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Case Study on the use of Integrated Approaches for Testing and Assessment for "Eye hazard identification" of "surfactants"

Ninth Review Cycle (2023)

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Environment Directorate

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Paris 2024

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Foreword

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by Cosmetics Europe for illustrating practical use of IATA and submitted to the 2023 review cycle of the IATA Case Studies Project. This case study was reviewed by the project team, and was approved by the Working Party on Hazard Assessment

This case study is an illustrative example, and its publication as an OECD monograph does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, this case study should not be interpreted as official regulatory decisions made by the authoring member countries.

This document is published under the responsibility of the Chemicals and Biotechnology Committee of the OECD.

Table of contents

FIGURES

TABLES

Abbreviations and Acronyms

CASRN	Chemical Abstracts Service Registry Number
Cat. 1	UN GHS classification for chemicals causing irreversible effects on the eye/serious damage to the eye
Cat. 2	UN GHS classification for chemicals causing reversible effects on the eye/eye irritation
CC	Conjunctival chemosis
CE	Cosmetics Europe
CO	Corneal opacity
Conj	Conjunctival effects
CR	Conjunctival redness
DA	Defined approach
DAL	Defined approach liquids
DASF	Defined approach surfactants
DIP	Data integration procedure
DRD	Draize eye test Reference Database
ECHA	European Chemicals Agency
EIT	Eye Irritation Test
GD	Guidance Document
GHS	Globally Harmonised System
GL	Guideline
HCE	Human Corneal Epithelium
IATA	Integrated approaches to testing and assessment
IR	Iritis
ITS	Integrated Testing Strategy
MoA	Modes of Action
MW	Molecular weight
NA	Not applicable
NAM	New approach methodology
No Cat.	Not requiring UN GHS Classification
OECD	Organisation for Economic Cooperation and Development
RhCE	Reconstructed human Cornea-like Epithelium
ST	Surface tension
STE	Short Time Exposure
TG	Test Guideline
UN GHS	United Nations Globally Harmonized System
VRM	Validated Reference Method
WoE	Weight of Evidence

Executive summary

The assessment of eye irritation/serious eye damage originally involved the use of albino rabbits according to the Draize eye test method (OECD Test Guideline 405). Considerable progress has been made in the partial replacement of the regulatory *in vivo* Draize eye test (Draize et al., 1944). Currently, two defined approaches (DA) are accepted by the Organisation for Economic Cooperation and Development (OECD TG 467) for the hazard identification i.e. discrimination between three United Nations Globally Harmonized System of Classification (UN GHS) categories i.e., Category 1 (Cat. 1) on “serious eye damage”; Category 2 (Cat. 2) on “eye irritation” and No Category (No Cat.) for chemicals “not requiring classification and labelling” for eye irritation or serious eye damage. The current work illustrates a new DA that was developed to predict this endpoint for liquid, semi-solid and solid chemicals having surfactant properties (DASF). The DASF is based on the combination of Reconstructed human Cornea-like Epithelium test methods (RhCE, OECD TG 492, EpiOcular™ EIT or SkinEthic™ HCE EIT) and a modification of the Short Time Exposure test method (Alépée et al., 2023). The eye hazard potential of two neat surfactants (one Cat. 1 and one No Cat.) and different dilutions of one surfactant covering the three UN GHS categories was determined based on the results of DASF.

The data interpretation procedure (DIP) applied uses the readout of the prediction models of the individual test method. For each chemical, the final prediction using the bottom-up or top-down approach of the DAs was concordant with the *in vivo* classification. In conclusion, these case studies illustrate the applicability of the DAs and their potential to successfully distinguish between the 3 UN GHS categories for eye hazard identification.

1 Introduction

The assessment of eye irritation/serious eye damage originally involved the use of albino rabbits according to the Draize eye test method (OECD Test Guideline 405) (OECD TG 405, 2021a). The hazard potential of a test chemical was determined based on its effect on corneal opacity (CO), iritis (IR), conjunctival redness (CR), and conjunctival chemosis (CC). Based on the severity of effects and/or the timing of their reversibility, classifications are derived according to the serious eye damage/eye irritation classification criteria defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (UN 2023). According to the UN GHS classification system, Category 1 (Cat. 1) is defined as causing irreversible effects (not fully reversible within 21 days) on the eye/serious damage to the eye. Category 2 (Cat. 2) is defined as causing reversible effects (fully reversible within 21 days) on the eye/eye irritation. When none of the Cat. 1 or Cat. 2 classification criteria are met, the test chemical does not require classification which corresponds to No Category (No Cat.).

A number of Test Guidelines (TGs) on *in vitro* new approach methodologies (NAMs) have been adopted for the identification of test chemicals inducing serious eye damage (UN GHS Cat. 1) or for the identification of test chemicals not requiring classification for eye irritation and serious eye damage hazards (UN GHS No Cat.), notably OECD TG 437, TG 438, TG 460, TG 491, TG 492, TG 494, and TG 496 (OECD, 2023a, 2023b, 2023c, 2023d, 2023e, 2021, 2023f respectively). Data generated with these NAMs are proposed to be used together, as well as with information sources such as physicochemical properties, *in silico* and read-across predictions from chemical analogues, within integrated approaches to testing and assessment (IATA) or defined approaches (DAs) (Guidance Document (GD) No. 263; OECD, 2023g). The prediction from a DA may be used alone or along with further information as part of an IATA (OECD, 2023g).

The major difficulty for a single *in vitro* test method to fully replace the *in vivo* rabbit eye test (TG 405) is to predict the middle category (UN GHS Cat. 2) and it is therefore recommended to make use of testing strategies (e.g., Top-Down or Bottom-Up approach) that combine the strengths of individual *in vitro* test methods to address the required ranges of irritation potential (Scott et al., 2010). The determination of the most relevant *in vivo* endpoint(s), in particular the effects on cornea, iris or conjunctiva, is important for the development of adequate *in vitro* methods as it allows to better understand the relationship between the *in vitro* and the *in vivo* data (Adriaens et al., 2014; Barroso et al., 2017). For this reason, it is recommended to take into consideration the most important drivers for UN GHS Cat. 1 and Cat. 2 classifications as well as the distribution of *in vivo* effects for chemicals not requiring classification when selecting reference chemicals for the development, evaluation and/or validation of alternative methods and/or strategies for serious eye damage and eye irritation testing (OECD, 2023g). A detailed description of the drivers of classification and key criteria that should be considered when selecting reference chemicals for the development of NAMs is provided in the paper of Barroso and co-workers (2017).

The SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation test (EIT) has been adopted for test chemicals not requiring classification for eye irritation and serious eye damage hazards (OECD TG 492, 2023e). Recently, the SkinEthic™ HCE Time-to-Toxicity (TTT) test method has been developed using the same tissue construct. The TTT method allows to distinguish the three UN GHS categories (Alépée

et al., 2020, 2021, 2022). This stand-alone NAM was adopted by the OECD as a full replacement to the *in vivo* Draize eye test for classification of chemicals (TG 492B, 2022a). Likewise, two defined approaches (DAs) were developed to fully replace the Draize eye test for eye hazard identification of non-surfactant liquids (Alépée et al., 2019a, 2019b). The DAs are based on the combinations of methods as recommended in the Part 3 of the IATA GD No. 263 (OECD, 2023g) and are integrated in a new OECD TG 467 (OECD, 2022b). Background information supporting this TG is reported in OECD Guidance Document (GD) 354 (2022c). To date, no DA has been adopted to specifically address the eye hazard potential of surfactants across the three UN GHS categories. Here, a new DA is proposed which was developed to predict this endpoint for liquid, semi-solid and solid chemicals having surfactant[1] properties (Alépée et al., 2023). The DASF is based on the combination of Reconstructed human Cornea-like Epithelium test methods (RhCE, OECD TG 492, EpiOcular™ EIT or SkinEthic™ HCE EIT) and a modification of the Short Time Exposure test method (Alépée et al., 2023).

Since the DA's applicability domain is limited to surfactants only, there exists only a limited database with good quality Draize eye test data. A total of 47 surfactants (22 Cat. 1, 8 Cat. 2, and 17 No Cat.) were identified in the Cosmetics Europe Draize Eye Test Reference Database (DRD) (Table 8). Surfactants are classified based on the composition of their head and are divided into four classes: non-ionic (no charge), cationic (+ charge), anionic (- charge), and amphoteric (opposite charge). The set of surfactants assessed with the DASF comprises the four different classes. Each class is represented by mono-constituent and multi-constituent or UVCBs. Several families are represented in the set (e.g. alcohol ethoxylates, alkyl sulfates, sulfosuccinates, …). The DRD contained only 8 diluted Cat. 2 surfactants of which only 6 were available for testing (Barroso et al., 2017). Two additional diluted Cat. 2 surfactants with historical Draize eye test were identified from other databases (Blazka et al., 2000 and Scheel et al., 2011). Thus, only 8 diluted Cat. 2 surfactants were available to assess the predictive performance of the DASF for this category. Further efforts were made by consulting regulatory agencies and stakeholders, and reviewing the Cosing (Cosmetic Ingredient) database, but so far no additional Cat. 2 surfactants could be identified that meet the predefined requirements.

The modes of action for eye irritation are unknown for most chemicals, the mechanism is better understood for surfactants. Due to their amphiphilic (polar and lipophilic properties) nature, surfactants form micelles in an aqueous environment. When biological tissues, such as the cornea, are exposed to surfactants, a series of events takes place including cell membrane lysis, protein extraction, and ultimately cell membrane disruption (Helenius and Simons, 1975). Since the focus is on this specific group of chemicals with similar properties, there is greater confidence in the predictions.

[1] 'Surfactant' means any substance and/or mixture, which has surface-active properties and which consists of one or more hydrophilic and one or more hydrophobic groups of such a nature and size that it is capable of reducing the surface tension of water (< 45 N/m), and of forming spreading or adsorptive monolayers at the water-air interface, and of forming emulsions and/or microemulsions and/or micelles, and of adsorption at water-solid interfaces (modified from Regulation (EC) No. 648/2004 on Detergents).

2 Purpose

2.1. Purpose of use

The purpose of this case study is to show how results from multiple information sources can be used together in a DASF to evaluate the eye hazard potential of surfactants (neat and in dilution). The workflow of the DA is illustrated with two neat surfactants and several dilutions of one surfactant covering the 3 UN GHS categories for eye hazard identification.

2.2. Target chemical(s)

The target chemicals are surfactants. The following three surfactants were selected to illustrate the case study: Ethylhexyl acid phosphate ester (CASRN 12645-31-7), Tween 80 (9005-65-6), and Cetylpyridinium bromide (140-72-7). The availability of existing NAM information for the surfactants including a defined chemical structure and *in vitro* data (OECD TG 492 and the modified STE test method) (OECD, 2023e: Alépée et al., 2023) allowed illustration of the DASF that follow the workflow recommended in the IATA (GD No. 263; OECD, 2023g). Note that no human data or animal data were considered in the applicability of the DA. The surfactants represent the different classes (cationic, anionic, and non-ionic) from different families (a quaternary ammonium chemical, a fatty alcohol phosphoric acid ester, and a sorbitan ethoxylated fatty acid ester) and include a mono-, a multi-constituent, and an UVCB. This existing information is summarized in Table 2. The prediction results obtained with the DASF were compared with the classification based on historical *in vivo* Draize eye test data.

2.3. Endpoint(s)

The endpoint of interest is serious eye damage and eye irritation. The intended regulatory purpose is: hazard identification, i.e. discrimination between three UN GHS categories i.e., Category 1 (Cat. 1) on “serious eye damage”; Category 2 (Cat. 2) on “eye irritation” and No Category (No Cat.) for chemicals “not requiring classification and labelling” for eye irritation or serious eye damage.

3 Hypothesis for performing IATA

The DA described in this case study (Alépée et al., 2023), is based on the recommendations and combinations of modules as stipulated in the IATA GD No. 263 for serious eye damage and eye irritation (OECD, 2023g). The IATA groups various individual information sources in "modules" according to the type of information provided and is divided into three Parts. Part 1 on existing and non-testing data and Part 2 on a weight of evidence (WoE) analysis are not discussed in this document. Only Part 3 on the generation of new test data is illustrated in this case study. A detailed description of the different modules within the three parts is given in IATA GD No. 263 (OECD, 2023g).

Part 3 of the IATA GD No. 263 recommends the integration of *in vitro* test methods into testing strategies that combine the strengths of individual *in vitro* test methods to address the required ranges of irritation potential and/or chemical classes (Scott et al., 2010). Two tiered approaches are recommended for eye hazard identification: a Top-Down approach, starting with *in vitro* test methods that can identify test chemicals causing serious and/or irreversible eye damage (UN GHS Cat. 1) with low false positive predictions and the highest possible accuracy and a Bottom-Up approach, starting with *in vitro* test methods that can identify test chemicals not requiring classification for eye hazard (UN GHS No Cat.) with low false negative predictions and the highest possible accuracy. These tiered testing approaches can be considered as DAs to Testing and Assessment and can be used as a component within the IATA.

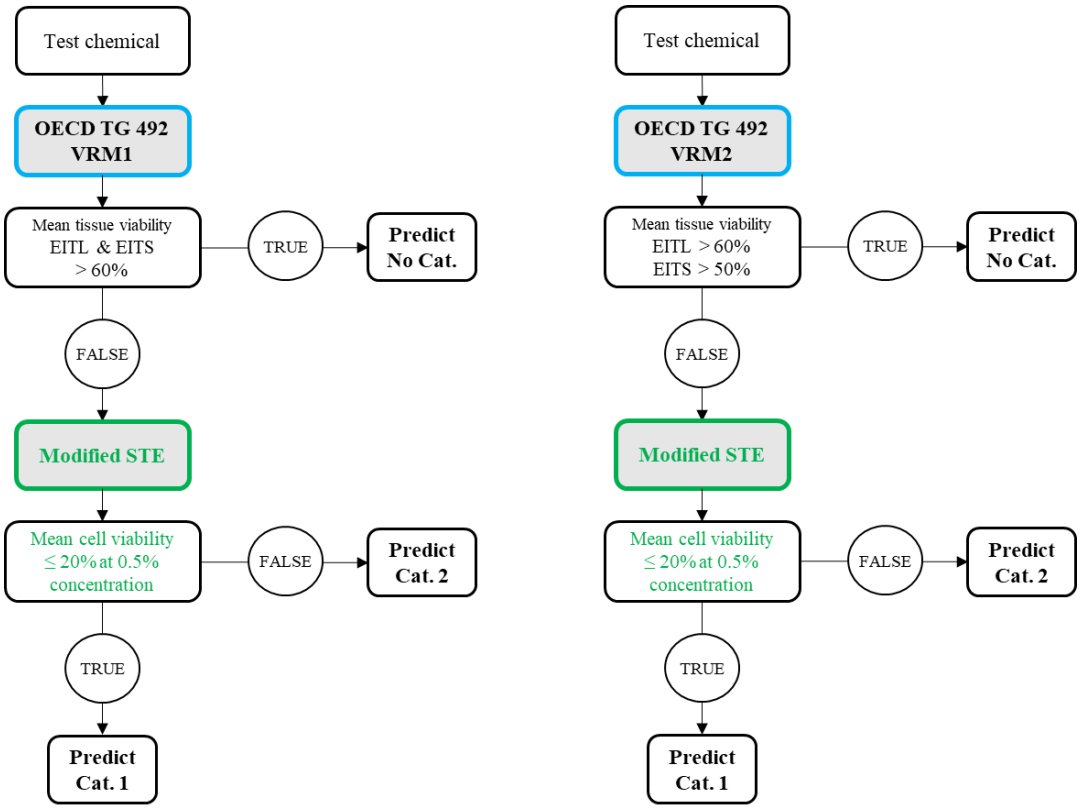
4 Defined Approach for Surfactants - DASF

A rule-based DA for surfactants is described in this document for the purpose of classification and labelling without the use of animal testing. A fixed data interpretation procedure (DIP) is applied to *in vitro* data to derive a prediction without the need for expert judgment since all the components of the DA are well defined. The DA uses method combinations intended to overcome some of the limitations of the individual NAMs in order to provide increased confidence in the overall result obtained.

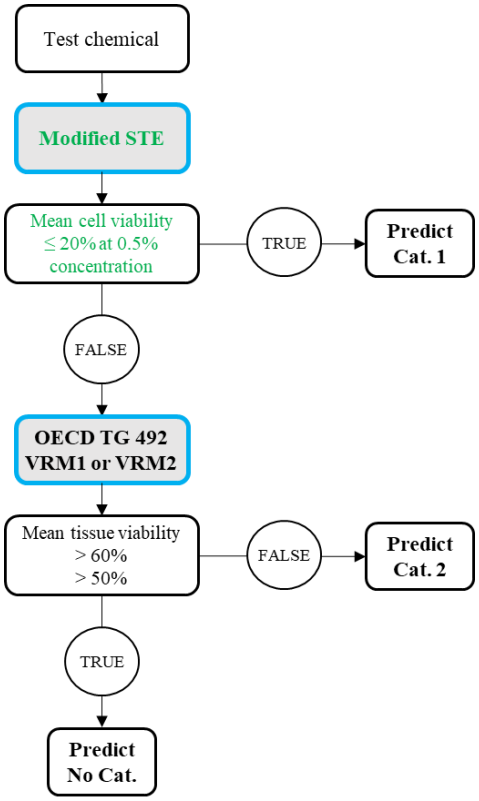
The DASF (Figure 1) is based on a combination of Reconstructed human Cornea-like Epithelium (RhCE) test methods described in OECD TG 492 (2023e) and a modification of the Short Time Exposure (STE) test method (Alépée et al., 2023).

Figure 1. Bottom-up and Top-Down Schemes of the DASF

Bottom-Up approach: For the RhCE test methods, two different protocols are used, one for liquids (EITL) and one for solids (EITS). Left scheme: start with RhCE test method Validated Reference Method 1 (VRM1: EpiOcular™ EIT) followed by the modified STE test method. Right scheme: start with VRM2 (SkinEthic™ HCE EIT) followed by the modified STE test method.



Top-Down approach: start with the modified STE test method followed by RhCE test method (same cut-offs are applicable for liquids and solids as shown in the Bottom-Up approach for VRM1 and VRM2).



4.1. Rationale underlying the construction of the defined approach

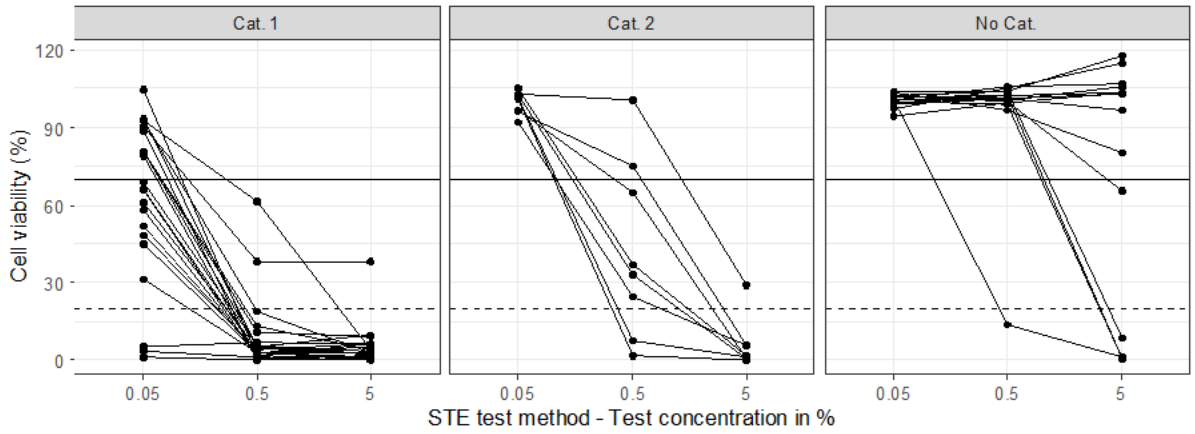
The use of viability of the RhCE tissues after topical exposure to a test chemical to discriminate UN GHS No Cat. chemicals from those requiring classification and labelling (UN GHS Cat. 1 and UN GHS Cat. 2) is based on the assumption that all chemicals inducing serious eye damage or eye irritation will induce cytotoxicity in the corneal epithelium and/or conjunctiva (OECD TG 492 paragraph 22, 2023e). Jester and co-authors (1998) and Maurer and co-authors (2002) have shown that cytotoxicity plays an important mechanistic role in determining the overall serious eye damage and eye irritation response of a chemical regardless of the physicochemical processes underlying tissue damage. The STE test method measures the cytotoxic effect of chemicals on a confluent monolayer of Statens Seruminstitut Rabbit Cornea (SIRC) cells. The cytotoxic effect of test chemicals on corneal epithelial cells is an important mode of action leading to corneal epithelium damage and eye irritation.

The STE test method conducted according to OECD TG 491 uses a 5% and 0.05% test concentration to identify UN GHS Cat. 1 and No Cat. substances with 70% cell viability as cut-off values (OECD, 2023d). Several UN GHS Cat. 1 surfactants resulted in a cell viability above 70% for the 0.05% test concentration resulting in No Prediction Can be Made (NPCM). This was also observed for UN GHS Cat. 2 surfactants, meaning that it is difficult to distinguish *in vivo* Cat. 1 from *in vivo* Cat. 2 surfactants (Figure 2). Based on concentration response studies (5%, 0.5%, and 0.05% test concentration) it was shown that a 0.5% concentration in combination with 20% cell viability as cut-off value improved the correct identification of Cat. 1 surfactants substantially. The optimization of the test protocol for UN GHS Cat. 1 identification resulted in the “modified STE test method” (Alépée et al., 2023), considering the 0.5% test concentration only which has not yet been OECD-approved. As specified in the IATA GD No. 263 for serious eye damage and eye irritation (module 4), both OECD-adopted and non-OECD adopted

alternative test methods can be used as elements of an IATA (OECD, 2023g). In fact, results obtained with optimized non-OECD adopted test methods might be used to identify UN GHS Cat. 1 (OECD, 2019c).

Figure 2. Profile plots of the cell viability measured at 0.05%, 0.5%, and 5% test concentration of the surfactants grouped by UN GHS category.

The solid line at 70% cell viability is the threshold value used in the original prediction model of the STE test method (OECD adopted method, TG 491). The dashed line at 20% cell viability is the threshold value used for the modified STE test method.



4.2. Description of the individual information sources used

The RhCE test methods evaluate the eye hazard potential of a test chemical based on its ability to induce cytotoxicity in a RhCE tissue construct. The viability of the RhCE tissue following exposure to a test chemical is determined in comparison to tissues treated with the negative control substance (% viability), and is then used to predict the eye hazard potential of the test chemical. Two different treatment protocols are used, one for liquids (Eye Irritation Test for Liquids - EITL) and one for solids (EITS) (Table 1). Liquids that result in a tissue viability > 60% are classified No Cat., liquids that result in a tissue viability ≤ 60% require further testing. For solid surfactants the EITS protocol should be used, the threshold cell viability value used to identify No Cat. is > 60% for VRM1 and > 50% for VRM2. Solids that result in a tissue viability less than or equal to the threshold value require further testing. The protocols are published and publicly available through the DB-ALM dataset (DB-ALM Protocol n◦ 164: EpiOcular™ EIT SOP, Version 9, 2015; DB-ALM Protocol n◦ 190 and n° 191: SkinEthic™ HCE EITL and EITS, respectively).

Table 1. RhCE Prediction models according to UN GHS classification

OECD TG 492 Validated reference method (VRM)	No Category	No prediction can be made
EpiOcular™ (VRM1)		
EITL[a] and EITS[b]	Mean tissue viability > 60%	Mean tissue viability = 60%
SkinEthic™ HCE (VRM2)		
EITL[a]	Mean tissue viability > 60%	Mean tissue viability = 60%
EITS[b]	Mean tissue viability > 50%	Mean tissue viability = 50%

[a] EITL is used for pipetteable substances (OECD TG 492, Annex II)
[b] EITS is used for not pipetteable substances (OECD TG 492, Annex II)

The STE test method predicts the eye hazard potential of a test chemical based on its ability to induce cytotoxicity of the treated SIRC cells after 5 minutes exposure. In the modified STE test method, a surfactant is classified as UN GHS Cat. 1 when the 0.5% test concentration results in a relative cell viability ≤ 20%. Surfactants that result in a relative cell viability > 20% require further testing (Alépée et al., 2023). The protocol is essentially the same as the one described in the STE test protocol (Version 1.9E) published on the National Toxicology Program website with the exception that surfactants should be tested at a 0.5% test concentration only (1 deviation only).

5 Data/Information gathering

A cationic, an anionic and a non-ionic surfactant that cover the different UN GHS eye hazard categories, were selected for this case study. All input data (*in vitro* test methods) for the DASF was available.

5.1. Identify chemical of interest and molecular structure

The molecular structure and surface tension for Cetylpyridinium bromide (CAS RN 140-72-7), Ethylhexyl acid phosphate ester (CAS RN 12645-31-7), and Tween 80 (CAS RN 9005-65-6) were identified (Table 2).

5.2. Identify existing hazard information

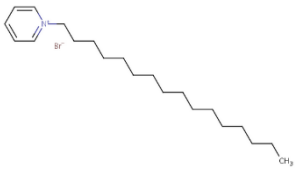
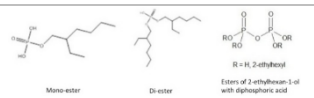
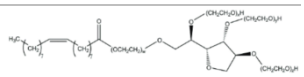
The existing *in vitro* results for Cetylpyridinium bromide, Ethylhexyl acid phosphate ester, and Tween 80 are presented in Table 3.

5.3. Identify analogues/suitability assessment and existing data

Not applicable. We purposely decided to restrict decision making solely on available NAM data as read across was beyond the scope for this IATA case study.

Table 2. Existing information collected for the DA – surfactant characteristics.

Name	Cetylpyridinium bromide	Ethylhexyl acid phosphate ester	Tween 80
CAS RN	140-72-7	12645-31-7	9005-65-6
SMILES	CCCCCCCCCCCCCCCC[N+]1=CC=CC=C1.[Br-]	CCCCC(CC)COP(='O)(O)O' (mono-ester) CCCCC(CC)COP(='O)(O)OCC(CC)CCCC' (di-ester)	~ 20 EO (average ethoxylation number)
Type of substance	Mono-constituent	Multi-constituent: mixture of mono- and di-esters	UVCB
Molecular Weight	MW: 384.4 Da	MW: 210.21 – 322.42 Da	MW: ~ 1310 Da
Surface tension of aqueous solutions	44.3 dyne/cm	44.4 dyne/cm	42.5 dyne/cm

measured according to OECD GL 115			
Surfactant class	Cationic	Anionic	Non-ionic
Family	Quaternary ammonium compound	Fatty alcohol phosphoric acid esters	Sorbitan ethoxylated fatty acid ester
			 A mixture of oleate esters of sorbitol and sorbitol anhydrides, consisting predominantly of the monoester, condensed with approximately 20 moles of ethylene oxide (EO). It conforms generally to the formula in the cell above

UVCB: substances of Unknown or Variable composition, Complex reaction products or Biological materials MW: Molecular Weight
Cetylpyridinium bromide (CAS RN 140-72-7) was diluted in water.
The surface tension of Cetylpyridinium bromide (CAS RN 140-72-7) is actually this of Cetylpyridinium chloride (CAS RN 6004-24-6), a structural analogue to Cetylpyridinium bromide. Surface tension reported for Cetylpyridinium chloride and Ethylhexyl acid phosphate ester (CAS RN 12645-31-7) were obtained from ECHA registration dossiers. The surface tension of Tween 80 (CAS RN 9005-65-6) was obtained from the Handbook of pharmaceutical excipients (2020).

5.4. Hypothesis generation

In case data on all the *in vitro* test methods are available and are within the applicability domain of each test method, the Bottom-Up or Top-Down approach can be used. Based on a WoE analysis on all information collected, as described in part 1 and 2 of IATA GD 263, the most likely hazard (classified versus not-classified) is identified and the Top-Down or Bottom-Up approach is followed.

a. If it is assumed that the surfactant is a severe eye irritant it is recommended to follow the Top-Down approach, and start with the modified STE test method that can identify test chemicals causing serious and/or irreversible eye damage (UN GHS Cat. 1). Further testing is required with an RhCE method (VRM1 or VRM2) in case the chemical does not result in severe eye damage based on the modified STE.

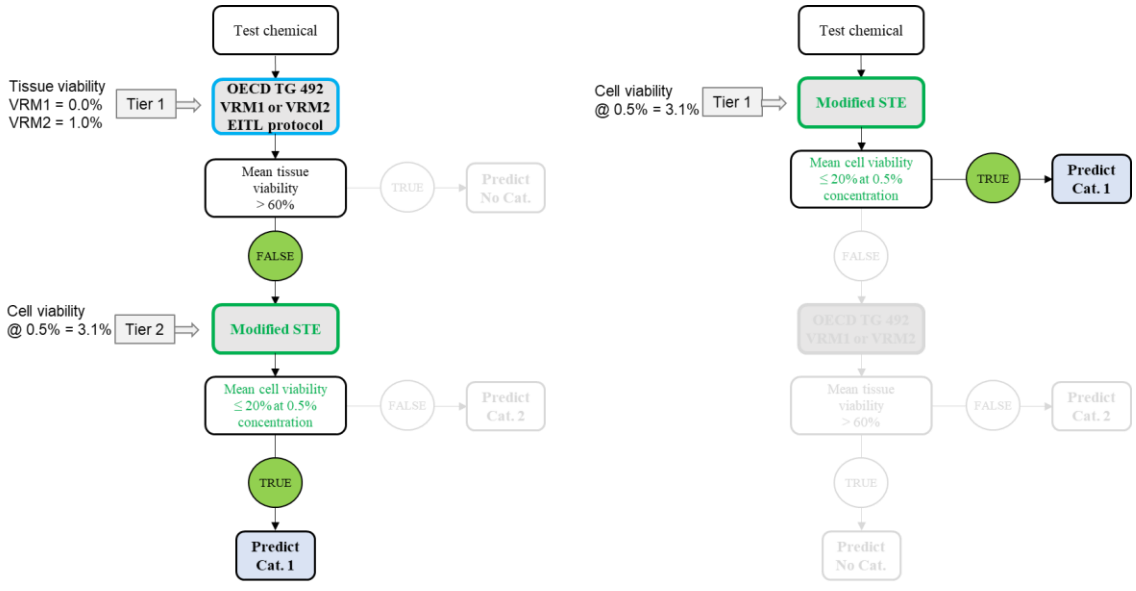
b. If the surfactant is assumed not to cause eye irritation, it is recommended to start with an RhCE method (VRM1 or VRM2). Further testing with the modified STE method is required if the surfactant is not predicted No Cat. with VRM1 and/or VRM2.

6 Data interpretation procedure applied

The DIP applied uses the readout of the individual NAMs described in paragraph 16 and 17. The DASF is constructed as a tiered approach with a decision point at the end of each tier, allowing stepwise and efficient information gathering. The first tier can be either the RhCE test method (Bottom-Up) or the modified STE test method (Top-Down). In the Bottom-Up approach, an RhCE test method is used to distinguish No Cat. from classified substances. In case the surfactant results in a positive call based on an RhCE method, the modified STE method is used to further sub-categorize into Cat. 1 (positive call) or Cat. 2 (negative call). A scheme of the Bottom-Up and Top-Down approach for the DASF is presented in Figure 1 and illustrated with an example in Figure 3.

Application of the DASF for the variations of the DIP are illustrated in Figure 3 with ethylhexyl acid phosphate ester (CAS RN 12645-31-7), a liquid surfactant. Applying the Bottom-Up approach, the EITL protocol for the RhCE test method is used in Tier 1 to identify surfactants with no serious eye damage or eye irritation potential. Both RhCE methods (VRM1 & VRM 2) result in tissue viability much lower than 60% (not a No Cat.). Further testing is required with the modified STE in Tier 2 to classify the surfactant as UN GHS Cat. 1 or Cat. 2. The final prediction corresponds with UN GHS Cat. 1, the viability for the 0.5% concentration is below 20%. When the Top-Down approach is used, only one tier is required since the modified STE induced low cell viability (< 20%) for the 0.5% test concentration resulting in a UN GHS Cat. 1 prediction. It can be concluded that regardless of the DIP version used, the prediction for ethylhexyl acid phosphate ester corresponds to UN GHS Cat. 1 (Table 3).

Figure 3. Bottom-Up and Top-Down approach of the DASF for ethylhexyl acid phosphate ester (CASRN 12645-31-7)



The prediction of the 3 surfactants discussed in this case study are presented in Table 3. The DIPs of Cetylpyridinium bromide and Tween 80 are illustrated in Annex A and B.

Table 3. Results of the individual test methods and the DASF prediction for the surfactants of the case study.

Chemical	CASRN	Conc.	RhCE tissue viability		RhCE prediction	STE cell viability @ 0.5%	STE prediction	DASF prediction	BU/TD [a]
			VRM1	VRM2					
Ethylhexyl acid phosphate ester	12645-31-7	Neat	0.0%	1.0%	NPCM	3.1%	Cat. 1	Cat. 1	Yes
Cetylpyridinium bromide	140-72-7	10%	3.9%	1.4%	NPCM	4.3%	Cat. 1	Cat. 1	Yes
		6%	8.5%	1.8%	NPCM	13.1%	Cat. 1	Cat. 1	Yes
		1%	49.6%	9.5%	NPCM	71.9%	Not Cat. 1	Cat. 2	Yes
		0.1%	80.9%	90.3%	No Cat.	96.4%	Not Cat. 1	No Cat.	Yes
Tween 80	9005-65-6	Neat	72.3%	74.5%	No Cat.	96.5%	Not Cat. 1	No Cat.	Yes

NPCM: No Prediction Can be Made

[a] *BU/TD: Bottom-Up/Top-Down concordance in prediction.*

In vitro data were retrieved from public sources (Alépée et al., 2018, 2020, 2023; Abo et al., 2018; Kaluzhny et al., 2011; Kandarova et al., 2018; Takahashi et al., 2010).

7 Application of the Defined Approach

7.1. Performance of the DASF

The predictive performance considering the three UN GHS categories (Cat. 1, Cat. 2, No Cat.) of the DASF is reported for 47 surfactants (Table 4). Since not all surfactants were tested with both RhCE methods, the results of VRM1 and VRM2 were combined. Note that the concordance in predictions for the surfactants tested with both methods was 97.7% (42/43). The performance of the DA was assessed against the minimum performance of 75% for Cat. 1, 50% for Cat. 2 and 70% for No Cat. as agreed by the OECD experts for the DAs for non-surfactant liquids (OECD SD 354, 2022c). The DASF met the acceptance criteria, 90.9% of Cat. 1 (N = 22), 75.0% of Cat. 2 (N = 8), and 76.0% of No Cat. (N = 17) were correctly predicted. In addition, the performance for the *in vivo* classified surfactants was similar for the Bottom-Up and Top-down approach (Annex D, Table 11). Since STE results were missing for 2/17 *in vivo* No Cat. substances predicted as No Cat. using an RhCE test method, the performance of the Top-Down approach for this category could not be assessed.

Table 4. Performance of the DASF (N=47)

UN GHS	Prediction DASF		
	Cat. 1	Cat. 2	No Cat.
Cat. 1 (N='22),' %[a] (n/N)	≥75% 90.9% (20/22)	≤ 25% [a] 9.1% (2/22)	≤ 5% [a] 0.0% (0/22)
Cat. 2 (N='8),' %[a] (n/N)	≤ 30% [a] 25.0% (2/8)	≥ 50% [a] 75.0% (6/8)	≤ 30% [a] 0.0% (0/8)
No Cat. (N='17),' %[a] (n/N)	≤ 5% [a] 5.9% [b] (1/17)	≤ 30% [a] 18.1% (3.1/17) [c]	≥ 70% [a] 76.0% (12.9/17) [c]

[a] OECD minimum acceptance criteria

[b] It is acknowledged that the over-prediction rate for UN GHS No Cat. predicted as Cat. 1 is slightly higher (5.9%) than the value accepted by the OECD (5%). However, this is an exceedance that is negligible (i) given that the correct No Cat. prediction of 76% is above the minimum of 70% and the No Cat. over-prediction rate of 18.1% is below the maximum of 30% and (ii) the fact that the surfactant (3% Sodium lauryl sulfate, CAS RN 151-21-3) also resulted in some irritation in the Draize eye study. The substance resulted in corneal opacity above the classification threshold of CO mean ≥ 1 in 1 out of 6 animals (subgroup CO > 0 **).

[c] The final prediction of the DASF is based on all prediction results (weighted calculation which takes into account all individual results, one surfactant resulted once in NPCM and 11 times in No Cat. with the RhCE test method). In all other cases where multiple results exist for a given chemical, the prediction was concordant. To improve the readability of the numbers in the table, the numbers n/N have been rounded, so they may deviate slightly from the percentage corresponding to the weighted calculation.
Note: The performance of the UN GHS Cat. 1 and UN GHS Cat. 2 is the same for the two versions of the DIP (TD/BU approaches) (Figure 1).

Optimization of the STE test method resulted in a significant improvement of the correct Cat. 1 prediction compared to the OECD-adopted version of the STE test method (OECD TG 491), which predicted only 54.5% Cat.1 correctly (Table 11, Annex D).

The set of reference chemicals used to assess the performance of the DASF was composed of 31 unique surfactants (neat and diluted, Table 8, Annex D). Out of the 31 surfactants, 9 were tested neat, 13 were tested at a single dilution and 9 were tested at multiple dilutions (at least 2 dilutions per surfactant). The set contained all available surfactants with historical good quality Draize eye test data (paragraph 5). The set contains cationic, anionic, non-ionic and amphoteric surfactants from different families (Table 7, Annex C). More detailed information on the development and performance of the DASF and the predictions of the NAMs for all surfactants (N=47) are provided in Annex D.

7.2. Limitations in the application of the defined approach

The DASF is applicable to neat and diluted surfactants, including, mono- and multi-constituent substances and UVCBs. The DASF is not applicable to non-surfactants. Because the reference set for Cat. 2 contains only surfactants tested in dilution, the confidence of a Cat. 2 prediction for neat surfactants is unknown.

The strengths and limitations of individual test methods are described in the corresponding OECD TG 491 and TG 492 (OECD, 2023d; OECD, 2023e). The limitations of the OECD adopted version of the STE also apply to the modified STE test method. Users should refer to the limitations of the individual *in vitro* test methods as specified in their respective TGs, which are revised as new data become available and should be consulted regularly. Currently, there are no restrictions on the testing of surfactants described in TG 491 and TG 492.

It is important to separate the limitations into (1) technical limitations and (2) limitations in the predictivity for UN GHS categories. For example, for the STE test method the test item should dissolve or form a stable suspension for at least five minutes in a selected solvent. Therefore, upfront consideration should be given to verify if a chemical is testable in the STE and this may thus limit the DASF applicability domain. No technical limitation is known for the RChE methods for the evaluation of surfactants.

7.3. Considerations of uncertainties associated with the application of the defined approach

7.3.1. Uncertainty of the information sources used within the DASF

Transferability, within- and between-laboratory reproducibility (WLR and BLR) of the OECD TG 492 adopted test methods have been assessed during their respective validation studies (EC EURL ECVAM, 2014; Alépée et al., 2016a and 2016b). Transferability, within- and between-laboratory reproducibility (WLR and BLR) of OECD TG 491 (test concentration of 5% and 0.05%) has been assessed during the respective validation studies (STE review document ICCVAM, 2013). During these validation studies, STE cell viability was also measured at 0.5% (results not published), so

reproducibility at this concentration could be assessed. The BLR for a concentration of 0.5% with 20% cell viability as cut-off to distinguish Cat. 1 from not Cat. 1, based on 71 compounds (16 surfactants, 15 solids, and 40 liquids), was 97.2%. BLR at 0.5% in terms of predictions was demonstrated for 8 surfactants (Annex D, Table 11) resulting in 100% concordance in predictions.

7.3.2. Impact of uncertainty on the DIP's prediction

The uncertainty of this case study has been qualitatively assessed by reviewing and discussing the uncertainty in each element of the DASF. Table 5 describes the uncertainty along each step of the DA.

Table 5. Uncertainties associated with the individual components of the DASF and how they affect the overall uncertainty of the final outcome/prediction

DASF element	Comment
General considerations	The reproducibility and reliability of the *in vitro* test methods was assessed during the validation studies. Therefore, the uncertainty of the predictions is considered minimal. Similar reproducibility and reliability were reported for both RhCE VRMs. The BLR was at least 92%. The accuracy to distinguish No Cat. from classified chemicals was 82% to 84%, with a specificity of at least 63% and sensitivity of at least 95% (EC EURL ECVAM, 2014; Alépée et al., 2016a and 2016b). The reproducibility and reliability of the OECD-adopted STE test method was assessed during the validation studies. The BLR of the modified STE test method was 98% (paragraph 35). The accuracy of the modified STE test method to distinguish Cat. 1 from not Cat. 1 based on the set of surfactants was 89.4% (42/47), with 90.9% (20/22) sensitivity and 88.0% (22/25) specificity. .
Case study chemicals	The surfactants of the case study are all well characterized. Cetyl pyridinium bromide (CASRN 140-72-7) is a mono-constituent cationic surfactant with a purity of 98%. Ethylhexyl acid phosphate ester (CASRN 12645-31-7) is a multi-constituent (mixture of mono- (42.8%) and di-esters (51.0%)) anionic surfactant. Tween 80 (CAS RN 9005-65-6) is a non-ionic UVCB. Low uncertainty of the DASF prediction for new surfactants that fall within the range of surfactant properties (classes and families listed in Table 7, Annex C) used to develop the DASF.
NAM data used in DASF	No technical issues were reported while testing the chemicals in the *in vitro* methods.
DASF outcome/prediction	Surfactant 1 (Ethylhexyl acid phosphate ester, CASRN 12645-31-7): This chemical was tested neat and induced low viability in both RhCE methods indicating that the surfactant causes severe cytotoxicity of the reconstructed tissues. This is confirmed by the high cytotoxicity that was observed in the modified STE test method. Viability for the RhCE and modified STE test methods was well below the classification cut-off, so the level of confidence in the DASF prediction is high. Surfactant 2 (Cetyl pyridinium bromide, CASRN 140-72-7): was tested at 4 dilutions. • A 10% and 6% w/v concentration resulted in low viability in both RhCE methods and in the modified STE test method. Because tissue viability was well below the classification cut-off, the level of confidence in the DASF prediction is high. • A 1% w/v concentration resulted in low (VRM1) to moderate (VRM2) viability, but clearly below the cut-off value. Only limited cytotoxicity was observed in the modified

	STE, which was clearly above the cut-off, so the level of confidence in the DASF prediction is high. • A 0.1% w/v concentration resulted in high viability in both RhCE models, those methods have low false negative predictions (OECD TG 492, 2023e). Since the viability was clearly above the cut-off the level confidence in the No Cat. prediction based on the stand-alone method is high. • Note that for all test methods the cytotoxicity increased with increasing surfactant concentration. In general, viability was well below or above the cut-off value for classification for the different components of the DASF, so the level of confidence in the DASF prediction is high. Surfactant 3 (Tween 80, CASRN 9005-65-6): Tween 80 was tested neat and resulted in high viability in both RhCE models. Since the viability was clearly above the cut-off, the level confidence in the No Cat. prediction based on the stand-alone method is high.
Overall uncertainty for DASF	The different variations of the DIP result in the same hazard assessment conclusion with a low uncertainty.

7.3.3. Hazard assessment

The prediction of the surfactants discussed in this case study was compared with the UN GHS category that was derived from the Draize Eye test and the results are presented in Table 6.

Table 6. Comparison between UN GHS category and DASF predictions

Chemical	CASRN	Concentration	UN GHS [a]	Prediction DASF [b]	Figures
Ethylhexyl acid phosphate ester	12645-31-7	Neat	Cat. 1	Cat. 1	Figure 3
Cetylpyridinium bromide	140-72-7	10%	Cat. 1	Cat. 1	Annex A
		6%	Cat. 1	Cat. 1	Annex A
		1%	Cat. 2	Cat. 2	Annex A
		0.1%	No Cat.	No Cat.	Annex A
Tween 80	9005-65-6	Neat	No Cat.	No Cat.	Annex B

[a] Barroso et al, 2017 - DRD Supplementary Material 1
[b] The different variations of the DIP come to the same hazard assessment conclusion with a low uncertainty.

Hazard assessments based upon predictions from the DASF concluded that (1) Ethylhexyl acid phosphate ester (CASRN 12645-31-7) and 10% and 6% Cetylpyridinium promide (CASRN 140-72-7) are classified UN GHS Cat. 1, (2) 1% Cetylpyridinium promide (CASRN 140-72-7) is classified UN GHS Cat. 2 and (3) 0.1% Cetylpyridinium promide (CASRN 140-72-7) and Tween 80 (CASRN 9005-65-6) are classified UN GHS No Cat. This is in agreement with the UN GHS categories that were assigned based on historical *in vivo* Draize eye test studies.

7.3.4. Strategy and integrated hazard assessment conclusion

This case study, with two neat surfactants (1 liquid, 1 solid) and one surfactant (solid in solvent) tested at different concentrations (0.1% to 10%), was performed to illustrate the use of the DASF and the potential to successfully distinguish between the 3 UN GHS categories for eye hazard identification. The case study followed the tiers and steps outlined in the DASF (Figure 1). The data of the different information sources (*in vitro* test methods) were integrated in the DA and both the Bottom-Up and Top-Down approach was illustrated.

This case study illustrates an approach on how to move from animal testing into an evaluation of new surfactant (neat or diluted) ingredients based on examples of application of a DA on an IATA for safety purposes of surfactants.

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Annex A. Cetylpyridinium bromide (CASRN 140-72-7)

Figure 4. Bottom-Up and Top-Down approach of the DASF for Cetylpyridinium bromide 10% (CASRN 140-72-7)

Left scheme (Bottom-Up): Tier 1 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat and Tier 2 starts with the modified STE to identify Cat. 1. Right scheme (Top-Down): Tier 1 starts with the modified STE to identify Cat. 1.

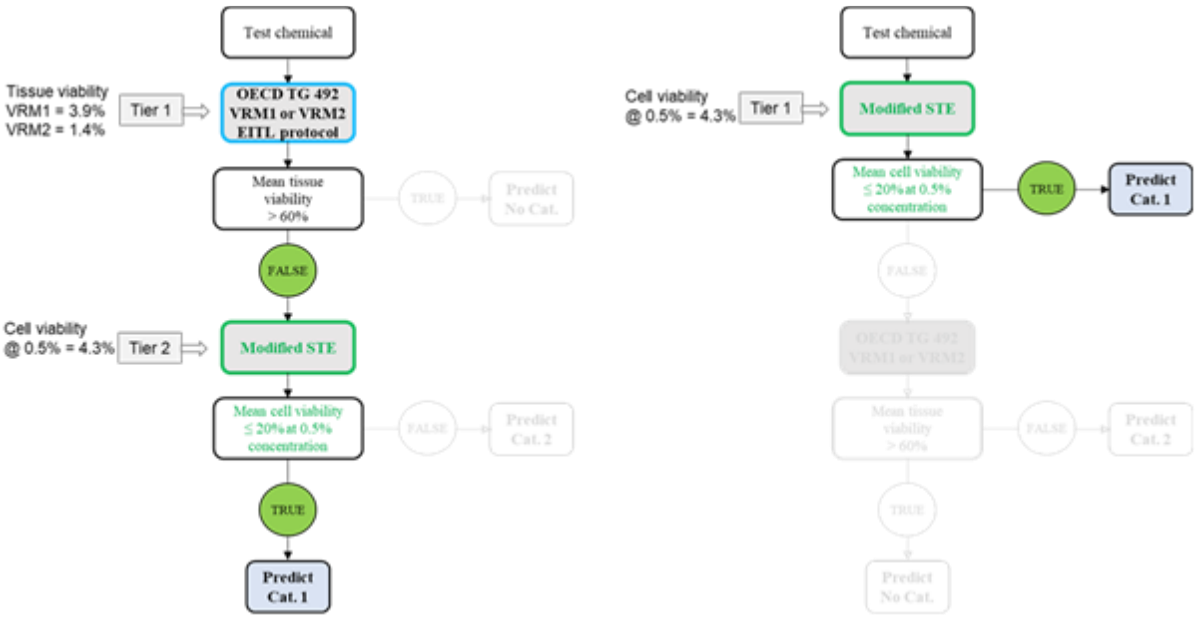


Figure 5. Bottom-Up and Top-Down approach of the DASF for Cetylpyridinium bromide 6% (CASRN 140-72-7)

Left scheme (Bottom-Up): Tier 1 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat and Tier 2 starts with the modified STE to identify Cat. 1. Right scheme (Top-Down): Tier 1 starts with the modified STE to identify Cat. 1.

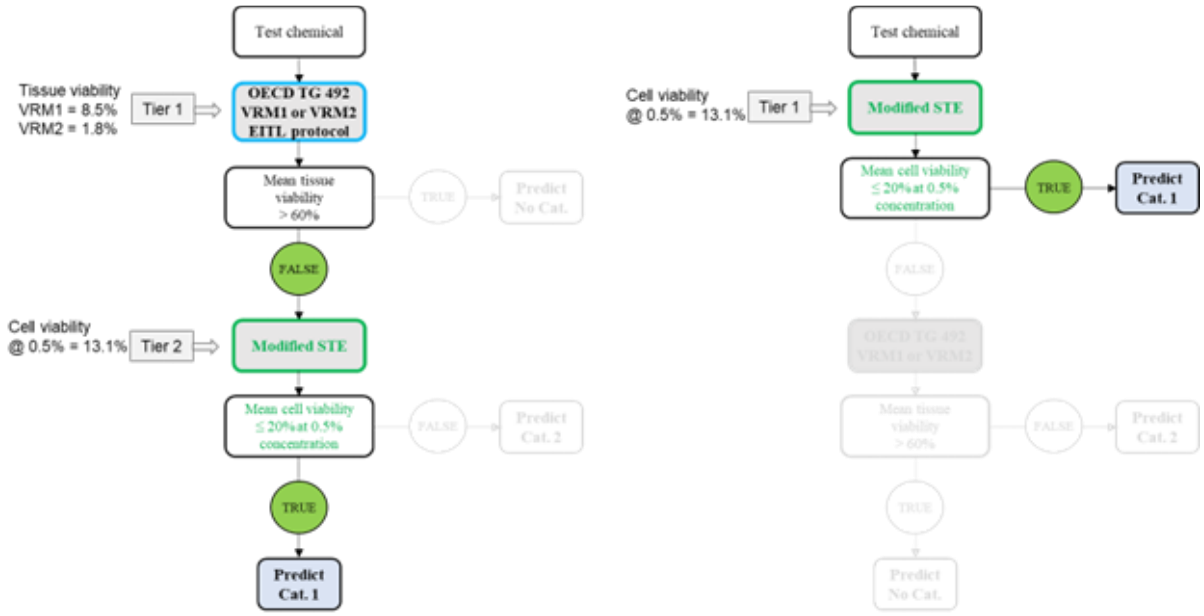


Figure 6. Bottom-Up and Top-Down approach of the DASF for Cetylpyridinium bromide 1% (CASRN 140-72-7)

Left scheme (Bottom-Up): Tier 1 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat and Tier 2 starts with the modified STE to identify Cat. 1. Right scheme (Top-Down): Tier 1 starts with the modified STE to identify Cat. 1 and Tier 2 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat.

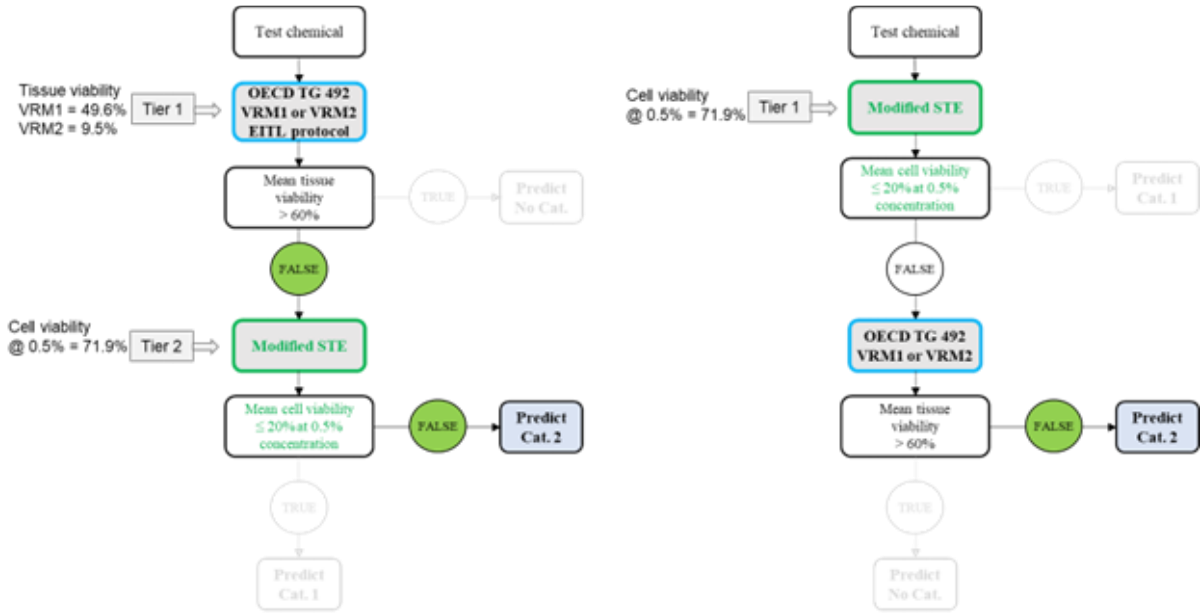
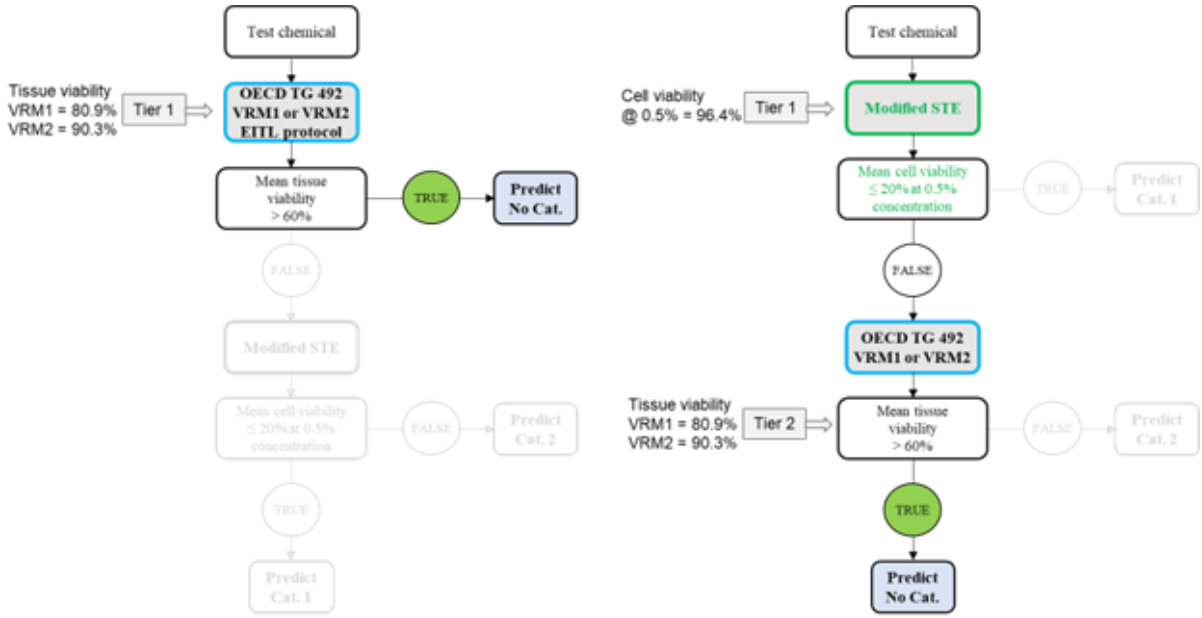


Figure 7. Bottom-Up and Top-Down approach of the DASF for Cetylpyridinium bromide 0.1% (CASRN 140-72-7)

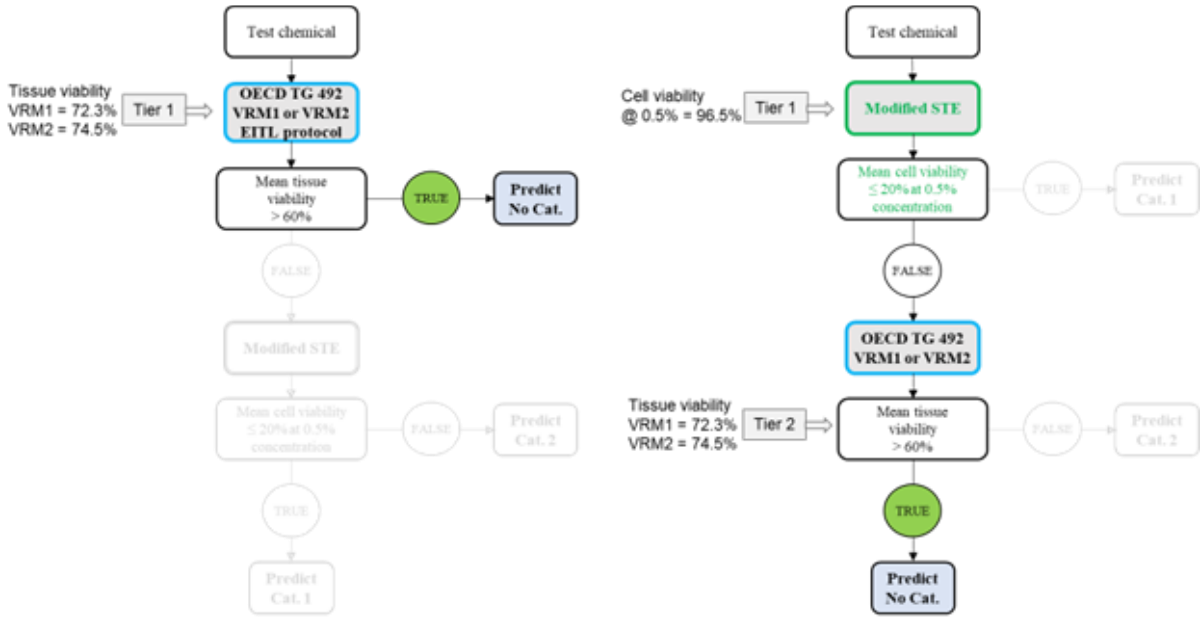
Left scheme (Bottom-Up): Tier 1 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat and Tier 2 starts with the modified STE to identify Cat. 1. Right scheme (Top-Down): Tier 1 starts with the modified STE to identify Cat. 1 and Tier 2 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat.



Annex B. Tween 80 (CASRN 9005-65-6)

Figure 8. Bottom-Up and Top-Down approach of the DASF for Tween 80 (CASRN 9005-65-6)

Left scheme (Bottom-Up): Tier 1 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat and Tier 2 starts with the modified STE to identify Cat. 1. Right scheme (Top-Down): Tier 1 starts with the modified STE to identify Cat. 1 and Tier 2 starts with an RhCE test method (Liquids protocol: EITL) to identify No. Cat.



Annex C. Surfactants information

Table 7. Listing of the surfactants that were used to assess the performance of the DASF

Name	CAS RN	Physical form	Type	Class	Family	Surface tension	Concentrations tested and their corresponding UN GHS (based on Draize add in footnote)		
							Cat. 1	Cat. 2	No Cat.
Cetyltrimethyl ammonium bromide	57-09-0	L (tested in solvent, available as S)	mono-constituent	Cationic	Quaternary ammonium compound	39.0	10%		
Stearyltrimethylammonium chloride	112-03-8	L (tested in solvent, available as S)	mono-constituent	Cationic	Quaternary ammonium compound	38.0	10%		
Didecyl dimethyl ammonium chloride	7173-51-5	L (tested in solvent, available as S)	mono-constituent	Cationic	Quaternary ammonium compound	27.0	1%		
Benzalkonium chloride	63449-41-2	L (tested in solvent, available as S)	UVCB	Cationic	Quaternary ammonium compound	28.3	10%, 5%, 1%		
Domiphen bromide	538-71-6	L (tested in solvent, available as S)	mono-constituent	Cationic	Quaternary ammonium compound	NA	10%		
Di(2-ethylhexyl)sodium sulphosuccinate	577-11-7	L (tested in solvent, available as S)	mono-constituent	Anionic	Sulphosuccinates	30.7	10%		

Coco amidopropyl betaine	61789-40-0	Liquid	UVCB	Amphoteric	Quaternary ammonium compound	35.0	25%		
Cetylpyridinium chloride	6004-24-6	L (tested in solvent, available as S)	mono-constituent	Cationic	Alkyl pyridinium	44.3	10%		0.1%
Sodium lauryl sulphate	151-21-3	L (tested in solvent, available as S)	mono-constituent	Anionic	Alkyl sulfates	25.2	15%		3%, 1%
Ethylhexyl acid phosphate ester	12645-31-7	Liquid	multi-constituent	Anionic	Fatty alcohol phosphoric acid esters	44.4	Neat		
Distearyldimethylammonium chloride	107-64-2	Solid	mono-constituent	Cationic	Quaternary ammonium compound	11.0	Neat		
Ethyl lauroyl arginate HCl	60372-77-2	Solid	mono-constituent	Cationic	Ammonium salt	25.4	Neat		
Surfonic HDL-1 (Chemical name: Nonyl phenol ethoxylate, branched)	9016-45-9	Liquid	UVCB	Nonionic	Nonylphenols, ethoxylated with triethanolamine	31.5	Neat		
Triton X-100	9002-93-1	Liquid	UVCB	Nonionic	Octylphenol, ethoxylated	30.0	Neat, 10%	5%	1%
1-Hexadecanaminium, N,N,N-trimethyl-, chloride	112-02-7	L (tested in solvent, available as S)	mono-constituent	Cationic	Quaternary ammonium compound	33.0	25%	2%	
Cetyl pyridinium bromide	140-72-7	L (tested in solvent, available as S)	mono-constituent	Cationic	Alkyl pyridinium	NA	10%, 6%	1%	0.1%
Benzethonium chloride	121-54-0	L (tested in solvent, available as S)	mono-constituent	Cationic	Quaternary ammonium compound	33.4.	10%	1%	
N-Lauroyl sarcosine Na salt	137-16-6	L (tested in solvent, available as S)	mono-constituent	Anionic	Amino acid surfactant	31.4	30%	10%	3%

Deoxycholic acid Na salt	302-95-4	L (tested in solvent, available as S)	mono-constituent	Anionic	Steroid	41.8		10%	
Lauryl sulphobetaine	14933-08-5	L (tested in solvent, available as S)	mono-constituent	Amphoteric	Sulphobetaine	35.5		10%	
Methyl N,N,N-trimethyl-4-[(4,7,7-trimethyl-3-oxobicyclo[2.2.1]hept-2-ylidene)methyl]anilinium sulphate	52793-97-2	L (tested in solvent, available as S)	mono-constituent	Cationic	Quaternary ammonium compound	NA		30%	
Polyethylene glycol monolaurate (10EO)	9004-81-3	Liquid	UVCB	Nonionic		NA			10%
Steareth-10 allyl ether/acrylates copolymer	109292-17-3		multi-constituent	Anionic		NA			40%
Polyoxyethylene 23 lauryl ether (Brij-35)	9002-92-0	L (tested in solvent, available as S)	UVCB	Nonionic	Alcohol ethoxylate	36.6			10%
Polyoxyethylene 8-stearate (Myrj-45)	9004-99-3	L (tested in solvent, available as S)	UVCB	Nonionic	Alcohol ethoxylate	NA			10%
Tween 80	9005-65-6	Liquid	UVCB	Nonionic	Sorbitan ethoxylated fatty acid ester	24.0			Neat, 10%
Tween 20	9005-64-5	Liquid	UVCB	Nonionic	Sorbitan ethoxylated fatty acid ester	28.8			Neat
Polyglyceryl-3-diisooctadecanoate	63705-03-3	Liquid	UVCB	Nonionic		NA			Neat
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	Solid	UVCB	Nonionic	Alkoxylated fatty acids	NA			Neat
Cellulose,2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride	68610-92-4	Solid		Cationic	polymeric quaternary ammonium salt of hydroxyethyl cellulose	NA			Neat

Myristyl myristate	3234-85-3	Solid	mono-constituent	Nonionic		NA			Neat

NA: not available

Annex D. Background information on the development and performance of the DASF

Two rule-based defined approaches (DAs) were developed to fully replace the Draize eye test for eye hazard identification of non-surfactant liquids (Alépée et al., 2019a, 2019b). The DAs are based on the combinations of methods as recommended in the Part 3 of the IATA GD No. 263 (OECD, 2023g) and are integrated in a new OECD TG 467 (OECD, 2022b). Background information supporting TG 467 is reported in OECD GD 354 (2022c). The DAL-1 is based on the use of a combination of test methods described in OECD TG 437 (Bovine Corneal Opacity and Permeability (BCOP) using the laser light-based opacitometer (LLBO), 2023a) and TG 492 (EpiOcular™ EIT or SkinEthic™ HCE EIT, OECD, 2023e) as well as the physicochemical properties of the test chemical. DAL-2 in contrast, is based on the use of a combination of test methods described in the OECD TG 437 (BCOP LLBO, 2023a) and TG 491 (OECD, 2023d). The DAL-1 is applicable to neat non-surfactant liquids. The DAL-2 is applicable to non-surfactant neat liquids, liquids and solids dissolved in water.

It is important to note that the restriction of the applicability domain of DAL-1 to neat non-surfactant liquids was based on a number of observations that are briefly outlined. During the development of the DAs, all chemicals (liquids, solids, and surfactants) were considered and the result showed that the predictivity was better for liquids as compared to solids (Alépée et al., 2019a). Furthermore, for the BCOP LLBO test method, a difference in predictivity was observed between non-surfactants liquids and surfactants. The correct identification of *in vivo* Cat. 2 non-surfactant liquids was higher when only the opacity endpoint was considered, while this did not affect the predictive value for *in vivo* Cat. 1. This is in contrast to what was observed for *in vivo* Cat. 1 surfactants, for which approximately 40% (N=14) were underpredicted as Cat. 2 based on the BCOP LLBO. Therefore, surfactants were excluded from the applicability domain of the DAL-1. In the DAL-2, the STE (OECD TG 491, 2023d) test method is used to identify No Cat. liquids while the BCOP based on the LLBO opacity endpoint is used to identify Cat. 1 liquids. Surfactants were also excluded based on the same reason described for DAL-1. Furthermore, non-surfactant solids were excluded since those chemicals are outside the applicability domain of the STE test method when used to identify No Cat. (OECD, 2023d).

Subsequently, initial analyses based on the original dataset used to develop DAL-1 and DAL-2 showed that a combination of the RhCE (EpiOcular™ EIT or SkinEthic™ HCE EIT, OECD, 2023e) test methods with a modification of the STE test method (5 min exposure to a 0.5% concentration) seemed the most promising approach. As such, the original set of 22 surfactants was further expanded to provide a more comprehensive set of 47 test items having surfactant properties. Since the applicability domain is limited to surfactants only, there exists only a limited database with good quality Draize eye test data. Especially the set of available *in vivo* Cat. 2 surfactants (N = 8) is small. Further efforts were made by consulting regulatory agencies and stakeholders, and reviewing the Cosing (Cosmetic Ingredient) database, but so far no additional Cat. 2 surfactants could be identified that meet the predefined requirements. Only surfactants for which individual raw data of the Draize eye study are available and that have no conflicting data in case multiple *in vivo* studies are available, should be considered as candidates for assessing the performance of the DASF.

In the next paragraphs, the performance of the individual NAMs are discussed and the performance of the combination of TG 492 with TG 491 and the DASF are compared.

The performance of the RhCE test methods are provided in Table 8. The details on the viability and predictions for the individual surfactants are provided in Table 11. EpiOcular™ EIT and SkinEthic™ HCE EIT results were available for 46 and 44 surfactants, respectively. Results on 43 surfactants were available for both RhCE test methods, and for the remaining 4 surfactants, results were available for one RhCE. The concordance in predictions between the RhCE test methods was 97.7%, 42 out of 43 surfactants that were tested with both methods, were predicted identically. Only one surfactant (Cellulose,2-(2-hydroxy-3-(trimethylammonium)propoxy) ethyl ether chloride, CASRN 68610-92-4) showed discordant predictions. With the EpiOcular™ EIT, this substance was two times predicted No Cat. and once "no stand-alone prediction can be made" (NPCM). This surfactant was nine times predicted as No Cat. with the SkinEthic™ HCE EIT method. For several surfactants multiple results were available for each test method, the concordance in prediction was 95.2% (20/21) for EpiOcular™ EIT and 100% (21/21) for SkinEthic™ HCE EIT. The false negative rate for both methods was 0%, with 72.9% (EpiOcular™ EIT, N = 16) and 75.0% (SkinEthic™ HCE EIT, N = 16) of the *in vivo* No Cat. being correctly identified (Table 8). Therefore, OECD TG 492 (EpiOcular™ EIT or SkinEthic™ HCE EIT) can be used in a first tier of a Bottom-Up approach or in the last tier of a Top-Down approach for eye hazard assessment of surfactants.

Table 8. Performance of the RhCE test methods (VRM1 and VRM2, OECD TG 492)

UN GHS	TG 492					
	EpiOcular™ HCE			SkinEthi™ HCE EIT		
	N	NPCM	No Cat.	N	NPCM	No Cat.
Cat. 1	22	100%	0%	18	100%	0%
Cat. 2	8	100%	0%	8	100%	0%
No Cat.	16	27.1%	72.9%	16	25.0%	75.0%

The performance of the STE according to TG 491 and according to the modified STE is provided in Table 9. The cell viability for the individual surfactants at 5%, 0.5%, and 0.05% is provided in Table 11. Note that several Cat. 1 surfactants resulted in a cell viability >70% at a 0.05% test concentration and all these Cat. 1 surfactants had a cell viability <70% at a 5% test concentration (see also Figure 2), and are therefore categorized as NPCM. This was also observed for almost all Cat. 2 surfactants, meaning that it is difficult to distinguish *in vivo* Cat. 1 from *in vivo* Cat. 2 surfactants. The majority of the *in vivo* No Cat. surfactants resulted in a cell viability >70% at both the 5% and 0.05% test concentration. When the OECD TG 491 prediction model was applied, 54.5% Cat. 1 (N = 22) and 61.7% No Cat. (N = 15) surfactants were correctly identified (Table 9). These values are below the OECD minimum acceptance criteria of 75% for Cat. 1 and 70% for No Cat. Therefore, it is not recommended to use TG 491 in a first tier of the Top-Down or Bottom-Up approach for eye hazard assessment of surfactants. Based on concentration response studies (5%, 0.5%, and 0.05% test concentration) it was shown that a 0.5% concentration in combination with 20% cell viability as cut-off value improved the correct identification of Cat. 1 surfactants substantially. This cut-off value of 20% that best discriminates between the two categories (Cat. 1 vs Not Cat. 1) was identified by classification trees based. The modified STE identified 90.9% of the Cat. 1 (N = 22) correctly with an acceptable over-prediction of *in vivo* Cat. 2 and

No Cat. predicted as Cat. 1 (Table 9), it is therefore recommended to use the modified STE in a first tier of the Top-Down approach or the last tier in the Bottom-Up approach.

Table 9. Performance of the STE and modified STE test method

UN GHS	STE					
		TG 491			Modified STE	
	N	Cat. 1	NPCM	No Cat.	Cat. 1	Not Cat. 1
Cat. 1	22	54.5%	45.5%	0.0%	90.9%	9.1%
Cat. 2	8	0.0%	100%	0.0%	25.0%	75.0%
No Cat.	15	0.0%	38.3%	61.7%	6.7%	93.3%

The performance of the individual test methods showed that a combination of a RhCE test method (OECD TG 492) and the modified STE test method seemed most promising to identify the eye hazard potential of surfactants. The EpiOcular™ EIT or SkinEthic™ HCE EIT test methods are used in a first step to identify No Cat. surfactants. In case the viability of the RhCE method is below 60%, the modified STE test method is proposed to identify Cat. 1 surfactants. In order to assess the predictivity of the DASF based on the set of 47 surfactants, the results of EpiOcular™ EIT and SkinEthic™ HCE EIT were combined since not all surfactants were tested in both methods. Note that the concordance in predictions for the surfactants that were tested with both RhCE methods was almost 97.7% (based on 43 surfactants). Furthermore, the performance of the DASF was the same for the Bottom-Up and Top-Down approach (Table 11). The DASF met the OECD minimum acceptance criteria, 90.9% of Cat. 1 (N=22), 75.0% of Cat. 2 (N=8), and 76.0% of No Cat. (N=17) were correctly predicted (Table 4). The performance of the DA based on the combination of OECD TG 492 test methods (VRM1 or VRM2) and the OECD TG 491 test method based on the same set of surfactants is shown in Table 10. The performance of the DASF that combines OECD TG 492 (VRM1 or VRM2) with the modified STE test method (test concentration of 0.5%) is substantially better than the combination of OECD TG 492 and OECD TG 491 (STE test method with test concentration of 5% and 0.05%). The DASF identified 90.9% of Cat. 1 correctly versus 54.5% for OECD TG 491 in combination with OECD TG 492.

Table 10. Performance of the DA based on the combination of the RhCE test methods (VRM1 and VRM2, OECD TG 492) and the STE adopted test method (OECD TG 491) (N=47)

UN GHS	Prediction combination TG 492 and TG 491		
	Cat. 1	Cat. 2	No Cat.
Cat. 1 (N='22),' %[a] (n/N)	≥ 75% 54.5% (12/22)	≤ 25% 43.9% (9.7/22)	≤ 5% 1.5% (0.3/22)
Cat. 2 (N='8),' %[a] (n/N)	≤ 30% 0.0%	≥ 50% 100%	≤ 30% 0.0%

	(0/8)	(8/8)	(0/8)
No Cat. (N='17),' %[a] (n/N)	≤ 5% 0.0% (0/17)	≤ 30% 24.0% (4.1/17) [a]	≥ 70% 76.0% (12.9/17) [a]

OECD minimum acceptance criteria

[a] The final prediction is based on all prediction results (weighted calculation which takes into account all individual results, one surfactant resulted once in NPCM and 11 times in No Cat. with the RhCE test method). In all other cases where multiple results exist for a given chemical, the prediction was concordant. To improve the readability of the numbers in the table, the numbers n/N have been rounded, so they may deviate slightly from the percentage corresponding to the weighted calculation.

In conclusion the DASF is the best strategy for eye hazard identification of surfactant.

Table 11. Predictions for the individual test methods, the combination of TG 492 with TG 491 and the DASF.

Name	CAS RN	EpiOcular™ EIT (TG 492)				SkinEthic™ HCE EIT (TG 492)				STE									Combination	DASF	UN GHS	Agreement in prediction
		N	Tissue viability	Prediction		N	Tissue viability	Prediction		N	Cell viability			OECD TG 491			modified STE		TG491 with TG492	Prediction	Draize	
			min - ma	NPCI	No Ca			NPCI	No Ca		5%	0.5%	0.05%	Cat.	NPC	No C	Cat.	Not Ca				TP/BU
Cetyltrimethyl ammonium bromide (10%)	57-09-0	3	6.0 - 17.3	1.00	0.00	11	1.6 - 4.2	1.00	0.00	1	2.5	5.9	69	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Stearyltrimethylammonium chloride (10%)	112-03-8	1	12.1	1.00	0.00	3	3.9 - 6.9	1.00	0.00	1	3.2	4.9	80.7	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
Didecyl dimethyl ammonium chloride (1%)	7173-51-5	1	33.1	1.00	0.00	1	21.6	1.00	0.00	1	2.6	18.9	92.9	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
Benzalkonium chloride (10%)	63449-41-2	3	4.1 - 7.2	1.00	0.00	1	1.5	1.00	0.00	1	6.5	4.1	48.3	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Benzalkonium chloride (5%)	63449-41-2	2	3.0 - 4.0	1.00	0.00	1	1.9	1.00	0.00	1	8.9	10.9	80.7	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
Benzalkonium chloride (1%)	63449-41-2	1	2.4	1.00	0.00	9	1.7 - 3.1	1.00	0.00	1	4.2	61.6	92.5	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 1	Yes
Domiphen bromide (10%)	538-71-6	3	5.0 - 6.6	1.00	0.00	11	1.7 - 3.7	1.00	0.00	1	5.2	2.9	51.6	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Di(2-ethylhexyl)sodium sulphosuccinate (10%)	577-11-7	1	4	1.00	0.00	9	2.5 - 4.7	1.00	0.00	1	9.6	5.0	93.4	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
Coco amidopropyl betaine (25%)	61789-40-0	9	3.6 - 7.6	1.00	0.00	1	0.3	1.00	0.00	1	1.5	0.7	79.3	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
Cetylpyridinium chloride (10%)	6004-24-6	1	4.5	1.00	0.00	1	1.1	1.00	0.00	1	1.4	3.1	65.7	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Sodium lauryl sulphate (15%)	151-21-3	2	2.0	1.00	0.00	Not tested				1	0.1	0.8	65.7	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Ethylhexyl acid phosphate ester	12645-31-7	2	0	1.00	0.00	2	0	1.00	0.00	1	2.7	3.1	45.1	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Distearyldimethylammonium chloride	107-64-2	2	9.0 - 31.1	1.00	0.00	2	2.0 - 3.2	1.00	0.00	2	19.2 - 57.6	27.4 - 49.0	78.6 - 101.7	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 1	Yes
Ethyl lauroyl arginate HCl	60372-77-2	3	15.0 - 20.1	1.00	0.00	Not tested				1	6.3	6.7	5.4	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Surfonic HDL-1 (Nonyl phenol ethoxylate, branched)	9016-45-9	1	27.1	1.00	0.00	1	0.5	1.00	0.00	1	-0.3	1	3.4	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Triton X-100	9002-93-1	1	1.5	1.00	0.00	1	0.8	1.00	0.00	3	-0.2 - 1.2	-0.2 - 0.7	0.1 - 3.4	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Triton X-100 (10%)	9002-93-1	1	6.6	1.00	0.00	9	0.6 - 1.0	1.00	0.00	1	0.5	2	104.6	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
1-Hexadecanaminium, N,N,N-trimethyl-, chloride (25%)	112-02-7	2	6.9 - 11.9	1.00	0.00	2	0.9 - 1.7	1.00	0.00	1	0.7	3	31.4	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Cetyl pyridinium bromide (10%)	140-72-7	1	3.9	1.00	0.00	3	1.4 - 2.4	1.00	0.00	1	2.5	4.3	60.7	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
Cetyl pyridinium bromide (6%)	140-72-7	2	8.5 - 35.1	1.00	0.00	3	1.8 - 5.7	1.00	0.00	1	4.1	13.1	80.9	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
Benzethonium chloride (10%)	121-54-0	1	3.9	1.00	0.00	9	1.4 - 2.5	1.00	0.00	1	6.2	1.9	58.3	1.00	0.00	0.00	1.00	0.00	Cat. 1	Cat. 1	Cat. 1	Yes
N-Lauroyl sarcosine Na salt (30%)	137-16-6	1	Not published	1.00	0.00	1	0.5	1.00	0.00	2	-0.3 - 0.4	- 0.3 - -0.6	83.5 - 93.5	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 1	Yes
Triton X-100 (5%)	9002-93-1	4	3.0 - 5.8	1.00	0.00	1	5	1.00	0.00	1	1.8	37.1	105.2	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 2	Yes
1-Hexadecanaminium, N,N,N-trimethyl-, chloride (1%)	112-02-7	1	7.2	1.00	0.00	1	3.7	1.00	0.00	1	5.5	24.6	92	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 2	Yes
Cetyl pyridinium bromide (1%)	140-72-7	1	49.6	1.00	0.00	3	9.5 - 40.4	1.00	0.00	2	3.7 - 7.3	71.9 - 78.0	92 - 100.6	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 2	Yes
Benzethonium chloride (1%)	121-54-0	1	3.3	1.00	0.00	1	2	1.00	0.00	1	0.8	64.9	96.5	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 2	Yes
N-Lauroyl sarcosine Na salt (10%)	137-16-6	1	Not published	1.00	0.00	1	1.8	1.00	0.00	1	0.1	2	101.1	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 2	Yes
Methyl N,N,N-trimethyl-4-[(4,7,7-trimethyl-3-oxobicyclo[2.2.1]hept-2-ylidene)methyl]anilinium sulphate	52793-97-2	9	9.9 - 17.9	1.00	0.00	1	4.6	1.00	0.00	1	29.2	100.7	103.2	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 2	Yes
Deoxycholic acid Na salt (10%)	302-95-4	1	5.5	1.00	0.00	1	0.6	1.00	0.00	2	0.9	5.9 - 9.4	101.6 - 101.8	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	Cat. 2	Yes
Lauryl sulphobetaine (10%)	14933-08-5	1	3	1.00	0.00	1	0.4	1.00	0.00	1	0.8	33.2	103.2	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	Cat. 2	Yes
Cetylpyridinium chloride (0.1%)	6004-24-6	1	83.2	0.00	1.00	1	87	0.00	1.00	1	65.4	100.2	102.4	0.00	1.00	0.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Sodium lauryl sulphate (3%)	151-21-3	2	3.0 - 6.0	1.00	0.00	1	7.7	1.00	0.00	1	1.1	13.6	100.4	0.00	1.00	0.00	1.00	0.00	Cat. 2	Cat. 1	No Cat.	Yes
Sodium lauryl sulphate (1%)	151-21-3	1	21.3	1.00	0.00	1	16.3	1.00	0.00	1	0.5	102.4	99.6	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	No Cat.	Yes
Triton X-100 (1%)	9002-93-1	1	18	1.00	0.00	3	1.2 - 10.1	1.00	0.00	1	8.4	104.1	102.6	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	No Cat.	Yes
Cetyl pyridinium bromide (0.1%)	140-72-7	1	80.9	0.00	1.00	3	88.8 - 99.0	0.00	1.00	1	80.2	96.4	103.4	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
N-Lauroyl sarcosine Na salt (3%)	137-16-6	1	Not published	1.00	0.00	1	8.5	1.00	0.00	1	0.4	99	100.2	0.00	1.00	0.00	0.00	1.00	Cat. 2	Cat. 2	No Cat.	Yes
Polyethylene glycol monolaurate (10EO) (10%)	9004-81-3	1	89.9	0.00	1.00	1	84.1	0.00	1.00	1	106.7	105.6	100.8	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Steareth-10 allyl ether/acrylates copolymer (30%)	109292-17-3	9	69.6 - 112.3	0.00	1.00	Not tested				Not tested									No Cat.	No Cat.	No Cat.	NA
Polyoxyethylene 23 lauryl ether (Brij-35) (10%)	9002-92-0	Not tested				3	72.5 - 89.4	0.00	1.00	1	118	104.2	103.8	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Polyoxyethylene 8-stearate (Myrj-45)	9004-99-3	1	85.3	0.00	1.00	1	94.6	0.00	1.00	1	115	105.3	97.5	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Tween 80	9005-65-6	4	63.4 - 85.5	0.00	1.00	2	74.5 - 76.7	0.00	1.00	2	91 - 114	96 - 105	99 - 105	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Tween 80 (10%)	9005-65-6	1	104.1	0.00	1.00	1	87.9	0.00	1.00	1	96.7	101	98.9	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Tween 20	9005-64-5	5	79.8 - 102.8	0.00	1.00	9	75.1 - 90.8	0.00	1.00	4	21.1 - 82.9	95 - 105	91 - 104	0.00	0.75	0.25	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Polyglyceryl-3-diisooctadecanoate	63705-03-3	9	86.9 - 103.9	0.00	1.00	1	91.4	0.00	1.00	1	105.8	100.8	100.8	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	3	65.2 - 89.2	0.00	1.00	9	73.9 - 115.3	0.00	1.00	4	91 - 118	98 - 103	94 - 108	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes
Cellulose,2-(2-hydroxy-3-(trimethylammonium)propoxy)ethyl ether chloride	68610-92-4	3	59.8 - 66	0.33	0.67	9	63.6 - 79.6	0.00	1.00	Issue [a]									No Cat.	No Cat.	No Cat.	NA
Myristyl myristate	3234-85-3	3	98.6 - 136	0.00	1.00	9	87.9 - 112.8	0.00	1.00	1	103.4	102.1	102	0.00	0.00	1.00	0.00	1.00	No Cat.	No Cat.	No Cat.	Yes

TP/BU: Top-Down/Bottom-Up approach,
[a] Applicable but test article couldn't be washed from the plate