

3.0 SUBSTANCES USED FOR VALIDATION OF THE HET-CAM TEST METHOD

3.1 Rationale for the Substances Selected for Use

In vitro ocular test method validation studies should, ideally, evaluate an adequate sample of test substances and products from chemical and product classes that would be evaluated using the *in vivo* rabbit eye test method. Test substances with a wide range of *in vivo* ocular responses (e.g., corrosive/severe irritant to nonirritant) also should be assessed to determine any limit to the range of responses that can be evaluated by the *in vitro* test method.

In general, both criteria were used to select the substances used in the studies considered in this BRD: CEC (1991); Gettings et al. (1991, 1994, 1996); Bagley et al. (1992); Vinardell and Macián, (1994); Balls et al. (1995); Kojima et al. (1995); Gilleron et al. (1996, 1997); Spielmann et al. (1996); and Hagino et al. (1999).

3.1.1 CEC (1991)

The selection of substances used in this evaluation was based on the following criteria:

- The substances should be representative of currently used industrial chemicals and should represent a range of chemical structures.
- The substances should cover the range of eye effects from nonirritant to severe irritant.
- The *in vivo* rabbit eye studies should have been conducted in accordance with European Economic Commission (EEC) criteria and the animal data should be sufficient to allow an irritancy classification to be definitively assigned to the test substance.
- Whenever possible, the substances should have been used in previous validation studies.

3.1.2 Gettings et al. (1991, 1994, 1996)

The studies described in this set of papers focused evaluating the ability of alternative test methods to identify ocular corrosives and severe irritants that are developed by the cosmetic, toiletry, and fragrance industries. Therefore, for this evaluation a set of formulations were developed that were representative of cosmetic, toiletry, and fragrance formulations used at the time of the study.

3.1.3 Bagley et al. (1992)

The studies described in paper focused evaluating the ability of alternative test methods to identify ocular corrosives and severe irritants. Therefore, substances that were (1) raw materials commonly used in the cosmetics/toiletries and household cleaning product industries, and (2) formulations representing products from these industries were evaluated.

3.1.4 Balls et al. (1995)

In the EC/HO validation study (Balls et al. 1995), the test substances were initially selected from the 1992 European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Reference Data Bank for ocular irritation (ECETOC 1992) based on the following criteria:

- Substances should be single chemicals (no mixtures).
- Substances should be available at high purity and stable when stored.
- The *in vivo* rabbit eye test data should have been generated since 1981 according to the OECD TG 405 and in compliance with GLP guidelines.

Other criteria specific to the conduct of the studies are noted in the study report (Balls et al. 1995).

Originally, 60 substances that met the established criteria were found in the ECETOC data bank. However, this selection was determined to be inadequate due to the low number of solids, the insufficient number of moderate to severe irritants, and the lack of pesticides. To avoid additional animal testing, the validation study management team attempted to locate high quality rabbit eye study data within the commercial sector. Subsequently, based on the availability of additional data that met the established criteria (obtained primarily from unpublished studies), the original list was modified to include more solids, some pesticides, and substances representing moderate to severe degrees of irritation. During the validation study, it was discovered that 14 of the reference substances had been tested by a protocol that involved rinsing or removing the solid material from the eye one hour after application, rather than allowing it to remain continuously. Thus, the study protocol for these substances had not adhered to OECD TG 405. These 14 substances were retested *in vivo* and it was found that one, thiourea, was extremely toxic, killing the three rabbits on which it was tested. Based on this response, thiourea was excluded from the list of reference substances.

The final list of test substances included a total of 51 substances, four of which were tested at two different concentrations and two of which were tested at three concentrations, for a total of 59 different tests.

3.1.5 Vinardell and Macián (1994)

There was no specific rationale for the selection of substances used by Vinardell and Macián (1994) provided in the literature reference.

3.1.6 Kojima et al. (1995)

Kojima et al. (1995) evaluated substances that were major ingredients in cosmetic formulations and preparations. These substances included surfactants and solvents.

3.1.7 Gilleron et al. (1996, 1997)

Gilleron et al. (1996, 1997) selected substances that represented a broad spectrum of ocular irritancies, chemical classes, and chemical structures. Substances also were selected on the basis of availability of historical *in vivo* data, to avoid conducting additional tests for the validation study.

3.1.8 Spielmann et al. (1996)

Spielmann et al. (1996) selected substances that represented a broad spectrum of ocular irritancies, chemical classes, and chemical structures. Substances also were selected on the basis of availability of historical *in vivo* data, to avoid conducting additional tests for the validation study.

3.19 Hagino et al. (1999)

Hagino et al. (1999) evaluated substances that were major ingredients in cosmetic formulations and preparations. These substances included surfactants and solvents.

3.2 Rationale for the Number of Substances Tested

No rationale was provided for the number of substances tested in these studies except for Spielmann et al. (1996). Spielmann et al. (1996) noted that the Amden validation workshop recommended that approximately 200 substances should be used to assess the performance of an alternative test method. Therefore, they originally selected a total of 200 substances for their effort to validate HET-CAM (Phase I and Phase II). The number was reduced to a total of 118 substances, after substances were excluded due to unacceptable *in vivo* or *in vitro* data quality.

3.3 Chemicals or Products Evaluated

Physicochemical properties for each of the tested substances was obtained from information provided in the published reports and submitted data. No attempt was made to review original records in order to obtain additional information about each substance. For each substance tested in HET-CAM, **Appendix B** provides information on its CASRN, chemical and/or product class. **Appendix C** provides information on the physicochemical properties (e.g., pH, physical form tested), where available from the published reports or submitted data.

Chemical and product classes were assigned for each test substance based on information found in the study report. If a chemical class was not assigned in the study report, such information, when available, was retrieved from the National Library of Medicine's ChemID Plus database, or assigned based on chemical structure. Chemical classes were assigned to each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system (available at <http://www.nlm.nih.gov/mesh>) that ensures consistency in classifying substances among all *in vitro* ocular test methods under consideration. If a product class was not assigned in the study report, such information, when available, was retrieved from publicly available sources that discussed the substance. A substance could be assigned to more than one chemical or product class.

Table 3-1 provides the chemical class information on the test substances evaluated with HET-CAM. The chemical classes with the greatest number of substances tested are alcohols, carboxylic acids, and organic salts. Of the substances included in **Appendix B**, 53 were formulations. For some of the test substances that were identified as formulations, components of the formulation and the relative concentrations of the components were available. Summaries of the relative concentrations of each component in these formulations are provided in **Appendices B-2 to B-4**.

Table 3-1 Chemical Classes Tested in the HET-CAM Test Method

Chemical Class	# of Substances	Chemical Class	# of Substances
Acyl halide	2	Inorganic salt	14
Alcohol	75	Imide	4
Aldehyde	9	Ketone	15
Alkali	4	Lactone	5
Amide	2	Nitrile	3
Amidine	6	Nitro compound	3
Amine	34	Onium compound	22
Amino acid	7	Organic salt	50
Carbohydrate	1	Organometallic compound	2
Carboxylic acid	51	Organophosphorous compound	1
Ester	34	Organosilicon compound	6
Ether	38	Phenol	4
Formulation	53	Polycyclic compound	11
Heterocyclic compound	37	Organic sulfur compound	18
Hydrocarbon, Acyclic	5	Unknown	28
Hydrocarbon, Cyclic	5	Urea	3
Inorganic boron compound	2		

Table 3-2 provides the product class information on the test substances evaluated with HET-CAM. The most common product classes tested are solvent, shampoo, surfactants, and cosmetics. Of the substances included in **Appendix B**, 167 were not be classified within a product class.

3.3.1 Substances Evaluated in Reviewed Studies

3.3.1.1 *CEC (1991)*

This report described the results of a study commissioned by the Division Control of Chemicals, Industrial Risks and Biotechnologies of Directorate General Environment, Nuclear Safety, Civil Protection and the Health and Safety Directorate of Directorate General Employment Industrial Relations and Social Affairs. In this study, 21 substances were evaluated. All substances appear to have been tested as 100% or 10% concentrations. The authors provided purity of the tested substances but not other physicochemical properties. The authors used the IS(B) analysis method to evaluate the irritancy potential of the test substances.

Table 3-2 Product Classes Tested in the HET-CAM Test Method

Product Class	# of Substances
Aerosol formulation ingredient	1
Anti-freezing agent	1
Anti-infective agent, Anti-bacterial agent	2
Anti-perspirant	1
Bactericide, Biocide, Fungicide, Germicide	4
Beverage	1
Cationic surface active agent	1
Chemical intermediate	6
Cleaner	1
Conditioner, Hair	2
Cosmetics	14
Cream	1
Disinfectant	1
Drug vehicle	1
Emollient	2
Fertilizer	1
Flavor ingredient	5
Fragrances	4
Industrial explosive	1
Laboratory reagent	7

Product Class	# of Substances
Lotion	3
Lubricant	1
Mouthwash	1
Neurotransmitter	2
Pesticide	5
Pharmaceutical agent, Pharmaceutical intermediate, Pharmaceutical metabolite	4
Plasticizer	2
Polymer	1
Preservative	1
Raw material	1
Shampoo, Hair	13
Solvent	13
Sunscreen	3
Surfactant	17
Synthetic flavor ingredient, Flavor ingredient	4
Synthetic intermediate	1
Unknown	167

Information (e.g., CASRN, chemical and/or product class, physiochemical properties) was extracted for 15 test substances. Chemical classes of the tested substances included, but were not limited to, alcohols, esters, and carboxylic acids. Product classes of the tested substances included, but were not limited to, bactericide and surfactant.

3.3.1.2 *Gettings et al. (1991)*

This report described results from Phase I of the Cosmetic, Toiletry, and Fragrance Association (CTFA) Evaluation of Alternatives Program, a program that evaluated promising *in vitro* alternative test methods for the *in vivo* rabbit eye test. Each phase of the program evaluated a specific product type. Phase I (1991) evaluated 10 hydroalcoholic formulations. Formulations were generic formulations that represented formulations used at the time of the study (e.g., facial cleaner). All formulations in Phase I were tested undiluted. The IS(B) analysis method was used to evaluate the irritancy potential of the test substances. The

product classes of the tested formulations included, but were not limited to, fragrances, mouthwash, and sunscreen.

Information (e.g., formulation components, physicochemical properties) was extracted for all formulations evaluated. Information on the components of the 10 formulations was obtained from the literature; this information is provided in **Appendix B-2**.

3.3.1.3 *Gettings et al. (1994)*

This report described results from Phase II CTFA Evaluation of Alternatives Program. Each phase of the program evaluated a specific product type. Phase II evaluated 18 oil/water formulations. Formulations were generic formulations used at the time of the study (e.g., conditioner). All formulations in Phase II were tested undiluted. The authors used the IS(A) and IS(B) analysis methods to evaluate the irritancy potential of the test substances. The product classes of the tested formulations included, but were not limited to, conditioner, sunscreen, and cream.

Information (e.g., formulation components, physicochemical properties) was extracted for all formulations evaluated. Information on the components of the 18 formulations was obtained from the literature; this information is provided in **Appendix B-3**.

3.3.1.4 *Gettings et al. (1996)*

This report described results from Phase III CTFA Evaluation of Alternatives Program. Each phase of the program evaluated a specific product type. Phase III evaluated 25 surfactant-based personal cleaning formulations. Formulations were generic formulations used at the time of the study (e.g., shampoo). In Phase III, nine of the substances were evaluated at a concentration of 25% (v/v) in distilled water. The IS(A) and IS(B) analysis methods were used to evaluate the irritancy potential of the test substances. The product class of the tested formulations was shampoo.

Information (e.g., formulation components, physicochemical properties) was extracted for all formulations evaluated. Information on the components of the 25 formulations was obtained from the literature; this information is provided in **Appendix B-4**.

3.3.1.5 *Bagley et al. (1992)*

In this study, 32 substances were evaluated; 12 were raw materials commonly used in cosmetics, toiletries, and household products and 20 were prepared formulations. All substances appear to have been tested as neat liquids or solutions. The authors did not provide information on the constituents of the formulations or the physicochemical properties of any of the tested substances in the reviewed study. The range of MAS values for the substances was 0.3 to 44.7. The source of the raw materials and the concentration tested were provided in the report. The authors used the IS(A) analysis method to evaluate the irritancy potential of the test substances.

Information (e.g., CASRN, chemical and/or product class, physicochemical properties) was extracted for two of the raw materials. The chemical classes of these raw materials were ether and alcohol/amine. The range of MAS values of the substances extracted from this

study was 2.7 to 40.0. All substances appear to have been tested as neat liquids or solutions *in vitro*.

3.3.1.6 *Vinardell and Macián (1994)*

The study evaluated six vehicles and six commercial disinfectant products. The substances were tested at concentrations ranging from 0.1% to 100%. Other than pH of the tested solutions, which ranged from 3.3 to 13.02, no other physicochemical properties were provided. All substances appear to have been tested as neat liquids or solutions. In the study report, the authors did not provide any information about the ingredients of the disinfectant products. The IS(B) analysis method was used to evaluate the irritancy potential of the test substances.

Information (e.g., CASRN, chemical and/or product class, physiochemical properties) was extracted for two vehicles. The pH values of these two vehicles were 3.3 and 7.2; both were tested as neat liquids *in vitro*.

3.3.1.7 *Balls et al. (1995)*

The study evaluated 51 substances with the HET-CAM test method. The substances were evaluated at concentrations ranging from 0.1% to 100%. Of these substances, 30 were water-soluble, 18 were water insoluble, and 12 were classified as surfactants by the study authors. Fourteen substances were tested as solutions, 20 were tested as solids, and 26 were tested as liquids *in vitro* and *in vivo*. For each substance, the authors provided in the report the CASRN, chemical class, source, catalog number, purity, and form tested. The S-Score and Q-Score analysis methods were used to evaluate the irritancy potential of the test substances. The chemical classes evaluated included, but were not limited to, amine, carboxylic acid, and organic salt.

Information (e.g., CASRN, chemical and/or product class, physiochemical properties) was extracted for all substances.

3.3.1.8 *Kojima et al. (1995)*

In this study, 24 substances were evaluated with the HET-CAM test method. Solubility and other physicochemical properties of the test substances were not provided in the paper. For each substance, the authors provided in the report information on its source and the concentration tested (10% solution). The authors used the IS(B) analysis method to evaluate the irritancy potential of the test substances.

Information (e.g., CASRN, chemical and/or product class, physiochemical properties) was extracted for five substances. The chemical classes of the extracted test substances were alcohol, organic salt, carboxylic acid salt, onium, and ether. All substances appeared to have been tested as solutions.

3.3.1.9 *Gilleron et al. (1996)*

The 46 evaluated substances were classified by the authors as solids (17), liquids (21), and surfactants (8). Chemical classes of the substances tested included alcohols, carboxylic acid, heterocyclic, and amine. Solubility and other physicochemical properties of the test

substances were not provided in the paper. For each substance, the CASRN and source were provided in Gautheron et al. (1994). The authors used the IS(B) analysis method to evaluate the irritancy potential of the test substances.

Information (e.g., CASRN, chemical and/or product class, physicochemical properties) was extracted for all tested substances.

3.3.1.10 *Spielmann et al. (1996)*

In this study, 200 substances from the pharmaceutical and chemical industries were initially selected for evaluation; 34 substances were evaluated in Phase I and 166 substances were evaluated in Phase II of the study. All substances were tested at various concentrations to determine the threshold concentration for inducing an effect (ITC). Chemical classes of the tested substances included alcohol, amine, ester, ether, heterocyclic, and organic salt. Several analysis methods were used to assess the irritancy potential of the tested substances including, but not limited to, the IS and ITC analysis method and the mtc10 analysis method.

Information (e.g., CASRN, physicochemical properties) for all the substances evaluated by this study is provided in Spielmann et al. (1996). Information for 112 substances evaluated in the HET-CAM BRD was extracted for the IS(B)-10 and IS(B)-100 analysis methods.

3.3.1.11 *Gilleron et al. (1997)*

In this study, the 52 different substances were tested *in vitro* and compared to 60 *in vivo* studies. Solubility and other physicochemical properties of the test substances were not provided in the paper. The CASRN and physicochemical properties of the tested substances were detailed in Balls et al. (1995). Chemical classes of the tested substances included alcohol, amine, ester, ether, carboxylic acid, and heterocyclic. The authors used the IS(B) analysis method to evaluate the irritancy potential of the test substances.

Information (e.g., CASRN, chemical and/or product class, physicochemical properties) was extracted for all tested substances.

3.3.1.12 *Hagino et al. (1999)*

In Phase III of a three-part validation study, 14 cosmetic ingredients were evaluated. For each substance, the authors provided in the report its chemical class, the concentration tested, and its physical form. The chemical classes of the tested substances included, but were not limited to, alcohol, carboxylic acid, organic salt, onium, and amine/amidine. Of these 14 substances, 12 were tested as a solution and two were tested as a suspension. The pH of the tested substances ranged from 2.4 to 12.48. The MAS of the tested substances ranged from 0 to 102.7. CASRN were obtained from the National Library of Medicine's ChemID Plus database. In the study, the authors used the IS(A) analysis method to evaluate the irritancy potential of the test substances.

Information (e.g., CASRN, chemical and/or product class, physicochemical properties) was extracted for all 14 cosmetic ingredients discussed above.

3.4 Coding Procedures Used in the Validation Studies

The coding procedures used in the studies considered in this BRD were evaluated by the information provided in the published reports. No attempt was made to obtain original study records to assess these procedures. Based on the available information, the reports that identified using coded chemicals were Gettings et al. (1991, 1994, 1996), Bagley et al. (1992), Balls et al. (1995), Spielmann et al. (1996), and Hagino et al. (1999).

3.4.1 Gettings et al. (1991, 1994, 1996)

A two-part system was developed to ensure that the identity of the test substances remained unknown during testing. The first part of the identification consisted of a Sample ID that was specific for each distribution of the sample. The Sample ID consisted of a two letter and one number combination. If additional samples were needed, the number was increased in sequence. The two-letter code was chosen at random, but was unique to each sample and laboratory. The second part of the identification consisted of a Sample Number (which ranged from 1 to 12). The Sample Numbers corresponded to the substances provided in each shipment to the participating laboratories.

3.4.2 Bagley et al. (1992)

The samples were transferred from original containers at a central coordinating point and then randomly coded from 1 to 32 prior to shipping to the participating laboratories.

3.4.3 Balls et al. (1995)

Test substances and participating laboratories were each assigned a numeric code in order for subsequent data analysis to be performed without knowledge of the identities of the test substance or laboratory. The total number of aliquots of each test substance required for the full study was determined. Computer software was then used to generate random codes for the total number of samples, so that a unique number could be assigned to each sample.

3.4.4 Spielmann et al. (1996)

The substances were coded prior to distribution to the participating laboratories. No information was provided in the report on how the substances were coded and/or tracked.

3.4.5 Hagino et al. (1999)

The Japanese Cosmetic Industry Association provided the test substances to the Test Substance Control Committee. The substances were then coded and distributed to the participating laboratories. No information was provided in the report on how the substances were coded and/or tracked.