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Case Study on the use of Integrated Approaches for Testing and Assessment for "Eye hazard identification" of "non-surfactant neat liquids"

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NO. 375

Case Study on the use of Integrated Approaches for Testing and Assessment for “Eye hazard identification” of “non-surfactant neat liquids”

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

Environment Directorate

ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT

Paris 2023

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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 2022 review cycle of the IATA Case Studies Project. This case study was reviewed by the project team.

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.

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Abo, T., Adriaens, E., Alépée, N., Bagley, D., Giusti, A., Magby, J., Mewes, K.R.

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Abbreviations and acronyms

BCOP	Bovine Corneal Opacity and Permeability
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
DIP	Data integration procedure
DRD	Draize eye test Reference Database
ECHA	European Chemicals Agency
EIT	Eye Irritation Test
GHS	Globally Harmonised System
GL	Guideline
HCE	Human Corneal Epithelium
IATA	Integrated approaches to testing and assessment
IR	Iritis
ITS	Integrated Testing Strategy
LLBO	Laser light-based opacitometer
LogP	Octanol-water partition coefficient
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
VP	Vapour pressure
VRM	Validated Reference Method
WoE	Weight of evidence
WS	Water solubility

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 the application of both DAs to assess the eye hazard potential of non-surfactant neat liquids (hereinafter referred to as DAL-1 and DAL-2) according to the three UN GHS categories. The eye hazard potential of four neat liquids (two No Cat., one Cat. 2, and one Cat. 1) was determined based on the results of physicochemical properties and the results of OECD TG *in vitro* test methods. DAL-1, applicable to neat liquids only, combines 4 physicochemical properties with a reconstructed human cornea-like epithelium (RhCE) test method (EpiOcular™ EIT or SkinEthic™ HCE EIT; OECD TG 492), and the Bovine Corneal Opacity and Permeability laser light-based opacitometer (BCOP LLBO) test method (OECD TG 437). DAL-2, applicable to neat liquids and to liquids and solids dissolved in water, is based on the combination of the BCOP LLBO and the Short Time Exposure test method (STE, OECD TG 491).

The data interpretation procedure (DIP) applied uses the readout of the prediction models of the individual test method as defined by the Test Guidelines and/or information on the physicochemical properties retrieved from publicly available databases or QSAR models.

For each chemical, the final prediction using variants of the DAs was concordant with the *in vivo* classification. In conclusion, these case study demonstrate 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, 2020a). 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 2021). 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* methods 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, 2020b, 2018, 2017, 2020c, 2019a, 2019b, 2019c, respectively). Data generated with these *in vitro* methods 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) (OECD, 2019d). The prediction from a DA may be used alone or along with further information as part of an IATA (OECD, 2019d).

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 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, 2019d).

A comprehensive in-depth analysis of historical *in vivo* rabbit eye data provided insight into which of the observed *in vivo* effects are important in driving the classification of chemicals for serious eye damage/eye irritation according to the UN GHS, concluding that full replacement of *in vivo* testing for eye hazard will require accounting for the impact of the *in vivo* tissue effects which drive classification (Adriaens et al., 2014; Barroso et al., 2017). The analyses identified different criteria from the four *in vivo* tissue effects (CO, IR, CR, and CC) that can each independently drive the classification of a chemical (Barroso et al., 2017). The most important drivers for Cat. 1 classification are CO severity (CO mean ≥ 3, days 1–3) and CO persistence on day 21 in the absence of severity. The most important drivers for Cat. 2 classification are CO (CO mean ≥ 1) and conjunctival effects (CR and/or CC mean ≥ 2). Regarding chemicals that do

not require classification for serious eye damage/eye irritation (No Cat.), four subgroups were identified. The analysis revealed that majority of the No Cat. chemicals did not cause any effect on CO, so all CO scores were equal to 0 (CO = 0) in all animals and all observed time points. The second group consists of chemicals that induced CO > 0, but below the classification cut-off. Some chemicals induced a mean of the scores of days 1–3 above the classification cut-off for at least one endpoint but not in enough animals to generate a classification (borderline cases), those studies are marked with ** (CO = 0**, CO > 0**). A detailed description of the drivers of classification and use of the terms CO, IR and Conj to describe key effects is provided in the paper of Barroso and co-workers (2017).

Over the last decades, many efforts were made to develop New Approach Methodologies (NAMs) that follow recommendations and combinations of modules as specified in the IATA (OECD, 2019d). Cosmetics Europe developed two DAs for eye hazard identification of non-surfactant liquids to distinguish between the three UN GHS categories (Alépée et al., 2019a, 2019b). The DAs are based on the combinations of methods as recommended in the IATA (OECD, 2019d) and are integrated in a new OECD TG 467 (OECD, 2022a). Background information supporting this TG is reported in OECD GD 354 (2022b). Likewise, OECD TG 492B proposes a new stand-alone method for eye hazard identification according to the three UN GHS categories using a reconstructed human corneal epithelium (SkinEthic™ Human Corneal Epithelium (HCE) Time-to-Toxicity (TTT) test method) (OECD, 2022c). The assessment of the performance of the test methods and DAs against the *in vivo* Draize eye test has been conducted based on reference chemicals selected according to key criteria, as defined by Barroso and co-workers (2017), such that the important drivers of classification for each UN GHS category and a wide range of organic functional groups are represented. Modes of action for eye irritation are unknowable for the majority of chemicals and do not provide additional insight in evaluation of the test methods and DAs, and thus they were not considered for the analysis of the test chemicals for the DAs.

Only the conceptual approach that follows the principles of the IATA will be illustrated in this document. The purpose is to show how results from multiple information sources can be used together in DAs to predict the eye hazard potential of test chemicals. The workflow of the DAs is exemplified with a case study with four chemicals.

2 Purpose

2.1. Purpose of use

The aim of this case study is to illustrate the applicability of the DAs for serious eye damage and eye irritation integrated in the OECD TG 467 (OECD, 2022a). The specific purpose of this case study is to identify the eye hazard potential of non-surfactant neat liquids and to demonstrate the applicability of the DA to distinguish between the three UN GHS categories for eye hazard identification.

2.2. Target chemical(s)

The target chemicals are non-surfactant neat liquids. The following four chemicals were selected to illustrate the case study: 2-Hydroxy iso-butyric acid ethyl ester (CASRN 80-55-7), iso-Butanal (CASRN 78-84-2), n-Butyl acetate (CASRN 123-86-4), and Glycerol (CASRN 56-81-5). The availability of existing NAM information for the liquids including a defined chemical structure, physicochemical properties, and *in vitro* data (OECD TG 437, 491, and 492) (OECD, 2020b, 2020c, 2019a) allowed illustration of two DAs that follow the workflow recommended in the IATA (GD No. 263) to assess the eye hazard potential of the case study. Note that no human data or animal data were considered in the applicability of the DA. Both the top-down and bottom-up approach were conducted for the four chemicals. Furthermore, the chemicals, which cover different organic functional groups, are general chemicals and not typical cosmetic ingredients that were selected to illustrate the DAs. The prediction results obtained by DAL-1 and DAL-2 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 DAs described in this case study, which are integrated in the OECD TG 467 (OECD, 2022a), are based on the recommendations and combinations of modules as stipulated in the IATA for serious eye damage and eye irritation (OECD, 2019d). 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 GD 263 (OECD, 2019d).

Part 3 of the IATA 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 Approaches used

Two rule-based DAs for non-surfactant liquids, that were recently adopted by the OECD (TG 467, 2022a), are 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 data (e.g *in silico* predictions, *in chemico*, *in vitro* data) to derive a prediction without the need for expert judgment. The DAs use method combinations intended to overcome some of the limitations of the individual, stand-alone methods in order to provide increased confidence in the overall result obtained. The DAs provide information that can be used for eye hazard identification.

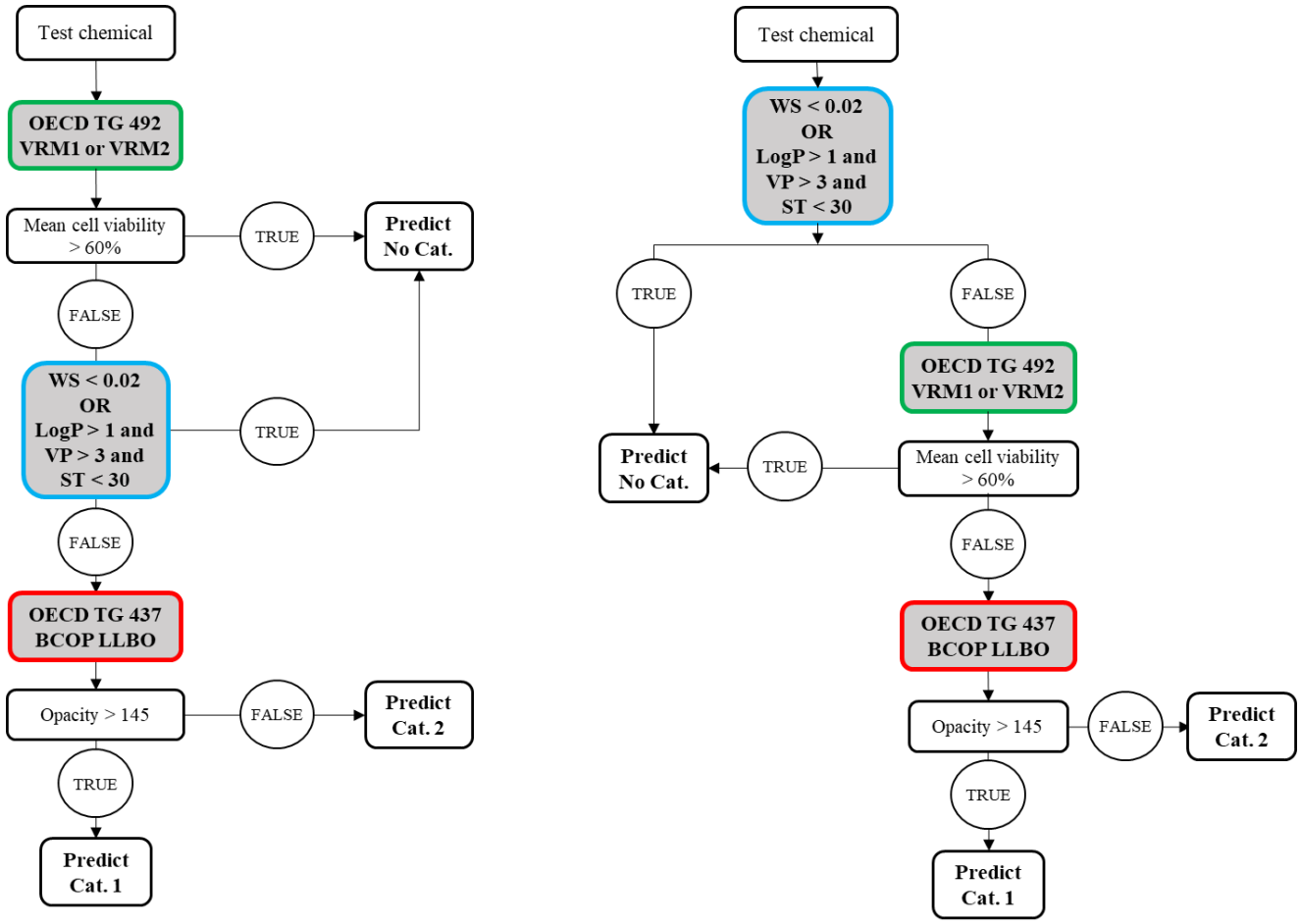
4.1. Defined approach 1 (DAL-1)

The DAL-1 is based on the use of a combination of two *in vitro* test methods described in OECD TG 437 (Bovine Corneal Opacity and Permeability (BCOP) using the laser light-based opacitometer (LLBO)) and TG 492 (Reconstructed human Cornea-like Epithelium, RhCE) as well as four physicochemical properties of the test chemical. The DIP applied uses the readout of the prediction models of the individual test method as defined by the TGs and/or information on the physicochemical properties. A scheme of the Bottom-Up approach for DAL-1 as proposed in TG 467 is presented in Figure 1. Both options of the Bottom-UP DAL-1 are illustrated with an example in Annex A. An example of the Top-Down approach is also given in Annex A.

4.2. Defined approach 2 (DAL-2)

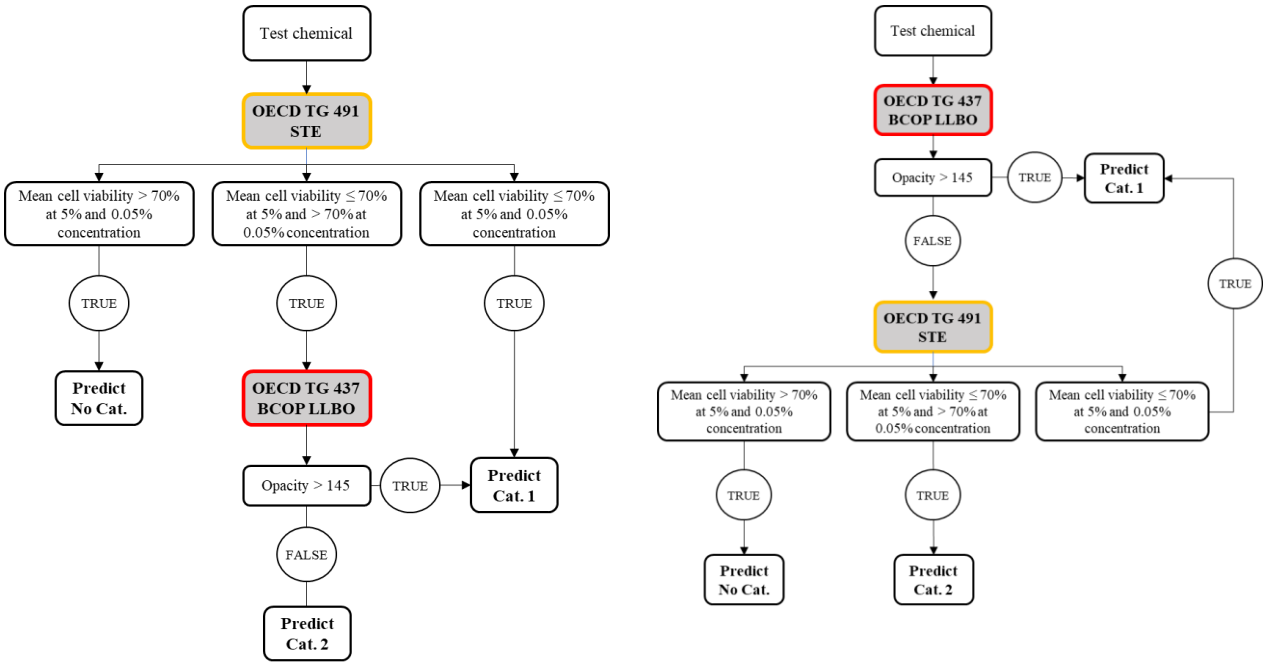
The DAL-2 describes the combination of two *in vitro* test methods described in OECD TG 491 (Short Time Exposure, STE) and TG 437 (BCOP LLBO). A scheme of DAL-2 is presented in Figure 2. An example of the Bottom-Up and Top-Down approach is given in Annex A.

Figure 1. Bottom-Up approach of the DAL-1



(left) option 1: step 1 RhCE test method used to identify No Cat., step 2 physicochemical exclusion rules (WS: water solubility in mg/mL; or LogP: octanol-water partition coefficient / VP: vapour pressure in mm Hg / ST: surface tension in dyne/cm) to identify No Cat., and step 3 BCOP LLBO used to identify Cat. 1
(right) option 2: step 1 physicochemical exclusion rules to identify No Cat., step 2 RhCE test method used to identify No Cat., and step 3 BCOP LLBO used to identify Cat. 1

Figure 2. Schemes of the DAL-2



(left) Bottom-Up approach: start with the STE test method followed by the BCOP LLBO test method and
(right) Top-Down approach: start with the BCOP LLBO test method followed by the STE test method.

5 Data/Information gathering

Four non-surfactant liquids that cover the different UN GHS eye hazard categories, were selected for this case study. All input data (physicochemical properties and outcome of the *in vitro* test methods) for the DAL-1 and DAL-2 were available.

5.1. Identify chemical of interest and molecular structure

The molecular structure and physicochemical properties for 2-Hydroxy iso-butyric acid ethyl ester, iso-Butanal, n-Butyl acetate, and Glycerol were identified (Table 1).

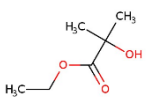
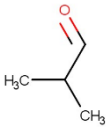
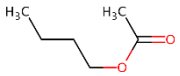
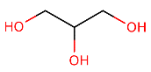
5.2. Identify existing hazard information

The existing *in vitro* results for 2-Hydroxy iso-butyric acid ethyl ester, iso-Butanal, n-Butyl acetate, and Glycerol are presented in Table 1.

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 1. Existing information collected for the DAs.

Name	2-Hydroxy iso-butyric acid ethyl ester	iso-Butanal	n-Butyl acetate	Glycerol
CAS RN	80-55-7	78-84-2	123-86-4	56-81-5
SMILES	CCOC(=O)C(C)(C)O	CC(C)C=O	CCCCOC(C)=O	OCC(O)CO
				
Physicochemical properties	MW: 132.16 Da	MW: 72.11 Da	MW: 116.16 Da	MW: 92.09 Da

	LogP: 0.14 (**) VP: 0.55 mmHg (**) ST: 27.8 dyn/cm (**) WS: 166.5 mg/mL (**)	LogP: 0.77 (*) VP: 172 mmHg (**) ST: 23.2 dyn/cm (**) WS: 60 mg/mL (*)	LogP: 2.30 (*) VP: 8.4 mmHg (*) ST: 24.9 dyn/cm (**) WS: 5.3 mg/mL (*)	LogP: -1.75 (*) VP: 0.0 mmHg (**) ST: 37.4 dyn/cm (**) WS: 1105 mg/mL (**)
OECD TG 492 **EpiOcular™ EIT (Viability)**	13.9%	3.1%	17.9%	97.6%
OECD TG 492 **SkinEthic™ EIT (Viability)**	2.3%	0.9%	2.1%	88.1%
OECD TG 437 **BCOP LLBO (Opacity)**	172.2	104.3	30.8	18.5
OECD TG 491 **STE (Viability)** **@ 5%** **@ 0.05%**	6.4% 102.4%	6.7% 94.6%	103.2% 106.7%	89.8% 97.2%

In vitro data were retrieved from public sources (Kaluzhny et al., 2011; Takahashi et al., 2011; Alépée et al., 2016a; Abo et al., 2018; Kandarova et al., 2018; Verstraelen et al., 2013; Verstraelen et al., 2018). The purity of the test items tested with the different in vitro methods was at least 98%. Physicochemical properties were obtained from ECHA registration dossiers and the EPA Comptox Dashboard https://comptox.epa.gov/dashboard. The values correspond with measured properties according to the OECD GLs (*) or QSAR predictions (**) that are based on the 5 OECD principles for QSAR models and that have a QMRF (QSAR Model Reporting Format). The QSAR models used for LogP, VP, and WS are the OPERA models and T.E.S.T. was used for ST. 28.
MW: Molecular Weight; LogP: Octanol/water partition coefficient @ 20-25°C ; VP : Vapor Pressure @ 20-25°C; ST: surface tension @ 20-25°C; WS: water solubility @ 20-25°C

5.4. Hypothesis generation

In case data on all the components (i.e. *in vitro* test methods and physicochemical properties) of the DAs are available and are within the applicability domain of each component, the Bottom-Up or Top-Down approach of both DAs can be used.

In case no data is available, it is recommended to verify if the physicochemical properties can be estimated with QSAR models. If the exclusion rules are met, the chemical is predicted No Cat. based on physicochemical properties only. If the exclusion rules are not met, further testing is required and depending on the assumption to Top-Down (1) or Bottom-Up (2) approach is recommended.

(1) If it is assumed that the chemical is a severe eye irritant than it is recommended to follow the Top-Down approach, and start with an *in vitro* test method that can identify test chemicals causing serious and/or irreversible eye damage (UN GHS Cat. 1) as such it is recommended to start with

the BCOP LLBO test method. Further testing is required (DAL-1 or DAL-2) in case the chemical does not result in severe eye damage based on the BCOP LLBO.

(2) If the chemical is assumed not to cause eye irritation, it is recommended to follow the Bottom-Up approach starting with an *in vitro* test method that can identify test chemicals not requiring classification for eye hazard (UN GHS No Cat.) such as the RhCE test method (DAL-1) or STE test method (DAL-2). Further testing with the BCOP LLBO) is required if the chemical is not predicted No Cat.

The present case study is illustrated with four chemicals for which all components are available (Table 1).

6 Application of the Defined Approaches

6.1. Defined Approaches 1 (DAL-1), based on physicochemical properties and *in vitro* data, for neat non-surfactant liquids

Summary

The DAL-1 describes the combination of one and/or a combination of three physicochemical properties with the results of two OECD adopted *in vitro* test methods (Alépée et al., 2019a). The *in vitro* test methods used in DAL-1 are the BCOP LLBO according to the OECD TG 437 (2021b) and the Reconstructed human Cornea-like Epithelium (RhCE) according to the OECD TG 492 (2019a). The RhCE-based test methods that are part of DAL-1 are the EpiOcular™ Eye Irritation Test (EIT) and the SkinEthic™ Human Corneal Epithelium (HCE) EIT and are referred to as Validated Reference Methods (VRM) - EpiOcular™ EIT (VRM1; OECD TG 492) and SkinEthic™ HCE EIT (VRM2; OECD TG 492). The DA 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 by either the physicochemical exclusion rules, the RhCE test method, or the BCOP LLBO test method. The DAL-1 is intended for the identification of the eye irritation hazard of non-surfactant liquid substances primarily for the purpose of classification and labelling without the use of animal testing, i.e. UN GHS Cat. 1 vs. UN GHS Cat. 2 vs. UN GHS No Cat.

Rationale underlying the construction of the defined approach

The BCOP LLBO test method addresses corneal effects, which are one of the major drivers of classification *in vivo* when considering the UN GHS classification (Adriaens et al., 2014; Barroso et al., 2017). 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 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. 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 underlying rationale of applying physicochemical property exclusion rules to identify liquid chemicals with no serious eye damage or eye irritation potential is that several *in vivo* No Cat. liquids with specific properties [low water solubility (WS < 0.02 mg/mL) and/or a combination of octanol-water partition coefficient (LogP > 1), vapor pressure (> 3 mmHg), and surface tension (ST < 30 dyne/cm)], are over-predicted (false positive) with the RhCE test methods. Such chemicals can still be predicted correctly based on the physicochemical property exclusion rules.

Description of the individual information sources used

The measurements of the physicochemical properties should be performed according to the OECD GLs and test reports are required providing the information requested in the corresponding GLs. More detailed information on the GLs that should be used for the measurements are provided in Annex E of the OECD supporting document No. 354 of TG 467 (OECD, 2022a and 2022b). Regarding the prediction of physicochemical properties, models that are based on the 5 OECD principles for QSAR models (1) and that have a QMRF (QSAR Model Reporting Format) should be used. The OPERA models are QSAR tools that were developed based on those principles and a QMRF (QSAR Model Reporting Format) is available for LogP, Vapor pressure and Water solubility. The OPERA predictions are available at the NTP Integrated Chemical Environment https://ice.ntp.niehs.nih.gov/ and the EPA Comptox Dashboard https://comptox.epa.gov/dashboard. For local use, the OPERA application can also be downloaded from the NIEHS GitHub repository https://github.com/NIEHS/OPERA. For Surface tension the toxicity-estimation-software-tool-test (T.E.S.T.) can be used and is available at https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test. The T.E.S.T. model for Surface tension is a QSAR tool that was developed based on the 5 principles for QSAR models. The predictions for Surface tension are also available at the EPA Comptox Dashboard https://comptox.epa.gov/dashboard. A calculation report and a QMRF is available.

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. Liquids that result in a tissue viability > 60% are classified No Cat., liquids that result in a tissue viability ≤ 60% require further testing.

The BCOP LLBO test method measures the eye hazard potential of a test chemical by its ability to induce opacity and permeability in an isolated bovine cornea. Note that only opacity measurement is considered in the DAL-1. Liquids inducing an opacity > 145 are predicted Cat. 1, in case the opacity is ≤ 145, further testing is required.

Data interpretation procedure applied

The DIP applied uses the outcome of the individual test methods as defined by the TGs and/or information on the physicochemical properties. Because of the sequential nature of the different information sources that are part of the DAL-1, one can either start with the physicochemical properties (Bottom-Up approach), an RhCE (Bottom-Up approach) or the BCOP LLBO test method (Top-Down approach).

Application of the DAL-1 for the variations of the DIP are illustrated with 2-Hydroxy iso-butyric acid ethyl ester. Applying the Bottom-Up approach option 1 (Annex A), the RhCE test method is used in Tier 1 to identify liquid chemicals with no serious eye damage or eye irritation potential. Both RhCE methods result in tissue viability much lower than 60%, hence further testing is required. In Tier 2 physicochemical property exclusion rules based on WS or a combination of LogP, VP and ST of the pure liquid are used to identify chemicals not requiring classification. Since the WS is clearly > 0.02 mg/mL (116.5 mg/mL) and the exclusion rules for LogP and VP are also not met, further testing is required. The final prediction corresponds with Cat. 1 and is based on the BCOP LLBO in Tier 3 (opacity of 172.2 > 145) (Annex A, Figure 3). Three different options of the Bottom-Up approach are possible (Annex A), they all result in the same conclusion that 2-Hydroxy iso-butyric acid ethyl ester is a severe eye irritatant (Cat. 1). The first two variations of the DIP require the results of all components of the DAL-1 (three Tiers). In the third option of the Bottom-Up approach, the prediction is based on two tiers, the results of the RhCE methods (OECD adopted method to identify No Cat.) and the results of the BCOP LLBO (OECD adopted method to identify Cat. 1). When the Top-Down approach is used, only one tier is required since the BCOP LLBO results in

a high opacity resulting in a Cat. 1 prediction and no further testing is required, the prediction is based on a stand-alone test method. In conclusion, independent of the DIP version that is used, the prediction for 2-Hydroxy iso-butyric acid ethyl ester corresponds with Cat. 1.

Performance of the DAL-1

The predictive performance considering the three UN GHS categories (Cat. 1, Cat. 2, No Cat.) of DAL-1 is reported for 94 liquids with VRM1 (Table 2) and for 86 liquids with VRM2 (Table 3), and yielded balanced accuracies of 68.7% and 75.0%, respectively (OECD, 2022a). Note that physicochemical properties, EpiOcular™ EIT and/or SkinEthic™ HCE EIT, BCOP LLBO predictions (except see *Note) and UN GHS categories based on Draize Eye test data are available. The performance was the same for the different variations of the Bottom-Up approach (Figure 1). Furthermore, the performance for the in vivo classified chemicals was the same for the different variations the Top-down approach. *Note that BCOP LLBO results were missing for 17/55 and 11/46 in vivo No Cat. substances that were predicted No Cat. with VRM1 and VRM2, respectively. As such, the performance of the Top-Down approach could not be assessed for this category.

Table 2. Performance of the DAL-1 with VRM 1 (N=94)

UN GHS	Prediction DAL-1 with VRM1		
	Cat. 1	**Cat. 2**	**No Cat.**
Cat. 1 (N='17),' %[a] (n/N)	76.5% (13.0/17.0)	23.5% (4.0/17.0)	0.0% (0.0/17.0)
Cat. 2 (N='22),' %[a] (n/N)	27.3% (6.0/22.0)	**59.1%** **(13.0/22.0)**	13.6% (3.0/22.0)
No Cat. (N='55),' %[a] (n/N)	5.5% (3.0/55.0)	24.0% (13.2/55.0)	70.5% (38.8/55.0)

[a] The proportion given is based on a weighted calculation which takes into account (where they exist) multiple results from an individual information source for a given chemical, and applying a correction factor so that all chemicals have a weight of 1. 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 is the same for the two versions of the DIP (Figure 1).

Table 3. Performance of the DAL-1 with VRM 2 (N=86)

UN GHS	Prediction DAL-1 with VRM2		
	Cat. 1	**Cat. 2**	**No Cat.**
Cat. 1 (N=17), %[a] (n/N)	76.5% (13.0/17.0)	23.5% (4.0/17.0)	0.0% (0.0/17.0)
Cat. 2 (N=23), %[a] (n/N)	30.4% (7.0/23.0)	68.7% (15.8/23.0)	0.9% (0.2/23.0)
No Cat. (N=46), %[a] (n/N)	3.1% (1.4/46.0)	17.2% (7.9/46.0)	79.7% (36.7/46.0)

[a] The proportion given is based on a weighted calculation which takes into account (where they exist) multiple results from an individual information source for a given chemical, and applying a correction factor so that all chemicals have a weight of 1. 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 is the same for the two versions of the DIP (Figure 1).

Limitations in the application of the defined approach

DAL-1 is not applicable for surfactants and solids. The DAL-1 is applicable to neat liquids, excluding mixtures (> 20% impurity), UVCBs and multi-constituent substances. For impurities with concentration > 5% and < 20%, the physicochemical properties of the impurities also need to be determined, and only when all components meet the exclusion criteria, the liquid is predicted No Cat., in all other cases, proceed with an RhCE test method. (OECD TG 467, 2022a).

The strengths and limitations of individual test methods are described in the corresponding OECD TG 437 and TG 492. 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. The most up-to-date published version of the respective TGs should always be used.

It is important to separate the limitations into (1) technical limitations and (2) limitations in the predictivity for UN GHS categories. For example, technical limitations may make a chemical not testable in one or more components of DAL-1 and may thus limit its applicability domain.

- Strong colourants that stain the bovine cornea result in interference of the opacity measurement and are considered outside the applicability domain of the BCOP LLBO test method (technical limitation). Such chemicals can still be assessed with the RhCE test method (OECD TG 492) using HPLC/UPLC-spectrophotometry (OECD, 2019a). Note that the RhCE method is not capable to distinguish between UN GHS Cat. 1 and UN GHS Cat. 2, but can identify UN GHS No Cat. (limitation in the predictivity).

The limitations of the individual methods for measuring the physicochemical properties are specified in their respective GLs (GL 104, GL 105, GL 107, GL 115, GL 117, GL 123).

- Before performing the measurement, it is important to verify the stability of the chemical since this may affect the level of confidence of the measurement.

- It is important to consider the bulk amount of the chemical needed to measure the property, the cost of measuring the properties and the time needed to obtain the results. Measuring LogP, VP and WS may take several weeks.

The level of confidence is low for QSAR predicted physicochemical properties that fall outside the applicability domain. If there is uncertainty regarding the QSAR prediction of the physicochemical properties, measuring these properties could be considered. Furthermore, , other information sources (RhCE and/or BCOP LLBO) will be needed to assess the hazard potential of the chemical. Measured and predicted values are available for n-butyl acetate, the QSAR predicted LogP = 1.78 and the measured = 2.3, the QSAR predicted VP = 11.6 mmHg and the measured = 8.4 mmHg), the QSAR predicted WS = 7.5 mg/mL and the measured = 5.3 mg/mL. Regarding the application of the exclusion rules, both measured and QSAR predicted values come to the same conclusion.

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

Uncertainty of the information sources used within the DAL-1

Assessment on how accuracy of predictions from the different QSAR models (OPERA and T.E.S.T.) can be evaluated, is provided in Annex E of the OECD supporting document No. 354 of TG 467 (OECD, 2022a and 2022b). For the physicochemical properties that are predicted with the OPERA models (LogP, vapor pressure, and water solubility), applicability domain indices (global and local) are provided and they give an indication on the reliability of the prediction. Furthermore, a confidence level index ranging from 0 to 1 is provided, the higher this index, the more the prediction is likely to be reliable. The performance statistics of the QSAR model for surface tension are reported in the User's Guide for T.E.S.T (Toxicity Estimation Software Tool) Version 5.1. The software also provides predictions for similar chemicals in the test set. If similar chemicals in the test set are predicted well, this provides more confidence in the predicted value. More information can be found in the corresponding QMRFs.

Experimental methods for measuring LogP are described in OECD GL 107, GL 117, and GL 122. The procedure for measuring vapor pressure and water solubility is provided in OECD GL 104 and GL 105, respectively. Note that for surface tension, OECD GL 115 should be used, and that the measurement should be performed on pure liquids only. In general, the measurements are only valid when the chemical falls within the applicability domain, each GL contains a section on initial considerations that should be taken into account before performing the measurement.

Transferability, within- and between-laboratory reproducibility (WLR and BLR) of OECD TG 492 and TG 437 adopted test methods have been assessed during their respective validation studies (EC EURL ECVAM, 2014; Alépée et al., 2016a and 2016b, Adriaens et al., 2020).

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 DAL-1. Table 4 describes the uncertainty along each step of the DA.

Table 4. Uncertainties associated with the individual components of the DAL-1 and how they affect the overall uncertainty of the final outcome/prediction

DAL element	Uncertainty (low, medium, high)	Comment
General considerations	Low	Data quality is considered high since the physicochemical properties were measured/predicted according to OECD GLs/QSAR models that are based on the 5 OECD principles and have a QMRF and with *in vitro* data that were generated based on OECD validated test methods. The reproducibility and reliability of the *in vitro* test methods was assessed during the validation studies, the values that are reported are for liquids only. Similar reproducibility and reliability was reported for both VRMs. The WLR and BLR was at least 92%. The accuracy to distinguish No Cat. from classified liquids was 82% to 84%, with a specificity of at least 66% and sensitivity of at least 98% (EC EURL ECVAM, 2014; Alépée et al., 2016a and 2016b). The WLR for the BCOP LLBO (opacity only) was 93%, the accuracy to distinguish Cat. 1 from not Cat. 1 was 79% with 81% specificity and 76% sensitivity.
Case study chemicals	Low	The chemicals of the case study are all well characterized with a purity of at least 98%
Physicochemical properties	Low	The chemicals of the case study lie within the applicability domain of the QSAR models, as such the user will have higher confidence in the predictions. Measured properties according to OECD GLs with no restriction or accepted restrictions.
NAM data used in DAL-1	Low	No technical issues were reported while testing the chemicals in the *in vitro* methods.
DAL-1 outcome/prediction	Low	Chemical 1 (2-Hydroxy iso-butyric acid ethyl ester, CASRN 80-55-7): PCP exclusion rules for the single property (WS) and combination properties (two out of three: LogP and VP) were not met. The low viability in both RhCE methods indicate that the chemical causes severe cytotoxicity of the reconstructed tissues. This is confirmed by the high opacity that was observed in the BCOP LLBO. In general, no borderline predictions were observed for the different components of the DAL-1, so the level of confidence in the DAL-1 prediction is high. Chemical 2 (iso-Butanal, CASRN 78-84-2): PCP exclusion rules for the single property (WS) and combination of properties (one out of three: LogP) were not met. The low viability in both RhCE methods indicate that the chemical causes severe cytotoxicity of the reconstructed tissues. The opacity measured in the BCOP LLBO was moderate resulting in a Cat. 2 prediction. In general, no borderline predictions were observed for the different components of the DAL-1, so the level of confidence in the DAL-1 prediction is high. Chemical 3 (n-Butyl acetate, CASRN 123-86-4): The PCP exclusion rule was met for the combination of the properties (LogP, VP, and ST) with no borderline values resulting in a No Cat. prediction. The level of confidence in the prediction based on the PCP exclusion rule is high. Chemical 4 (Glycerol, CARN 56-81-5): Glycerol resulted in high viability in both RhCE models, those methods have low false negative predictions (OECD TG 492,

		2019a). Since the viability was clearly above the cut-off the level of confidence in the No Cat. prediction based on the stand-alone method is high.
Overall uncertainty for DAL-1	Low	The different variations of the DIP come to the same hazard assessment conclusion with a low uncertainty.

Hazard assessment

The prediction of the four chemicals 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 4.

Table 5. Comparison between UN GHS category and DAL-1 predictions

Chemical	CASRN	UN GHS [a]	Prediction DAL-1 with VRM1 [b]	Prediction DAL-1 with VRM2 [b]	Figures
2-Hydroxy iso-butyric acid ethyl ester	80-55-7	Cat. 1	Cat. 1	Cat. 1	Annex A
iso-Butanal	78-84-2	Cat. 2	Cat. 2	Cat. 2	Annex B
n-Butyl acetate	123-86-4	No Cat.	No Cat.	No Cat.	Annex C
Glycerol	56-81-5	No Cat.	No Cat.	No Cat.	Annex D

[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.

6.3. Defined Approaches 2 (DAL-2), based on *in vitro* data, for non-surfactant neat liquids, liquids and solids dissolved in water

Summary

The DAL-2 describes the combination of two *in vitro* test methods (STE: OECD TG 491 and BCOP LLBO: OECD TG 437) (Alépée et al., 2019b). The DA 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 by either the STE test method or the BCOP LLBO test method. The DAL-2 is intended for the identification of the eye irritation hazard of non-surfactant neat liquids, liquids and solids dissolved in water, primarily for the purposes of classification and labelling without the use of animal testing, i.e. UN GHS Cat. 1 vs. UN GHS Cat. 2 vs. UN GHS No Cat.

Since the BCOP LLBO is also a component of DAL-1, the respective information is provided in section 6.1 and will not be repeated in the different subsections of DAL-2.

Rationale underlying the construction of the defined approach

The STE test method measures the cytotoxic effect of chemicals on a confluent monolayer of Statens Seruminstitut Rabbit Cornea (SIRC) cells. The cytotoxic effects of test chemicals on corneal epithelial cells is an important mode of action leading to corneal epithelium damage and eye irritation.

Description of the individual information sources used

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. A test chemical is classified as UN GHS Cat. 1 when both the 5% and 0.05% concentrations result in a relative cell viability ≤ 70%. Conversely, a test chemical is predicted as UN GHS No Cat. when both the 5% and 0.05% concentrations result in a relative cell viability > 70%. For liquids that result in a mean cell viability ≤ 70% at 5% concentration but > 70% at 0.05%, further testing is required.

Data interpretation procedure applied

The DIP applied uses the outcome of the individual test methods as defined by the TGs. Because of the sequential nature of the different information sources that are part of the DAL-2, one can either start with the STE test method (Bottom-Up approach) or the BCOP LLBO test method (Top-Down approach).

Application of the DAL-2 for the variations of the DIP are illustrated with 2-Hydroxy iso-butyric acid ethyl ester. Applying the Bottom-Up approach option 1 (Annex A), the STE test method is used in Tier 1 to identify liquid chemicals with no serious eye damage or eye irritation potential. Since the viability after 5 minutes exposure is below 70% for the 5% concentration and > 70% for the 0.05% concentration, further testing is required with the BCOP LLBO in Tier 2. The final prediction corresponds with Cat. 1 and is based on the BCOP LLBO (opacity of 172.2 > 145) (Annex A, Figure 5). When the Top-Down approach is used, only one tier is required since the BCOP LLBO induced a high opacity resulting in a Cat. 1 prediction. Hence, no further testing is required, the prediction is based on a stand-alone test method. In conclusion, independent of the version of the DIP that is used, the prediction for 2-Hydroxy iso-butyric acid ethyl ester corresponds with Cat. 1.

Performance of the DAL-2

The predictive performance considering the three UN GHS categories (Cat. 1, Cat. 2, No Cat.) of DAL-2 is reported for 164 liquids (Table 6) and yielded a balanced accuracy of 74.3% (OECD, 2022a). Note that STE predictions, BCOP LLBO predictions (except see *Note) and UN GHS categories based on Draize Eye test data are available. The performance for the *in vivo* classified chemicals was the same for the Bottom-Up and the Top-down approach (Figure 2). *Note that BCOP LLBO results were missing for 89/123 *in vivo* No Cat. substances that were predicted No Cat. with the STE test methods. As such, the performance of the Top-Down approach (start with BCOP LLBO) could not be assessed for this category.

Table 6. Performance of the DAL-2 (N=164)

UN GHS	Prediction DAL-2		
	Cat. 1	Cat. 2	No Cat.
Cat. 1 (N='17),' % [a] (n/N)	**81.2%** **(13.8/17.0)**	17.6% (3.0/17.0)	1.2% (0.2/17.0)
Cat. 2 (N='24),' % [a] (n/N)	30.2% (7.2/24.0)	**56.3%** **(13.5/24.0)**	13.5% (3.2/24.0)
No Cat. (N='123),' %[a] (n/N)	4.1% (5.1/123.0)	10.6% (13.0/123.0)	**85.3%** **(104.9/123.0)**

[a] The proportion given is based on a weighted calculation which takes into account (where they exist) multiple results from an individual information source for a given chemical, and applying a correction factor so that all chemicals have a weight of 1. 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.

Limitations in the application of the defined approach

DAL-2 is not applicable for surfactants and solids dissolved in water (OECD TG 467, 2022a).

The strengths and limitations on individual test methods are described in the corresponding OECD TG 437 and TG 491. 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. The most up-to-date published version of the respective TGs should always be used.

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

Uncertainty of the information sources used within the DAL-2

Transferability, within- and between-laboratory reproducibility (WLR and BLR) of the individual test methods have been assessed during their respective validation studies (STE review document ICCVAM, 2013, Adriaens et al., 2020).

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 DAL-2. Table 7 describes the uncertainty along each step of the DA.

Table 7. Uncertainties associated with the individual components of the DAL-2 and how they affect the overall uncertainty of the final outcome/prediction

DAL element	Uncertainty (low, medium, high)	Comment
General considerations	Low	The reproducibility and reliability of the STE test method was assessed during the validation studies, the values for reliability that are reported are for liquids only. The accuracy of the STE test method to distinguish Cat. 1 from not Cat. 1 was 85% with 99% specificity and 29% sensitivity. The accuracy to distinguish No cat. from classified was 86% with 82% specificity and 90% sensitivity. BCOP LLBO see Table 4
Case study chemicals	Low	The chemicals of the case study are all well characterized with a purity of at least 98%
NAM data used in DAL-2	Low	No technical issues were reported while testing the chemicals in the *in vitro* methods.
DAL-2 outcome/prediction	Low	Chemical 1 (2-Hydroxy iso-butyric acid ethyl ester, CASRN 80-55-7): This chemical induced a high opacity in the BCOP LLBO. No borderline predictions were observed for the STE and BCOP LLBO test method, so the level of confidence in the DAL-2 prediction is high. Chemical 2 (iso-Butanal): The STE and BCOP LLBO predicted this chemical as moderately irritating (Cat. 2) and since no borderline predictions were observed, the level of confidence in the DAL-2 prediction is high. Chemical 3 (n-Butyl acetate): This chemical resulted in a high viability in the STE at both test concentrations. Since the viability was clearly above the cut-off, the level of confidence in the No Cat. prediction based on the stand-alone method is high. Chemical 4 (Glycerol): This chemical resulted in a high viability in the STE at both test concentrations. Since the viability was clearly above the cut-off, the level of confidence in the No Cat. prediction based on the stand-alone method is high.
Overall uncertainty for DAL-2	Low	The different variations of the DIP come to the same hazard assessment conclusion with a low uncertainty.

Hazard assessment

The prediction of the four chemicals 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 8.

Table 8. Comparison between UN GHS category and DAL-2 predictions

Chemical	CASRN	UN GHS[a]	Prediction DAL-2 [b]	Figures
2-Hydroxy iso-butyric acid ethyl ester	80-55-7	Cat. 1	Cat. 1	Annex A
iso-Butanal	78-84-2	Cat. 2	Cat. 2	Annex B
n-Butyl acetate	123-86-4	No Cat.	No Cat.	Annex C
Glycerol	56-81-5	No Cat.	No Cat.	Annex D

[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.

7 Strategy and integrated hazard assessment conclusion

This illustrative case study, with four non-surfactant liquids, was performed to demonstrate the applicability of the DAs and their 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 DAL-1 (Figure 1) and DAL-2 (Figure 2). The data of the different information sources (physicochemical properties and *in vitro* test methods) were integrated in two DAs and both the Bottom-Up and Top-Down approach was illustrated.

Hazard assessments based upon predictions from DAL-1 and DAL-2 concluded that (1) 2-Hydroxy iso-butyric acid ethyl ester (CASRN 80-55-7) is classified Cat. 1, (2) iso-Butanal (CASRN 78-84-2) is classified Cat. 2 and (3) n-Butyl acetate (CASRN 123-86-4) and Glycerol (CASRN 56-81-5) are classified No Cat. This is in agreement with the UN GHS categories that were assigned based on historical *in vivo* Draize eye test studies.

This case study reflects approaches on how to move from animal testing into an evaluation of new non-surfactant liquids ingredients based on examples of application of DAs on an IATA for safety purposes of ingredients.

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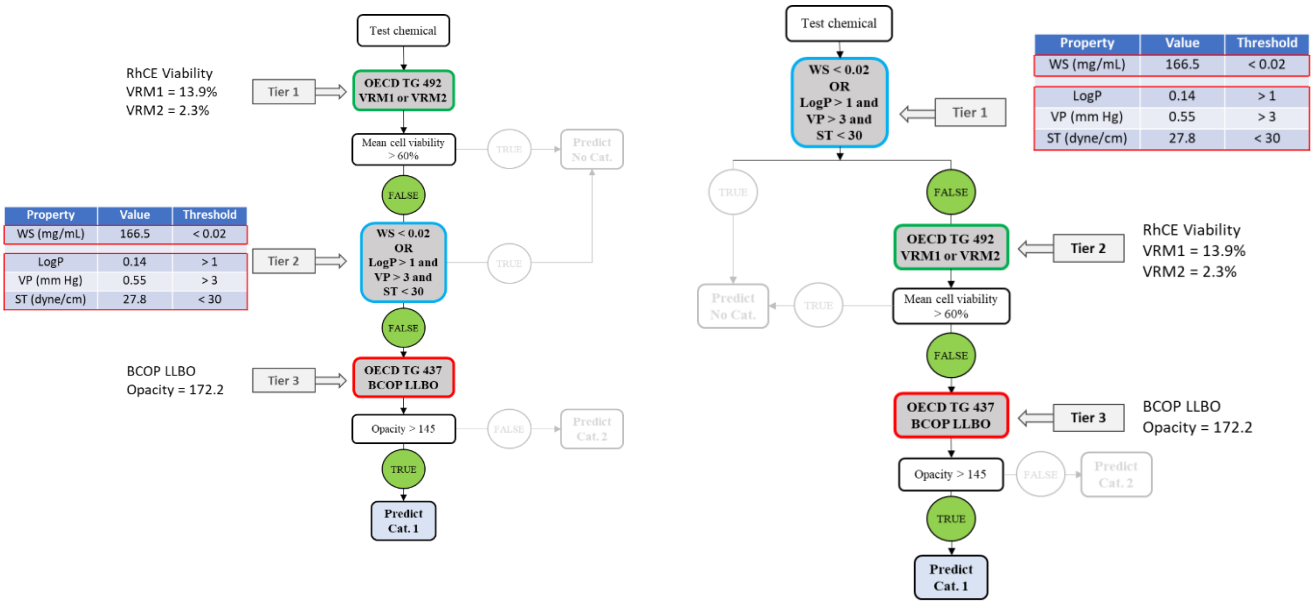
Annex A: 2-Hydroxy iso-butyric acid ethyl ester (CASRN 80-55-7)

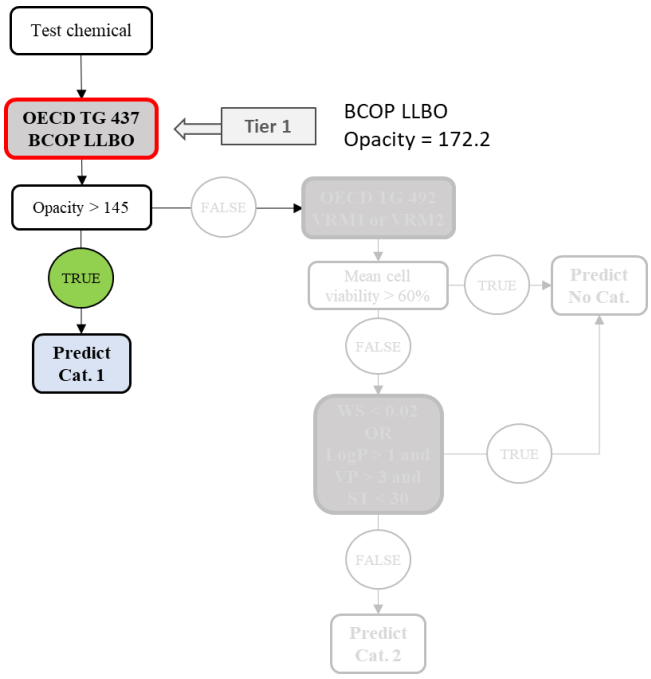
Figure 3. Bottom-Up approach of the DAL-1



(left) for 2-Hydroxy iso-butyric acid ethyl ester (CASRN 80-55-7). Left scheme: Tier 1 RhCE test method used to identify No Cat., Tier 2 physicochemical exclusion rules (WS: water solubility in mg/mL; or LogP: octanol-water partition coefficient / VP: vapour pressure in mm Hg / ST: surface tension in dyne/cm) to identify No Cat., and Tier 3 BCOP LLBO used to identify Cat. 1

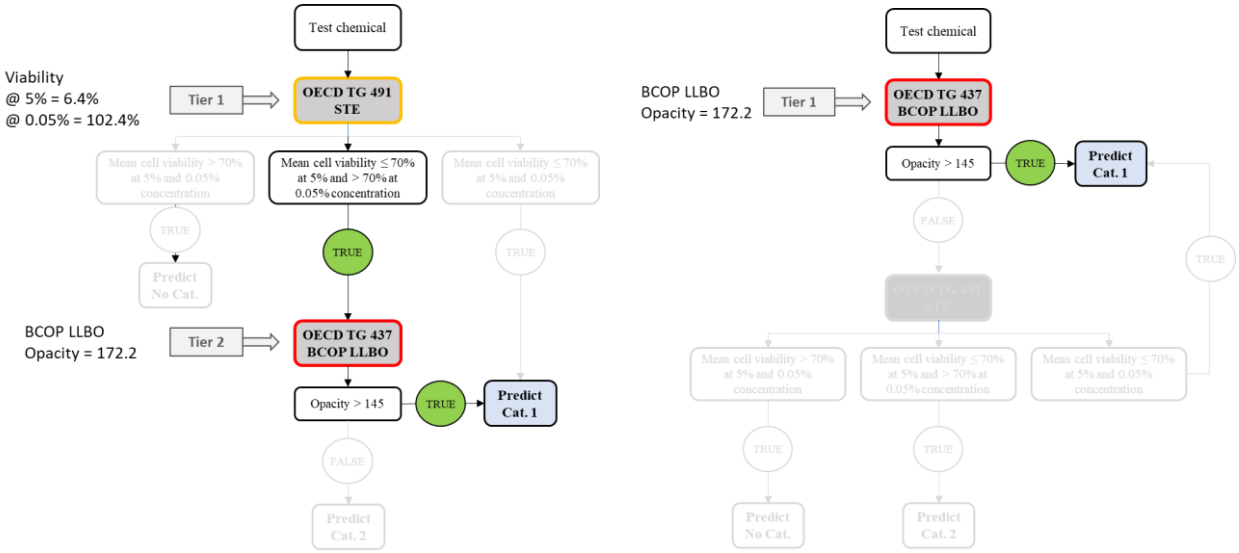
Right scheme: Tier 1 physicochemical exclusion rules to identify No Cat., Tier 2 RhCE test method used to identify No Cat., and Tier 3 BCOP LLBO used to identify Cat. 1

Figure 4. Top-Down approach of the DAL-1 for 2-Hydroxy iso-butyric acid ethyl ester (CASRN 80-55-7).



Tier 1 LLBO test method used to identify Cat. 1

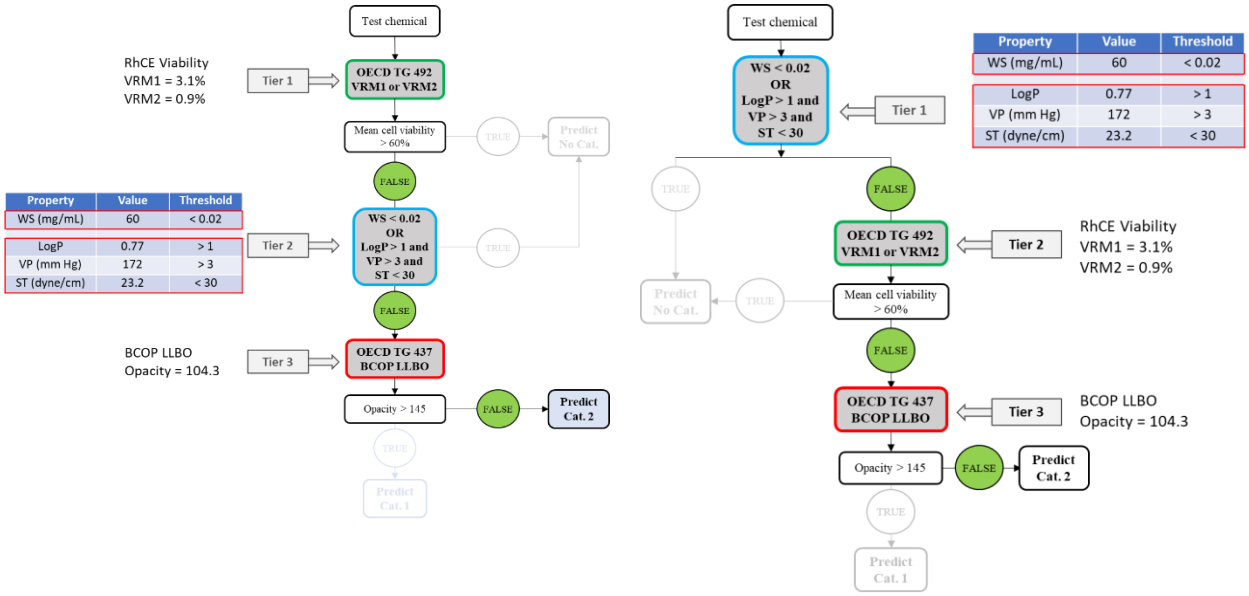
Figure 5. Schemes of the DAL-2



(left) Bottom-Up approach: Tier 1 start with the STE test to identify No Cat., Tier 2 BCOP LLBO test method to identify Cat. 1 and (right) Top-Down approach: Tier 1 start with the BCOP LLBO test method to identify Cat. 1.

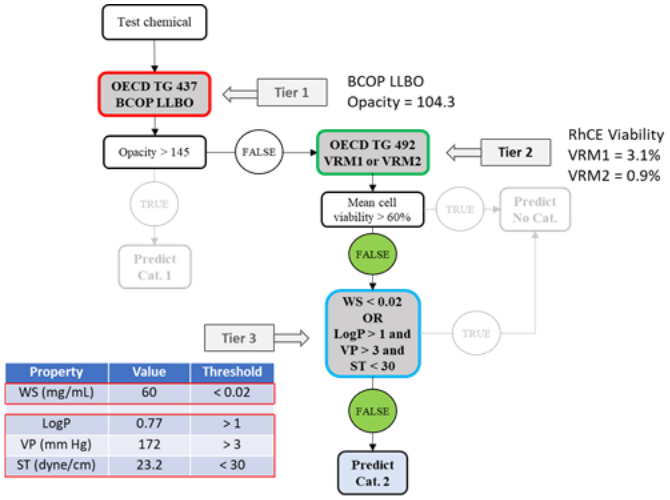
Annex B: iso-Butanal (CASRN 78-84-2)

Figure 6. Bottom-Up approach of the DAL-1



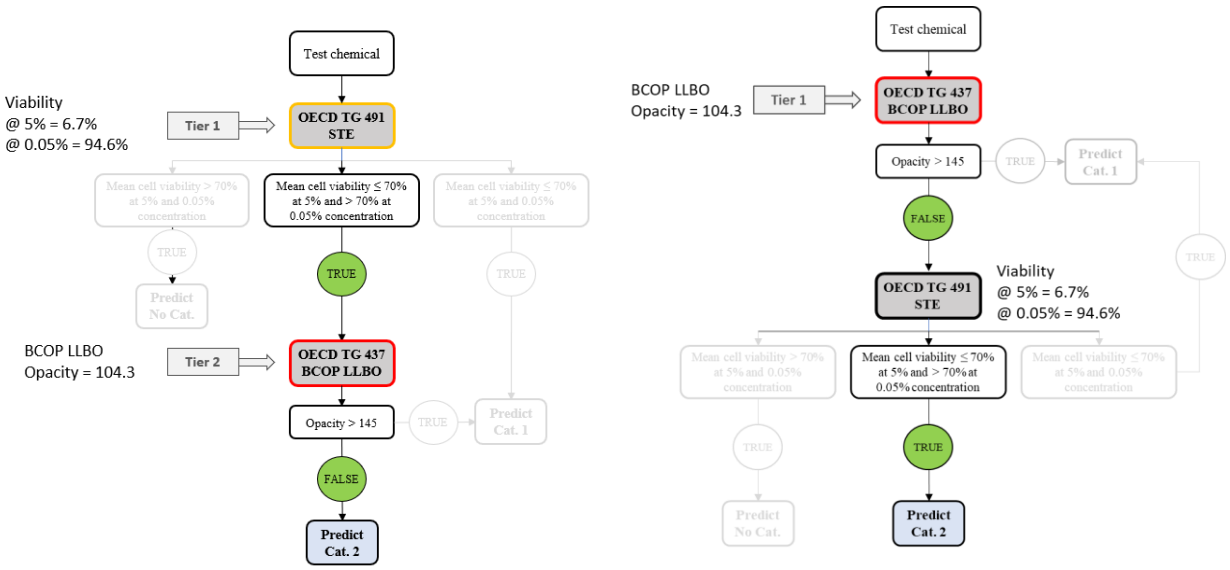
(left) for iso-Butanal (CASRN 78-84-2). Left scheme: Tier 1 RhCE test method used to identify No Cat., Tier 2 physicochemical exclusion rules (WS; or LogP/ VP /ST) to identify No Cat., and Tier 3 BCOP LLBO used to identify Cat. 1
Right scheme: Tier 1 physicochemical exclusion rules to identify No Cat., Tier 2 RhCE test method used to identify No Cat., and Tier 3 BCOP LLBO used to identify Cat. 1

Figure 7. Top-Down approach of the DAL-1 for iso-Butanal (CASRN 78-84-2).



Tier 1 LLBO test method used to identify Cat. 1. Tier 2 RhCE test method used to identify No Cat., Tier 3 physicochemical exclusion rules (WS; or LogP/ VP /ST) to identify No Cat.

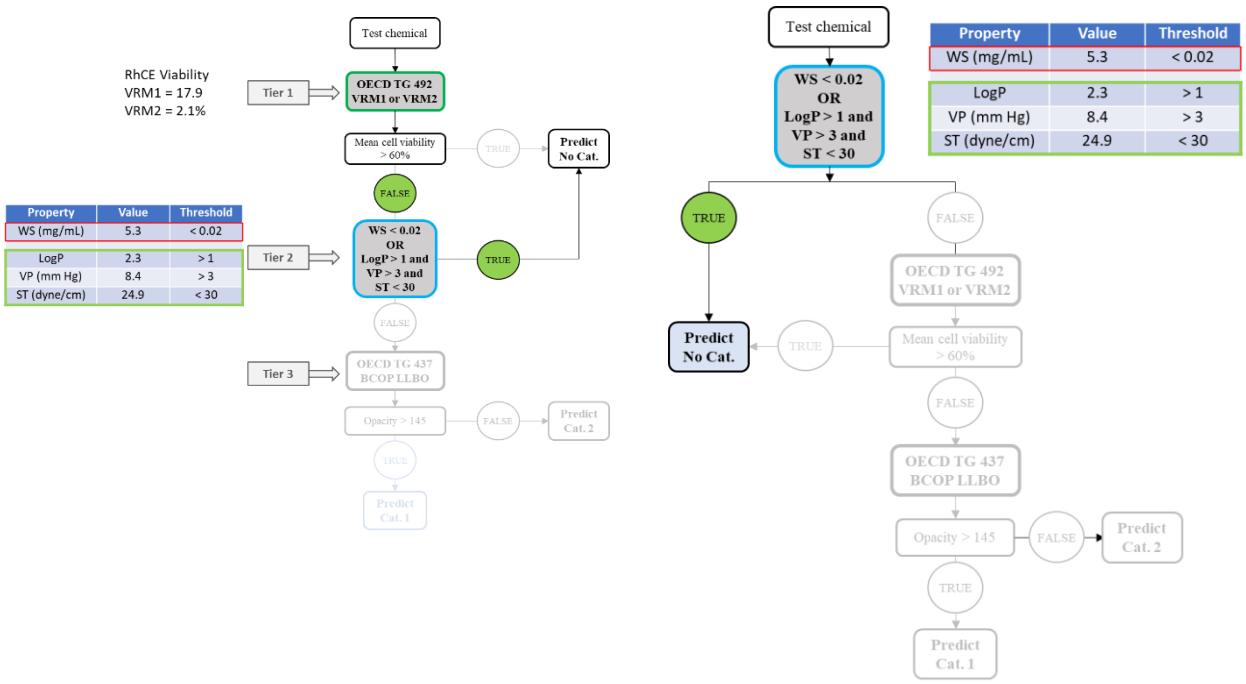
Figure 8. Schemes of the DAL-2 (left) Bottom-Up approach:



Tier 1 start with the STE test to identify No Cat., Tier 2 BCOP LLBO test method to identify Cat. 1 and (right) Top-Down approach: Tier 1 start with the BCOP LLBO test method to identify Cat. 1.

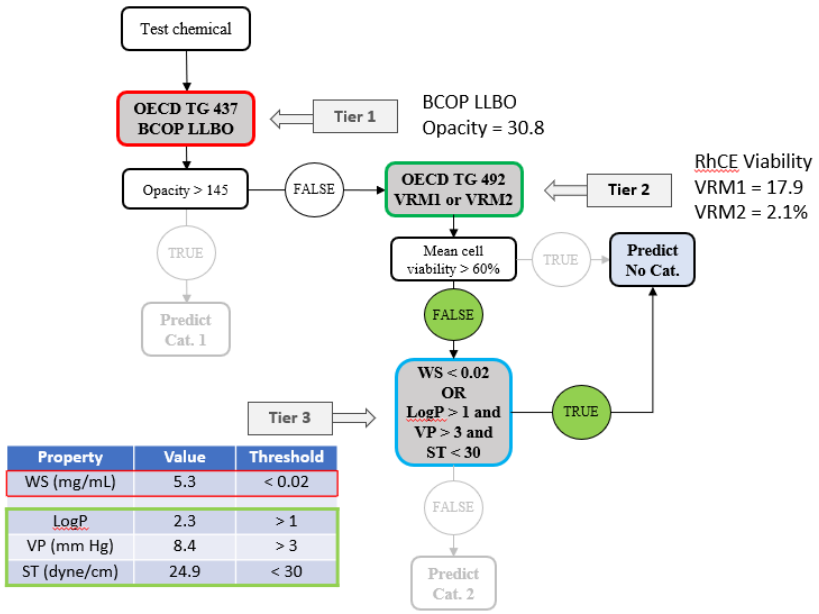
Annex C: n-butyl acetate (CASRN 123-86-4)

Figure 9. Bottom-Up approach of the DAL-1 (left) for n-butyl acetate (CASRN 123-86-4).



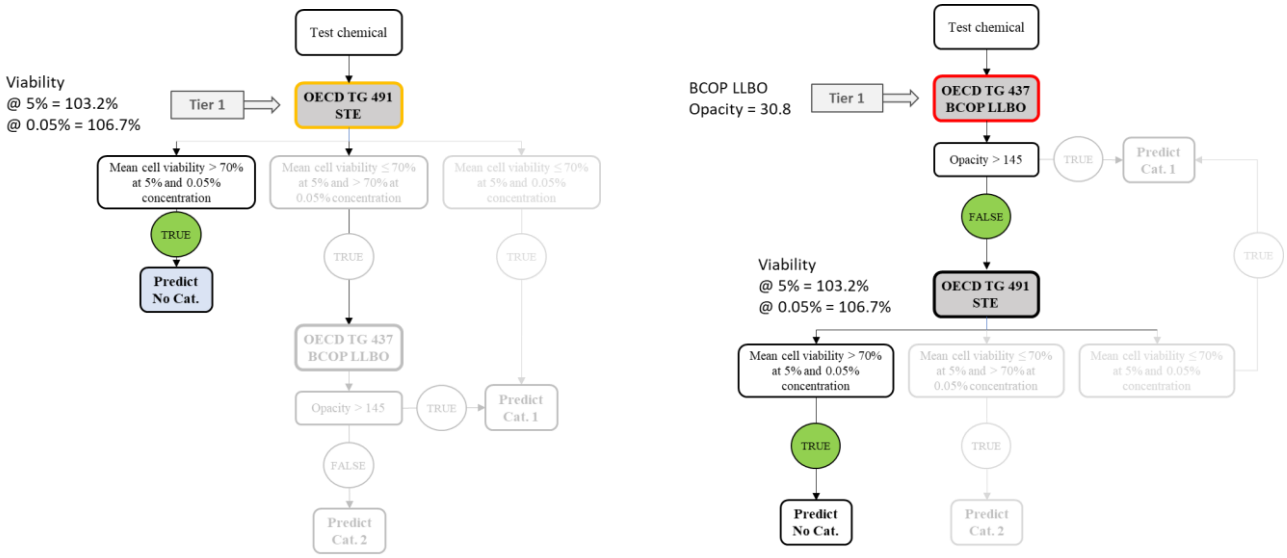
Left scheme: Tier 1 RhCE test method used to identify No Cat., Tier 2 physicochemical exclusion rules (WS; or LogP/ VP /ST) to identify No Cat
Right scheme: Tier 1 physicochemical exclusion rules to identify No Cat

Figure 10. Top-Down approach of the DAL-1 for n-butyl acetate (CASRN 123-86-4).



Tier 1 LLBO test method used to identify Cat. 1. Tier 2 RhCE test method used to identify No Cat., Tier 3 physicochemical exclusion rules (WS; or LogP/ VP /ST) to identify No Cat.

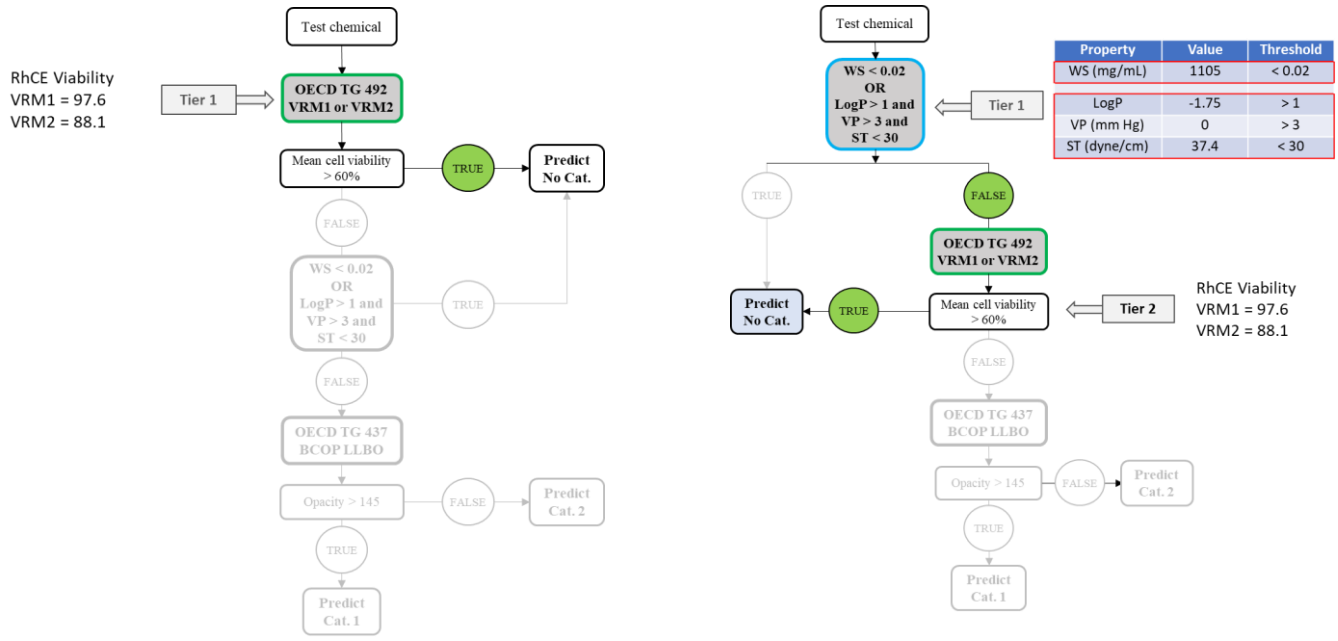
Figure 11. Schemes of the DAL-2 (left) Bottom-Up approach:



Tier 1 start with the STE test to identify No Cat.; Top-Down approach: Tier 1 start with the BCOP LLBO test method to identify Cat. 1., Tier 2 STE test to identify No Cat.

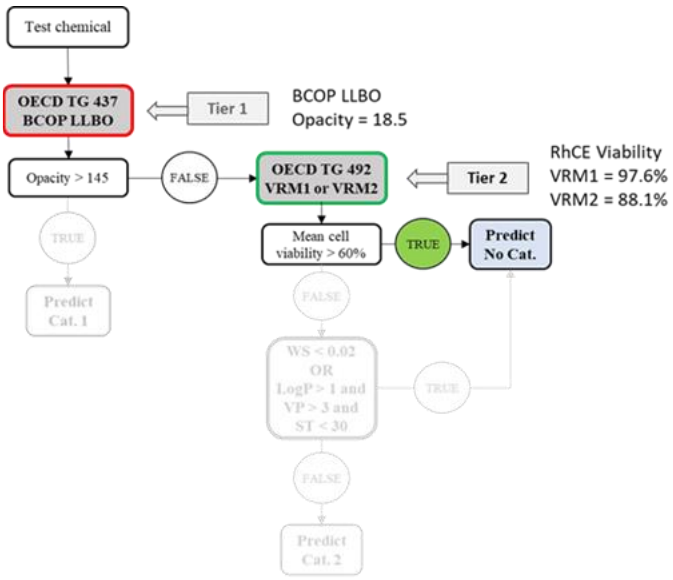
Annex D: glycerol (CASRN 56-81-5)

Figure 12. Bottom-Up approach of the DAL-1 (left) for glycerol (CASRN 56-81-5).



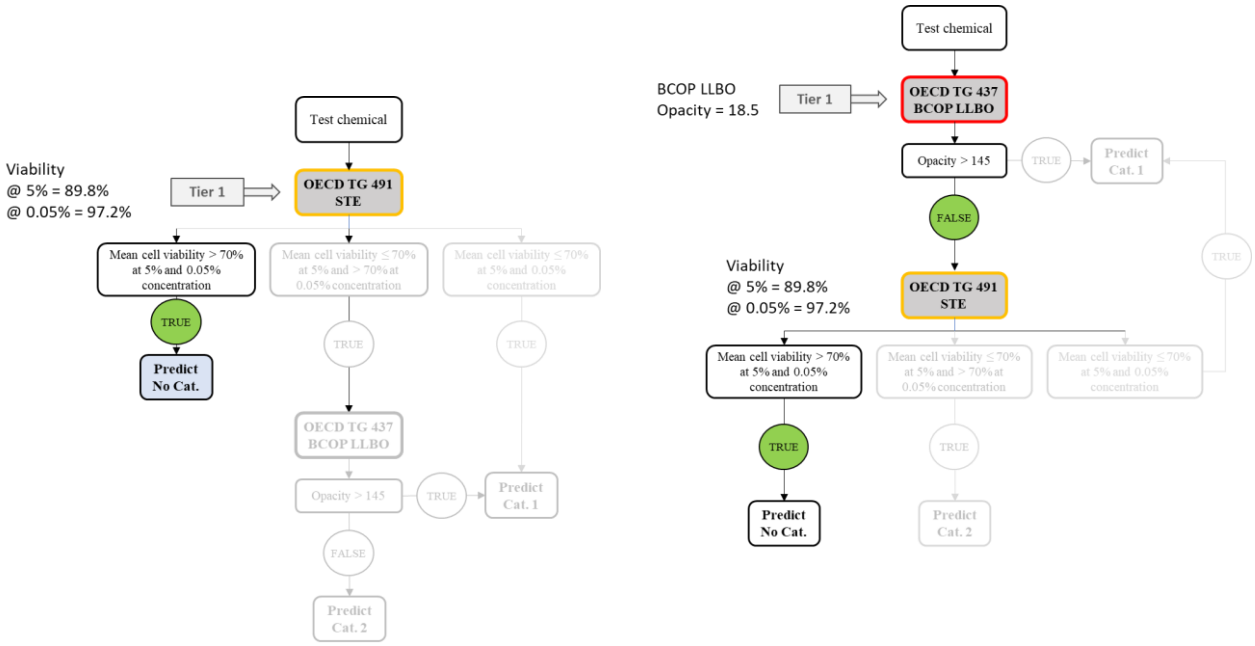
Left scheme: Tier 1 RhCE test method used to identify No Cat.;
Right scheme: Tier 1 physicochemical exclusion rules to identify No Cat., Tier 2 RhCE test method used to identify No Cat.

Figure 13. Top-Down approach of the DAL-1 for glycerol (CASRN 56-81-5).



Tier 1 LLBO test method used to identify Cat. 1

Figure 14. Schemes of the DAL-2



(left) Bottom-Up approach: Tier 1 start with the STE test to identify No Cat.
(right) Top-Down approach: Tier 1 start with the BCOP LLBO test method to identify Cat. 1, Tier 2 STE test to identify No Cat.