B studies respectively **(Fig. 1B)**. The numbers in the bars correspond to the number of studies / the number of unique test chemicals. The individual drivers appearing in each study were distributed in different groups depending if they occurred alone (single driver) or together with other Cat 1 or Cat 2A/2B drivers of classification (multiple drivers) and on the physical state of the chemical as tested (Liquid, Solid or Unknown).

(Fig. 1A) Cat 1



(Fig. 2B) Cat 2A/2B

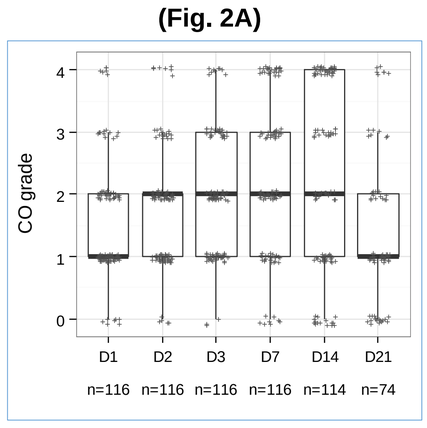


Results: Studies Classified Cat 1 Based Only on Persistence

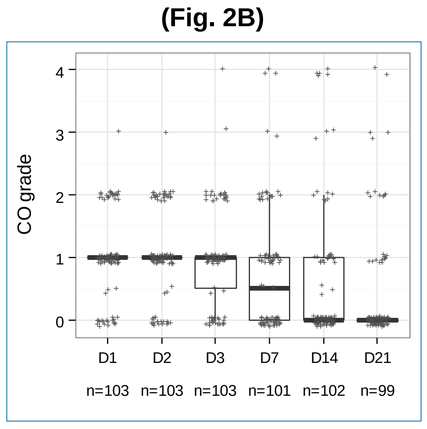
Figure 2. Boxplots presenting the distribution of individual animal CO grades at 1, 2, 3, 7, 14 and 21 days after instillation of the test chemical for **(Fig. 2A)** Cat 1 studies showing CO persistence in the majority of the animals but with CO mean < 3 and IR mean ≤ 1.5 in the majority of animals (32 studies with 116 animals), **(Fig. 2B)** Cat 1 studies showing CO persistence in the minority of the animals but with CO mean < 3 and IR mean ≤ 1.5 in the majority of animals (25 studies with 104 animals), and **(Fig. 2C)** Cat 2A studies showing persistence of CO on day 7 in at least one animal (28 studies with 104 animals). The symbols (+) present individual observations.

In general, the CO scores of the Cat 1 chemicals classified based on CO persistence in the minority of the animals **(Fig. 2B)** have a similar distribution as those of the Cat 2A chemicals showing CO persistence on day 7 **(Fig. 2C)**. In fact, based on the CO scores observed over the first three days, it is not possible to distinguish the Cat 1 studies with CO persistence in the minority of the animals **(Fig. 2B)** from the Cat 2A studies (**Fig. 2C**). The same is true for CR and CC (data not shown). Persistence of effects in a minority of the animals should therefore not be used to drive a Cat 1 classification, nor should isolated extreme effects (CO = 4) appearing late in the study, as these are most probably not related to the test chemical itself.

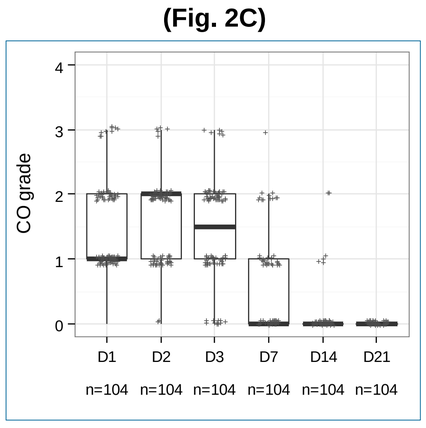
(Fig. 2A)



(Fig. 2B)



(Fig. 2C)



Key Conclusions

The analysis of the DRD clearly demonstrates the importance of understanding the *in vivo* tissue effects which drive eye damage/irritation classification according to the UN GHS/EU CLP systems. This builds on recent more general publications in this area (i.e. Barroso *et al*. 2013**; Adriaens *et al*., 2014***). Key conclusions drawn from the current analysis are:

- The most important drivers for Cat 1 Classification are CO mean ≥ 3 (days 1-3) (severity) and CO persistence on day 21 in the absence of severity corresponding to 22% and 47% of studies respectively.
- The most important drivers for Cat 2 classification are CO mean ≥ 1 and conjunctival redness mean ≥ 2 corresponding to 71% and 84% of studies respectively though the latter appears to hold a higher weight because it occurs alone more often than corneal opacity does (29 % vs. 16 %).
- It is shown that all classifiable effects (including persistence and CO = 4) should be present in >60% of the animals to drive a classification. As a consequence, our analyses suggest the need for a critical revision of the UN GHS/EU CLP decision criteria for the Cat 1 classification of chemicals.
- A number of key criteria are identified that should be taken into consideration when selecting reference chemicals for the development, evaluation and/or validation of alternative methods and/or strategies for serious eye damage/eye irritation testing. These considerations are detailed in a manuscript that will be progressed for publication in a peer reviewed journal.

References

*: 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.

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***: Adriaens *et a*l. 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.