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Abbreviations

ATCC	American Type Culture Collection
BCOP	Bovine corneal opacity and permeability
CVS	Cell-Crystal Violet Staining
EU	European Union
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FDA	Food and Drug Administration
IC_{50}	50% of Inhibitory concentration
GHS	Globally Harmonized Systems of Classification and Labeling
GLP	Good Laboratory Practice
ICATM	International Cooperation on Alternative Test Methods
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICE	Isolated Chicken Eye
JaCVAM	Japanese Centre for the Validation of Alternative Methods
MEM	Eagle's Minimal Essential Medium
MAS	Maximal average Draize Total Score
MW	molecular weight
NI	Non-irritant
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organization for Economic Co - operation and Development
SIRC	Statens Seruminstitut Rabbit Cornea
SOP	Standard Operating Procedure
TEA	Triethanolamine
TG	Test guideline
UN	United Nations
VMT	Validation Management Team

1. Abstract

The SIRC-CVS ocular irritation test method was developed as a simplified[1] alternative to the Draize rabbit eye test for use in screening chemical substances used as ingredients in cosmetics and quasi-drugs for ocular irritation. The SIRC-CVS:TEA test method is a modified version of the SIRC-CVS test method that was validated in the 1990s under the Ministry of Health and Welfare Project[2] on alternatives to the Draize test, and this study was implemented at three participating laboratories in accordance with the spirit of GLP to validate intra- and inter-laboratory reproducibility as well as usefulness for distinguishing non-irritants from irritants in a bottom up approach.

The SIRC-CVS:TEA test method assesses cytotoxicity by measuring viable SIRC cells stained by crystal violet following a 72-hour exposure to test substances. The result is then used to calculate an IC_{50} value for the test substance, and if this value is smaller than the IC_{50} value of triethanloamine (TEA) as a relative control, the test substance is judged to be an irritant. The test substances were selected to provide a balanced representation of GHS categories and were coded prior to distribution to the participating laboratories.

Transferability of the test method was assessed using four test substances in Phase I. Intra-laboratory reproducibility was assessed using twenty test substances in Phase II. Inter-laboratory reproducibility was assessed using thirty test substances from Phases II and III at each of three participating laboratories, and predictive capacity was assessed using 115 test substances from Phases II and III.

The results demonstrated that the test method:

1. Was easily transferable to technically proficient laboratory technicians,

2. Demonstrated excellent intra-laboratory reproducibility (100%, 20/20) and inter-laboratory reproducibility (90%, 3/30),

3. Demonstrated an accuracy of 71.4% (30/42), sensitivity of 95.2% (20/21), and specificity of 47.6% (10/21) with a low false-negative rate of 4.8% (1/21) for test substances with a molecular weight of 180 or greater.

On the other hand, a significantly higher false-positive rate was observed for alcohols, esters, ketones, and other similar test substances.

From the above described results, we concluded that the SIRC-CVS:TEA test method demonstrated excellent intra- and inter-laboratory reliability and that, with a carefully defined applicability domain, it is a useful alternative to the Draize test for distinguishing cosmetic ingredients that are ocular non-irritants from those that are irritants.

2 Introduction

Assessing the ocular toxicity of the chemical substances used as cosmetic ingredients is an essential part of product development. The Draize eye irritation test has been commonly used to assess in vivo ocular damage to rabbit eyes caused by exposure to chemical substances (Draize et al., 1959). At present, however, animal welfare and other considerations point to the desirability of in vitro test methods that can be used in place of the Draize test. In fact, a variety of in vitro eye irritation test methods have heretofore been developed and validated. In September 2009, the bovine corneal opacity and permeability test and the isolated chicken eye test were adopted as the Test Guidelines 437 and 438, respectively, by the Organization for Economic Cooperation and Development as a means of assessing chemical substances for severe eye irritation potential. Both of the test guidelines were later revised and adopted by the OECD in July 2013 as a test method to assess non-irritants as well as severe eye irritants. Guidance describing the BCOP's contribution in assessing the safety of cosmetics and quasi-drugs was published by the Japanese Ministry of Health, Labour and Welfare in February 2014. Although OECD guidelines have been adopted for test methods that use isolated organs, there are as of yet no guidelines adopted for in vitro eye irritation test methods that assess non-irritants using corneal cells rather than organs.

The Statens Seruminstitut Rabbit Cornea Cell-Crystal Violet Staining (SIRC-CVS) cytotoxicity test uses an established SIRC cell line derived from the corneas of rabbit eyes. It assesses cytotoxicity by measuring viable cells stained by crystal violet following a 72-hour exposure to test substances. This

305 in vitro method has previously been considered for use as an alternative to the Draize test. Itagaki et
306 al. (1991) assessed the eye irritation potential of twelve surfactants using the SIRC-CVS test and
307 reported in vitro results that correlated well with in vivo results, thereby suggesting that the
308 SIRC-CVS test is useful for assessing the eye irritation potential of cosmetic ingredients. Based on
309 this potential, a three-phase validation study of the SIRC-CVS test was planned and performed with
310 the support of the Ministry of Health and Welfare (Ohno et al., 1999). Entitled *Studies on the test*
311 *methods to evaluate the safety of new ingredients of cosmetics*, the study was carried out by six
312 independent laboratories from 1991 to 1999. In this study, assessment of nine surfactants and saline
313 indicated good intra- and inter-laboratory reproducibility as well as good correlation between in
314 vitro and in vivo tests results (Itagaki et al., 1995). Also, a strong correlation ($r = -0.805$, $n = 29$)
315 between in vitro (cell viability measured as IC_{50}) and in vivo (the maximal average Draize total
316 score; MAS) was found for twenty-nine cosmetic ingredients, (Tani et al., 1999). The SIRC-CVS
317 test was later modified for use in distinguishing substances used as cosmetic ingredients which are
318 ocular non-irritants from those which are irritants, and polyoxyethylene sorbitan monolaurate
319 (20E.O.) was set as a reference substance for non-irritancy at a concentration of 10% (Ohno, 2004).
320 Data from the Japanese validation as reported by Tani et al. (1999) and the study reported by Hagino
321 et al. (2010) were re-analyzed using the cut-off value of triethanolamine (TEA) as a reference for
322 evaluating neat substances. A JaCVAM peer review of the SIRC-CVS test based on this data, which
323 was obtained between 2009 and 2011, concluded that this test could be used to identify non-irritants
324 but that a validation using the modified test protocol, known as SIRC-CVS:TEA, was still
325 necessary.
326 The purpose of this study is to validate intra- and inter-laboratory reproducibility as well as the
327 predictive capacity of the SIRC-CVS:TEA cytotoxicity test method. As a specific goal, this
328 validation study aims to clarify whether or not the SIRC-CVS:TEA cytotoxicity test method is a
329 useful alternative to the Draize test method in a bottom-up approach for distinguishing substances
330 used as cosmetic ingredients which are ocular non-irritants from those which are irritants under
331 GHS classification. To this end, we planned to validate the proposed SIRC-CVS:TEA cytotoxicity

test protocol using a sufficient number of coded substances for three laboratories to assess the eye irritation potential of the test substances. The validation began in 2011 and comprises three phases:

Phase I for validating technical transfer and training using four non-coded test substances

Phase II for validating intra- and inter-laboratory reproducibility using 20 coded test substances

Phase III for validating inter-laboratory reproducibility and predictive capacity using 100 coded test substances

3. Methods

3.1 Study Plan

3.1.1 Project plan

This validation study was implemented in September 2009 to assess the transferability, intra- and inter-laboratory reproducibility, and predictive capacity of a proposed SIRC-CVS:TEA test protocol. The specific goal of this study was to demonstrate that the proposed SIRC-CVS:TEA test method is a useful in vitro alternative to the in vivo Draize test method for identifying non-irritants under the Globally Harmonized System of Classification and Labeling of Chemicals (GHS). This validation study comprises three phases to confirm the reproducibility and the predictive capacity of the SIRC-CVS:TEA cytotoxicity test method. These study plans were organized and approved by the members of the Validation Management Team (VMT) and the participating laboratories.

3.1.2 Organization

The validation study was organized as shown in Fig. 1 to assure scientific pertinence and smooth implementation.

The SIRC-CVS:TEA validation management team (VMT) comprises a chairperson, members of the chemical management group, the data analysis group, the record management group, and a representative of test development lead laboratory. Support to participating laboratories was provided by the lead laboratory. A representative of ICCVAM acted as a liaison to the VMT and the representatives of the participating laboratories were observers. The VMT prepared, reviewed, and

finalized all draft study plans and protocols. In addition, the VMT managed the validation study by following its progress, assuring the quality of its records, contacting and coordinating with participants, and handling other administrative duties as necessary. Table 1 shows the organization of the VMT.

3.1.2.1 Chairperson

A chairperson was elected by vote of the VMT members and was responsible for preparing draft study plans, the study protocol, and the test substance list as well as for convening ad hoc VMT meetings for review and finalization of the study plan, the study protocol, and the test substance list. The chairperson was also responsible for other administrative duties related to the validation study.

3.1.2.2 Chemical management group

The chemical management group comprised two members selected from the VMT and was responsible for preparing list of test substances as well as conferring with the chairperson to finalize the test substances used in the validation study. It also prepared and distributed lists of non-coded or coded test substances to chemical distributors.

3.1.2 3. Data analysis group

The data analysis group comprised one member selected from the VMT and was responsible for providing objective analysis of data obtained in this validation study from a third-party standpoint as well as for statistical processing of data.

3.1.2.4. Record management group

The record management group comprised one member selected from the VMT and was responsible for preparing the protocol, test substance preparation sheets, blank data sheets, and other necessary materials as well as for distributing these materials to the participating research laboratories. It also collected the completed forms and data sheets after testing, reviewed the records for errors and

omissions, and requested correction as necessary.

3.1.2.5. Research laboratories

The following three laboratories participated in the assessment of test substances using the SIRC-CVS: TEA test method.

1. Bozo Research Center Inc. of Japan
2. Nihon Kolmar Co., Ltd of Japan
3. Biotoxtech Co., Ltd of Korea

Representatives of the participating laboratories were observers to the VMT and were responsible for carrying out testing according to the study protocol as well as for filling out and submitting all necessary records and forms upon completion of testing.

3.1.3. Study design

The SIRC-CVS:TEA test method measures viable cells stained by crystal violet. Crystal violet staining is with a variety of cultured cells and produces relatively stable results. Validation of the SIRC-CVS:TEA test method was carried out in three phases, as detailed in Appendix 8.1.

3.1.3.1 Training of participating personnel

A technical transfer workshop focusing on the principles of and protocol for the SIRC-CVS test method was held on Thursday, Nov. 11, 2011, with personnel from all three laboratories in attendance. Instructors from the lead laboratory explained the test method by demonstrating the protocol as shown in a video presentation, which was provided to all three laboratories after the workshop in a DVD.

3.1.3.2 Phase I study

The Phase I study was designed to assess transferability using four non-coded test substances. (Study Plan version 1.1). Each test substance was judged either positive or negative based on

obtaining consistent results from each of three runs.

3.1.3.3 Phase II study

Phase II was designed to assess intra- and inter-laboratory reproducibility using twenty coded substances (Study Plan IIA; version 1.51, Study Plan IIB; version1.53), but was split into two parts: Phases IIA and IIB.

Phase IIA was designed to assess the intra- and inter-laboratory reproducibility of five test substances, after which Phase IIB was designed to validate an additional fifteen test substances. Each test substance was judged either positive or negative based on three runs per set for each of three sets.

3.1.3.4 Phase III study

Phase III was designed to assess the inter-laboratory reproducibility and predictive capacity of the SIRC-CVS:TEA test method for one hundred coded test substances. Each laboratory tested one common set of ten test substances and one unique set of 30 test substances, as shown in Table 2 (Study Plan version 1.56). Each test substance was judged either positive or negative based on two runs. When the results of the first and second runs were consistent, judgment was based on these first two runs alone, and no third run was performed. When the results of the first and second runs were inconsistent, a third run was performed and the test substance judged according to the results of the final run.

3.1.3.5 Test substances

The test substances were selected to ensure that a variety of substances were represented, including various eye-irritant levels per GHS and EPA categories, physical state, chemical classes, and eye lesions produced. Substances for which high-quality in vivo data, especially data including results from individual animals, was available were given preference, such as substances listed in ICCVAM or ECVAM Eye Irritation Validation Studies. All selected test substances are available commercially.

A total of more than one hundred test substances were used in this validation study. These substances were selected by the chemical management group and approved by the VMT. All substances tested in Phase III were coded, and their names provided only after completion of the study. Each of the three laboratories tested a total of forty substances, ten of which were tested in common by all three laboratories, as shown in Table 3.

3.1.3.6 Study duration

Testing was performed from the of September 2011 until September 2013

Phase I, from September 2011 to March 2012 (Study Plan ver. 1.1)

Phase II-A, from March 2012 to September 2012 (Study Plan ver. 1.51)

Phase II-B, September 2012 to March 2013 (Study Plan ver. 1.53)

Phase III, March 2013 to September 2013 (Study Plan ver. 1.56)

3.1.4 Success criteria

Success criteria for intra- and inter-laboratory reproducibility was 80%, for accuracy was 80%, and for false negatives was less than 5%, as determined by the VMT prior to testing. Other acceptance criteria are described in section 3.2.8. *Quality Control*. The data file used at the participating laboratories was developed by the data analysis group, and entering data from test results automatically calculates values for IC_{50} using a dose-response plot as well as several other quality control criteria described in protocol Ver. 2.13E.

3.2 Summary of protocol

An overview of the SIRC-CVS test method is shown in Fig. 2. In addition, the current test protocol, Ver. 2.13E, is shown in Appendix 8.2 document. The procedures are described in greater detail below.

3.2.1 Cells

SIRC cells (Statens Seruminstitut rabbit corneal cells: ATCC NO. CCL-60) were obtained from

American Type Culture Collection. The cells were used within 3 months of the start of cultivation and were managed per the criteria stipulated in Section 3.2.8 *Quality Control.*

SIRC cells were cultured in a culture flask at 37°C, 5% CO_2 in Eagle's Minimal Essential Medium (MEM), containing 10% (v/v) fetal bovine serum, and 1% (v/v) antibiotic solution of 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL Amphotericin B. Confluent cells were dispersed in culture to single cells using trypsin-EDTA solution, after which they were transferred to a culture flask.

3.2.2 Stability of the test substance in the medium

The solubility of each test substance in the medium was confirmed in advance, using the procedure shown in Fig. 2. The test substance was dissolved or suspended uniformly in the medium at a concentration of 10,000 μg/mL (1% w/v). A vortex mixer, water bath, or sonicator as necessary. If the test substance could not be dissolved or suspended uniformly in the medium alone, it was mixed with DMSO at a concentration of 10,000 μg/mL in the medium. If the test substance could not be dissolved or suspended uniformly with DMSO in the medium, the test substance was mixed with ethanol a concentration of 10,000 μg/mL in the medium. If the test substance could not be dissolved or suspended uniformly with ethanol in the medium, the concentration was lowered, and it was mixed with DMSO at a concentration of 5,000 μg/mL in the medium. If the test substance could not be dissolved or suspended uniformly with DMSO at a concentration of 5,000 μg/mL in the medium, it was mixed with ethanol at a concentration of 5,000 μg/mL in the medium. Any test substance that still could not be suspended uniformly was judged to be unsuitable for the SIRC-CVS:TEA test. Judgment of suspensibility was performed macroscopically.

3.2.3 Preparation of the test substances

Each test substance was dissolved or suspended uniformly in the medium using the solvent identified per the procedure given in Section 3.2.3 *Stability of the test substance in the medium.* Test substances that could not be dissolved or suspended uniformly at a concentration of 10,000 μg/mL

in the medium alone were mixed with DMSO at a concentration of 10,000 μg/mL in the medium, with ethanol at a concentration of 10,000 μg/mL in the medium, with DMSO at a concentration of 5,000 μg/mL in the medium, or with ethanol at a concentration of 5,000 μg/mL in the medium. Any test substance that still could not be suspended uniformly was judged to be unsuitable for the SIRC-CVS:TEA test. The final maximal concentrations of both the test substances and the appropriate solvent (DMSO or ethanol) were 5,000 μg/mL, respectively, after dilution by the medium containing the SIRC cells. The final maximal concentration of the substances and the appropriate solvent was 2,500 μg/mL (0.25% w/v) and 5,000 μg/mL, respectively, when the low concentration of the substance was selected. Wells that exhibited precipitation or other problems any time after the test substance and cells were mixed, especially prior to the end of the 72-hr incubation period, were rejected.

3.2.4 Application of the test substance

1. PBS(-), negative control, dilution series of the test substance, positive control, and relative control were prepared in a 96-well microplates per the layout shown in Fig. 3.
2. One tenth mL of the $2x10^5$ cells/mL suspension was added to the wells as shown in Fig. 4.1and 4.2..
3. A microplate lid and wrap film were used to prevent contamination from volatile test substances. The six quality control criteria were used to check for contamination from volatile test substances. If contamination of a volatile test substance was found, the test was redone using the dilution series.
4. After adding the test substance and the cell suspension, each microplate was left to stand still for 20 minutes on a clean bench until the cells adhered to the bottom of the well. After that, the microplates were moved to the CO_2 incubator.

   The microplates were incubated for 72 hrs at 37degC and 5% CO_2.

3.2.5 Crystal violet staining

1. After incubation, the medium containing the test substance was removed by gently tilting the microplates.
2. 200 μL of PBS(-) was added, the microplates shaken gently, and the PBS(-) removed by gently tilting the microplates. This step was performed twice.
3. 100 μL of crystal violet methanol solution was added to each well, and let stand for 30 minutes to stain the cells.
4. After the staining, the crystal violet methanol solution was removed by gently tilting the microplates. After a thorough washing with tap water, any residual water was removed by blotting with paper towels and the cells was then allowed to dry naturally or placed in a dryer.
5. Absorbance as measured using a microplate reader at 588 nm or 570 nm, depending upon available equipment.

3.2.6 Calculation of IC50

Absorbance of control wells containing no test substance was assumed to be 100%, and the percentage absorbance for each well was calculated. The concentration at which the growth of cells was inhibited to 50% of the control (IC_{50}) was calculated using two concentrations around the predicted concentration of 50% cell viability per the following formula.

$LogIC_{50} = \{(50-y1)Logx2-(50-y2)Logx1\}/(y2-y1)$

where x1 is the lower concentration, x2 the higher concentration, y1 cell viability at the lower concentration, y2 cell viability at the higher concentration, and Log the common logarithm (log_{10}).

If cell viability was > 50% at the maximal concentration of 5,000 μg/mL, the IC_{50} of the test substance was recorded as > 5,000 μg/mL. Also, if the cell viability was < 50% at the concentration of 39.1 μg/mL (the lowest concentration tested), IC_{50} of the test substance was recorded as < 39.1 μg/mL.

In spreadsheets, the cell viability value was rounded to the nearest tenths.

3.2.7 Evaluation

549 The eye irritation of test substances was assessed using TEA as a relative control. TEA (100%) is
550 classified as No Category under GHS, per data published by Ohno et al. Test substances with an IC_{50}
551 greater than or equal to that of TEA were judged negative (GHS No Category). Those with an IC_{50}
552 less than that of TEA were judged positive (GHS Category 1 or 2). When the results of the first two
553 runs did not match, a third run was performed and judgment was based on that result. In cases where
554 standard deviation was calculated, three runs were made.
555
556 **3.2.8 Quality control**
557 Quality control was based on six criteria. If measurements did not satisfy quality control criteria, the
558 test substance was retested. In cases where volatile test substances caused discrepancies, the test
559 substance was retested at a lower concentration.
560
561 1. The absolute OD obtained from the negative control was used as an index of normal
562 proliferation for SIRC cells seeded at the concentration of 1×10^4 cells/well and incubated
563 for 72 hrs. The mean OD of the negative control wells must be > 0.4 for the test results to
564 be accepted.
565 2. Sodium dodecyl sulfate (SDS) was used as a positive control. The IC_{50} of SDS must be
566 77–259 μg/mL when tested by the standard protocol for the test results to be accepted.
567 3. TEA was used as a relative control. The IC_{50} range of TEA must be 1,000–2,500 μg/mL
568 when tested by the standard protocol for the test results to be accepted.
569 4. The difference between two dilution series of the substance should be confirmed. The IC_{50}
570 of the first series and the second series must be within ±20% of the mean IC_{50} of the two
571 series for the test results to be accepted. If the IC_{50} was lower than 39.1 μg/mL (the lowest
572 concentration tested), 39.1 μg/mL was adopted as the IC50. If the IC_{50} was higher than
573 5000 μg/mL (the highest concentration tested), 5,000 μg/mL was adopted as the IC50.
574 5. The mean ODs of the left and right wells must be within ± 15% of the mean OD of all
575 negative control wells for the test results to be accepted.

6. There should be no more than a two-fold difference between the mean values of the IC_{50} positive controls of two separate runs.

3.3 Test substances

3.3.1 Selection of test substances for the Phases I, II, and III studies

3.3.1.1 Test substances for phase I study

To confirm transferability of the SIRC-CVS:TEA test, sodium dodecyl sulfate was used as a positive control, TEA as a relative control, and four un-coded substances were assessed at the three participating laboratories, each of which performed one run using an identical protocol. The four un-coded substances were ethyl-2-methyl acetoacetate (water solubility), safflower oil (oil solubility), 3-chloropropionitrile (high volatility and cytotoxicity), and sodium dehydroacetate (cytotoxicity) (Table 4). The results from the three participating laboratories were compared with the data from the lead laboratory.

3.3.1.2 Test substances for phase II study

For Phase II, the Chemical Management Group and VMT selected 20 substances, as shown in Table 5, which had previously been assessed using the Draize eye test and classified under both GHS. The test substances were coded prior to distribution to the three participating laboratories (Appendix 8.3).

3.3.1.3 Test substances for Phase III study

For Phase III, the Chemical Management Group and VMT selected 100 substances, as shown in Table 6, which had previously been assessed using the Draize eye test and classified under GHS. The test substances were coded before being assessed at the three participating laboratories. A set of 10 common test substances and a set of 30 unique test substances were allocated to each of the three participating laboratories, as shown in Table 2.

3.3.2 Test substances selected for the validation study

The 120 test substances listed in Tables 2 and 3 of Appendix 8.3 were used to analyze the predictive capacity of the SIRC-CVS:TEA assay. One of these, 3,3-dithiodipropionic acid, was duplicated in distribution, so one entry was eliminated from the list. Three other test substances—citric acid, hexyl cinnamic aldehyde, and potassium sorbate—were excluded due to a lack of individual animal data with a clear source. Thus, there were a total of 116 test substances with individual animal data.

The physical state, chemical class, and classification per both GHS and EPA for each of the 116 test substances is shown in Table 4 of Appendix 8.3. The VMT considers the structural diversity of the selected test substances to cover the physical chemical properties as well as the full range of ocular irritation potential represented in GHS categories.

3.3.3 Purchase, coding, and distribution of test substances

All of the test substances used in Phases I, II, and III were obtained from commercial sources, as shown in Table 4 of Appendix 8.3. Test substances used in the Phases II and III were coded and distributed to the participating laboratories by JaCVAM.

3.4 Quality assurance

The participating laboratories conducted all tests in accordance with the principles of Good Laboratory Practice (GLP, OECD 1999), which were documented together with a discussion of any impact on study results. Preparation of test substances was recorded using a format developed for this validation by the lead laboratory. Researchers in participating laboratories recorded information such as the code name of each test substance, solvent name, date of the preparation, solubility or suspensibility, and concentration of the sample solution. These records were sent from the participating laboratories to JaCVAM, where their validity and accuracy were checked. These records are maintained by JaCVAM.

3.5 Record collection and analysis

630 Data collection and analysis was performed in close collaboration with biostatisticians. The data
631 files used by the participating laboratories were developed by the data analysis group. Independent
632 biostatisticians collected and organized data using specialized data collection software that
633 calculates the value of IC_{50} using a dose-response plot and quality control criteria. The data was
634 decoded and analyzed statistically. The data management procedures and the statistical tools were
635 approved by the chairperson and the data analysis group. Any deviations found in the analysis were
636 documented together with a discussion of any impact on study results. The eye irritation potential of
637 the test substances were evaluated by using TEA as a relative control in accordance with protocol
638 Ver. 2.13 of Appendix 8.2. Test results were evaluated against with GHS classification based on an
639 analysis of specific IC_{50} criteria.
640 Predictive capacity of the SIRC-CVS:TEA test method was evaluated using data from Phase II and
641 III. First, an analysis was performed to assess predictive capacity using the TEA IC_{50} to determine
642 GHS classification for either a bottom-up or a top-down approach. Further analysis was then
643 performed to reduce false negatives by limiting the applicability domain using chemical classes and
644 properties of interest. Chemical classes with at least six representative substances were examined:
645 alcohols, carboxylic acids, esters, ethers, halogen compounds, heterocyclic compounds,
646 hydrocarbons, ketones, organic salts, phenols, surfactants, and thiol compounds. Physical chemical
647 properties of interest were molecular weight, physical state, purity, water solubility, distribution
648 coefficient (log D), and vapor pressure. Criteria and rationale for selection of these properties of
649 interest are shown in Table 7.
650
651 **4. Results**
652 **4.1 Phase I study**
653 Phase I was designed to assess transferability and intra-laboratory reproducibility of the
654 SIRC-CVS:TEA eye irritation test method. Prior to this validation study, the lead laboratory
655 carried out technical training using the test protocol ver. 1.7.1E. The four non-coded substances
656 selected for the phase I study were ethyl-2-methyl acetoacetate (water solubility), safflower oil (oil