

SECTION IV

**HEN'S EGG TEST - CHORIOALLANTOIC
MEMBRANE (HET-CAM) TEST METHOD
ACCURACY AND RELIABILITY REANALYSIS**

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2614 **1.0 INTRODUCTION**

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2616 On November 1, 2004, NICEATM released draft BRDs on the current status of four *in vitro*
2617 test methods for detecting ocular corrosives and severe irritants (see
2618 http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm). The test methods reviewed
2619 were the BCOP, the HET-CAM, the IRE, and the ICE assays. On January 11-12, 2005,
2620 ICCVAM convened an Expert Panel to independently evaluate the validation status of these
2621 four *in vitro* test methods for identifying ocular corrosives or severe irritants. The Expert
2622 Panel Report, *Evaluation of the Current Validation Status of In Vitro Test Methods for*
2623 *Identifying Ocular Corrosives and Severe Irritants*, can be obtained by contacting
2624 NICEATM or electronically from <http://iccvam.niehs.nih.gov/methods/eyeirrit.htm>. Public
2625 comments at the meeting revealed that additional data could be made available that had not
2626 yet been provided in response to earlier requests for data. The Expert Panel subsequently
2627 recommended that the additional data be requested and that a reanalysis of the accuracy and
2628 reliability of each test method be conducted, to the extent possible.

2629

2630 In response to this recommendation, a second *FR* notice was published on February 28, 2005
2631 (*FR* Vol. 70, No. 38, pp. 9661-9662; <http://iccvam.niehs.nih.gov/methods/eyeirrit.htm>)
2632 requesting all available *in vitro* data on these four *in vitro* ocular irritancy test methods and
2633 corresponding *in vivo* rabbit eye test method data, as well as any human exposure data (either
2634 via ethical human studies or accidental exposure). The first *FR* notice requesting these data
2635 had been published on March 24, 2004 (*FR* Vol. 69, No. 57, pp. 13859-13861;
2636 <http://iccvam.niehs.nih.gov/methods/eyeirrit.htm>). Also, a request for relevant data was re-
2637 sent directly to the primary developers or users of each test method, and sent to other
2638 scientists who participated in or attended the Expert Panel Meeting on January 11-12, 2005
2639 and who had indicated a desire to provide additional data. No human exposure data was
2640 obtained for the substances evaluated in the HET-CAM test method, and therefore no
2641 calculations could be made for the accuracy of the HET-CAM test method for predicting
2642 human severe ocular irritancy.

2643

2644 Other factors also necessitated a reanalysis of the accuracy of HET-CAM for detecting ocular
2645 corrosives and severe irritants. First, clarification regarding the rules for classification of
2646 severe irritants was obtained subsequent to the release of the four BRDs that resulted in
2647 changes to the hazard classification of some of the substances used in the original analysis.
2648 For the original analysis, reversibility of ocular effects for all EU and GHS hazard
2649 classification systems was considered to be achieved if, by post-exposure day 21, the
2650 endpoint scores fell below the threshold that resulted in a test substance being classified as a
2651 severe irritant (EU [2001]; UN [2003]). The new information obtained indicated that
2652 reversibility of ocular effects is achieved only when all scores reach zero on post-exposure
2653 day 21. This change resulted in two substances previously classified as GHS nonsevere
2654 irritants now being classified as GHS severe irritants.

2655

2656 Second, the chemical classes assigned to each test substance were revised to reflect a
2657 standardized classification scheme (based on MeSH [www.nlm.nih.gov/mesh]) that would
2658 ensure consistency in classifying substances among all *in vitro* ocular test methods under
2659 consideration. This resulted in some chemicals being reclassified. The accuracy of the HET-

2660 CAM test method, by chemical class and using the GHS classification system (UN [2003]),
2661 has been reanalyzed to reflect these changes.

2662
2663 Finally, an additional accuracy analysis was conducted. In this analysis, the accuracy of each
2664 *in vitro* ocular irritancy test method for detecting ocular corrosives or severe irritants,
2665 depending on whether the classification was based on the severity of the response and/or its
2666 persistence to day 21 post-treatment, was determined.

2667
2668 For the HET-CAM test method, the changes to the existing database that resulted from using
2669 the appropriate persistence classification criteria and any new data and/or information
2670 received subsequent to the release of the draft BRD are summarized in **Table IV-1**.

2671 Additional HET-CAM test method data and corresponding *in vivo* rabbit eye test data were
2672 received from the German Center for Documentation and Evaluation of Alternative Methods
2673 to Animal Experiments (ZEBET) for substances that were originally described in Spielmann
2674 et al. (1996) (Spielmann and Liebsch [2005a]). HET-CAM test data previously discussed in
2675 Section 9.0 of the draft HET-CAM BRD also were included in this reanalysis (Gilleron et al.
2676 [1996, 1997]). Results from control studies run concurrently with HET-CAM studies also
2677 were provided (Vanparys and VanGoethem [2005b]; Spielmann and Liebsch [2005b]). In
2678 addition, replicate intralaboratory and interlaboratory HET-CAM test data were obtained
2679 (Vanparys and VanGoethem [2005a]). The efforts of Dr. P. Vanparys, Dr. F. Van Goethem,
2680 Dr. M. Liebsch, and Dr. med. H. Spielmann who provided additional data and/or information
2681 are gratefully acknowledged.

2682

2683 **2.0 ACCURACY OF THE HET-CAM TEST METHOD - REANALYSIS**

2684

2685 The ability of the HET-CAM test method to correctly identify ocular corrosives and severe
2686 irritants, as defined by the GHS, EPA, and EU classification systems was evaluated (EPA
2687 [1996]; EU [2001]; UN [2003])¹. The three regulatory ocular hazard classification systems
2688 considered during this analysis use different classification systems and decision criteria to
2689 identify ocular corrosives and severe irritants based on *in vivo* rabbit eye test results. All
2690 three classification systems are based on individual animal data in terms of the magnitude of
2691 the response and on the extent to which induced ocular lesions fail to reverse by day 21.
2692 However, there are differences among the three classifications systems with regard to the
2693 criteria used by NICEATM for distinguishing between a severe and a nonsevere response
2694 (See **Appendix A**). Thus, to evaluate the accuracy of the HET-CAM test method for
2695 identifying ocular corrosives and severe irritants, individual rabbit data collected at the
2696 different observation times was needed for each substance.

¹ For the purposes of this analysis, an ocular corrosive or severe irritant was defined as a substance that would be classified as Category 1 according to the GHS classification system (UN [2003]), as Category I according to the EPA classification system (EPA [1996]), or as R41 according to the EU classification system (EU [2001]).

2697 **Table IV-1. Summary of HET-CAM Database Changes**

2698

Data Source	Data Set	Analysis Method	Number of Available Substances	Number of Acceptable Substances by Ocular Irritancy Classification System			Comments
				EPA ¹	EU ²	GHS ³	
				Cat I/Total	R41/Total	Cat 1/Total	
Bagley et al. (1992)	New ⁴	IS(A) ⁵	32	0/2 ⁶	0/2	0/2	
	Old ⁴	IS(A)	32	0/3	0/3	0/3	
Balls et al. (1995)	New	Q-Score ⁵ S-Score ⁵	59	14/45 9/15	13/39 4/14	12/43 4/16	The decrease, where present, in the total number of usable substances is due to excluding substances from consideration due to insufficient rabbit eye test data for classification (See Appendix A). The increase, where present, in the number of corrosives and severe irritants is due to reclassification of substances.
	Old	Q-Score S-Score	59	10/40 2/12	14/48 4/19	15/45 4/17	
CEC (1991)	New	IS(B) ⁵		-	15/21	-	Data previously described in an Addendum to the draft HET-CAM BRD which was released to the public on November 16, 2004. The decrease, where present, in the total number of usable substances is due to excluding substances from consideration due to insufficient rabbit eye test data for classification (See Appendix A).
	Old	IS(B)		-	21/21	-	
Gettings et al. (1991)	New	IS(B)	9	3/9	3/8	3/9	The decrease, where present, in the total number of usable substances is due to excluding substances from consideration due to insufficient rabbit eye test data for classification (See Appendix A). The increase, where present, in the number of corrosives and severe irritants is due to reclassification of substances.
	Old	IS(B)	9	3/9	2/9	3/9	
Gettings et al. (1994)	New	IS(A) IS(B)	18	1/18 1/18	1/18 1/18	1/18 1/18	

Data Source	Data Set	Analysis Method	Number of Available Substances	Number of Acceptable Substances by Ocular Irritancy Classification System			Comments
				EPA ¹	EU ²	GHS ³	
				Cat I/Total	R41/Total	Cat 1/Total	
	Old	IS(A) IS(B)	18	1/18 1/18	1/18 1/18	1/18 1/18	
Gettings et al. (1996)	New	IS(A) IS(B)	25	3/25 9/25	3/23 8/23	3/23 8/23	The decrease, where present, in the total number of usable substances reflects the exclusion of substances from consideration due to insufficient rabbit eye test data for classification (See Appendix A). The increase, where present, in the number of corrosives and severe irritants is due to reclassification of substances.
	Old	IS(A) IS(B)	25	3/25 9/25	1/25 6/25	3/23 8/23	
Gilleron et al. (1996)	New	IS(B)		-	2/43	-	Data previously described in Section 9.0 of the draft HET-CAM BRD. Data were included in the reanalysis for the ability of the test method to accurately classify test substances according to the EU classification system.
	Old	IS(B)	0	-	-	-	
Gilleron et al. (1997)	New	IS(B)	60	16/53	16/48	19/54	Data previously described in Section 9.0 of the draft HET-CAM BRD. Data were included in the reanalysis for the ability of the test method to accurately classify test substances according to the GHS, EPA, and EU classification system.
	Old	IS(B)	0	-	-	-	
Hagino et al. (1999)	New	IS(A)	17	7/15	7/15	8/12	The decrease, where present, in the total number of usable substances reflects the exclusion of substances from consideration due to insufficient rabbit eye test data for classification (See Appendix A). The increase, where present, in the number of corrosives and severe irritants is due to reclassification of substances.
	Old	IS(A)	17	6/14	7/17	8/16	
Kojima et al. (1995)	New	IS(A)	24	2/5	2/4	2/5	The decrease, where present, in the total number of usable substances is due to excluding substances from consideration due to insufficient rabbit eye test data for
	Old	IS(A)	24	2/5	2/5	2/5	

Data Source	Data Set	Analysis Method	Number of Available Substances	Number of Acceptable Substances by Ocular Irritancy Classification System			Comments
				EPA ¹	EU ²	GHS ³	
				Cat I/Total	R41/Total	Cat 1/Total	
							classification (See Appendix A).
Spielmann et al. (1996)	New	mtc10 ⁵	142	-	25/142	-	
	New	mtc10	189	-	30/189	-	
	New	IS(B)-10 ⁵	120	11/73	14/71	19/77	Previous ocular irritancy calls only available for EU classification system. Additional <i>in vivo</i> and <i>in vitro</i> data received which allowed for an accuracy evaluation when compared to all three classification systems.
		IS(B)-100 ⁵	120	13/70	16/69	21/75	
Old	IS(B)-10	0	-	-	-		
	IS(B)-100	0	-	-	-		
Vinardell and Macián (1994)	New	IS(B)	13	0/2	0/2	0/2	
	Old	IS(B)	13	0/2	0/2	0/2	

2699 ¹EPA = U.S. Environmental Protection Agency (EPA [1996]).

2700 ²EU = European Union (EU [2001]).

2701 ³GHS = Globally Harmonized System (UN [2003]).

2702 ⁴New = accuracy statistics based on the revised analysis; Old = accuracy statistics based on the previous analysis included in the draft HET-CAM BRD.

2703 ⁵IS(A) = method described in Luepke (1985); IS(B), IS(B)-10, and IS(B)-100 = method described in Kalweit et al. (1987); Q = Q-Score, method described in Balls et al. (1995); S = S-Score, method described in Balls et al. (1995); mtc10 = mean time to coagulation after administration of a 10% solution, method described in Spielmann et al. (1996).

2706 ⁶First number (before forward slash) refers to the number of substances in each study that were classified as a severe irritant according to each classification system (EPA, EU, and GHS). The second number (after the forward slash) refers to the number of substances in were classified, based on animal data, for each classification system (EPA, EU, GHS).

2708

2709 The ability of the HET-CAM test method to correctly identify ocular corrosives and severe
2710 irritants, as defined by the GHS, EPA, and EU classification systems (EPA [1996]; EU
2711 [2001]; UN [2003]), was evaluated using two approaches. In the first approach, the accuracy
2712 of HET-CAM was assessed separately for each *in vitro-in vivo* comparative study (i.e.,
2713 publication) reviewed in Sections 4.0 and 5.0 and some studies reviewed in Section 9.0 of the
2714 draft HET-CAM BRD. For this accuracy analysis, the HET-CAM ocular irritancy potential
2715 of each substance in each report was determined. When the same substance was evaluated in
2716 multiple laboratories within the same study (e.g., Balls et al. [1995]), the HET-CAM ocular
2717 irritancy potential for each independent test result was determined. Subsequently, an overall
2718 HET-CAM ocular irritancy classification was assigned for each substance in the study based
2719 on the majority of ocular irritancy classification calls (e.g., if two laboratories classified a
2720 substance as a nonirritant and three laboratories classified a substance as a severe irritant; the
2721 overall *in vitro* irritancy classification for the substance would be severe irritant). When
2722 there was an even number of different irritancy classifications for substances (e.g., two
2723 laboratories classified a substance as a nonirritant and two laboratories classified a substance
2724 as a severe irritant), the more severe irritancy classification was used for the overall
2725 classification for the substance (severe irritant, in this case; see **Appendix IV-A**). Once the
2726 ocular irritancy potential classification was determined for each substance in each of the
2727 studies, the ability of the HET-CAM test method to identify ocular corrosives and severe
2728 irritants, as defined by the GHS (UN [2003]), EPA (1996), and EU (2001) classification
2729 systems.

2730

2731 In the second approach to evaluating the accuracy of HET-CAM, results from the different
2732 studies using the same HET-CAM analysis approach were combined. As noted in the draft
2733 HET-CAM BRD There is no standardized data collection method for HET-CAM studies and
2734 several different data collection methods have been developed (i.e., IS, Q-Score, S-Score).
2735 Since conversion of the values obtained by one data collection method to another method
2736 (i.e., conversion of Q-Score to IS) was not possible, the accuracy assessments conducted in
2737 this section were evaluated according to each of the data collection methods described. Once
2738 the ocular irritancy classification was determined for each substance, the ability of the HET-
2739 CAM test method to identify ocular corrosives and severe irritants, as defined by the GHS
2740 (UN [2003]), EPA (1996), and EU (2001) classification systems, was determined for each
2741 analysis method (**Appendix IV-A**). Since the test methods protocols used in different studies
2742 to generate HET-CAM test results are not identical, care should be used when interpreting
2743 the results of these analyses.

2744 Based on the revisions made to the HET-CAM test method database, a revised accuracy
2745 analysis has been conducted. The calculations were performed as described previously in
2746 Section 6.0 of the draft HET-CAM BRD. To allow for a comparison of the results obtained
2747 in the revised analysis relative to those obtained previously, the data tables include accuracy
2748 statistics from both analyses. However, the discussion of the results in the sections that
2749 follow relate to the revised analysis only.

2750

2751 **2.1 GHS Ocular Hazard Classification System**

2752

2753 Ten studies (Gettings et al. [1991, 1994, 1996]; Bagley et al. [1992]; Vinardell and Macián
2754 [1994]; Balls et al. [1995]; Kojima et al. [1995]; Spielmann et al. [1996]; Gilleron et al.

2755 [1997]; Hagino et al. [1999]) contained HET-CAM test data on 376 substances, 260 of which
2756 had sufficient *in vivo* data to be assigned an ocular irritancy classification as defined by the
2757 GHS classification system (UN [2003])². Based on results from *in vivo* rabbit eye
2758 experiments, 92³ of the 260 substances were classified as severe irritants (i.e., Category 1)
2759 and 119 substances were classified as nonsevere irritants (either Category 2A, 2B) or
2760 nonirritants. The remaining 49 substances that could not be classified according to the GHS
2761 classification system due to the lack of adequate animal data are noted in **Appendix IV-A**.
2762

2763 For one set of data (Spielmann et al. [1996]) a large number of substances were available to
2764 compare the accuracy of the test method when substances were evaluated at a 10% and 100%
2765 concentration *in vitro* and 100% *in vivo*. Therefore, a comparison of the accuracy statistics
2766 of these two *in vitro* concentrations was possible. To include the additional HET-CAM test
2767 data, which were tested at 10% and 100% concentrations, appropriate data were combined
2768 with each of the Spielmann et al. (1996) data sets. These combined data sets were used to
2769 evaluate the overall accuracy of the IS(B) test method, when using a 10% (IS(B)-10) and
2770 100% (IS(B)-100) concentration *in vitro*, in predicting the effect produced *in vivo* at 100%
2771 concentration. As a corollary to this evaluation, the accuracy of the IS(A) method, when
2772 substances were tested at 10% or 100% concentration *in vitro*, in predicting the effect
2773 produced *in vivo* at 100% concentration also was evaluated.
2774

2775 Based on the data provided in the ten reports and when results across multiply tested
2776 substances were combined to generate a single consensus call per test substance, the HET-
2777 CAM test method has an accuracy in predicting substances classified as corrosives or severe
2778 irritants, according to the GHS classification system (UN [2003]), of 41% to 83%, a
2779 sensitivity of 20% to 100%, a specificity of 33% to 100%, a false positive rate of 0% to 67%,
2780 and a false negative rate of 0% to 80%. The performance characteristics for each report are
2781 provided in **Table IV-2**.
2782

2783 The overall performance statistics, arranged by HET-CAM data analysis method, are
2784 provided in **Table IV-3**. Based on the combined test result approach, the HET-CAM test
2785 method has an accuracy in predicting substances classified as corrosives or severe irritants,
2786 according to the GHS classification system (UN [2003]), of 44% to 85%, a sensitivity of
2787 25% to 100%, a specificity of 39% to 100%, a false positive rate of 0% to 61%, and a false
2788 negative rate of 0% to 75%.

² For the purpose of this accuracy analysis, *in vivo* rabbit study results were used to identify GHS Category 1 irritants (i.e., severe irritants); substances classified as GHS Category 2A and 2B irritants were identified as nonsevere irritants.

³ Two chemicals (benzalkonium chloride and sodium lauryl sulfate) were tested *in vivo* twice. The results from these studies were discordant with respect to GHS classification. According to one test, the classification was Category 1, while results from the other test yielded a Category 2B for both chemicals. The accuracy analysis was performed with the substances classified as Category 1.

2789 **Table IV-2. Evaluation of the Performance of the HET-CAM Test Method in Predicting Ocular Corrosives and Severe**
 2790 **Irritants Compared to the *In Vivo* Rabbit Eye Test Method, as Defined by the GHS¹ Classification System, by**
 2791 **Study**
 2792

Data Source	Data Set	Anal. ²	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
				%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Gettings et al. (1991)	New ⁵	IS(B)	9/10	78	7/9	100	3/3	67	4/6	60	3/5	100	4/4	33	2/6	0	0/3
	Old ⁵	IS(B)	9/10	78	7/9	100	3/3	67	4/6	60	3/5	100	4/4	33	2/6	0	0/3
Gettings et al. (1994)	New	IS(A)	18/18	83	15/18	25	1/4	100	14/14	100	1/1	82	14/17	0	0/14	75	3/4
	Old	IS(A)	18/18	83	15/18	100	1/1	82	14/17	25	1/4	100	14/14	18	3/17	0	0/1
Gettings et al. (1994)	New	IS(B)	18/18	78	14/18	20	1/5	100	13/13	100	1/1	76	13/17	0	0/13	80	4/5
	Old	IS(B)	18/18	78	14/18	100	1/1	76	13/17	20	1/5	100	13/13	24	4/17	0	0/1
Gettings et al. (1996)	New	IS(A)	24/25	50	12/24	25	4/12	100	8/8	100	4/4	40	8/12	0	0/8	75	12/16
	Old	IS(A)	23/25	78	18/23	38	3/8	100	15/15	100	3/3	75	15/20	0	0/15	63	5/8
Gettings et al. (1996)	New	IS(B)	24/25	71	17/24	56	9/16	100	8/8	100	9/9	53	8/15	0	0/8	44	7/16
	Old	IS(B)	23/25	100	23/23	100	8/8	93	14/15	89	8/9	100	14/14	7	1/15	0	0/8
Bagley et al. (1992)	New	IS(A)	2/32	0	0/2	-	-	0	0/2	0	0/2	-	-	100	2/2	-	-
	Old	IS(A)	2/32	0	0/2	-	-	0	0/2	0	0/2	-	-	100	2/2	-	-
Vinardell and Macián (1994)	New	IS(B)	2/13	50	1/2	0	0/1	100	1/1	0	0/1	-	-	0	0/1	100	1/1
	Old	IS(B)	2/13	50	1/2	-	-	50	1/2	0	0/1	100	1/1	50	1/2	-	-
Balls et al. (1995)	New	Q	43/59	63	27/43	100	12/12	43	12/28	48	15/31	100	12/12	57	16/28	0	0/12
	Old	Q	45/59	62	28/45	100	15/15	43	13/30	47	15/32	100	13/13	57	17/30	0	0/15
Balls et al. (1995)	New	S	16/59	44	7/16	36	4/11	60	3/5	67	4/6	30	3/10	40	2/5	64	7/11
	Old	S	17/59	47	8/17	36	4/11	67	4/6	67	4/6	36	4/11	33	2/6	64	7/11
Kojima et al. (1995)	New	IS(A)	5/24	60	3/5	100	2/2	33	1/3	50	2/4	100	1/1	67	2/3	0	0/2
	Old	IS(A)	5/24	80	4/5	67	2/3	100	2/2	100	2/2	67	2/3	0	0/2	33	1/3
Spielmann et al. (1996)	New	IS(B)-10	77/120	68	52/77	79	19/24	62	33/53	49	19/39	87	33/38	38	20/53	21	5/24
	New	IS(B)-100	75/120	55	41/75	88	21/24	39	20/51	40	21/52	87	20/23	61	31/51	13	3/24
Gilleron et al. (1997)	New	IS(B)	54/60	41	22/54	40	19/48	50	3/6	86	19/22	9	3/32	50	3/6	60	29/48
Hagino et al. (1999)	New	IS(A)	15/17	80	12/15	73	8/11	100	4/4	100	8/8	57	4/7	0	0/4	27	3/11
	Old	IS(A)	16/17	75	12/16	100	8/8	50	4/8	67	8/12	100	4/4	50	4/8	0	0/8

2793 ¹GHS = Globally Harmonized System (UN [2003]).

2794 ²Anal. = data collection/analysis method used to transform the sample data into HET-CAM scores. IS(A) = method described in Luepke (1985); IS(B), IS(B)-
2795 10, and IS(B)-100 = method described in Kalweit et al. (1987); Q = Q-Score, method described in Balls et al. (1995); S = S-Score, method described in Balls et
2796 al. (1995).
2797 ³N = number of substances included in this analysis/the total number of substances in the study.
2798 ⁴Data used to calculate the percentage.
2799 ⁵New = accuracy statistics based on the revised analysis; Old = accuracy statistics based on the previous analysis included in the draft HET-CAM BRD.
2800

2800 **Table IV-3. Evaluation of the Performance of the HET-CAM Test Method in Predicting Ocular Corrosives and Severe**
 2801 **Irritants Compared to the *In Vivo* Rabbit Eye Test Method, as Defined by the GHS¹ Classification System, by**
 2802 **HET-CAM Analysis Method**
 2803

Analysis Method ²	Data Set	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
			%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
IS(A)-100 ⁵	New ⁶	20	85	17/20	100	2/2	83	15/18	40	2/5	100	15/15	17	3/18	0	0/2
IS(A)-10 ⁵	New	24	50	12/24	25	4/12	100	8/8	100	4/4	40	8/20	0	0/8	75	12/16
IS(A)	New	64	66	42/64	52	14/29	77	27/35	65	15/23	66	27/41	23	8/35	48	15/29
IS(A)	Old ⁶	61	75	46/61	67	12/18	79	34/43	57	12/21	85	34/40	21	9/43	33	6/18
IS(B)-100 ⁵ (Entire database)	New	143	53	76/143	85	35/41	40	41/102	36	35/96	87	41/47	60	61/102	15	6/41
IS(B)-100 ⁵ (Spielmann et al. 1996)	New	75	55	41/75	88	21/24	39	20/51	40	21/31	87	20/23	61	31/51	13	3/24
IS(B)-10 ⁵ (Entire database)	New	101	68	69/101	70	28/40	67	41/61	58	28/48	77	41/53	33	20/61	30	12/40
IS(B)-10 ⁴ (Spielmann et al. 1996)	New	77	68	52/77	79	19/24	62	33/53	49	19/39	87	33/38	38	20/53	21	5/24
IS(B)	New	107	57	61/107	76	32/42	45	29/65	47	32/68	74	29/39	55	36/65	24	10/42
IS(B)	Old	52	85	44/52	100	12/12	80	32/40	60	12/20	100	32/32	20	8/40	0	0/12
Q-Score	New	43	63	27/43	100	12/12	43	12/28	48	15/31	100	12/12	57	16/28	0	0/12
	Old	45	63	28/45	100	15/15	43	13/30	47	15/32	100	13/13	57	17/30	0	0/15
S-Score	New	16	44	7/16	36	4/11	60	3/5	67	4/6	30	3/10	40	2/5	64	7/11
	Old	17	47	8/17	36	4/11	67	4/6	67	4/6	36	4/11	33	2/6	64	7/11

2804 ¹GHS = Globally Harmonized System (UN [2003]).

2805 ²IS(A), IS(A)-10, IS(A)-100 = method described in Luepke (1985); IS(B), IS(B)-10, IS(B)-100 = method described in Kalweit et al. (1987); Q = Q-Score,
 2806 method described in Balls et al. (1995); S = S-Score, method described in Balls et al. (1995).

2807 ³N = number of substances evaluated in each study.

2808 ⁴Data used to calculate the percentage.

2809 ⁵The analysis compares the ability of the specified concentration tested *in vitro* (IS(A)-10 represents the 10% concentration tested *in vitro*) to predict the effect
2810 produced by the undiluted test substance tested *in vivo*.

2811 ⁶New = accuracy statistics based on the revised analysis; Old = accuracy statistics based on the previous analysis included in the draft HET-CAM BRD.

2812 The IS(A)-100 analysis method (substances were tested *in vitro* at a concentration of 100%
2813 and compared to substances tested *in vivo* at 100%) had the highest accuracy for predicting
2814 ocular corrosives and severe irritants (85%; 17/20). It is noted that for the IS(A)-100 analysis
2815 method evaluation represents 20 substances that are mostly formulations. Comparatively, the
2816 IS(B) approach (which has a larger database and contains many individual chemicals) had
2817 the highest accuracy when 10% concentration tested *in vitro* was compared to 100%
2818 concentration tested *in vivo*. The false positive and false negative rates for this analysis
2819 method were 33% (20/41) and 30% (12/40), respectively.

2820

2821 2.2 EPA Ocular Hazard Classification System

2822

2823 Ten studies (Gettings et al. [1991, 1994, 1996]; Bagley et al. [1992]; Vinardell and Macián
2824 [1994]; Balls et al. [1995]; Kojima et al. [1995]; Spielmann et al. [1996]; Gilleron et al.
2825 [1997]; Hagino et al. [1999]) contained HET-CAM test data on 376 substances, 256 of which
2826 had sufficient *in vivo* data to be assigned an ocular irritancy classification as defined by the
2827 EPA classification system (EPA [1996])⁴. Based on results from the *in vivo* rabbit eye test,
2828 76⁵ of these 256 substances were classified as severe irritants (i.e., Category I), while the
2829 other 127 substances were classified as nonsevere irritants or nonirritants (Categories II, III,
2830 or IV). The remaining 127 substances that could not be classified according to the EPA
2831 classification system are so noted in **Appendix IV-A**.

2832

2833 As described in the previous section (see **Section IV-2.1**), a large number of substances were
2834 available to compare the accuracy of the test method when substances were evaluated at a
2835 10% and 100% concentration *in vitro* and 100% *in vivo*. As conducted previously,
2836 appropriate data, which were tested at 10% and 100% concentration, were combined with
2837 each of the Spielmann et al. (1996) data sets. These combined data sets were used to
2838 evaluate the overall accuracy of the IS(B) test method, when using a 10% (IS(B)-10) and
2839 100% (IS(B)-100) concentration *in vitro*, in predicting the effect produced *in vivo* at 100%
2840 concentration. As a corollary to this evaluation, the accuracy of the IS(A) method, when
2841 substances were tested at 10% or 100% concentration *in vitro*, in predicting the effect
2842 produced *in vivo* at 100% concentration was evaluated.

2843

2844 Based on the data provided in the ten reports and when results across multiply tested
2845 substances were combined to generate a single consensus call per test substance, the HET-
2846 CAM test method has an accuracy in predicting substances classified as corrosives or severe
2847 irritants, according to the EPA classification system (EPA [1996]), of 57% to 83%, a
2848 sensitivity of 24% to 100%, a specificity of 39% to 100%, a false positive rate of 0% to 61%,
2849 and a false negative rate of 0% to 80%. The performance characteristics for each report are
2850 provided in **Table IV-4**.

2851

⁴ For the purpose of this accuracy analysis, *in vivo* rabbit study results were used to identify GHS Category I irritants (i.e., severe irritants); substances classified as EPA Category II, III, and IV were identified as nonsevere irritants.

⁵ One chemical (sodium lauryl sulfate) was tested *in vivo* twice. The results from these studies were discordant with respect to EPA classification. According to one test, the classification was Category I, while results from the other test yielded a Category II. The accuracy analysis was performed with the substances classified as Category I.

2851 The overall performance statistics, arranged by HET-CAM data analysis method, are
2852 provided in **Table IV-5**. Based on the combined test result approach, the HET-CAM test
2853 method has an accuracy in predicting substances classified as corrosives or severe irritants,
2854 according to the EPA classification system (EPA [1996]), of 51% to 85%, a sensitivity of
2855 24% to 100%, a specificity of 39% to 100%, a false positive rate of 0% to 61%, and a false
2856 negative rate of 0% to 76%.

2857

2858 The IS(A)-100 analysis approach, when substances were tested *in vitro* at a concentration of
2859 100% and compared to substances tested *in vivo* at 100%, had the highest accuracy for
2860 predicting ocular corrosives and severe irritants (85%; 17/20), as classified by the EPA (EPA
2861 [1996]). It is noted that the database used for the IS(A)-100 analysis method evaluation
2862 represents 20 substances that are mostly formulations. Comparatively, the IS(B) approach
2863 (which has a larger database and contains many individual chemicals) had the highest
2864 accuracy when 10% concentration tested *in vitro* was compared to 100% concentration tested
2865 *in vivo*. The false positive and false negative rates for this analysis method were 36% (24/67)
2866 and 32% (10/31), respectively.

2867

2868 **2.3 EU Ocular Hazard Classification System**

2869

2870 Twelve studies (CEC [1991]; Gettings et al. [1991, 1994, 1996]; Bagley et al. [1992];
2871 Vinardell and Macián [1994]; Balls et al. [1995]; Kojima et al. [1995]; Spielmann et al.
2872 [1996]; Gilleron et al. [1996, 1997]; Hagino et al. [1999]) contained HET-CAM test data on
2873 381 substances, 312⁶ of which had sufficient *in vivo* data to be assigned an ocular irritancy
2874 classification as defined by the EU classification system (EU [2001])⁷. Based on results from
2875 the *in vivo* rabbit eye test, 85 of these 312 substances were classified as severe irritants (i.e.,
2876 R41), while the other 156 substances were classified as nonsevere irritants (i.e., R36) or
2877 nonirritants. The remaining 71 substances that could not be classified according to the EU
2878 classification system are so noted in **Appendix IV-A**.

2879

2880 As described in **Section IV-2.1** of this addendum, a large number of substances were
2881 available to compare the accuracy of the test method when substances were evaluated at a
2882 10% and 100% concentration *in vitro* and 100% *in vivo*. As conducted previously,
2883 appropriate data, which were tested at 10% and 100% concentrations, were combined with
2884 each of the Spielmann et al. (1996) data sets. These combined data sets were used to
2885 evaluate the overall accuracy of the IS(B) test method, when using a 10% (IS(B)-10) and
2886 100% (IS(B)-100) concentration *in vitro*, in predicting the effect produced *in vivo* at 100%
2887 concentration. As a corollary to this evaluation, the accuracy of the IS(A) method, when

⁶ Two chemicals (benzalkonium chloride and sodium lauryl sulfate) were tested *in vivo* twice. The results from these studies were discordant with respect to EU classification. According to one test, the classification was R41, while results from the other test yielded a nonsevere (R36 or nonirritant) for both chemicals. The accuracy analysis was performed with the substances classified as R41.

⁷ For the purpose of this accuracy analysis, *in vivo* rabbit study results were used to identify EU R41 irritants (i.e., severe irritants); substances classified R36 and nonirritants were identified as nonsevere irritants.

2888 **Table IV-4. Evaluation of the Performance of the HET-CAM Test Method in Predicting Ocular Corrosives and Severe**
 2889 **Irritants Compared to the *In Vivo* Rabbit Eye Test Method, as Defined by the EPA¹ Classification System, by**
 2890 **Study**
 2891

Data Source	Data Set	Anal. ²	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
				%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Gettings et al. (1991)	New ⁵	IS(B)	9/10	78	7/9	100	3/3	67	4/6	60	3/5	100	4/4	33	2/6	0	0/3
	Old ⁵	IS(B)	9/10	78	7/9	100	3/3	67	4/6	60	3/5	100	4/4	33	2/6	0	0/3
Gettings et al. (1994)	New	IS(A)	18/18	83	15/18	25	1/4	100	14/14	100	1/1	82	14/17	0	0/14	75	3/4
	Old	IS(A)	18/18	83	15/18	100	1/1	82	14/17	25	1/4	100	14/14	18	3/17	0	0/1
Gettings et al. (1994)	New	IS(B)	18/18	78	14/18	20	1/5	100	13/13	100	1/1	76	13/17	0	0/13	80	4/5
	Old	IS(B)	18/18	78	14/18	100	1/1	76	13/17	20	1/5	100	13/13	24	4/17	0	0/1
Gettings et al. (1996)	New	IS(A)	25/25	48	12/25	24	4/17	100	8/8	100	4/4	38	8/21	0	0/8	76	13/17
	Old	IS(A)	25/25	68	17/25	30	3/10	93	14/15	75	3/4	67	14/21	7	1/15	70	7/10
Gettings et al. (1996)	New	IS(B)	25/25	72	18/25	59	10/17	100	8/8	100	10/10	53	8/15	0	0/8	41	7/17
	Old	IS(B)	25/25	92	23/25	90	9/10	93	14/15	90	9/10	93	14/15	7	1/15	10	1/10
Bagley et al. (1992)	New	IS(A)	2/32	0	0/2	-	-	0	0/2	0	0/2	-	-	100	2/2	-	-
	Old	IS(A)	3/32	0	0/3	-	-	0	0/3	0	0/3	-	-	100	3/3	-	-
Vinardell and Macián (1994)	New	IS(B)	2/13	50	1/2	0	-	100	1/1	-	-	50	1/2	0	0/2	100	1/1
	Old	IS(B)	2/13	50	1/2	-	-	50	1/2	0	0/1	100	1/1	50	1/2	-	-
Balls et al. (1995)	New	Q	44/59	61	27/44	100	14/14	43	13/30	45	14/17	100	13/13	57	17/30	0	0/14
	Old	Q	40/59	58	23/40	100	10/10	43	13/30	37	10/27	100	13/13	57	17/30	0	0/14
Balls et al. (1995)	New	S	14/59	57	8/14	50	4/8	67	4/6	67	4/6	50	4/8	33	2/6	50	4/8
	Old	S	12/59	50	6/12	33	2/6	67	4/6	50	2/4	50	4/8	33	2/6	67	4/6
Kojima et al. (1995)	New	IS(A)	5/24	80	4/5	100	2/2	67	2/3	67	2/3	100	2/2	33	1/3	0	0/2
	Old	IS(A)	5/24	80	4/5	67	2/3	100	2/2	100	2/2	67	2/3	0	0/2	33	1/3
Spielmann et al. (1996)	New	IS(B)-10	73/120	63	46/73	79	11/14	59	35/59	31	11/35	92	35/38	41	24/59	21	3/14
	New	IS(B)-100	70/120	50	35/70	93	13/14	39	22/56	28	13/34	96	22/23	61	34/56	7	1/14
Gilleron et al. (1997)	New	IS(B)	53/60	38	20/53	35	16/46	57	4/7	84	16/19	12	4/34	43	3/7	65	30/46
Hagino et al.	New	IS(A)	15/17	73	11/15	64	7/11	100	4/4	100	7/7	50	4/8	0	0/4	36	4/7

Data Source	Data Set	Anal. ²	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
				%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
(1999)	Old	IS(A)	14/17	71	10/14	100	6/6	50	4/8	60	6/10	100	4/4	50	4/8	0	0/6

2892

¹EPA = U.S. Environmental Protection Agency (EPA [1996]).

2893

²Anal. = data collection/analysis method used to transform the sample data into HET-CAM scores. IS(A) = method described in Luepke (1985); IS(B), IS(B)-10, and IS(B)-100 = method described in Kalweit et al. (1987); Q = Q-Score, method described in Balls et al. (1995); S = S-Score, method described in Balls et al.

2894

(1995).

2895

2896

³N = number of substances included in this analysis/the total number of substances in the study.

2897

⁴Data used to calculate the percentage.

2898

⁵New = accuracy statistics based on the revised analysis; Old = accuracy statistics based on the previous analysis included in the draft HET-CAM BRD.

2899

2899 **Table IV-5 Evaluation of the Performance of the HET-CAM Test Method in Predicting Ocular Corrosives and Severe**
 2900 **Irritants Compared to the *In Vivo* Rabbit Eye Test Method, as Defined by the EPA¹ Classification System, by**
 2901 **HET-CAM Analysis Method**
 2902

Analysis Method ²	Data Set	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
			%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
IS(A)-100 ⁵	New ⁶	20	85	17/20	100	2/2	83	15/18	40	2/5	100	15/15	17	3/18	0	0/2
IS(A)-10 ⁵	New	25	48	12/25	24	4/17	100	8/8	100	4/4	38	8/13	0	0/8	76	13/17
IS(A)	New	65	65	42/65	50	14/28	76	28/37	61	14/23	67	28/42	24	9/37	50	14/28
	Old ⁶	61	70	43/61	56	10/18	77	33/43	50	10/20	80	33/41	23	10/43	44	8/18
IS(B)-100 ⁵ (Entire database)	New	138	51	70/138	87	26/30	41	44/108	29	26/90	92	44/48	59	64/108	13	4/30
IS(B)-100 ⁵ (Spielmann et al. 1996)	New	70	50	35/70	93	13/14	39	22/56	28	13/47	96	22/23	61	34/56	7	1/14
IS(B)-10 ⁵ (Entire database)	New	98	65	64/98	68	21/31	64	43/67	47	21/45	81	43/53	36	24/67	32	10/31
IS(B)-10 ⁵ (Spielmann et al. 1996)	New	73	63	46/73	79	11/14	59	35/59	31	11/35	92	35/38	41	24/59	21	3/14
IS(B)	New	107	56	60/107	75	30/40	45	30/67	45	30/67	75	30/40	55	30/67	25	10/40
IS(B)	Old	54	83	45/54	93	13/14	80	32/40	62	13/21	97	32/33	20	8/40	7	1/14
Q-Score	New	44	61	27/44	100	14/14	43	13/30	45	14/17	100	13/13	57	17/30	0	0/14
	Old	40	58	23/40	100	10/10	43	13/30	37	10/27	10	13/13	57	17/30	0	0/10
S-Score	New	14	57	8/14	50	4/8	67	4/6	67	4/6	50	4/8	33	2/6	50	4/8
	Old	12	50	6/12	33	2/6	67	4/6	50	2/4	50	4/8	33	2/6	67	4/6

2903 ¹EPA=U.S. Environmental Protection Agency (EPA [1996])

2904 ²IS(A), IS(A)-10, IS(A)-100 = Method described in Luepke (1985); IS(B), IS(B)-10, IS(B)-100 = Method described in Kalweit et al. (1987); Q = Q-Score,
 2905 Method described in Balls et al. (1995); S = S-Score, Method described in Balls et al. (1995).

2906 ³N = Number of substances evaluated in each study.

2907 ⁴Data used to calculate the percentage.

2908 ⁵The analysis compares the ability of the specified concentration tested *in vitro* (IS(A)-10 represents the 10% concentration tested *in vitro*) to predict the effect
 2909 produced by the undiluted test substance tested *in vivo*.

2910 ⁶New = accuracy statistics based on the revised analysis; Old = accuracy statistics based on the previous analysis included in the draft HET-CAM BRD.

2911 substances were tested at 10% or 100% concentration *in vitro*, in predicting the effect
2912 produced *in vivo* at 100% concentration was evaluated.

2913
2914 Based on the data provided in the twelve reports and when results across multiply tested
2915 substances were combined to generate a single consensus call per test substance, the HET-
2916 CAM test method has an accuracy in predicting substances classified as corrosives or severe
2917 irritants, according to the EU classification system (EU [2001]), of 40% to 96%, a sensitivity
2918 of 20% to 100%, a specificity of 38% to 100%, a false positive rate of 0% to 62%, and a false
2919 negative rate of 0% to 90%. The performance characteristics for each report are provided in
2920 **Table IV-6**.

2921

2922 The overall performance statistics, arranged by HET-CAM data analysis method, are
2923 provided in **Table IV-7**. Based on the combined test result approach, the HET-CAM test
2924 method has an accuracy in predicting substances classified as corrosives or severe irritants,
2925 according to the EU classification system (EU [2001]), of 50% to 83%, a sensitivity of 25%
2926 to 100%, a specificity of 38% to 100%, a false positive rate of 0% to 62%, and a false
2927 negative rate of 0% to 80%.

2928

2929 The IS(A)-100 analysis approach, when substances were tested *in vitro* at a concentration of
2930 100% and compared to substances tested *in vivo* at 100%, had the highest accuracy for
2931 predicting ocular corrosives and severe irritants (85%; 17/20), as classified by the EU (EU
2932 [2001]). It is noted that the database used for the IS(A)-100 analysis method evaluation
2933 represents 20 substances that are mostly formulations. Comparatively, the IS(B) approach
2934 (which has a larger database and contains many individual chemicals) had the highest
2935 accuracy when 10% concentration tested *in vitro* was compared to 100% concentration tested
2936 *in vivo*. The false positive and false negative rates for this analysis method were 34% (21/61)
2937 and 30% (10/53), respectively.

2938

2939 In addition to the accuracy evaluations conducted as previously described in Section 6.0 of
2940 the draft HET-CAM BRD, accuracy analyses conducted using a different HET-CAM
2941 endpoint are included in **Table IV-6** and **IV-7**⁸. In the study by Spielmann et al. (1996),
2942 discriminant analyses were used to select HET-CAM endpoints with the highest power and
2943 to develop models for the prediction of severe irritants as classified by the EU classification
2944 system (EU [1996]). In this evaluation, it was shown that the mean detection time for the
2945 appearance of coagulation on the chorioallantoic membrane (CAM) obtained with a 10%
2946 solution of the test substance (termed mtc10) was the endpoint with the greatest power in
2947 distinguishing severe irritants from nonsevere test substances.

⁸ Data described in these rows were taken directly from Spielmann et al. (1996); no additional analyses of these studies were conducted.

2948 **Table IV-6. Evaluation of the Performance of the HET-CAM Test Method In Predicting Ocular Corrosives and Severe**
 2949 **Irritants Compared to the *In Vivo* Rabbit Eye Test Method, as Defined by the EU¹ Classification System, by**
 2950 **Study**
 2951

Data Source	Data Set	Anal. ²	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
				%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
CEC (1991)	New ⁵	IS(B)	26/32	62	16/26	86	6/7	53	10/19	40	6/15	91	10/11	47	9/19	14	1/7
	Old ⁵	IS(B)	32/32	65	21/32	91	10/11	52	11/21	50	10/20	92	11/12	48	10/21	9	1/11
Gettings et al. (1991)	New	IS(B)	8/10	88	7/8	100	3/3	80	4/5	75	3/4	100	4/4	20	1/5	0	0/3
	Old	IS(B)	9/10	67	6/9	100	2/2	57	4/7	40	2/5	100	4/4	43	3/7	0	0/2
Gettings et al. (1994)	New	IS(A)	18/18	83	15/18	25	1/4	100	14/14	100	1/1	82	14/17	0	0/14	75	3/4
	Old	IS(A)	18/18	83	15/18	100	1/1	82	14/17	25	1/4	100	14/14	18	3/17	0	0/1
Gettings et al. (1994)	New	IS(B)	18/18	78	14/18	20	1/5	100	13/13	100	1/1	76	13/17	0	0/13	80	4/5
	Old	IS(B)	18/18	78	14/18	100	1/1	76	13/17	20	1/5	100	13/13	24	4/17	0	0/1
Gettings et al. (1996)	New	IS(A)	24/25	50	12/24	25	4/16	100	8/8	100	4/4	40	8/20	0	0/8	75	12/16
	Old	IS(A)	25/25	68	17/25	17	1/6	84	16/19	25	1/4	76	16/21	16	3/19	83	5/6
Gettings et al. (1996)	New	IS(B)	24/25	71	17/24	56	9/16	100	8/8	100	9/9	53	8/15	0	0/8	44	7/16
	Old	IS(B)	25/25	84	21/25	100	6/6	79	15/19	60	6/10	100	15/15	21	4/19	0	0/6
Bagley et al. (1992)	New	IS(A)	2/32	0	0/2	-	-	0	0/2	0	0/2	-	-	100	2/2	-	-
	Old	IS(A)	3/32	0	0/3	-	-	0	0/3	0	0/3	-	-	100	3/3	-	-
Vinardell and Macián (1994)	New	IS(B)	2/13	50	1/2	0	0/1	100	1/1	-	-	50	1/2	0	0/1	100	1/1
	Old	IS(B)	2/13	50	1/2	-	-	50	1/2	0	0/2	100	1/1	50	1/2	-	-
Balls et al. (1995)	New	Q	39/49	64	25/39	100	13/13	46	12/26	48	13/27	100	12/12	54	14/26	0	0/13
	Old	Q	48/59	58	28/48	100	14/14	41	14/34	41	14/34	100	14/14	59	20/34	0	0/14
Balls et al. (1995)	New	S	14/59	50	7/14	44	4/5	60	3/5	67	4/6	38	3/8	40	2/5	56	5/9
	Old	S	19/59	47	9/19	36	4/11	63	5/8	57	4/7	42	7/11	38	3/8	64	7/11
Kojima et al. (1995)	New	IS(A)	4/24	75	3/4	100	2/2	50	1/2	67	1/3	100	1/1	50	1/2	0	0/2
	Old	IS(A)	5/24	80	4/5	67	2/3	100	2/2	100	2/2	67	2/3	0	0/2	33	1/3
Spielmann et al. (1996)	New	IS(B)-10	71/120	66	47/71	82	14/17	61	33/54	40	14/35	92	33/36	39	21/54	18	3/17
	New	IS(B)-100	69/120	52	32/69	94	16/17	38	20/52	33	16/48	95	20/21	62	32/52	6	1/17
	New ⁶	mtc10	142	76	108/142	52	25/48	88	83/94	70	25/36	78	83/106	12	11/94	48	23/48
	New ⁶	mtc10	189	77	145/189	53	30/57	87	115/132	64	30/47	81	115/142	13	17/132	47	27/57

Data Source	Data Set	Anal. ²	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
				%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Gilleron et al. (1996)	New	IS(B)	43/46	57	26/43	10	2/21	96	24/25	67	2/3	56	24/43	4	1/25	90	19/21
Gilleron et al. (1997)	New	IS(B)	48/60	40	19/48	37	16/43	60	3/5	89	16/18	10	3/30	40	2/5	63	27/43
Hagino et al. (1999)	New	IS(A)	15/17	73	11/15	64	7/11	100	4/4	100	7/7	50	4/8	0	0/4	36	4/11
	Old	IS(A)	17/17	65	11/17	100	7/7	40	4/10	54	7/13	100	4/4	60	6/10	0	0/7

2952 ¹EU = European Union (EU [2001]).

2953 ²Anal. = data collection/analysis method used to transform the sample data into HET-CAM scores. IS(A) = method described in Luepke (1985); IS(B), IS(B)-10,
2954 and IS(B)-100 = method described in Kalweit et al. (1987); Q = Q-Score, method described in Balls et al. (1995); S = S-Score, method described in Balls et al.
2955 (1995).

2956 ³N = number of substances included in this analysis/the total number of substances in the study.

2957 ⁴Data used to calculate the percentage.

2958 ⁵New = accuracy statistics based on the revised analysis; Old = accuracy statistics based on the previous analysis included in the draft HET-CAM BRD.

2959 ⁶Results were calculated based on the results presented in Spielmann et al. (1996)(pages 765 and 767). Classification of *in vivo* results is described in Spielmann
2960 et al. (1996).

2961 **Table IV-7. Evaluation of the Performance of the HET-CAM Test Method In Predicting Ocular Corrosives and Severe**
 2962 **Irritants Compared to the *In Vivo* Rabbit Eye Test Method, as Defined by the EU¹ Classification System, by**
 2963 **HET-CAM Analysis Method**
 2964

Analysis Method ²	Data Set	N ³	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
			%	No. ⁴	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
IS(A)-100 ⁵	New ⁶	20	85	17/20	100	2/2	83	15/18	40	2/5	100	15/15	17	3/18	0	0/2
IS(A)-10 ⁵	New	24	50	12/24	25	4/16	100	8/8	100	4/4	40	8/16	0	0/8	75	12/16
IS(A)	New	62	66	41/62	54	14/26	75	27/36	61	14/23	69	27/39	25	9/36	46	14/26
	Old ⁶	64	69	44/64	60	9/15	71	35/49	39	9/23	85	35/41	29	14/49	40	6/15
IS(B)-100 ⁵ (Entire database)	New	178	54	96/178	89	81/35	45	65/143	28	31/109	94	65/69	55	78/143	11	4/35
IS(B)-100 ⁵ (Spielmann et al. 1996)	New	69	52	36/69	94	16/17	38	20/52	33	16/48	95	20/21	62	32/52	6	1/17
IS(B)-10 ⁵ (Entire database)	New	95	67	64/95	70	23/33	66	41/62	52	23/44	80	41/51	34	21/61	30	10/53
IS(B)-10 ⁵ (Spielmann et al. 1996)	New	71	66	47/71	82	14/17	61	33/54	40	17/35	92	33/36	39	21/54	18	3/17
IS(B)	New	173	58	101/173	77	37/48	51	64/125	38	37/98	85	64/75	49	61/125	23	11/48
IS(B)	Old	86	73	63/86	95	19/20	67	44/66	44	19/43	98	44/45	33	22/66	5	1/20
Q-Score	New	39	64	25/39	100	13/13	46	12/26	48	13/27	100	12/12	54	14/26	0	0/13
	Old	48	58	28/48	100	14/14	41	14/34	41	14/34	100	14/14	59	20/34	0	0/14
S-Score	New	14	50	7/14	44	4/5	60	3/5	67	4/6	38	3/8	40	2/5	56	5/9
	Old	19	47	9/19	36	4/11	63	5/8	57	4/7	42	7/11	38	3/8	64	7/11
mtc10 ⁷	New	142	76%	108/142	52	25/48	88	83/94	70	25/36	78	83/106	12	11/94	48	23/48
mtc10 ⁷	New	189	77%	145/189	53	30/57	87	115/132	64	30/47	81	115/142	13	17/132	47	27/57

2965 ¹EU=European Union (EU [2001]).

2966 ²IS(A), IS(A)-10, IS(A)-100 = method described in Luepke (1985); IS(B), IS(B)-10, IS(B)-100 = method described in Kalweit et al. (1987); Q = Q-Score,
 2967 method described in Balls et al. (1995); S = S-Score, method described in Balls et al. (1995).

2968 ³N = number of substances evaluated in each study.

2969 ⁴Data used to calculate the percentage.

2970 ⁵The analysis compares the ability of the specified concentration tested *in vitro* (IS(A)-10 represents the 10% concentration tested *in vitro*) to predict the effect
2971 produced by the undiluted test substance tested *in vivo*.
2972 ⁶New = accuracy statistics based on the revised analysis; Old = accuracy statistics based on the previous analysis included in the draft HET-CAM BRD.
2973 ⁷Results were calculated based on the results presented in Spielmann et al. (1996) (pages 765 and 767). Classification of *in vivo* results is described in
2974 Spielmann et al. (1996).

2975 **2.4 Accuracy of the HET-CAM IS(B) Analysis Method for the GHS Ocular**
2976 **Hazard Classification System, by Chemical Class and Property of Interest -**
2977 **Reanalysis**

2978

2979 In order to further evaluate discordant responses of the HET-CAM test method relative to the
2980 *in vivo* hazard classification, several accuracy sub-analyses were performed. These included
2981 specific classes of chemicals with sufficiently robust numbers of substances ($n \geq 5$), as well
2982 as certain properties of interest considered relevant to ocular toxicity testing (e.g., pesticides,
2983 surfactants, pH, physical form). Because the international community will soon adopt the
2984 GHS classification system for hazard labeling (UN [2003]), and considering that there were
2985 only modest differences in overall HET-CAM test method accuracy among the three
2986 regulatory classification systems (i.e., EPA, EU, GHS), these sub-analyses were focused only
2987 on the GHS system.

2988

2989 Due to the various concentrations of test substances evaluated in this test method, different
2990 permutations of these sub-analyses are provided for comparative purposes. The overall false
2991 positive and false negative rates for the test substances evaluated are provided for two
2992 different groups: (a) substances tested at a 10% concentration in the entire database and (b)
2993 substances tested at a 100% concentration in the entire database. As is shown in **Table IV-8**,
2994 the false negative rate of the IS(B) analysis method is higher when test substances are tested
2995 at a 10% concentration (30%, 12/40) when compared to 100% (15%, 6/40). However, the
2996 false positive rate of the IS(B) analysis method is lower for the 10% concentration (33%,
2997 20/61) compared to the 100% concentration (60%, 61/102).

2998

2999 As indicated in **Table IV-8**, there were some notable trends in the performance of the HET-
3000 CAM test method among subgroups of the tested substances. The chemical class of
3001 substances that was most consistently overpredicted according the GHS classification system
3002 (i.e., were false positives) by both analysis methods is alcohols. Nine out of 10 (90%) and 10
3003 out of 11 alcohols (91%) were overpredicted by the IS(B)-10 and IS(B)-100 analysis
3004 methods, respectively. The remaining chemical classes represented among both analysis
3005 methods as being overpredicted were ethers, amines, organic salts, and heterocycles.
3006 Formulations appeared to have the lowest false positive rates for both analysis methods (0%
3007 [0/8] and 19% [6/31]). The chemical classes that were underpredicted by both the IS(B)-10
3008 and IS(B)-100 analysis methods were amines and ethers. Generally, the false negative and
3009 false positive rates for the same chemical class were higher for the IS(B)-100 analysis
3010 method when compared to the IS(B)-10 analysis method.

3011

3012 With regard to physical form of the substances overpredicted by the IS(B)-10 analysis
3013 method, the false positive and false negative rates were 34% (12/62) and 30% (10/33),
3014 respectively for liquids. Since only diluted chemicals were tested for the IS(B)-10 analysis
3015 method, there were no solids to evaluate for this analysis method. For the IS(B)-100 analysis
3016 method liquids performed better than solids (see **Table IV-8**).

3017

3017 **Table IV-8. False Negative and False Positive Rates of the HET-CAM Test Method,**
 3018 **by Chemical Class and Properties of Interest, for the GHS¹ Classification**
 3019 **System**
 3020

Category	N ²	False Positive Rate ³		False Negative Rate ³	
		%	No.	%	No.
Overall IS(B)-10 (Entire database)	101	33	20/61	30	12/40
Overall IS(B)-100 (Entire database)	143	60	61/102	15	6/41
<i>Chemical Class⁴-IS(B)-10</i>					
Alcohol	17	90	9/10	25	2/7
Amine	7	60	3/5	50	1/2
Ether	14	50	5/10	50	2/4
Formulation	24	0	0/8	44	7/16
Heterocycle	6	83	5/6	-	-
Organic salt	7	57	4/7	-	-
<i>Chemical Class⁴-IS(B)-100</i>					
Alcohol	20	91	10/11	11	1/9
Aldehyde	6	80	4/5	0	0/1
Amine	10	83	5/6	50	2/4
Ester	14	83	10/12	0	0/2
Ether	20	60	9/15	20	1/5
Formulation	51	19	6/31	35	7/13
Heterocycle	10	75	6/8	-	-
Inorganic salt	5	100	2/2	0	0/3
Ketone	6	67	4/6	-	-
Onium	7	100	2/2	0	0/5
Organic salt	8	88	7/8	-	-
<i>Properties of Interest</i>					
Physical Form: IS(B)-10					
Liquid	95	34	21/62	30	10/33
Solid	-	-	-	-	-
Physical Form: IS(B)-100					
Liquid	85	60	36/60	28	7/25
Solid	40	76	16/21	26	5/19
Surfactant – Total IS(B)-100	3	66	2/3	-	-
-nonionic	3	66	2/3	-	-
-anionic	0	-	-	-	-
-cationic	0	-	-	-	-
Surfactant-Based Formulation – IS(B)-10	24	0	0/8	44	7/16
pH – IS(B)-10⁵	35	58	11/19	13	2/16
- acidic (pH < 7.0)	24	50	7/14	20	2/10
- basic (pH > 7.0)	11	80	4/5	0	0/6
pH – IS(B)-100⁵	35	68	13/19	13	2/16
- acidic (pH < 7.0)	23	69	9/13	10	1/10
- basic (pH > 7.0)	12	67	4/6	17	1/6

Category	N ²	False Positive Rate ³		False Negative Rate ³	
		%	No.	%	No.
Category 1 Subgroup- IS(B)-10⁶					
- Total	40	-	-	30	12/40
- 4 (CO=4 at any time)	13	-	-	15	2/13
- 3 (severity/persistence)	0	-	-	-	-
- 2 (severity)	0	-	-	-	-
- 2-4 combined ⁷	13	-	-	15	2/11
- 1 (persistence)	27	-	-	37	10/27
Category 1 Subgroup- IS(B)-100⁶					
- Total	37	-	-	11	4/37
- 4 (CO=4 at any time)	19	-	-	11	2/19
- 3 (severity/persistence)	2	-	-	0	0/2
- 2 (severity)	2	-	-	0	0/2
- 2-4 combined ⁷	23	-	-	9	2/23
- 1 (persistence)	18	-	-	11	2/18

3021 ¹GHS = Globally Harmonized System (UN [2003]).

3022 ²N=number of substances

3023 ³False Positive Rate = the proportion of all negative substances that are falsely identified as positive *in vitro*; n =
3024 number of substances; False Negative Rate = the proportion of all positive substances that are falsely identified
3025 as negative *in vitro*.

3026 ⁴Chemical classes included in this table are represented by at least five substances tested in the HET-CAM test
3027 method and assignments are based on the MeSH categories (www.nlm.nih.gov/mesh). See **Appendix B**.

3028 ⁵Total number of GHS Category 1 substances for which pH information was obtained.

3029 ⁶NICEATM-defined subgroups assigned based on the lesions that drove classification of a GHS Category 1
3030 substance. 1: based on lesions that are persistent; 2: based on lesions that are severe (not including Corneal
3031 Opacity [CO]=4); 3: based on lesions that are severe (not including CO=4) and persistent; 4: CO = 4 at any time.

3032 ⁷Subcategories 2 to 4 combined to allow for a direct comparison of GHS Category 1 substances classified *in vivo*
3033 based on some lesion severity component and those classified based on persistent lesions alone.

3034

3035 Information regarding the pH of test substances was only available for a subset of the
3036 substances evaluated by the IS(B)-10 and IS(B)-100 analysis methods. Among all the
3037 substances that were tested at a 10% concentration, 2 out of 35 test substances were
3038 underpredicted (false negative rate: 13%; 2/16). Among these two, both were acidic (pH <
3039 7.0). For all substances tested at a 100% concentration, 2 out of 35 test substances were
3040 underpredicted. Of these substances, one was acidic (pH < 7.0) and one was basic (pH >
3041 7.0). For substances that were overpredicted, basic substances were more overpredicted than
3042 acidic substances when tested at a 10% concentration *in vitro* (false positive rate of basic
3043 substances = 80% [4/5] vs. false positive rate of acidic substances: 50% [7/14]). The false
3044 positive rate of acidic and basic substances, when tested at 100% concentration *in vitro*, was
3045 approximately the same (see **Table IV-8**).

3046

3047 Finally, substances were more likely to be underpredicted if (a) the *in vivo* effect was based
3048 on a persistent lesion and (b) if the concentration of the test substance *in vitro* was 10%
3049 (**Table IV-8**).

3050

3050 **2.5 Accuracy of the HET-CAM Test Method for Identifying Ocular Corrosives**
3051 **and Severe Irritants – Summary of Reanalysis**
3052

3053 As detailed in **Section VI-1.0**, additional or new relevant HET-CAM test method data was
3054 received after the Expert Panel meeting on January 11 and 12, 2005 that increased the size of
3055 the comparative HET-CAM *in vivo* rabbit eye test database for the GHS classification system
3056 (UN [2003]), EPA classification system (EPA [1996]), and EU classification system (EU
3057 [2001]). The reanalysis of the accuracy of the HET-CAM test method for identifying ocular
3058 corrosives and severe irritants based on the additional data and the reclassification of some
3059 nonsevere irritants as severe irritants resulted in changes in the accuracy, sensitivity, and
3060 specificity of the HET-CAM test method.
3061

3062 The previous accuracy analysis of the IS(B) analysis method, which included substances
3063 used at a variety of concentrations, had an accuracy of 83% to 85%, a false positive rate from
3064 20% to 27%, and a false negative rate from 0% to 7%. When the reanalysis was conducted,
3065 the accuracy rates decreased and the false positive and false negative rates increased for all
3066 three classification systems (see rows labeled IS(B)-10 and IS(B)-100 in **Tables IV-3, IV-5,**
3067 **and IV-7**).
3068

3069 When new analyses were conducted with the IS(A) and IS(B) methods, wherein substances
3070 tested at either 10% or 100% concentration were compared only against *in vivo* studies which
3071 were conducted with undiluted test substances, several interesting patterns were noted. For
3072 the IS(A) analysis method, these evaluations showed that accuracy increased when
3073 substances were evaluated at 100% concentration *in vitro* compared to the 10% concentration
3074 (e.g., 85% [17/20] for IS(A)-100 vs. 50% [12/24] for IS(A)-10; GHS classification system).
3075 Comparatively, the opposite was observed for the IS(B) analysis method. The IS(B)-10
3076 method had a higher accuracy and lower false positive and false negative rate when
3077 compared to the IS(B)-100 analysis method.
3078

3079 Unlike the original analysis, where only formulations were evaluated by the IS(B) method,
3080 additional chemical classes were available for this assessment. The revised analysis
3081 indicated that several chemical classes are overpredicted by the HET-CAM IS(B) analysis
3082 methods. These chemical classes include alcohols, ethers, amines, organic salts, and
3083 heterocycles. Additionally, the IS(B)-100 analysis method overpredicted esters. The
3084 chemical class that was consistently underpredicted by the IS(B)-10 and IS(B)-100 analysis
3085 methods was formulations.
3086

3087 As noted in **Section IV-2.4**, an evaluation based on the physical form of the test substance
3088 was dependent on the analysis method being evaluated. Liquids could only be evaluated for
3089 the IS(B)-10 analysis method while solids and liquids could be evaluated for the IS(B)-100
3090 analysis method. In the case of the IS(B)-100 evaluation, solids had a higher false positive
3091 rate than compared to liquids (76% [16/21] vs. 60% [36/60]). Comparatively, the false
3092 negative rates for solids and liquids were 26% (5/19) and 28% (7/25), respectively, for the
3093 IS(B)-100 analysis method (see **Table IV-5**). The false positive and false negative rate for
3094 liquids (when tested by the IS(B)-10 method) also were 34% (21/62) and 30% (10/33),
3095 respectively.
3096

3096 Using the expanded database, an analysis was conducted of the ability of the HET-CAM test
 3097 method to identify ocular corrosives and severe irritants, depending on the nature of the *in*
 3098 *vivo* ocular lesions (i.e., severity and/or persistence) responsible for classification of a
 3099 substance as an ocular corrosive/severe irritant. As indicated in **Table IV-8**, the
 3100 underpredicted substances were more likely to be substances classified *in vivo* based on
 3101 persistent lesions (false negative rates = 37% [10/27] for IS(B)-10 and 11% [2/18] for IS(B)-
 3102 100).

3103

3104 A new analysis not included original evaluation was an assessment of accuracy related to
 3105 acidic or basic pH. For all the Category 1 substances in the database, pH information was
 3106 only for 35 substances tested by the IS(B)-10 and IS(B)-100 methods. Among the two
 3107 underpredicted substances that were tested at a 10% concentration for which pH information
 3108 was available, both were acidic (pH < 7.0). Between the two underpredicted substances that
 3109 were tested at a 100% concentration for which pH information was available, one was acidic
 3110 and one was basic (pH > 7.0).

3111

3112 **Tables IV-9** and **IV-10** provide a breakdown of the *in vivo* and *in vitro* irritancies of the
 3113 substances tested using the IS(B)-10 and IS(B)-100 analysis methods. These tables indicate
 3114 that the false positives for both analysis methods were typically nonirritants (18 substances
 3115 for the IS(B)-10 method and 39 substances for the IS(B)-100 method). Category 2A and 2B
 3116 substances made up a smaller proportion of the substances that were classified as false
 3117 positives (2 and 22 substances for the IS(B)-10 and IS(B)-100 methods, respectively).

3118

3119

3120 **Table IV-9. Overall Accuracy of the HET-CAM Test Method in Predicting the**
 3121 **Irritancy of a Substance as Defined by the GHS¹ Classification System**
 3122 **(IS(B)-10 Analysis Method)**

3123

<i>In Vivo</i> Classification	<i>In Vitro</i> Classification			
	Severe	Moderate	Slight	Nonirritant
Category 1	28	8	3	1
Category 2A	1	-	-	-
Category 2B	1	5	3	-
Nonirritant	18	8	18	7
Total	48	21	24	8

3124

¹GHS = Globally Harmonized System (UN [2003]).

3125

3125 **Table IV-10. Overall Accuracy of the HET-CAM Test Method in Predicting the**
 3126 **Irritancy of a Substance as Defined by the GHS¹ Classification System**
 3127 **(IS(B)-100 Analysis Method)**
 3128

<i>In Vivo</i> Classification	<i>In Vitro</i> Classification			
	Severe	Moderate	Slight	Nonirritant
Category 1	35	3	2	1
Category 2A	15	-	1	-
Category 2B	7	1	1	1
Nonirritant	39	15	16	6
Total	96	19	20	8

3129 ¹GHS = Globally Harmonized System (UN [2003]).

3130

3131

3132 Among the analysis methods re-evaluated, the IS(A)-100 had the greatest accuracy rate for
 3133 the GHS classification system (85%; 17/20). Compared to the draft HET-CAM BRD, the
 3134 IS(B) analysis method GHS classification system accuracy rate decreased (from 85% (44/25)
 3135 to 57% (61/107) while the overall database increased.

3136 3.0 RELIABILITY OF THE HET-CAM TEST METHOD - REANALYSIS

3137

3138 An assessment of test method reliability (intralaboratory repeatability and inter- and intra-
3139 laboratory reproducibility) is an essential element of any evaluation of the performance of an
3140 alternative test method (ICCVAM [2003]). Repeatability refers to the closeness of
3141 agreement between test results obtained within a single laboratory when the procedure is
3142 performed on the same substance under identical conditions within a given time period
3143 (ICCVAM [1997, 2003]). Intralaboratory reproducibility refers to the determination of
3144 whether qualified people within the same laboratory can successfully replicate results using a
3145 specific test protocol at different times (ICCVAM [1997, 2003]). Interlaboratory
3146 reproducibility refers to the extent to which a test method can be transferred successfully
3147 among laboratories (ICCVAM [1997, 2003]). A reliability assessment includes determining
3148 the rationale for selecting the substances used to evaluate test method reliability, a discussion
3149 of the extent to which the substances tested represent the range of possible test outcomes, and
3150 a quantitative and/or qualitative analysis of repeatability and intra- and inter-laboratory
3151 reproducibility. In addition, measures of central tendency and variation are summarized for
3152 historical control data (negative, vehicle, positive), where applicable. This section provides
3153 the results of a more detailed analysis of HET-CAM test method reliability, based on the
3154 additional data provided subsequent to the previous analysis described in Section 7 of the
3155 draft HET-CAM BRD (http://iccvam.niehs.nih.gov/methods/ocudocs/ocu_brd.htm).

3156

3157 3.1 Substances Used to Re-evaluate the Reliability of the HET-CAM Test Method

3158

3159 There was limited information on the rationale for substance selection used in various
3160 multilaboratory studies to evaluate the reliability of the HET-CAM test method. Most
3161 reports indicated that substances were selected for inclusion based on available *in vivo* rabbit
3162 eye data for comparison, to cover the range of ocular irritation potential, and to include
3163 substances with different physicochemical properties (e.g., solids, liquids). The rationale for
3164 substance selection for CEC (1991), Balls et al. (1995), and Hagino et al. (1999) remain the
3165 same as in the draft HET-CAM BRD.

3166

3167 Gilleron et al. (1996, 1997) selected substances that had been tested previously and where
3168 existing data was available. Additionally, substances evaluated in the Gilleron et al. (1997)
3169 study were the same as those previously evaluated by Balls et al. (1995).

3170

3171 Spielmann et al. (1996) conducted an extensive evaluation of the accuracy of the HET-CAM
3172 test method. Substances selected for the evaluation were representative of the spectrum of
3173 chemicals produced by participating companies from the pharmaceutical and chemical
3174 industries.

3175

3.2 Reanalysis of HET-CAM Test Method Intralaboratory Repeatability

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An analysis of interlaboratory repeatability has included such approaches as:

3178

- a CV analysis, which is a statistical measure of the deviation of a variable from its mean (e.g., Holzhütter et al. [1996])

3179

3180

- ANOVA methods (e.g., Holzhütter et al. [1996]; ASTM [1999]) that would detect whether there are significant differences among replicate (in this case) eggs within an experiment.

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Two of the reports discussed in this section include intralaboratory repeatability data (Gilleron et al. [1996, 1997]). For both sets of reports, quantitative HET-CAM test method data were made available for replicate eggs within individual experiments. Using these data, the consistency of HET-CAM IS(B) results obtained among identically-treated eggs within an experiment was evaluated using a CV analysis. Considering the number of replicate eggs tested in each experiment, no attempt was made to use ANOVA to determine if any individual egg differed from any other egg.

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3.2.1 Gilleron et al. (1996)

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Individual egg results for 46 substances analyzed by the HET-CAM IS(B) analysis method and reported on by Gilleron et al. (1996) were received from Dr. P. Vanparys and Dr. F. Van Goethem in response to a request from NICEATM. In the data provided to NICEATM, the test results for nine of the 46 substances included in the 1996 publication (laurylsulfobetaine, deoxycholic acid, ethylacetoacetate, methyl isobutyl ketone, methanol, N-laurylsarcosine, promethazine hydrochloride, 2-methoxyethanol, benzethonium chloride, and imidazole) were no longer available. Since alternative HET-CAM test data generated by this laboratory were available for these substances, these data were provided to NICEATM. The overall replicate egg mean and median %CV values were evaluated with and without the inclusion of these data.

For each test substance, three different eggs were used in each of at least three replicate experiments. For this evaluation, the %CV values were determined for each endpoint evaluated (hemorrhage, lysis, coagulation) and for the overall *in vitro* IS(B) score. For each of the endpoints, there were a number of experiments where the test substance did not produce any effects (i.e., the average score of the three replicate eggs and standard deviation [SD] of the scores were both 0) (see **Table IV-11**). For the hemorrhage and lysis endpoints, 69 of 146 experiments (47%) resulted in an average score and SD of zero for the three replicate eggs, while, for the coagulation endpoint, 47 of 146 experiments (32%) resulted in an average score and SD of zero for the three replicate eggs. For the overall *in vitro* IS(B) score, 21 of 146 experiments (14%) resulted in an average score and SD of zero for the three replicate eggs. Three test substances (anthracene, ethylenediaminetetraacetic acid [EDTA] dipotassium, and iminodibenzyl) produced no response in any of the three endpoint evaluated in the three replicate eggs in each of three replicate experiments. The replicate egg repeatability %CV values for individual experiments, excluding studies where such values could not be calculated, ranged from 0.12 to 173.21 for hemorrhage, from 0.25 to 173.21 for lysis, from 0.00 to 173.21 for coagulation, and from 0.25 to 173.21 for the overall *in vitro* IS(B) score. The mean and median replicate egg repeatability %CV values for the overall *in*

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3221 **Table IV-11. Intralaboratory Repeatability Results for HET-CAM IS(B) Data of Gilleron et al. (1996)**
 3222

Substance	Test Number	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Allyl Alcohol	86	0.00	0.00		3.91	3.39	86.63	8.82	0.08	0.90	12.73	3.37	26.44
	95	0.62	1.07	173.21	1.38	2.38	173.21	8.03	0.18	2.19	10.02	3.52	35.09
	99	0.00	0.00		6.25	0.30	4.82	8.27	0.10	1.17	14.52	0.40	2.72
2-Aminophenol	91	0.00	0.00		0.00	0.00		1.42	1.27	89.14	1.42	1.27	89.14
	96	0.00	0.00		0.00	0.00		1.09	1.71	156.82	1.09	1.71	156.82
	101	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Anthracene	91	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	95	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	99	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Butyrolactone	88	4.98	0.01	0.12	0.00	0.00		7.89	0.48	6.04	12.87	0.47	3.69
	95	4.94	0.04	0.82	0.00	0.00		7.84	0.23	2.97	12.78	0.20	1.58
	100	4.72	0.34	7.16	4.55	0.51	11.18	6.67	0.78	11.70	15.94	1.07	6.72
Cyclohexanone	89	4.34	0.42	9.61	0.00	0.00		7.92	0.42	5.30	12.26	0.24	1.94
	98	4.64	0.47	10.16	6.61	0.18	2.69	7.59	0.68	8.93	18.85	1.01	5.38
	104	4.63	0.19	4.11	5.46	2.01	36.75	2.20	1.76	80.22	12.29	3.65	29.67
Deoxycholic acid, sodium salt	89	1.96	1.97	100.26	6.09	0.44	7.21	8.34	0.34	4.05	16.39	2.59	15.78
	97	1.27	1.32	103.73	5.55	0.57	10.32	0.00	0.00		6.82	0.75	10.92
	102	0.00	0.00		5.89	0.53	8.94	0.00	0.00		5.89	0.53	8.94
Diacetone alcohol	89	3.79	0.98	25.83	0.00	0.00		8.13	0.97	11.88	11.92	0.58	4.87
	98	4.90	0.03	0.61	5.57	0.29	5.17	6.53	0.86	13.19	17.00	1.02	6.02
	104	4.84	0.05	1.07	6.10	0.17	2.74	5.28	1.79	33.88	16.22	1.88	11.57
Dibenzoyl-L-tartaric acid	90	1.38	2.40	173.21	0.00	0.00		0.00	0.00		1.38	2.40	173.21
	93	4.83	0.03	0.60	0.00	0.00		0.00	0.00		4.83	0.03	0.60
	102	4.72	0.11	2.25	1.59	1.73	108.99	0.00	0.00		6.30	1.81	28.65

Substance	Test Number	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
2,4-Dichloro-5-sulfamoylbenzoic acid	91	1.61	2.78	173.21	0.00	0.00		0.00	0.00		1.61	2.78	173.21
	93	4.82	0.04	0.73	0.00	0.00		1.21	1.13	93.77	6.03	1.15	19.01
	100	3.67	0.99	27.03	0.00	0.00		0.00	0.00		3.67	0.99	27.03
Dimethyl biguanidine	92	0.00	0.00		0.00	0.00		8.07	0.53	6.61	8.07	0.53	6.61
	93	0.00	0.00		4.02	1.89	46.97	6.76	1.83	27.07	10.78	2.00	18.55
	103	0.00	0.00		6.46	0.12	1.80	0.00	0.00		6.46	0.12	1.80
Dimethyl sulfoxide	88	0.00	0.00		6.38	0.26	4.02	8.49	0.22	2.55	14.87	0.21	1.41
	93	0.39	0.68	173.21	0.00	0.00		7.00	0.36	5.11	7.39	0.85	11.49
	101	0.00	0.00		6.15	0.38	6.15	5.36	0.62	11.50	11.51	0.43	3.74
Ethanol	89	0.00	0.00		0.00	0.00		8.43	0.22	2.57	8.43	0.22	2.57
	97	0.00	0.00		5.79	0.08	1.40	5.68	0.35	6.08	11.47	0.42	3.64
	102	0.00	0.00		6.27	0.45	7.12	7.80	0.29	3.71	14.07	0.40	2.83
2-Ethoxyethanol	86	0.85	1.47	173.21	0.42	0.73	173.21	8.62	0.35	4.08	9.89	1.85	18.75
	95	1.82	2.35	128.66	0.00	0.00		6.22	0.23	3.62	8.04	2.14	26.55
	99	0.00	0.00		5.63	0.29	5.11	7.81	0.40	5.12	13.44	0.18	1.35
Ethylenediaminetetraacetic acid, dipotassium	91	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	94	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	99	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Furan	88	0.00	0.00		0.43	0.74	173.21	4.08	3.02	73.96	4.51	2.28	50.59
	95	0.92	1.60	173.21	1.74	1.55	89.39	3.88	2.37	61.19	6.54	2.65	40.51
	100	0.00	0.00		5.06	0.48	9.47	0.44	0.55	123.92	5.50	0.48	8.74
Gluconolactone	91	0.00	0.00		0.00	0.00		8.17	0.48	5.89	8.17	0.48	5.89
	93	0.00	0.00		2.08	3.61	173.21	0.00	0.00		2.08	3.61	173.21
	101	0.00	0.00		3.40	2.09	61.51	0.00	0.00		3.40	2.09	61.51
DL-Glutamic acid	91	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	95	2.03	1.78	87.69	0.00	0.00		0.00	0.00		2.03	1.78	87.69

Substance	Test Number	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
	100	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
3-Glycidopropyl-trimethoxysilane	99	3.63	0.48	13.10	0.00	0.00		0.00	0.00		3.63	0.48	13.10
	100	3.63	0.16	4.33	0.00	0.00		1.79	0.81	45.01	5.42	0.75	13.87
	105	3.90	0.11	2.88	0.00	0.00		0.00	0.00		3.90	0.11	2.88
Hexadecyltrimethyl-ammonium bromide	90	4.59	0.19	4.15	4.22	1.19	28.26	7.58	0.41	5.37	16.39	1.05	6.43
	97	2.81	2.44	86.70	4.85	1.11	22.91	0.00	0.00		7.66	2.03	26.43
	103	0.00	0.00		6.32	0.19	2.93	2.15	1.95	90.78	8.47	1.93	22.78
Hexane	86	1.24	0.78	63.10	0.00	0.00		4.62	0.19	4.06	5.86	0.80	13.63
	93	0.00	0.00		0.00	0.00		0.57	0.99	173.21	0.57	0.99	173.21
	105	0.00	0.00		2.82	2.48	87.96	0.00	0.00		2.82	2.48	87.96
Iminodibenzyl	92	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	96	0.00	0.00		0.00	0.00		0.09	0.16	173.21	0.09	0.16	173.21
	102	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Magnesium carbonate	92	0.00	0.00		0.00	0.00		3.53	1.33	37.76	3.53	1.33	37.76
	101	0.00	0.00		0.00	0.00		0.76	1.32	173.21	0.76	1.32	173.21
	106	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Methyl isobutyl ketone	88	2.09	1.85	88.54	0.00	0.00		7.16	0.48	6.73	9.25	2.15	23.26
	96	3.79	0.77	20.40	2.39	0.45	19.01	5.98	1.76	29.46	12.16	1.17	9.61
	105	3.46	0.44	12.77	6.60	0.19	2.81	0.00	0.00		10.06	0.48	4.78
MYRJ 45	92	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	97	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	102	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
1-Nitropropane	86	0.42	0.73	173.21	0.00	0.00		2.64	2.32	88.05	3.06	2.86	93.20
	87	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	95	1.32	0.88	66.81	1.55	2.68	173.21	2.51	2.41	95.92	5.37	0.71	13.25
Octanol	88	2.01	1.75	87.26	1.51	2.62	173.21	4.47	1.66	37.08	7.99	2.35	29.37

Substance	Test Number	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
	102	3.56	0.51	14.33	3.84	0.12	3.02	2.90	0.17	5.88	10.29	0.58	5.67
	106	3.37	0.15	4.56	5.73	0.48	8.42	2.14	1.50	70.17	11.25	1.32	11.71
2,4-Pentanedione	86	4.41	0.33	7.60	0.00	0.00		5.86	1.97	33.57	10.27	2.01	19.58
	87	4.37	0.19	4.44	0.00	0.00		6.39	0.90	14.08	10.76	1.07	9.90
	93	4.17	0.23	5.62	0.00	0.00		1.20	2.08	173.21	5.37	2.05	38.06
1-Phenyl-3-pyrazolidone	91	0.00	0.00		0.00	0.00		0.60	1.04	173.21	0.60	1.04	173.21
	96	0.00	0.00		0.00	0.00		0.79	1.05	132.77	0.79	1.05	132.77
	101	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Polyoxythethylene 23 lauryl ether	92	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	97	4.52	0.08	1.68	0.00	0.00		0.00	0.00		4.52	0.08	1.68
	103	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Propyl-4-hydroxybenzoate	92	1.11	1.92	173.21	0.00	0.00		0.38	0.66	173.21	1.49	2.58	173.21
	96	2.82	0.58	20.37	0.00	0.00		0.25	0.43	173.21	3.07	0.72	23.36
	101	1.07	0.87	81.17	0.00	0.00		0.00	0.00		1.07	0.87	81.17
Pyridine	89	2.97	2.61	87.69	0.00	0.00		8.68	0.23	2.62	11.65	2.39	20.51
	98	4.65	0.30	6.55	6.89	0.04	0.52	5.74	3.20	55.83	17.28	3.03	17.54
	104	4.34	0.87	19.93	6.74	0.28	4.17	7.31	0.51	6.99	18.39	0.44	2.37
Quinacrine	90	4.64	0.03	0.75	0.00	0.00		1.05	1.82	173.21	5.69	1.85	32.57
	93	4.82	0.05	0.96	0.00	0.00		0.00	0.00		4.82	0.05	0.96
	103	0.07	0.12	173.21	3.72	1.23	33.14	5.19	1.68	32.35	8.97	2.89	32.23
Tetraaminopyrimidine sulfate	92	1.59	2.75	173.21	0.00	0.00		0.00	0.00		1.59	2.75	173.21
	93	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	103	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Thiourea	90	3.88	0.09	2.19	6.82	0.07	1.06	7.48	0.53	7.07	18.18	0.50	2.76
	103	3.83	0.06	1.68	6.61	0.03	0.44	0.00	0.00		10.44	0.04	0.35
	107	4.02	0.13	3.11	6.70	0.06	0.87	0.00	0.00		10.72	0.13	1.21

Substance	Test Number	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
1,2,3-Trichloropropane	89	0.53	0.83	157.05	0.00	0.00		5.50	2.43	44.21	6.03	2.85	47.29
	98	3.70	0.55	14.76	0.81	0.96	118.49	4.99	0.60	11.94	9.50	1.11	11.74
	104	3.27	0.55	16.98	3.84	0.48	12.47	0.91	0.35	38.63	8.02	0.31	3.92
1,2,4-Trimethylbenzene	88	0.37	0.55	150.21	0.00	0.00		4.09	2.45	59.88	4.46	2.92	65.53
	95	0.62	1.07	173.21	0.00	0.00		0.88	1.52	173.21	1.50	2.59	173.21
	100	0.00	0.00		4.90	1.05	21.39	2.42	1.55	64.13	7.32	1.40	19.08
Triton X-155	92	0.25	0.43	173.21	0.00	0.00		1.03	1.53	148.70	1.28	1.33	103.67
	98	2.77	1.29	46.38	2.98	2.59	86.86	0.00	0.00		5.75	3.83	66.62
	104	0.00	0.00		4.74	0.17	3.48	0.00	0.00		4.74	0.17	3.48
Benzethonium chloride ³	90	4.71	0.18	3.83	0.00	0.00		7.71	0.47	6.08	12.42	0.63	5.08
	98	4.39	0.40	9.12	5.84	0.53	9.00	7.79	0.61	7.79	18.03	0.89	4.95
	104	0.00	0.00		6.53	0.06	0.90	0.37	0.33	89.68	6.90	0.28	4.06
	107	0.00	0.00		6.76	0.04	0.60	6.08	0.27	4.48	12.84	0.28	2.18
Ethylacetoacetate ³	89	3.97	0.16	4.12	0.00	0.00		3.52	0.58	16.56	7.49	0.68	9.08
	97	3.16	0.59	18.72	1.45	1.95	134.57	0.00	0.00		4.61	1.37	29.81
	102	3.99	0.45	11.23	4.57	0.30	6.47	5.04	0.67	13.26	13.61	0.42	3.05
Imidazole ³	96	4.41	0.34	7.63	4.31	0.87	20.16	8.91	0.00	0.00	17.63	1.20	6.80
	100	4.83	0.03	0.52	6.22	0.62	9.93	5.47	0.99	18.14	16.52	1.61	9.72
	105	4.90	0.03	0.59	6.89	0.03	0.42	7.29	0.53	7.28	19.09	0.49	2.55
	118	4.68	0.22	4.76	6.85	0.06	0.88	7.03	0.20	2.84	18.57	0.30	1.60
	115	4.90	0.03	0.51	6.56	0.25	3.78	8.48	0.33	3.90	19.94	0.11	0.54
	116	4.80	0.04	0.84	6.71	0.19	2.82	7.70	0.20	2.59	19.20	0.22	1.16
N-Laurylsarcosine, sodium salt ³	92	2.68	2.37	88.71	6.23	0.47	7.62	8.71	0.06	0.72	17.62	2.11	11.98
	94	2.91	2.52	86.74	6.34	0.23	3.70	1.13	1.26	111.58	10.38	1.69	16.24
	106	0.00	0.00		6.91	0.03	0.36	4.06	0.23	5.59	10.97	0.23	2.14
	103	1.46	2.53	173.21	6.63	0.03	0.38	0.24	0.42	173.21	8.33	2.33	27.95

Substance	Test Number	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Laurylsulfobetaine ³	92	0.00	0.00		6.14	0.16	2.55	2.92	0.83	28.29	9.06	0.94	10.40
	94	0.22	0.39	173.21	5.71	1.43	25.03	3.75	3.12	83.15	9.68	4.15	42.87
	104	0.00	0.00		6.83	0.02	0.25	0.00	0.00		6.83	0.02	0.25
Methanol ³	89	0.00	0.00		3.69	3.29	89.14	8.57	0.11	1.33	12.26	3.36	27.40
	93	0.00	0.00		0.00	0.00		7.97	0.12	1.57	7.97	0.12	1.57
	102	0.00	0.00		6.79	0.09	1.33	8.38	0.12	1.49	15.17	0.20	1.32
	105	0.00	0.00		6.84	0.05	0.66	5.81	1.59	27.37	12.65	1.55	12.22
2-Methoxyethanol ³	88	0.00	0.00		5.94	1.14	19.25	8.39	0.22	2.64	14.33	0.93	6.47
	89	0.00	0.00		5.09	1.22	24.00	8.45	0.30	3.59	13.54	1.27	9.40
	96	0.00	0.00		5.39	0.32	5.95	7.96	0.05	0.58	13.35	0.29	2.14
	101	0.00	0.00		6.02	0.29	4.84	5.66	0.92	16.25	11.68	0.71	6.11
Promethazine hydrochloride ³	85	0.00	0.00		6.69	0.11	1.64	8.37	0.71	8.47	15.06	0.80	5.30
	90	2.58	0.76	29.32	0.00	0.00		8.57	0.12	1.46	11.15	0.77	6.91
	102	0.00	0.00		6.71	0.07	1.03	8.51	0.02	0.20	15.22	0.05	0.34
	97	0.00	0.00		6.70	0.18	2.61	8.29	0.09	1.04	14.99	0.20	1.31
Triethanolamine ³	89	0.00	0.00		3.23	0.50	15.64	7.59	0.80	10.48	10.82	1.28	11.82
	104	0.00	0.00		6.70	0.18	2.61	5.72	0.88	15.35	12.42	0.91	7.34
	107	0.00	0.00		6.36	0.35	5.52	6.75	0.89	13.13	13.11	0.78	5.94
Mean (SD) for All Substances⁴		1.64 (1.93)			2.68 (2.88)			3.59 (3.44)			7.92 (5.84)		
Range for All Substances		0.12-173.21			0.25-173.21			0.00-173.21			0.25-173.21		
%CV for Substances⁵		117.56			107.52			95.69			73.74		
Number of Experiments		146			146			146			146		
Mean (SD) Excluding Nine Substances⁴		1.63 (1.90)			1.87 (2.57)			2.83 (3.25)			6.33 (5.43)		
Range Excluding Nine Substances		0.12-173.21			0.25-173.21			0.00-173.21			0.35-173.21		
%CV Excluding Nine Substances⁵		116.13			137.49			115.07			85.84		

Substance	Test Number	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Number of Experiments		111			111			111			111		
Mean Overall <i>In Vitro</i> Score %CV for All Substances		32.52											
Median Overall <i>In Vitro</i> Score %CV for all Substances		11.49											
Mean Overall <i>In Vitro</i> Score %CV Excluding Nine Substances		41.48											
Median Overall <i>In Vitro</i> Score %CV Excluding Nine Substances		17.54											

3223 ¹SD=standard deviation.

3224 ²%CV=percent coefficient of variation.

3225 ³Data not originally presented in Gilleron et al. (1996).

3226 ⁴Mean was calculated using the values from the “Mean for 3 Eggs” column for each endpoint and the Overall *In Vitro* Score. The standard deviation (SD) was
3227 calculated based on the values in these individual columns.

3228 ⁵To avoid eliminating data for which the %CV (coefficient of variation) value could not be calculated (i.e., where the mean and SD both equaled 0), the %CV
3229 values were calculated using the mean and standard deviation calculated as described in footnote 4 of this table.

3230 *in vitro* IS(B) scores for the entire data set (last column in **Table IV-11**), including the data for
3231 the nine substances previously noted and excluding studies where such values could not be
3232 calculated, were 32.25 and 11.49, respectively. When the data for the nine substances noted
3233 were removed, the mean and median replicate egg repeatability %CV values for the overall
3234 IS(B) scores were 41.58 and 17.54, respectively.

3235

3236 3.2.2 Gilleron et al. (1997)

3237 Individual egg results for 60 substances evaluated by the HET-CAM IS(B) analysis method
3238 and reported on by Gilleron et al. (1997) were provided by the authors to NICEATM.

3239 Among the data, the test results for four of the 60 substances included in the 1997 publication
3240 (Maneb, 1-naphthalene acetic acid, Tween 20, and 1-naphthalene acetic acid, sodium salt) were
3241 no longer available. Since alternative HET-CAM test data were available for these
3242 substances, these data were provided to NICEATM. The overall replicate egg mean and
3243 median %CV values were evaluated with and without the inclusion of these data.

3244

3245 For each test substance, three different eggs were used in each of at least three replicate
3246 experiments. For this evaluation, the %CV values were determined for each endpoint
3247 evaluated (hemorrhage, lysis, coagulation) and for the overall *in vitro* IS(B) score. For each
3248 of the endpoints, there were a number of experiments where the test substance did not induce
3249 any effects (i.e., the average score of the three replicate eggs and thus the SD of the scores
3250 were both zero) (see **Table IV-12**). For the hemorrhage endpoint, 91 of 184 experiments
3251 (49%) resulted in an average score and SD of zero for the three replicate eggs; for the lysis
3252 endpoint, 22 of 184 experiments (12%) resulted in an average score and SD of zero for the
3253 three replicate eggs; while, for the coagulation endpoint, 16 of 184 experiments (9%) resulted
3254 in an average score and SD of zero for the three replicate eggs. For the overall *in vitro* IS(B)
3255 score, 6 of 184 experiments (3%) resulted in an average score and SD of zero for the three
3256 replicate eggs. Only one test substance (Maneb) produced no response in any of the three
3257 endpoints evaluated in the three replicate eggs in each of three replicate experiments. The
3258 replicate egg repeatability %CV values for individual experiments, excluding studies where
3259 such values could not be calculated, ranged from 0.23 to 173.21 for hemorrhage, from 0.00 to
3260 173.21 for lysis, from 0.37 to 173.21 for coagulation, and from 0.13 to 173.21 for the overall
3261 *in vitro* IS(B) score.

3262

3263 The mean and median replicate egg repeatability %CV values for the overall *in vitro* IS(B)
3264 scores for the entire data set (last column in **Table IV-12**), including the data for the four
3265 substances previously noted and excluding studies where such values could not be calculated,
3266 were 7.61 and 2.24, respectively. When the data for the four substances noted were removed
3267 the mean and median replicate egg repeatability %CV values for the overall IS(B) scores
3268 were 6.99 and 2.04, respectively.

3269 **Table IV-12. Intralaboratory Repeatability Results for HET-CAM IS(B) Data of Gilleron et al. (1997)**
 3270

Substance	Test #	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Acetone	131	0.00	0.00		6.94	0.02	0.33	8.74	0.06	0.71	15.68	0.09	0.54
	137	0.00	0.00		6.98	0.00	0.00	8.07	0.10	1.29	15.05	0.10	0.69
	144	0.00	0.00		6.89	0.07	1.05	8.33	0.38	4.56	15.22	0.40	2.60
Ammonium nitrate	117	1.04	0.91	87.03	6.76	0.04	0.53	6.28	1.11	17.61	14.08	0.22	1.53
	122	0.38	0.23	59.62	6.87	0.05	0.75	8.13	0.13	1.61	15.38	0.22	1.43
	126	1.67	0.26	15.47	6.86	0.02	0.29	8.02	0.07	0.86	16.55	0.29	1.75
L-Aspartic Acid	206	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	214	0.00	0.00		1.29	2.23	173.21	1.07	1.85	173.21	2.36	4.09	173.21
	220	0.00	0.00		0.00	0.00		0.81	1.40	173.21	0.81	1.40	173.21
Benzalkonium chloride (1%)	130	0.00	0.00		6.87	0.01	0.17	8.50	0.05	0.54	15.37	0.04	0.23
	136	0.00	0.00		6.94	0.05	0.74	8.23	0.03	0.42	15.17	0.06	0.36
	144	0.00	0.00		6.91	0.04	0.51	8.00	0.11	1.42	14.91	0.12	0.81
Benzalkonium chloride (10%)	129	0.00	0.00		6.92	0.01	0.17	8.21	0.15	1.88	15.13	0.15	0.96
	134	0.00	0.00		6.89	0.10	1.51	8.38	0.27	3.25	15.27	0.38	2.46
	143	0.00	0.00		6.91	0.00	0.00	7.83	0.05	0.66	14.74	0.05	0.35
Benzalkonium chloride (5%)	129	0.00	0.00		6.92	0.01	0.17	8.08	0.26	3.16	15.00	0.27	1.77
	135	0.00	0.00		6.94	0.05	0.74	8.27	0.15	1.79	15.21	0.11	0.73
	143	0.00	0.00		6.93	0.02	0.29	7.28	0.57	7.80	14.21	0.59	4.13
n-Butyl acetate	207	4.74	0.04	0.85	2.58	2.39	92.52	7.77	0.22	2.78	15.09	2.60	17.24
	211	4.79	0.06	1.27	6.02	0.30	5.00	7.35	0.20	2.68	18.16	0.55	3.00
	217	4.24	0.11	2.59	6.26	0.13	2.09	7.87	0.14	1.80	18.37	0.38	2.04
Gamma-butyrolactone	131	4.94	0.01	0.23	6.94	0.01	0.17	7.68	0.18	2.34	19.55	0.18	0.93
	137	4.92	0.04	0.73	6.96	0.04	0.58	8.25	0.20	2.38	20.13	0.27	1.34
	145	4.92	0.02	0.35	6.95	0.03	0.42	6.62	0.52	7.88	18.49	0.48	2.61
Captan 90 concentrate	115	0.00	0.00		6.86	0.04	0.59	8.18	0.26	3.12	15.04	0.24	1.63
	118	0.00	0.00		6.84	0.06	0.84	7.98	0.25	3.08	14.82	0.19	1.29
	124	0.00	0.00		6.80	0.07	1.04	8.56	0.08	0.88	15.36	0.15	0.95
4-Carboxybenzaldehyde	206	3.79	0.09	2.28	0.00	0.00		5.34	0.39	7.35	9.13	0.48	5.22
	214	2.71	2.35	86.62	0.00	0.00		0.00	0.00		2.71	2.35	86.62
	220	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	

Substance	Test #	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Cetylpyridinium-bromide (0.1%)	210	0.00	0.00		5.96	0.07	1.24	7.26	0.13	1.80	13.22	0.19	1.45
	216	0.00	0.00		6.33	0.03	0.40	7.52	0.11	1.40	13.84	0.13	0.94
	219	0.00	0.00		5.86	0.04	0.60	5.10	1.26	24.71	10.96	1.24	11.31
Cetylpyridinium-bromide (6%)	129	0.00	0.00		6.81	0.03	0.37	7.68	0.27	3.47	14.49	0.26	1.79
	135	0.00	0.00		6.88	0.03	0.37	8.20	0.14	1.65	15.08	0.15	0.97
	143	0.00	0.00		6.82	0.10	1.41	7.42	0.14	1.82	14.24	0.14	0.98
Cetylpyridinium bromide (10%)	129	0.00	0.00		6.90	0.04	0.52	7.76	0.20	2.52	14.66	0.22	1.52
	135	0.00	0.00		6.89	0.05	0.69	8.29	0.32	3.87	15.18	0.37	2.42
	143	0.00	0.00		6.88	0.04	0.59	3.87	0.76	19.66	10.75	0.80	7.45
Chlorhexidine	115	4.50	0.14	3.04	6.05	0.20	3.34	0.00	0.00		10.55	0.28	2.63
	118	4.62	0.07	1.42	5.67	0.10	1.84	7.30	0.35	4.75	17.59	0.32	1.79
	124	4.32	0.26	6.10	5.71	0.31	5.39	6.43	0.89	13.84	16.46	1.45	8.81
Cyclohexanol	131	4.19	0.09	2.04	6.82	0.06	0.82	7.85	0.24	3.11	18.86	0.37	1.95
	137	4.06	0.10	2.42	6.87	0.01	0.17	8.13	0.25	3.02	19.06	0.19	1.01
	144	4.63	0.10	2.18	6.87	0.07	1.03	7.46	0.70	9.43	18.96	0.79	4.15
Dibenzoyl-L-tartaric acid	206	4.21	0.10	2.33	0.00	0.00		4.39	0.19	4.39	8.60	0.15	1.76
	214	4.65	0.20	4.31	4.95	0.20	3.99	5.07	0.46	9.11	14.67	0.50	3.40
	220	4.76	0.05	1.09	5.10	0.01	0.23	6.90	0.05	0.75	16.76	0.04	0.24
Dibenzyl phosphate	116	0.00	0.00		6.97	0.02	0.25	8.71	0.07	0.80	15.68	0.08	0.51
	119	0.00	0.00		6.89	0.03	0.42	8.15	0.56	6.83	15.04	0.56	3.72
	124	0.00	0.00		6.81	0.05	0.66	7.96	0.28	3.50	14.77	0.32	2.18
2,6-Dichlorobenzoyl chloride	128	0.00	0.00		5.01	0.72	14.30	4.94	0.25	5.09	9.95	0.69	6.96
	133	0.00	0.00		6.60	0.03	0.38	6.13	0.56	9.11	12.73	0.55	4.29
	141	0.00	0.00		6.30	0.24	3.79	3.23	4.17	129.01	9.53	4.21	44.19
2,2-Dimethylbutanoic acid	127	0.00	0.00		6.71	0.10	1.47	8.34	0.19	2.25	15.05	0.28	1.85
	133	0.00	0.00		6.95	0.03	0.36	8.39	0.15	1.76	15.34	0.15	0.98
	141	0.00	0.00		6.91	0.04	0.51	8.62	0.02	0.20	15.53	0.04	0.24
2,5-Dimethyl-hexandiol	150	4.39	0.17	3.85	6.81	0.10	1.39	4.56	0.26	5.62	15.77	0.47	2.95
	122	4.08	0.32	7.80	6.27	0.15	2.32	4.68	1.05	22.45	15.03	1.26	8.36
	126	4.07	0.07	1.60	4.58	0.49	10.75	6.55	0.15	2.35	15.20	0.64	4.23
Ethanol	132	0.00	0.00		6.92	0.01	0.17	8.01	0.03	0.37	14.93	0.03	0.22
	140	0.00	0.00		6.85	0.12	1.69	8.01	0.17	2.09	14.86	0.08	0.54

Substance	Test #	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Ethyl acetate	145	0.00	0.00		6.88	0.03	0.37	8.01	0.37	4.68	14.89	0.38	2.58
	205	4.90	0.02	0.41	6.59	0.01	0.18	7.88	0.12	1.54	19.37	0.11	0.58
	209	4.01	0.10	2.51	6.82	0.06	0.82	8.52	0.10	1.22	19.35	0.23	1.19
	213	4.08	0.27	6.65	6.56	0.05	0.72	8.60	0.12	1.45	19.25	0.30	1.57
2-Ethyl-1-hexanol	131	4.18	0.22	5.14	0.00	0.00		6.36	0.56	8.74	10.54	0.48	4.54
	137	4.04	0.13	3.10	6.15	0.07	1.15	5.90	0.48	8.22	16.09	0.62	3.87
	145	3.93	0.09	2.23	6.03	0.26	4.32	1.54	0.92	59.64	11.49	0.73	6.38
Ethyl-2-methyl-acetoacetate	128	4.43	0.04	0.79	0.00	0.00		6.77	0.23	3.33	11.20	0.21	1.84
	134	4.48	0.15	3.29	6.49	0.25	3.79	5.36	0.86	16.09	16.33	0.82	5.05
	142	4.56	0.18	4.02	6.73	0.14	2.09	0.00	0.00		11.29	0.06	0.49
Ethyltrimethyl acetate	207	0.18	0.20	115.50	3.46	0.49	14.11	7.18	0.40	5.56	10.82	0.91	8.38
	211	3.06	0.34	11.07	3.94	0.61	15.34	7.51	0.47	6.32	14.51	1.07	7.38
	217	4.13	0.18	4.47	4.80	0.96	20.04	7.21	0.18	2.44	16.14	1.11	6.91
Fomesafen	117	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	123	0.00	0.00		0.00	0.00		1.07	0.62	58.36	1.07	0.62	58.36
	150	0.00	0.00		0.00	0.00		2.56	0.66	25.82	2.56	0.66	25.82
Glycerol	209	0.00	0.00		5.44	0.16	2.88	8.61	0.05	0.60	14.05	0.11	0.78
	216	0.00	0.00		6.77	0.04	0.52	8.78	0.03	0.39	15.55	0.07	0.44
	220	0.00	0.00		6.22	0.07	1.18	8.63	0.03	0.40	14.85	0.10	0.67
n-Hexanol	127	3.84	0.88	22.80	6.56	0.09	1.44	7.63	0.30	3.88	18.03	1.12	6.20
	133	4.39	0.06	1.37	6.92	0.02	0.33	6.00	0.37	6.14	17.31	0.43	2.47
	141	4.12	0.21	5.18	6.72	0.05	0.74	6.51	0.57	8.76	17.35	0.69	3.98
Imidazole	116	4.79	0.02	0.48	6.86	0.04	0.59	7.39	0.26	3.50	19.04	0.26	1.35
	121	4.64	0.11	2.28	6.91	0.03	0.36	7.73	0.09	1.12	19.28	0.18	0.95
	125	4.87	0.02	0.31	6.93	0.00	0.00	8.18	0.33	4.04	19.98	0.32	1.60
Isobutanol	127	4.42	0.11	2.50	6.76	0.05	0.77	8.15	0.20	2.45	19.33	0.26	1.35
	133	4.67	0.07	1.46	6.89	0.07	1.05	7.93	0.12	1.53	19.49	0.12	0.59
	141	4.73	0.11	2.28	6.92	0.05	0.74	7.75	0.32	4.18	19.40	0.41	2.11
Isopropanol	132	0.00	0.00		6.91	0.04	0.59	7.87	0.42	5.40	14.78	0.47	3.15
	137	0.00	0.00		6.97	0.02	0.25	8.05	0.08	0.94	15.02	0.06	0.40
	152	0.00	0.00		6.88	0.03	0.37	7.73	0.20	2.53	14.61	0.22	1.51
Methyl acetate	131	4.68	0.05	1.07	6.86	0.04	0.59	7.92	0.23	2.96	19.46	0.18	0.91

Substance	Test #	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
	137	4.30	0.19	4.34	6.97	0.04	0.52	8.10	0.36	4.49	19.37	0.52	2.68
	146	3.62	0.29	7.93	6.91	0.03	0.42	7.59	0.06	0.79	18.12	0.34	1.89
Methyl cyanoacetate	128	4.47	0.16	3.60	4.54	0.84	18.57	7.04	0.45	6.40	16.05	1.42	8.82
	142	4.82	0.06	1.33	6.57	0.08	1.22	3.83	1.00	26.08	15.23	1.00	6.59
	161	4.67	0.21	4.40	6.28	0.18	2.83	6.70	0.70	10.50	17.65	1.08	6.10
Methyl cyclopentane	128	3.59	0.75	20.84	6.84	0.06	0.86	8.16	0.24	2.92	18.59	0.56	3.01
	134	2.40	2.08	86.61	6.87	0.06	0.88	8.17	0.03	0.42	17.44	2.06	11.82
	151	1.45	2.51	173.21	6.70	0.17	2.50	7.23	0.35	4.79	15.38	2.95	19.17
Methyl ethyl ketone	205	4.96	0.01	0.23	6.68	0.08	1.13	8.58	0.03	0.35	20.22	0.10	0.47
	209	3.87	0.03	0.75	6.84	0.03	0.37	8.64	0.03	0.35	19.34	0.08	0.42
	213	0.00	0.00		6.85	0.04	0.53	8.64	0.08	0.92	15.49	0.12	0.74
Methyl isobutyl ketone	207	4.79	0.04	0.84	3.65	1.38	37.72	6.91	0.68	9.77	15.35	2.06	13.39
	211	4.86	0.04	0.83	6.35	0.09	1.38	8.48	0.02	0.20	19.68	0.09	0.44
	217	3.88	0.38	9.81	5.93	0.14	2.41	8.40	0.10	1.24	18.22	0.53	2.92
n-Octanol	205	4.68	0.14	2.97	5.26	0.27	5.14	7.69	0.35	4.56	17.63	0.74	4.20
	209	4.68	0.07	1.40	4.33	0.21	4.88	7.28	0.43	5.85	16.29	0.37	2.28
	213	4.02	0.33	8.28	5.64	0.37	6.65	6.53	0.06	0.98	16.18	0.02	0.13
Parafluoroaniline	131	2.15	1.86	86.67	6.85	0.01	0.17	8.21	0.30	3.68	17.21	1.61	9.36
	137	0.00	0.00		6.92	0.01	0.17	8.40	0.05	0.62	15.32	0.06	0.38
	145	0.00	0.00		6.92	0.01	0.17	7.04	0.65	9.18	13.96	0.66	4.70
PEG 400	210	0.00	0.00		6.22	0.83	13.27	7.83	1.09	13.94	14.05	1.92	13.64
	216	0.00	0.00		6.70	0.06	0.96	8.30	0.06	0.75	15.00	0.13	0.84
	219	0.00	0.00		6.41	0.29	4.50	8.58	0.14	1.60	14.99	0.42	2.78
Potassium cyanate	117	0.00	0.00		6.77	0.06	0.95	6.19	0.27	4.37	12.96	0.22	1.71
	122	0.00	0.00		6.86	0.02	0.29	8.22	0.12	1.46	15.08	0.10	0.66
	150	0.00	0.00		6.66	0.08	1.20	8.12	0.25	3.10	14.78	0.27	1.79
Pyridine	132	3.29	2.85	86.60	6.94	0.01	0.17	8.56	0.07	0.81	18.79	2.79	14.84
	140	0.00	0.00		6.91	0.04	0.59	8.10	0.08	0.98	15.01	0.08	0.51
	145	4.70	0.12	2.49	6.88	0.03	0.37	7.97	0.17	2.07	19.55	0.24	1.22
Promethazine	206	0.00	0.00		0.00	0.00		4.87	0.74	15.12	4.87	0.74	15.12
	214	0.00	0.00		2.32	1.27	54.85	3.92	0.42	10.72	6.24	0.92	14.72
	220	0.00	0.00		1.27	1.13	88.73	6.42	0.57	8.95	7.69	1.16	15.07

Substance	Test #	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Quinacrine	206	1.41	1.20	84.83	0.00	0.00		5.22	1.80	34.49	6.63	2.50	37.71
	214	1.46	0.23	15.68	0.00	0.00		5.96	0.14	2.38	7.42	0.35	4.69
	220	0.00	0.00		1.97	0.31	15.76	5.84	0.06	1.07	7.81	0.35	4.52
Sodium hydroxide (1%)	127	4.94	0.04	0.73	6.89	0.05	0.69	7.32	0.21	2.87	19.15	0.15	0.80
	132	4.94	0.01	0.23	6.94	0.01	0.17	7.10	0.41	5.77	18.99	0.40	2.11
	141	4.88	0.02	0.31	6.95	0.03	0.42	7.07	0.41	5.76	18.90	0.45	2.36
Sodium hydroxide (10%)	127	4.99	0.01	0.23	6.91	0.00	0.00	8.59	0.14	1.65	20.49	0.13	0.64
	132	4.99	0.01	0.23	7.00	0.00	0.00	8.65	0.09	1.06	20.64	0.08	0.39
	140	4.89	0.08	1.54	6.98	0.03	0.36	8.68	0.24	2.79	20.54	0.17	0.81
Sodium lauryl sulfate (3%)	130	0.00	0.00		6.71	0.18	2.65	5.77	0.82	14.15	12.48	0.78	6.25
	136	0.00	0.00		6.84	0.03	0.42	7.31	0.53	7.18	14.15	0.50	3.54
	143	0.00	0.00		6.81	0.11	1.54	3.70	1.07	28.83	10.51	1.17	11.15
Sodium lauryl sulfate (15%)	205	0.00	0.00		6.92	0.01	0.17	8.47	0.09	1.08	15.39	0.08	0.53
	210	0.00	0.00		6.88	0.04	0.51	8.01	0.32	3.96	14.89	0.33	2.21
	213	0.00	0.00		6.90	0.05	0.69	7.97	0.11	1.43	14.87	0.16	1.08
Sodium oxalate	116	3.87	0.24	6.20	6.45	0.12	1.80	3.07	1.07	34.99	13.39	1.28	9.55
	120	0.00	0.00		6.78	0.11	1.62	7.93	0.48	6.01	14.71	0.43	2.92
	125	0.00	0.00		6.77	0.04	0.52	7.74	0.36	4.65	14.51	0.34	2.35
Sodium perborate, 4H ₂ O	117	0.00	0.00		6.62	0.08	1.22	4.66	0.59	12.59	11.28	0.61	5.41
	121	0.00	0.00		6.76	0.08	1.12	6.71	0.31	4.59	13.47	0.24	1.79
	125	0.00	0.00		6.76	0.05	0.70	8.05	0.19	2.40	14.81	0.23	1.56
tetra-Aminopyrimidine sulfate	116	4.40	0.59	13.33	0.00	0.00		0.00	0.00		4.40	0.59	13.33
	120	4.07	0.43	10.50	0.00	0.00		0.00	0.00		4.07	0.43	10.50
	125	4.52	0.11	2.48	0.00	0.00		0.00	0.00		4.52	0.11	2.48
Thiourea	149	4.40	0.06	1.42	6.84	0.08	1.18	4.92	1.12	22.81	16.16	1.10	6.80
	121	4.00	0.06	1.56	6.90	0.02	0.25	7.56	0.36	4.83	18.46	0.34	1.85
	125	4.15	0.10	2.52	6.91	0.03	0.42	8.00	0.23	2.84	19.06	0.34	1.76
Toluene	207	0.00	0.00		6.70	0.04	0.52	8.46	0.05	0.61	15.16	0.06	0.40
	211	0.00	0.00		6.83	0.10	1.44	8.31	0.13	1.57	15.14	0.23	1.51
	217	4.01	0.63	15.67	6.87	0.04	0.52	7.08	0.34	4.81	17.96	0.29	1.61
Trichloroacetic acid (3%)	209	0.00	0.00		6.91	0.03	0.36	8.67	0.08	0.92	15.58	0.07	0.42
	216	0.00	0.00		6.98	0.03	0.36	8.89	0.06	0.70	15.87	0.09	0.55

Substance	Test #	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Trichloroacetic acid (30%)	217	0.00	0.00		6.94	0.02	0.33	8.78	0.05	0.52	15.72	0.07	0.43
	127	0.00	0.00		6.88	0.03	0.37	8.71	0.06	0.72	15.59	0.05	0.29
	133	0.00	0.00		6.91	0.05	0.68	8.89	0.06	0.70	15.80	0.11	0.68
	141	0.00	0.00		6.91	0.00	0.00	8.81	0.03	0.39	15.72	0.03	0.22
Triton X-100 (10%)	129	4.55	0.07	1.50	6.78	0.01	0.17	1.98	1.02	51.31	13.31	0.99	7.42
	135	3.71	0.55	14.73	6.90	0.02	0.25	3.65	1.15	31.54	14.26	1.26	8.81
	143	4.07	0.30	7.30	6.74	0.11	1.59	2.80	1.54	54.95	13.61	1.17	8.62
Triton X-100 (5%)	130	3.99	0.30	7.53	6.39	0.06	1.01	0.00	0.00		10.38	0.24	2.31
	143	4.27	0.13	3.11	6.67	0.06	0.96	0.91	0.80	87.86	11.86	0.82	6.92
	152	3.85	0.31	8.00	6.74	0.04	0.60	0.00	0.00		10.59	0.35	3.27
Maneb ³	117	0.00	0.00		5.09	0.29	5.72	0.00	0.00		5.09	0.29	5.72
	123	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	126	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
	150	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
1-Napthalene acetic acid ³	115	1.85	1.34	72.38	0.00	0.00		0.92	1.29	140.68	2.77	2.52	90.92
	118	2.19	0.38	17.19	4.00	0.44	11.11	3.90	1.04	26.55	10.09	1.41	14.02
	124	0.67	1.15	173.21	3.12	1.12	35.78	3.69	0.65	17.64	7.48	1.99	26.62
	149	2.84	0.60	21.03	0.00	0.00		0.00	0.00		2.84	0.60	21.03
1-Napthalene acetic acid, sodium salt ³	115	0.00	0.00		6.73	0.07	1.10	7.65	0.45	5.92	14.38	0.51	3.51
	124	0.00	0.00		6.92	0.02	0.33	8.44	0.14	1.68	15.36	0.15	0.98
	149	0.76	1.31	173.21	6.84	0.03	0.37	7.93	0.38	4.76	15.52	1.43	9.20
Tween 20 ³	130	0.00	0.00		6.42	0.16	2.54	7.05	0.89	12.69	13.47	1.04	7.73
	136	0.00	0.00		6.15	0.62	10.03	6.75	1.16	17.17	12.90	1.77	13.75
	144	0.00	0.00		6.88	0.04	0.51	8.89	0.06	0.70	15.77	0.10	0.62
	210	0.00	0.00		6.51	0.34	5.23	7.83	0.29	3.69	14.34	0.61	4.26
	219	1.97	0.33	16.78	5.95	0.63	10.54	8.33	0.12	1.46	16.25	0.46	2.83
Mean (SD) for All⁴		1.94 (2.12)			5.60 (2.31)			6.42 (2.68)			13.96 (4.89)		
Range for All		0.23-173.21			0.00-073.21			0.37-173.21			0.13-173.21		
%CV for All⁵		109.10			41.24			41.78			34.99		
Number of Experiments		184			184			184			184		
Mean (SD) Excluding Four Substances⁴		2.07 (2.16)			5.75 (2.19)			6.60 (2.49)			14.42 (4.48)		

Substance	Test #	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
		Mean for 3 Eggs	SD ¹ for 3 Eggs	%CV ² for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs	Mean for 3 Eggs	SD for 3 Eggs	%CV for 3 Eggs
Range Excluding Four Substances		0.23-173.21			0.00-073.21			0.37-173.21			0.13-173.21		
%CV Excluding Four Substances⁵		104.43			38.04			37.78			31.05		
Number of Experiments		168			168			168			168		
Mean Overall <i>In Vitro</i> Score %CV for All Substances		7.61											
Median Overall <i>In Vitro</i> Score %CV for All Substances		2.24											
Mean Overall <i>In Vitro</i> Score %CV Excluding Four Substances		6.99											
Median Overall <i>In Vitro</i> Score %CV Excluding Four Substances		2.04											

3271 ¹SD = standard deviation.3272 ²%CV = percent coefficient of variation.3273 ³Data not originally presented in Gilleron et al. (1997).3274 ⁴Mean was calculated using the values from the "Mean for 3 Eggs" column for each endpoint and the Overall *In Vitro* Score. The standard deviation was
3275 calculated based on the values in these individual columns.3276 ⁵To avoid eliminating data for which the %CV value could not be calculated (i.e., where the mean and SD both equaled 0), the %CV values were calculated
3277 using the mean and standard deviation calculated as described in footnote 4 of this table.

3.3 Reanalysis of HET-CAM Test Method Intralaboratory Reproducibility

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The authors of two articles provided HET-CAM IS(B) data that could be used to evaluate intralaboratory reproducibility (Gilleron et al. [1996, 1997]). For both sets of studies, quantitative endpoint HET-CAM test method data were available for studies repeated three to five times in a single laboratory.

3.3.1 Gilleron et al. (1996)

Individual experimental results for 46 substances evaluated by the HET-CAM IS(B) analysis method and reported on by Gilleron et al. (1996) were received from Dr. P. Vanparys and Dr. F. Van Goethem in response to a request from NICEATM. In the data provided to NICEATM, the test results for nine of the 46 substances included in the 1996 publication (laurylsulfobetaine, deoxycholic acid, ethylacetoacetate, methyl isobutyl ketone, methanol, N-laurylsarcosine, promethazine hydrochloride, 2-methoxyethanol, benzethonium chloride, and imidazole) were no longer available. Since alternative HET-CAM test data generated by this laboratory were available for these substances, these data were provided to NICEATM. The overall replicate experiment mean and median %CV values were evaluated with and without the inclusion of these data.

In these studies, three different eggs were used for each experiment, with three experiments conducted for each test substance, except for the nine substances that were not part of the original data set used for the 1996 publication. For these nine substances, data for three to five experiments were provided.

For each of the endpoints, there were a number of experiments where the test substance did not induce any effects (i.e., the average score of the repeated experiments and SD of the scores were both 0) (see **Table IV-13**). For the hemorrhage endpoint, 12 of 46 (26%) substances resulted in an average and SD of zero for the repeated experiments, the lysis endpoint 14 of 46 (30%) substances resulted in an average and SD of zero for the repeated experiments, and for the coagulation endpoint, 6 of the 46 (13%) substances resulted in an average and SD of zero for the repeated experiments. For the overall *in vitro* IS(B) score, three of 46 experiments (7%) resulted in an average score and SD of zero for the repeated experiments. One test substance (EDTA) produced no response in any of the three endpoint evaluated in the three replicate eggs in each of replicate experiments. The reproducibility %CV values for individual substances, excluding studies where such values could not be calculated, ranged from 2.59 to 173.21 for hemorrhage, from 1.55 to 173.21 for lysis, from 1.52 to 173.21 for coagulation, and from 6.66 to 173.21 for the overall *in vitro* IS(B) score.

The mean and median reproducibility %CV values for the overall *in vitro* IS(B) scores for the entire data set (last column in **Table IV-13**), including the data for the nine substances previously noted and excluding studies where such values could not be calculated, were 52.73 and 33.70, respectively. When the data for the nine substances noted were removed, the mean and median reproducibility %CV values for the overall IS(B) scores were 60.66 and 39.15, respectively.

3322 **Table IV-13. Intralaboratory Reproducibility Results for HET-CAM IS(B) Data of Gilleron et al. (1996)**

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Chemical	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
	Mean of Exp. ¹	SD ² of Exp.	%CV ³ of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.
1,2,3-Trichloropropane	2.50	1.72	68.87	1.55	2.03	130.62	3.80	2.52	66.20	7.85	1.74	22.19
1,2,4-Trimethylbenzene	0.33	0.31	94.63	1.63	2.83	173.21	2.46	1.61	65.17	4.42	2.91	65.81
1-Nitropropane	0.58	0.67	115.89	0.52	0.89	173.21	1.72	1.49	86.69	2.81	2.70	95.85
1-Phenyl-3-pyrazolidone	0.00	0.00		0.00	0.00		0.46	0.41	89.00	0.46	0.41	89.00
2,4-Dichloro-5-sulfamoyl-benzoic acid	3.37	1.63	48.34	0.00	0.00		0.40	0.70	173.21	3.77	2.21	58.68
2,4-Pentanedione	4.32	0.13	2.92	0.00	0.00		4.48	2.86	63.70	8.80	2.98	33.85
2-Aminophenol	0.00	0.00		0.00	0.00		0.84	0.74	88.82	0.84	0.74	88.82
2-Ethoxyethanol	0.89	0.91	102.38	2.02	3.14	155.52	7.55	1.22	16.17	10.46	2.74	26.23
3-Glycidopropyl trimethoxysilane	3.72	0.15	4.17	0.00	0.00		0.60	1.03	173.21	4.31	0.96	22.34
Allyl alcohol	0.21	0.36	173.21	3.85	2.44	63.34	8.37	0.41	4.84	12.42	2.26	18.21
Anthracene	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Butyrolactone	4.88	0.14	2.86	1.52	2.63	173.21	7.47	0.69	9.25	13.86	1.80	12.98
Cyclohexanone	4.54	0.17	3.84	4.02	3.53	87.78	5.90	3.21	54.40	14.47	3.79	26.23
Deoxycholic acid, sodium salt	1.08	0.99	92.34	5.84	0.27	4.64	2.78	4.82	173.21	9.70	5.81	59.92
Diacetone alcohol	4.51	0.62	13.84	3.89	3.38	86.87	6.65	1.43	21.49	15.05	2.74	18.18
Dibenzoyl-L-tartaric acid	3.64	1.96	53.75	0.53	0.92	173.21	0.00	0.00		4.17	2.53	60.52
Dimethyl biguanidine	0.00	0.00		3.49	3.26	93.37	4.94	4.33	87.61	8.44	2.18	25.90
Dimethyl sulfoxide	0.13	0.23	173.21	4.18	3.62	86.65	6.95	1.57	22.53	11.26	3.74	33.27
DL-Glutamic acid	0.68	1.17	173.21	0.00	0.00		0.00	0.00		0.68	1.17	173.21
Ethanol	0.00	0.00		4.02	3.49	86.81	7.30	1.44	19.73	11.32	2.82	24.92
Ethylenediaminetetraacetic acid dipotassium	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Furan	0.31	0.53	173.21	2.41	2.39	99.17	2.80	2.05	73.08	5.51	1.02	18.44
Gluconolactone	0.00	0.00		1.83	1.72	93.82	2.72	4.72	173.21	4.55	3.20	70.34
Hexadecyltrimethylammonium bromide	2.47	2.31	93.82	5.13	1.08	21.06	3.24	3.91	120.45	10.84	4.82	44.46
Hexane	0.41	0.72	173.21	0.94	1.63	173.21	1.73	2.52	145.61	3.08	2.65	86.07
Iminodibenzyl	0.00	0.00		0.00	0.00		0.03	0.05	173.21	0.03	0.05	173.21

Chemical	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
	Mean of Exp. ¹	SD ² of Exp.	%CV ³ of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.
Magnesium carobonate	0.00	0.00		0.00	0.00		1.43	1.86	129.93	1.43	1.86	129.93
Methyl isobutyl ketone	3.11	0.90	28.98	3.00	3.34	111.51	4.38	3.84	87.64	10.49	1.50	14.33
MYRJ 45	0.00	0.00		0.00	0.00		0.00	0.00		0.00	0.00	
Octanol	2.98	0.85	28.36	3.69	2.12	57.27	3.17	1.19	37.48	9.84	1.67	17.01
Polyoxythethylene 23 lauryl ether	1.51	2.61	173.21	0.00	0.00		0.00	0.00		1.51	2.61	173.21
Propyl-4-hydroxybenzoate	1.67	1.00	60.02	0.00	0.00		0.21	0.19	91.97	1.88	1.06	56.26
Pyridine	3.99	0.89	22.35	4.54	3.94	86.62	7.24	1.47	20.31	15.77	3.61	22.90
Quinacrine	3.18	2.69	84.83	1.24	2.15	173.21	2.08	2.74	131.92	6.50	2.19	33.70
Tetraaminopyrimidine sulfat	0.53	0.92	173.21	0.00	0.00		0.00	0.00		0.53	0.92	173.21
Thiourea	3.91	0.10	2.59	6.71	0.10	1.55	2.49	4.32	173.21	13.11	4.39	33.49
Triton X-155	1.01	1.53	152.23	2.57	2.39	93.09	0.34	0.59	173.21	3.92	2.34	59.77
2-Methoxyethanol ⁴	0.00	0.00		5.61	0.45	7.95	7.62	1.32	17.35	13.22	1.12	8.43
Benzethonium chloride ⁴	2.28	2.63	115.61	4.79	3.21	67.17	5.49	3.50	63.81	12.55	4.55	36.23
Ethylacetoacetate ⁴	3.71	0.47	12.74	2.01	2.34	116.49	2.85	2.59	90.61	8.57	4.59	53.62
Imidazole ⁴	4.75	0.19	3.91	6.26	0.98	15.71	7.48	1.21	16.23	18.49	1.23	6.66
Laurylsulfobetaine ⁴	0.07	0.13	173.21	6.23	0.56	9.07	2.22	1.97	88.59	8.53	1.50	17.60
Methanol ⁴	0.00	0.00		4.33	3.24	74.82	7.68	1.27	16.57	12.01	2.99	24.87
N-Laurylsarcosine, sodium salt ⁴	1.76	1.33	75.79	6.53	0.30	4.66	3.54	3.82	107.96	11.82	4.03	34.05
Promethazine hydrochloride ⁴	0.65	1.29	200.00	5.03	3.35	66.67	8.44	0.13	1.52	14.11	1.97	13.97
Triethanolamine ⁴	0.00	0.00		5.43	1.92	35.28	6.69	0.94	14.01	12.12	1.18	9.71
Mean (SD) for All⁵	1.64 (2.04)			2.68 (2.96)			3.59 (3.52)			7.51 (5.28)		
Range for All	2.59-173.21			1.55-173.21			1.52-173.21			6.66-173.21		
%CV for All⁶	124.12			110.41			97.92			70.35		
Mean (SD) Excluding Nine Substances⁵	1.63 (2.01)			1.87 (2.66)			2.83 (3.34)			6.33 (5.06)		
Range Excluding Nine Substances	2.59-173.21			1.55-173.21			4.84-173.21			14.33-173.21		
%CV Excluding Nine Substances⁶	123.08			142.31			118.37			79.92		
Mean Overall <i>In Vitro</i> Score %CV for All Substances	52.73											
Median Overall <i>In Vitro</i> Score	33.70											

Chemical	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
	Mean of Exp. ¹	SD ² of Exp.	%CV ³ of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.
%CV for All Substances												
Mean Overall <i>In Vitro</i> Score %CV Excluding Nine Substances												
60.66												
Median Overall <i>In Vitro</i> Score %CV Excluding Nine Substances												
39.15												

3324

¹Exp. = experiment

3325

²SD = standard deviation

3326

³%CV = percent coefficient of variation

3327

⁴Data not originally presented in Gilleron et al. (1996).

3328

⁵Mean was calculated using the values from the "Mean for 3 Eggs" column for each endpoint and the Overall *In Vitro* Score. The standard deviation was calculated based on the values in these individual columns.

3329

3330

⁶To avoid eliminating data for which the %CV value could not be calculated (i.e., where the mean and SD both equaled 0), the %CV values were calculated using the mean and standard deviation calculated as described in footnote 5 of this table.

3331

3332 3.3.2 Gilleron et al. (1997)

3333 Individual experimental results for 60 substances evaluated by the HET-CAM IS(B) analysis
3334 method and reported on by Gilleron et al. (1997) were provided by the authors to NICEATM.
3335 Among the data, the test results for four of the 60 substances included in the 1997 publication
3336 (Maneb, 1-naphthalene acetic acid, Tween 20, and 1-naphthalene acetic acid, sodium salt) were
3337 no longer available. Since alternative HET-CAM test data were available for these
3338 substances, these data were provided to NICEATM. The overall replicate egg mean and
3339 median %CV values were evaluated with and without the inclusion of these data.

3340
3341 In these studies, three different eggs were used for each experiment, with generally three
3342 experiments conducted for each test substance, except for the four substances that were not
3343 part of the original data set used for the publication. For these four substances, data for three
3344 to five test runs were provided. For this evaluation, the %CV values were determined for
3345 each endpoint evaluated (hemorrhage, lysis, coagulation) and for the overall *in vitro* IS(B)
3346 score. For each of the endpoints, there were a number of experiments where the test
3347 substance did not induce any effects (i.e., the average score of the three replicate eggs and
3348 thus the SD of the scores were both zero) (see **Table IV-14**). For the hemorrhage endpoint,
3349 25 of 60 substances (42%) resulted in an average score and SD of zero for the three replicate
3350 eggs; for the lysis endpoint, 3 of 60 substances (5%) resulted in an average score and SD of
3351 zero for the three replicate eggs; while, for the coagulation endpoint, 2 of 60 substances (3%)
3352 resulted in an average score and SD of zero for the three replicate eggs. For the overall *in*
3353 *vitro* IS(B) score, none of substances resulted in an average score and SD of zero for the
3354 three replicate eggs. The reproducibility %CV values for individual substances, excluding
3355 studies where such values could not be calculated, ranged from 0.20 to 173.21 for
3356 hemorrhage, from 0.12 to 200.00 for lysis, from 0.00 to 173.21 for coagulation, and from
3357 0.34 to 200.00 for the overall *in vitro* IS(B) score.

3358
3359 The mean and median reproducibility %CV values for the overall *in vitro* IS(B) scores for
3360 the entire data set (last column in **Table IV-14**), including the data for the nine substances
3361 previously noted and excluding studies where such values could not be calculated, were
3362 17.48 and 6.34, respectively. When the data for the nine substances noted were removed, the
3363 mean and median reproducibility %CV values for the overall IS(B) scores were 13.49 and
3364 5.25, respectively. Calculations of the %CV values using only substances identified as GHS
3365 Category 1 (UN [2003]) or EPA Category 1 (EPA [1996]) are similar to those described
3366 above.

3367
3368 **Table IV-14. Intralaboratory Reproducibility Results for HET-CAM IS(B) Data of Gilleron et al. (1997)**

Chemical	GHS ¹ Cat. ² 1	EPA ³ Cat. I	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
			Mean of Exp. ⁴	SD ⁵ of Exp.	%CV ⁶ of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.
2,2-Dimethyl- butanoic acid		X	0.00	0.00		6.86	0.13	1.90	8.45	0.15	1.77	15.31	0.24	1.59
2,5-Dimethyl- hexandiol	X	X	4.18	0.19	4.45	5.89	1.17	19.79	5.26	1.12	21.20	15.33	0.39	2.52
2,6-Dichloro- benzoyl chloride			0.00	0.00		5.97	0.85	14.20	4.77	1.46	30.58	10.74	1.74	16.22
2-Ethyl-1-hexanol			4.05	0.13	3.18	4.06	3.51	86.62	4.60	2.66	57.83	12.71	2.96	23.33
4-Carboxybenz- aldehyde			2.17	1.95	90.08	0.00	0.00		1.78	3.08	173.21	3.95	4.69	118.7 5
Acetone			0.00	0.00		6.94	0.05	0.65	8.38	0.34	4.03	15.32	0.32	2.12
Ammonium nitrate			1.03	0.65	62.49	6.83	0.06	0.87	7.48	1.04	13.88	15.34	1.24	8.07
Benzalkonium chloride (1%)	X	X	0.00	0.00		6.91	0.03	0.46	8.24	0.25	3.04	15.15	0.23	1.52
Benzalkonium chloride (10%)	X	X	0.00	0.00		6.91	0.02	0.24	8.14	0.28	3.46	15.05	0.28	1.83
Benzalkonium chloride (5%)	X	X	0.00	0.00		6.93	0.01	0.15	7.88	0.53	6.67	14.80	0.53	3.55
Captan 90 concentrate	X	X	0.00	0.00		6.83	0.03	0.41	8.24	0.29	3.58	15.07	0.27	1.80
Cetylpyridinium bromide (0.1%)			0.00	0.00		6.05	0.25	4.05	6.63	1.33	20.03	12.67	1.51	11.95
Cetylpyridinium bromide (10%)	X	X	0.00	0.00		6.89	0.01	0.12	6.64	2.41	36.35	13.53	2.42	17.89
Cetylpyridinium bromide (6%)	X		0.00	0.00		6.84	0.04	0.55	7.77	0.40	5.11	14.61	0.43	2.95
Chlorhexidine	X		4.48	0.15	3.41	5.81	0.21	3.59	4.58	3.99	87.12	14.86	3.78	25.44
Cyclohexanol	X	X	4.29	0.30	7.03	6.85	0.03	0.42	7.81	0.34	4.31	18.96	0.10	0.53
Dibenzoyl-L-tartaric acid	X		4.54	0.29	6.36	3.35	2.90	86.63	5.45	1.30	23.80	13.34	4.24	31.74
Dibenzyl phosphate			0.00	0.00		6.89	0.08	1.14	8.27	0.39	4.71	15.17	0.47	3.07

Chemical	GHS ¹ Cat. ² 1	EPA ³ Cat. I	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
			Mean of Exp. ⁴	SD ⁵ of Exp.	%CV ⁶ of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.
Ethanol			0.00	0.00		6.89	0.04	0.51	8.01	0.00	0.00	14.90	0.04	0.24
Ethyl acetate			4.33	0.49	11.38	6.66	0.14	2.13	8.33	0.39	4.74	19.32	0.07	0.34
Ethyl-2-methyl- acetoacetate			4.49	0.07	1.46	4.40	3.82	86.65	4.04	3.57	88.34	12.94	2.94	22.68
Ethytrimethyl acetate			2.45	2.04	83.27	4.07	0.68	16.68	7.30	0.18	2.50	13.82	2.73	19.72
Fomesafen			0.00	0.00		0.00	0.00		1.21	1.29	106.26	1.21	1.29	106.2 6
Gamma-butyro- lactone			4.93	0.01	0.20	6.95	0.01	0.14	7.52	0.83	11.00	19.39	0.83	4.29
Glycerol			0.00	0.00		6.14	0.67	10.93	8.67	0.09	1.07	14.82	0.75	5.09
Imidazole			4.77	0.12	2.43	6.90	0.04	0.54	7.77	0.40	5.10	19.43	0.49	2.51
Isobutanol	X	X	4.61	0.17	3.60	6.86	0.09	1.26	7.94	0.20	2.52	19.41	0.08	0.43
Isopropanol			0.00	0.00		6.92	0.04	0.65	7.88	0.16	2.03	14.80	0.20	1.38
L-Aspartic Acid			0.00	0.00		0.43	0.74	173.21	0.63	0.56	89.05	1.06	1.20	113.4 9
Methyl acetate			4.20	0.54	12.83	6.91	0.06	0.82	7.87	0.26	3.29	18.98	0.75	3.94
Methyl cyanoacetate			4.65	0.18	3.84	5.80	1.10	18.97	5.86	1.76	30.11	16.31	1.23	7.56
Methyl cyclopentane			2.48	1.07	43.31	6.80	0.09	1.31	7.85	0.54	6.87	17.14	1.62	9.48
Methyl ethyl ketone			2.94	2.60	88.56	6.79	0.09	1.36	8.62	0.03	0.40	18.35	2.52	13.71
Methyl isobutyl ketone			4.51	0.54	12.07	5.31	1.45	27.39	7.93	0.88	11.15	17.75	2.20	12.42
n-Butyl acetate			4.59	0.30	6.57	4.95	2.06	41.57	7.66	0.28	3.60	17.21	1.84	10.69
n-Hexanol			4.12	0.27	6.64	6.74	0.18	2.68	6.71	0.83	12.42	17.57	0.41	2.31
n-Octanol			4.46	0.38	8.61	5.08	0.67	13.19	7.17	0.59	8.25	16.70	0.81	4.83
Parafluoroaniline			0.72	1.24	173.21	6.90	0.04	0.64	7.88	0.74	9.34	15.50	1.63	10.51
PEG 400			0.00	0.00		6.44	0.24	3.70	8.24	0.38	4.60	14.68	0.54	3.70
Potassium cyanate			0.00	0.00		6.76	0.10	1.51	7.51	1.14	15.24	14.27	1.15	8.05
Promethazine	X	X	0.00	0.00		1.20	1.16	97.13	5.07	1.26	24.89	6.27	1.41	22.50
Pyridine	X	X	2.66	2.41	90.56	6.91	0.03	0.44	8.21	0.31	3.78	17.78	2.43	13.69
Quinacrine	X	X	0.96	0.83	86.65	0.66	1.14	173.21	5.67	0.40	7.00	7.29	0.60	8.24
Sodium hydroxide			4.92	0.03	0.69	6.93	0.03	0.43	7.16	0.14	1.91	19.01	0.13	0.68

Chemical	GHS ¹ Cat. ² 1	EPA ³ Cat. I	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
			Mean of Exp. ⁴	SD ⁵ of Exp.	%CV ⁶ of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.
(1%)														
Sodium hydroxide (10%)	X	X	4.95	0.06	1.17	6.96	0.05	0.67	8.64	0.05	0.53	20.56	0.08	0.37
Sodium lauryl sulfate (15%)	X	X	0.00	0.00		6.90	0.02	0.29	8.15	0.28	3.41	15.05	0.29	1.96
Sodium lauryl sulfate (3%)			0.00	0.00		6.79	0.07	0.99	5.59	1.81	32.39	12.38	1.83	14.74
Sodium oxalate	X	X	1.29	2.24	173.21	6.67	0.19	2.84	6.25	2.75	44.07	14.20	0.71	5.01
Sodium perborate, 4H ₂ O	X	X	0.00	0.00		6.71	0.08	1.15	6.47	1.71	26.38	13.19	1.78	13.49
Tetraamino-pyrimidine sulfate			4.33	0.23	5.40	0.00	0.00		0.00	0.00		4.33	0.23	5.40
Thiourea			4.18	0.20	4.83	6.88	0.04	0.60	6.83	1.67	24.40	17.89	1.53	8.57
Toluene			1.34	2.31	173.21	6.80	0.09	1.28	7.95	0.76	9.52	16.09	1.62	10.07
Trichloroacetic acid (3%)			0.00	0.00		6.94	0.04	0.51	8.78	0.11	1.25	15.72	0.15	0.92
Trichloroacetic acid (30%)	X	X	0.00	0.00		6.90	0.02	0.24	8.80	0.09	1.02	15.71	0.11	0.67
Triton X-100 (10%)	X		4.11	0.42	10.26	6.81	0.08	1.20	2.81	0.84	29.72	13.73	0.48	3.52
Triton X-100 (5%)			4.04	0.22	5.34	6.60	0.19	2.80	0.30	0.53	173.21	10.94	0.80	7.28
1-Napthalene acetic acid ⁷	X	X	1.89	0.91	48.25	1.78	2.09	117.21	2.13	1.96	92.30	5.79	3.61	62.36
1-Napthalene acetic acid, sodium salt ⁷	X	X	0.25	0.44	173.21	6.83	0.10	1.44	8.01	0.40	5.00	15.09	0.62	4.12
Maneb ⁷			0.00	0.00		1.27	2.54	200.00	0.00	0.00		1.27	2.54	200.00
Tween 20 ⁷			0.39	0.88	223.61	6.38	0.35	5.53	7.77	0.88	11.39	14.55	1.44	9.91
Mean (SD) for All⁸			1.94 (2.12)			5.60 (2.31)			6.42 (2.68)			13.96 (4.89)		
Range for All			0.20-173.20			0.12-200.00			0.00-173.21			0.34-200.00		
%CV for All⁹			109.10			41.24			41.78			35.00		
Mean (SD) Excluding Four Substances⁸			2.07 (2.16)			5.75 (2.18)			6.60 (2.50)			14.42 (4.48)		

Chemical	GHS ¹ Cat. ² 1	EPA ³ Cat. I	Hemorrhage			Lysis			Coagulation			Overall <i>In Vitro</i> Score		
			Mean of Exp. ⁴	SD ⁵ of Exp.	%CV ⁶ of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.	Mean of Exp.	SD of Exp.	%CV of Exp.
Range Excluding Four Substances			0.20-173.21			0.12-173.21			0.00-173.21			0.34-118.75		
%CV Excluding Four Substances⁹			104.43			38.04			37.78			31.05		
Mean Overall <i>In Vitro</i> Score %CV for All Substances			17.48											
Median Overall <i>In Vitro</i> Score %CV for All Substances			6.34											
Mean Overall <i>In Vitro</i> Score %CV Excluding Nine Substances			13.49											
Median Overall <i>In Vitro</i> Score %CV Excluding Nine Substances			5.25											

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¹GHS = Globally Harmonized System

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²Cat. = category

3371

³EPA = U.S. Environmental Protection Agency

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⁴Exp. = experiment

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⁵SD = standard deviation

3374

⁶%CV = percent coefficient of variation

3375

⁷Data not originally presented in Gilleron et al. (1997).

3376

⁸Mean was calculated using the values from the "Mean for 3 Eggs" column for each endpoint and the Overall *In Vitro* Score. The standard deviation was

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calculated based on the values in these individual columns.

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⁹To avoid eliminating data for which the %CV value could not be calculated (i.e., where the mean and SD both equaled 0), the %CV values were calculated

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using the mean and standard deviation calculated as described in footnote 8 of this table.

3.4 Reanalysis of HET-CAM Test Method Interlaboratory Reproducibility

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Generally, an analysis of interlaboratory variability has included such approaches as:

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- the extent of concordance among laboratories in assigning the same regulatory classification for a particular substance (e.g., Holzhütter et al. [1996])

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- a CV analysis, which is a statistical measure of the deviation of a variable from its mean (e.g., Holzhütter et al. [1996])

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- ANOVA (e.g., Holzhütter et al. [1996]; ASTM [1999]), which can be used to determine if the test results obtained for an individual laboratory is significantly different from those obtained from the other laboratories

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- bivariate scatter diagrams/correlation analyses for pairs of laboratories to assess the extent of divergence (e.g., Holzhütter et al. [1996])

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Several of the studies included interlaboratory data for at least a subset of the substances evaluated. Using this data, the ability of the HET-CAM test method to reproducibly identify ocular corrosives and severe irritants versus nonsevere irritants (i.e., moderate and slight irritant) and nonirritants was evaluated using two approaches.

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In the first approach, a qualitative assessment of reproducibility was conducted. In this evaluation, the individual laboratory *in vitro* ocular irritation classification for each substance was used to evaluate the extent of agreement among the participating laboratories in their ability to identify ocular corrosives/severe irritants versus nonsevere irritants/nonirritants.

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The reliability of HET-CAM was assessed separately for each study (i.e., publication) with multiple laboratory data (see CEC [1991]; Balls et al. [1995]; Spielmann et al. [1996];

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Hagino et al. [1999]). In an alternative approach, the reliability of HET-CAM was assessed after pooling data across comparative studies that used the same data analysis method (e.g.,

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IS(B)).

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Substances classified, based on HET-CAM test data, as corrosive/severe irritants or nonsevere irritants/nonirritants were further classified by their *in vivo* rabbit eye test results, as determined within the GHS (UN [2003]), EPA (1996), and EU (2001) classification systems.

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Because the focus of this reliability assessment is on the interlaboratory reproducibility of HET-CAM test method in identifying corrosives/severe irritants versus nonsevere

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irritants/nonirritants, considerable variability could exist among laboratories in their

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classification of substances as nonsevere irritants or nonirritants. For example, three

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laboratories could classify a chemical as a nonirritant and one laboratory could classify the

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same chemical as a moderate irritant. Within this analysis, where a nonirritant and moderate

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3423

irritant classification would be placed together, this distribution of classification calls would

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be considered as 100% agreement between laboratories.

In the second approach, a quantitative assessment of reproducibility was determined. %CVs for test substances, where laboratory scores were available, for substances tested were reported or determined. The reproducibility of HET-CAM was assessed for studies (i.e.,

3425 publication) where individual testing laboratory data was available (see CEC [1991]; Balls et
3426 al. [1995]; Spielmann et al. [1996]; Hagino et al. [1999]).

3427
3428 As discussed in Section 2.0 of the draft HET-CAM BRD, there is no standardized data
3429 collection method for HET-CAM studies and several different analysis methods have been
3430 developed (i.e., IS, Q-Score, S-Score). Therefore, the reliability assessments conducted in
3431 this section were evaluated according to each of the analysis methods described.

3432

3433 3.4.1 Qualitative Reanalysis of Interlaboratory Reproducibility

3434 3.4.1.1 *GHS Ocular Hazard Classification System*

3435 Interlaboratory reproducibility for the HET-CAM test method was evaluated for the
3436 following reports: Balls et al. (1995), Spielmann et al. (1996) and Hagino et al. (1999). The
3437 agreement of classification calls among participating laboratories and its relationship to the
3438 GHS *in vivo* classification (UN [2003]) for the substances tested in each report is provided in
3439 **Table IV-15**.

3440

3441 The participating laboratories were in 100% agreement in regard to the GHS ocular irritancy
3442 classification for 21 (45%) of the 47 substances tested when using the Q-Score (Balls et al.
3443 1995). The extent of agreement between testing laboratories was greatest for substances
3444 identified from *in vivo* rabbit eye data as GHS corrosives or severe irritants when compared
3445 to any other combination of *in vivo* and *in vitro* results (60% [9/15] accurately identified
3446 severe substances were shown to have 100% classification agreement among testing
3447 laboratories). Comparatively, greater disparity between individual substance classifications
3448 was observed for substances that were identified as false positives (i.e., positive *in vitro* but
3449 negative *in vivo*) and those substances accurately classified as nonsevere irritants. For
3450 instance, 56% (9/16) of the false positives and 58% (7/12) of the correctly identified
3451 nonsevere irritants exhibited less than 100% agreement in the GHS irritancy classifications
3452 among laboratories.

3453

3454 In addition to the Q-Score, Balls et al. (1995) evaluated irritancy potential for some
3455 substances by using an S-Score. The participating laboratories were in 100% agreement in
3456 regard to the GHS ocular irritancy classification (corrosive/severe irritant or nonsevere
3457 irritant/nonirritant) for 13 (68%) of the 19 tested substances. Substances that were classified
3458 as false negatives (i.e., negative *in vitro* but positive not *in vivo*) and false positives were
3459 shown to exhibit the most discordant results, with 29% (2/7) of the false negatives and 100%
3460 (2/2) of the false positives exhibiting less than 100% classification agreement between testing
3461 laboratories. There was 100% agreement among testing laboratories for substances classified
3462 as severe irritants or nonsevere/nonirritants, based on the GHS classification system (UN
3463 2003).

3464 **Table IV-15. Evaluation of the Reliability of the HET-CAM Test Method In Predicting Ocular Corrosives and Severe**
 3465 **Irritants as Defined by the GHS¹ Classification System, by Study**
 3466

Report	Anal ²	Classification (<i>In Vivo/In Vitro</i>) ³	# of Labs	N ⁴	Substances with 100% Agreement among Labs	Substances with 80% Agreement among Labs	Substances with 75% Agreement among Labs	Substances with 66% Agreement among Labs	Substances with 60% Agreement among Labs	Substances with ≤50% Agreement among Labs
Balls et al. (1995)	Q	+/+	2 4	4 11	3 (75%) ⁵ 6 (55%)	-	- 4 (36%)	-	-	1 (25%) 1 (9%)
		+/-	-	-	-	-	-	-	-	-
		-/+	4	16	4 (25%)	-	9 (56%)	-	-	3 (19%)
		-/-	2 4	1 11	1 (100%) 4 (36%)	-	- 7 (64%)	-	-	-
		?/-	2	1	1 (100%)	-	-	-	-	-
		?/+	3 4	1 2	1 (100%) 1 (50%)	-	- 1 (50%)	-	-	-
		Total	2-4	47	21 (45%)	-	21 (45%)	-	-	5 (10%)
Balls et al. (1995)	S	+/+	2	4	4 (100%)	-	-	-	-	-
		+/-	2 3 4	1 4 2	1 (100%) 2 (50%) 2 (100%)	-	-	- 2 (50%) -	-	-
		-/+	2 4	1 1	-	-	-	-	-	1 (100%) 1 (100%)
		-/-	3 4	1 2	1 (100%) 2 (100%)	-	-	-	-	-
		?/-	3	1	-	-	-	1 (100%)	-	-
		?/+	2	2	1 (50%)	-	-	-	-	1 (50%)
		Total	2-4	19	13 (68%)	-	-	3 (16%)	-	3 (16%)
Spielmann et al. (1996)	IS(B) -10	+/+	2 3	18 1	16 (89%) -	-	-	- 1 (100%)	-	2 (11%) -
		+/-	2 3	4 1	4 (100%) -	-	-	- 1 (100%)	-	-
		-/+	2 3	16 2	7 (44%) 1 (50%)	-	-	-	-	9 (56%) 1 (50%)
		-/-	2 3	31 2	30 (97%) 1 (50%)	-	-	- 1 (50%)	-	1 (3%) -

Report	Anal ²	Classification (<i>In Vivo/In Vitro</i>) ³	# of Labs	N ⁴	Substances with 100% Agreement among Labs	Substances with 80% Agreement among Labs	Substances with 75% Agreement among Labs	Substances with 66% Agreement among Labs	Substances with 60% Agreement among Labs	Substances with ≤50% Agreement among Labs
		?/-	2	10	10 (100%)	-	-	-	-	-
			3	2	1 (50%)	-	-	1 (50%)	-	-
		?/+	2	16	14 (88%)	-	-	-	-	2 (11%)
			3	4	1 (25%)	-	-	2 (50%)	-	1 (25%)
		Total		107	85 (79%)			5 (5%)		16 (15%)
Spielmann et al. (1996)	IS(B) -100	+/+	2	17	16 (94%)	-	-	-	-	1 (6%)
			3	2	1 (50%)	-	-	1 (50%)	-	-
		+/-	2	2	2 (100%)	-	-	-	-	-
			3	4	1 (25%)	-	-	-	-	7 (26%)
		-/+	2	27	20 (74%)	-	-	-	-	-
			3	4	1 (25%)	-	-	3 (75%)	-	-
		-/-	2	17	16 (94%)	-	-	-	-	1 (6%)
			3	2	2 (100%)	-	-	-	-	-
?/-	2	6	6 (100%)	-	-	-	-	-		
	3	2	2 (100%)	-	-	-	-	-		
?/+	2	18	15 (83%)	-	-	-	-	-	3 (17%)	
	3	4	2 (50%)	-	-	2 (50%)	-	-	-	
		Total		99	81 (82%)			6 (6%)		12 (12%)
Hagino et al. (1999)	IS(A)	+/+	5	8	5 (63%)	2 (25%)	-	-	1 (12%)	-
		+/-	-	-	-	-	-	-	-	-
		-/+	5	3	3 (100%)	-	-	-	-	-
		-/-	5	4	1 (25%)	1 (25%)	-	-	2 (50%)	-
		?/-	-	-	-	-	-	-	-	-
		?/+	5	2	2 (100%)	-	-	-	-	-
				Total	2-4	17	11 (64%)	3 (18%)	-	3 (18%)

3467 ¹GHS = Globally Harmonized System (UN [2003]).

3468 ²Anal = analysis method used to transform the sample data into HET-CAM scores. IS(A) = method described in Luepke (1985); Q = Q-Score, method described
3469 in Balls et al. (1995); S = S-Score, method described in Balls et al. (1995).

3470 ³A “+” indicates that the substance was assigned an overall classification of corrosive or a severe irritant (Category 1); a “-” indicates that the substance was
3471 assigned an overall classification of nonsevere irritant (Category 2A or 2B) or nonirritant; a “?” indicates that, due to the lack of appropriate *in vivo* data (e.g.,
3472 studies were terminated too early to assess reversibility of effects; insufficient dose volume), a GHS classification could not be made. See Section 6.1 of the
3473 Draft HET-CAM BRD for a description of the rules followed to classify the ocular irritancy of test substances tested multiple times *in vitro*.

3474 ⁴N indicates number of substances.

3475 ⁵Number in parentheses indicates percentage of tested chemicals.

3476 The participating laboratories were in 100% agreement in regard to the GHS ocular irritancy
3477 classification for 85 (79%) of the 107 substances tested when using the IS(B)-10 analysis
3478 method (Spielmann et al. [1996]). The extent of agreement between testing laboratories was
3479 greatest for substances correctly identified as GHS nonsevere irritants or nonirritants by
3480 HET-CAM (94% [31/33]). Substances listed as “-/-“ were shown to have 100% classification
3481 agreement among testing laboratories. Comparatively, greater disparity between individual
3482 substance classifications was observed for substances that were identified as false positives
3483 (56% [10/18] false positive had less than 100% concordance between testing laboratories).

3484
3485 For the IS(B)-100 analysis method (Spielmann et al. 1996), the participating laboratories
3486 were in 100% agreement in regard to the GHS ocular irritancy classification for 81 (82%) of
3487 the 99 substances tested. As with the IS(B)-10 analysis method, the extent of agreement
3488 between testing laboratories was greatest for substances correctly identified as GHS
3489 nonsevere irritants or nonirritants by HET-CAM (94% [16/17]). Substances listed as “-/-“
3490 were shown to have 100% classification agreement among testing laboratories. Greater
3491 disparity between individual substance classifications was observed for substances that were
3492 identified as false positives (32% [10/31] false positive had less than 100% concordance
3493 between testing laboratories).

3494
3495 For the report by Hagino et al. (1999), the analysis was not affected by the information
3496 received subsequent to the release of the draft BRD on November 1, 2004. All the
3497 information presented here are the same as previously described in the draft HET-CAM
3498 BRD.

3499
3500 The overall reliability statistics, arranged by HET-CAM data analysis method, for the S-
3501 Score, Q-Score, and IS(A) methods are similar to what was described previously in the draft
3502 HET-CAM BRD.

3503 3504 3.4.1.2 EPA Ocular Hazard Classification System

3505 Reliability analyses for the HET-CAM test method were evaluated for the following two
3506 reports: Balls et al. (1995), Spielmann et al. (1996), and Hagino et al. (1999). The agreement
3507 of classification calls among participating laboratories and its relationship to the EPA (1996)
3508 *in vivo* classification for the substances tested in each report is provided in **Table IV-16**.

3509
3510 The participating laboratories were in 100% agreement in regard to the EPA ocular irritancy
3511 classification for 21 (45%) of the 47 substances tested when using the Q-Score. The
3512 agreement concordance among laboratories was greatest for accurately identified
3513 corrosives/severe irritants when compared to any other combination of *in vivo* and *in vitro*
3514 results (70% [7/10] of the accurately identified corrosives/severe irritants exhibited 100%
3515 classification agreement among laboratories). Comparatively, greater disparity between
3516 individual laboratory substance classifications was observed for substances that were
3517 identified as false positives and those substances accurately classified as nonsevere
3518 irritants/nonirritants. For instance, 76% (13/17) of the false positives and 58% (7/12) of the
3519 correctly identified EPA nonsevere irritants/nonirritants exhibited less than 100% agreement
3520 in irritancy classifications among laboratories.

3521 **Table IV-16. Evaluation of the Reliability of the HET-CAM Test Method In Predicting Ocular Corrosives and Severe**
 3522 **Irritants as Defined by the EPA¹ Classification System, by Study**
 3523

Report	Anal ²	Classification (<i>In Vivo/In Vitro</i>) ³	# of Labs	N ⁴	Substances with 100% Agreement among Labs	Substances with 80% Agreement among Labs	Substances with 75% Agreement among Labs	Substances with 66% Agreement among Labs	Substances with 60% Agreement among Labs	Substances with 50% or Less Agreement among Labs
Balls et al. (1995)	Q	++	2	4	3 (75%) ⁵	-	-	-	-	1 (25%)
			4	10	7 (70%)	-	3 (30%)	-	-	-
		+/-	-	-	-	-	-	-	-	-
		-/+	4	17	4 (24%)	-	9 (52%)	-	-	4 (24%)
		-/-	2	1	1 (100%)	-	-	-	-	-
			4	11	4 (36%)	-	7 (64%)	-	-	-
		?/-	2	1	1 (100%)	-	-	-	-	-
?/+	3	1	1 (100%)	-	-	-	-	-		
	4	2	2 (50%)	-	-	-	-	-		
Total	2-4	47	21 (45%)	-	21 (45%)	-	-	-	5 (10%)	
Balls et al. (1995)	S	++	2	3	3 (100%)	-	-	-	-	-
		+/-	3	3	2 (66%)	-	-	1 (33%)	-	-
			4	2	2 (100%)	-	-	-	-	-
		-/+	2	1	-	-	-	-	-	1 (100%)
			4	1	-	-	-	-	-	1 (100%)
		-/-	3	1	1 (100%)	-	-	-	-	-
			4	2	2 (100%)	-	-	-	-	-
?/-	2	1	1 (100%)	-	-	-	-	-		
	3	2	2 (100%)	-	-	2 (100%)	-	-		
?/+	2	2	1 (50%)	-	-	-	-	-	1 (50%)	
Total	2-4	18	12 (66%)	-	-	3 (17%)	-	-	3 (17%)	
Spielman et al. (1996)	IS(B) -10	++	2	9	8 (89%)	-	-	-	-	1 (11%)
			3	1	-	-	-	1 (100%)	-	-
		+/-	2	3	3 (100%)	-	-	-	-	-
		-/+	2	18	9 (50%)	-	-	-	-	9 (50%)
	3	3	1 (33%)	-	-	1 (33%)	-	1 (33%)		
-/-	2	31	31 (100%)	-	-	-	-	-		
	3	2	1 (50%)	-	-	1 (50%)	-	-		

Report	Anal ²	Classification (<i>In Vivo/In Vitro</i>) ³	# of Labs	N ⁴	Substances with 100% Agreement among Labs	Substances with 80% Agreement among Labs	Substances with 75% Agreement among Labs	Substances with 66% Agreement among Labs	Substances with 60% Agreement among Labs	Substances with 50% or Less Agreement among Labs
		?/-	2 3	10 3	10 (100%) 1 (33%)	- -	- -	- 2 (66%)	- -	- -
		?/+	2 3	21 3	19 (90%) 1 (33%)	- -	- -	- 1 (33%)	- -	2 (10%) 1 (33%)
		Total	2-3	104	84 (81%)			6 (6%)		14 (13%)
Spielman et al. (1996)	IS(B) -100	+/+	2 3	10 1	9 (90%) 1 (100%)	- -	- -	- -	- -	1 (10%) -
		+/-	2	1	1 (100%)	-	-	-	-	-
		-/+	2 3	29 4	22 (76%) 1 (25%)	- -	- -	- 3 (75%)	- -	7 (24%) -
		-/-	2 3	17 1	16 (94%) 1 (100%)	- -	- -	- -	- -	1 (6%) -
		?/-	2 3	7 1	7 (100%) 1 (100%)	- -	- -	- -	- -	- -
		?/+	2 3	21 5	19 (90%) 2 (40%)	- -	- -	- 3 (60%)	- -	2 (10%) -
		Total	2-3	97	80 (82%)			6 (6%)		11 (11%)
Hagino et al. (1999)	IS(A)	+/+	5	7	5 (71%)	2 (29%)	-	-	-	-
		+/-	-	-	-	-	-	-	-	-
		-/+	5	4	4 (100%)	-	-	-	-	-
		-/-	5	3	1 (33%)	-	-	-	2 (66%)	-
		?/-	-	-	-	-	-	-	-	-
		?/+	5	2	1 (50%)	-	-	-	1 (50%)	-
		Total	-	16	11 (69%)	3 (27%)	-	-	3 (27%)	-

3524 ¹EPA = U.S. Environmental Protection Agency (EPA [1996]).

3525 ²Anal = analysis method used to transform the sample data into HET-CAM scores. IS(A) = method described in Luepke (1985); Q = Q-Score, method described
3526 in Balls et al. (1995); S = S-Score, method described in Balls et al. (1995).

3527 ³A “+” indicates that the substance was assigned an overall classification of corrosive or a severe irritant (Category I); a “-“ indicates that the substance was
3528 assigned an overall classification of nonsevere irritant (Category II, III, or IV); a “?” indicates that, due to the lack of appropriate *in vivo* data (e.g., studies were
3529 terminated too early to assess reversibility of effects; insufficient dose volume), an EPA classification could not be made. See Section 6.1 of the Draft HET-
3530 CAM BRD for a description of the rules followed to classify the ocular irritancy of test substances tested multiple times *in vitro*.

3531 ⁴N indicates number of substances.

3532 ⁵Number in parentheses indicates percentage of tested chemicals.

3533 In addition to the Q-Score, Balls et al. (1995) evaluated irritancy potential for some
3534 substances by using an S-Score. The participating laboratories were in 100% agreement in
3535 regard to the EPA ocular irritancy classification for 12 (66%) of the 18 tested substances.
3536 Substances that were classified as false negatives and false positives exhibited the most
3537 discordant results, with 20% (1/5) of false negatives and 100% (2/2) of false positives
3538 exhibiting less than 100% agreement among laboratories. Complete agreement was observed
3539 for all the substances that were classified as EPA corrosives/severe irritants (3/3) or as EPA
3540 nonsevere irritants/nonirritants (3/3).

3541
3542 The participating laboratories were in 100% agreement in regard to the EPA ocular irritancy
3543 classification 84 of the 104 (81%) substances tested when using the IS(B)-10 analysis
3544 method (Spielmann et al. 1996). The extent of agreement between testing laboratories was
3545 greatest for substances correctly identified as GHS nonsevere irritants or nonirritants by
3546 HET-CAM (100% [31/31]). Substances listed as “-/-“ were shown to have 100%
3547 classification agreement among testing laboratories. Comparatively, greater disparity
3548 between individual substance classifications was observed for substances that were identified
3549 as false positives (52% [11/21] false positive had less than 100% concordance between
3550 testing laboratories). For the IS(B)-100 analysis method (Spielmann et al. [1996]), the
3551 participating laboratories were in 100% agreement in regard to the GHS ocular irritancy
3552 classification for 80 (82%) of the 97 substances tested. As with the IS(B)-10 analysis
3553 method, the extent of agreement between testing laboratories was greatest for substances
3554 correctly identified as GHS nonsevere irritants or nonirritants by HET-CAM (94% [17/18]).
3555 Substances listed as “-/-“ were shown to have 100% classification agreement among testing
3556 laboratories. Greater disparity between individual substance classifications was observed for
3557 substances that were identified as false positives (33% [10/33] false positive had less than
3558 100% concordance between testing laboratories).

3559
3560 For the report by Hagino et al. (1999), there was 100% agreement in regard to the EPA
3561 ocular irritancy classification for 11 (69%) of the 16 substances. Significant discordance in
3562 the classification results was observed for substances that were correctly identified as EPA
3563 nonsevere irritants/nonirritants. Of the three correctly identified EPA nonsevere
3564 irritants/nonirritants, two substances had less than 100% classification agreement among the
3565 laboratories. For EPA severe irritants, there was 100% laboratory agreement for 71% (5/7)
3566 of the tested substances.

3567
3568 The overall reliability statistics, arranged by HET-CAM data analysis method, for the S-
3569 Score, Q-Score, and IS(A) methods are similar to what was described previously in the draft
3570 HET-CAM BRD.

3571
3572 3.4.1.3 *EU Ocular Hazard Classification System*

3573 Reliability analyses for the HET-CAM test method were evaluated for the following two
3574 reports: CEC (1991), Balls et al. (1995), Spielmann et al. (1996), and Hagino et al. (1999).
3575 The agreement of classification calls among participating laboratories and its relationship to
3576 the EU (2001) *in vivo* classification for the substances tested in each report is provided in
3577 **Table IV-17**.

3578 **Table IV-17. Evaluation of the Reliability of the HET-CAM Test Method In Predicting Ocular Corrosives and Severe**
 3579 **Irritants (as Defined by the EU¹ Classification System), by Study**
 3580

Report	Anal ²	Classification (<i>In Vivo/In Vitro</i>) ³	# of Labs	N ⁴	Substances with 100% Agreement among Labs	Substances with 75-99% Agreement among Labs	Substances with 50-74% Agreement among Labs	Substances with 25-49% Agreement among Labs
Balls et al. (1995)	Q	+/+	2	4	3 (75%) ⁵	-	1 (25%)	-
			4	9	6 (67%)	3 (37%)	-	-
		+/-	-	-	-	-	-	-
		-/+	4	14	4 (28%)	7 (50%)	3 (21%)	-
		-/-	2	1	1 (100%)	-	-	-
			4	11	4 (36%)	7 (63%)	-	-
		?/-	2	1	1 (100%)	-	-	-
		?/+	3	1	1 (100%)	-	-	-
	4	6	1 (17%)	4 (67%)	1 (17%)	-		
	Total	2-4	47	21 (47%)	21 (47%)	5 (6%)	-	
Balls et al. (1995)	S	+/+	2	3	3 (100%)	-	-	-
		+/-	2	1	1 (100%)	-	-	-
			3	3	2 (66%)	-	1 (33%)	-
			4	2	2 (100%)	-	-	-
		-/+	2	1	-	-	1 (100%)	-
			4	1	-	-	1 (100%)	-
		-/-	3	1	1 (100%)	-	-	-
			4	2	2 (100%)	-	-	-
?/-	3	2	-	-	2 (100%)	-		
?/+	2	2	1 (50%)	-	1 (50%)	-		
	Total	2-4	18	12 (66%)	-	6 (34%)	-	
CEC (1991)	IS(B)	+/+	3	3	3 (100%)	-	-	-
			5	1	-	-	1 (100%)	-
			6	2	-	1 (50%)	1 (50%)	-
		+/-	7	1	-	1 (100%)	-	-
		-/+	3	3	-	-	1 (33%)	2 (66%)
			7	6	-	1 (17%)	2 (34%)	3 (51%)
		-/-	3	6	3 (50%)	-	2 (33%)	1 (17%)
			7	4	-	2 (50%)	2 (50%)	-
?/-	-	-	-	-	-	-		

Report	Anal ²	Classification (<i>In Vivo/In Vitro</i>) ³	# of Labs	N ⁴	Substances with 100% Agreement among Labs	Substances with 75-99% Agreement among Labs	Substances with 50-74% Agreement among Labs	Substances with 25-49% Agreement among Labs
		?/+	-	-	-	-	-	-
		Total	3-7	26	6 (23%)	5 (19%)	9 (35%)	6 (23%)
Spielmann et al. (1996)	IS(B)-10	+/+	2	12	11 (92%)	-	1 (8%)	-
			3	1	-	-	1 (100%)	-
		+/-	2	3	3 (100%)	-	-	-
		-/+	2	17	7 (41%)	-	-	10 (59%)
			3	2	1 (50%)	-	1 (50%)	-
		-/-	2	31	30 (97%)	-	1 (3%)	-
			3	2	1 (50%)	-	1 (50%)	-
		?/-	2	11	11 (100%)	-	-	-
	3	3	1 (33%)	-	2 (66%)	-		
?/+	2	20	18 (90%)	-	2 (10%)	-		
	3	4	1 (25%)	-	2 (50%)	1 (25%)		
		Total	2-3	106	84 (79%)		11 (10%)	11 (10%)
Spielmann et al. (1996)	IS(B)- 100	+/+	2	12	11 (92%)	-	1 (8%)	-
			3	1	1 (100%)	-	-	-
		+/-	2	1	1 (100%)	-	-	-
		-/+	2	28	21 (75%)	-	-	7 (25%)
			3	4	1 (25%)	-	3 (75%)	-
		-/-	2	17	16 (94%)	-	-	1 (6%)
		?/-	2	7	7 (100%)	-	-	-
			3	2	2 (100%)	-	-	-
?/+	2	21	18 (86%)	-	-	3 (24%)		
	3	2	2 (100%)	-	-	-		
		Total	2-3	95	80 (84%)		4 (4%)	11 (11%)
Hagino et al. (1999)	IS(A)	+/+	5	7	6 (86%)	2 (14%)	-	-
		+/-	-	-	-	-	-	-
		-/+	5	5	3 (60%)	1 (20%)	1 (20%)	-
		-/-	5	3	-	1 (25%)	2 (50%)	-
		?/-	-	-	-	-	-	-
		?/+	5	2	2 (100%)	-	-	-
				Total	2-4	17	11 (64%)	4 (24%)

- 3582 ¹EU = European Union (EU [2001]).
- 3583 ²Anal = analysis method used to transform the sample data into HET-CAM scores. IS(A) = method described in Luepke (1985); Q = Q-Score, method described
- 3584 in Balls et al. (1995); S = S-Score, method described in Balls et al. (1995).
- 3585 ³A “+” indicates that the substance was assigned an overall classification of corrosive or severe irritant (Category R41); a “-“ indicates that the substance was
- 3586 assigned an overall classification of nonsevere irritant (Category R36) or nonirritant; a “?” indicates that, due to the lack of appropriate *in vivo* data (i.e.,
- 3587 insufficient dose volume), an EU classification could not be made. See Section 6.1 of the Draft HET-CAM BRD for a description of the rules followed to
- 3588 classify the ocular irritancy of test substances tested multiple times *in vitro*.
- 3589 ⁴N indicates number of substances.
- 3590 ⁵Number in parentheses indicates percentage of tested chemicals.

3591 The participating laboratories were in 100% agreement in regard to the EU ocular irritancy
3592 classification for 21 (45%) of the 47 substances tested when using the Q-Score. The extent
3593 of agreement among laboratories was greatest for accurately identified EU corrosives/severe
3594 irritants when compared to any other combination of *in vivo* and *in vitro* results (69% [9/13]
3595 of the identified EU corrosives/severe irritants exhibited 100% classification agreement
3596 among laboratories). Comparatively, greater disparity between individual substance
3597 classifications was observed for substances that were identified as false positives and those
3598 substances accurately classified as EU nonsevere irritants/nonirritants. For instance, 71%
3599 (10/14) of the false positives and 58% (7/12) of the correctly identified EU nonsevere
3600 irritants/nonirritants exhibited less than 100% agreement among laboratories in irritancy
3601 classifications.

3602
3603 In addition to the Q-Score, Balls et al. (1995) evaluated irritancy potential for some
3604 substances by using an S-Score. The participating laboratories were in 100% agreement in
3605 regard to the EU ocular irritancy classification for 12 (66%) of the 18 tested substances.
3606 Substances that were classified as false positives exhibited the most discordant results, with
3607 100% (2/2) of false positives exhibiting less than 100% classification agreement among
3608 laboratories.

3609
3610 For the CEC evaluation, the participating laboratories were in 100% agreement in regard to
3611 the EU ocular irritancy classification for 6 (23%) of the 26 substances tested when using the
3612 IS(B) analysis method. The extent of agreement among laboratories was greatest for
3613 accurately identified EU corrosives/severe irritants when compared to any other combination
3614 of *in vivo* and *in vitro* results (50% [3/6] of the identified EU corrosives/severe irritants
3615 exhibited 100% classification agreement among laboratories). Comparatively, greater
3616 disparity between individual substance classifications was observed for substances that were
3617 identified as false positives and those substances accurately classified as EU nonsevere
3618 irritants/nonirritants. For instance, 100% (9/9) of the false positives and 70% (7/10) of the
3619 correctly identified EU nonsevere irritants/nonirritants exhibited less than 100% agreement
3620 among laboratories in irritancy classifications.

3621
3622 The participating laboratories were in 100% agreement in regard to the EPA ocular irritancy
3623 classification 84 of the 106 (79%) substances tested when using the IS(B)-10 analysis
3624 method (Spielmann et al. [1996]). The extent of agreement between testing laboratories was
3625 greatest for substances correctly identified as GHS nonsevere irritants or nonirritants by
3626 HET-CAM (93% [31/33]). Substances listed as “-/-“ were shown to have 100%
3627 classification agreement among testing laboratories. Comparatively, greater disparity
3628 between individual substance classifications was observed for substances that were identified
3629 as false positives (58% [11/19] false positive had less than 100% concordance between
3630 testing laboratories).

3631
3632 For the IS(B)-100 analysis method (Spielmann et al. [1996]), the participating laboratories
3633 were in 100% agreement in regard to the GHS ocular irritancy classification for 80 (84%) of
3634 the 95 substances tested. As with the IS(B)-10 analysis method, the extent of agreement
3635 between testing laboratories was greatest for substances correctly identified as GHS
3636 nonsevere irritants or nonirritants by HET-CAM (94% [16/17]). Substances listed as “-/-“

3637 were shown to have 100% classification agreement among testing laboratories. Greater
3638 disparity between individual substance classifications was observed for substances that were
3639 identified as false positives (33% [10/33] false positive had less than 100% concordance
3640 between testing laboratories).

3641
3642 For the report by Hagino et al. (1999), there was 100% agreement in regard to the EU ocular
3643 irritancy classification for 11 (64%) of the 17 substances. Significant discordance in the
3644 classification results was observed for substances that were correctly identified as EU
3645 nonsevere irritants/nonirritants. Of the three correctly identified EU nonsevere
3646 irritants/nonirritants, all substances exhibited less than 100% classification agreement among
3647 laboratories. Of the seven correctly identified EU corrosives/severe irritants, six substances
3648 (86%) produced the same classification in all five laboratories. Another group of substances
3649 that showed a high degree of agreement among laboratories were false positive substances
3650 (60% [3/5]).

3651
3652 The overall reliability statistics, arranged by HET-CAM data analysis method, for the S-
3653 Score, Q-Score, and IS(A) methods are similar to what was described previously in the draft
3654 HET-CAM BRD.

3655
3656 3.4.2 Quantitative Reanalysis of Interlaboratory Reproducibility

3657 3.4.2.1 *CEC (1991)*

3658 Between three and five laboratories evaluated each substance tested in this report. For this
3659 evaluation, only substances tested by five laboratories were assessed. CEC (1991) used the
3660 IS(B) analysis method. The average and median %CV values for these substances were
3661 altered based on removal of some substances, whose *in vivo* classification were not based on
3662 *in vivo* rabbit data. The reanalysis is shown in **Table IV-18**.

3663
3664 3.4.2.2 *Balls et al. (1995)*

3665 Individual laboratory results for tested substances were obtained from ECVAM. Balls et al.
3666 (1995) used two different analysis methods; the S-Score and Q-Score. The average and
3667 median %CV values for all the substances evaluated with the Q-Score and S-Score were not
3668 affected by the information received subsequent to the release of the draft BRD on November
3669 1, 2004 (**Table IV-19** and **Table IV-20**).

3670

3670 **Table IV-18. %CV¹ Values for Substances Evaluated Using the IS(B) Analysis Method**
 3671 **(from CEC [1991])**
 3672

Substance ²	Conc. ³	IS(B) Value	SD ⁴	%CV Values
2-Butoxyethyl acetate	100%	4.76	0.31	6.6
Butanol	100%	11.44	1.0	8.7
Triacetin	100%	4.18	0.91	21.8
Glycerol	100%	9.32	2.62	28.1
Tributyltin chloride	100%	8.94	2.88	32.2
Dimethyl sulfoxide	100%	9.88	3.24	32.8
Sodium dodecyl sulfate	100%	10.02	3.33	33.3
Triethanolamine	100%	8.52	2.94	34.6
Toluene	100%	11.04	4.31	39.1
2-Methoxyethanol	100%	9.14	3.72	40.7
n-Hexane	100%	5.04	3.16	62.8
Brij 35	100%	5.58	4.18	74.9
Mean	-	-	-	34.6
Median	-	-	-	33.1
Range	-	-	-	6.6-74.9

3673 ¹%CV = percent coefficient of variation.

3674 ²Substances organized by increasing %CV values.

3675 ³Conc. = concentration tested.

3676 ⁴SD = standard deviation.

3677
 3678
 3679 The average and median %CV values for GHS Category 1 substances (UN 2003), based on
 3680 *in vivo* results, were 36.26 and 38.93 for the Q-Score. The average and median %CV values
 3681 for EPA Category I substances (EPA [1996]), based on *in vivo* results, were 33.59 and 34.81
 3682 for the Q-Score. The average and median %CV values for GHS Category 1 and EPA
 3683 Category I substances evaluated using the S-Score were not affected by the information
 3684 received subsequent to the release of the draft BRD (**Table IV-19** and **Table IV-20**).
 3685

3686 3.4.2.3 *Spielmann et al. (1996)*

3687 Individual laboratory results on tested substances were provided by Drs. Spielmann and
 3688 Liebsch in response to a request by NICEATM. The data provided were for test substances,
 3689 evaluated using the IS(B) analysis method and published in Spielmann et al. (1996). In the
 3690 evaluation, substances were evaluated at a 10% and 100% concentration in at least two
 3691 different testing laboratories. Therefore, evaluation of the reliability of the test method was
 3692 conducted for each concentration tested. Additionally, in order to resolve discrepancies in
 3693 results between testing laboratories, some substances were tested in one additional testing
 3694 laboratory (substances are italicized in **Table IV-21**). In order to determine if the substance
 3695 tested in three laboratories affected the overall %CV values, an evaluation of the overall
 3696 %CV values was conducted with these substances removed.
 3697

3698 The average and median %CV values for substances tested at 10% concentration were 60.17
 3699 and 42.65, respectively. For substances tested at 100% concentration, the average and

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3701
3702**Table IV-19. %CV¹ Values for Substances Evaluated Using the Q-Score Analysis Method (from Balls et al. [1995])**

Substance ²	Conc. ³	GHS ⁴ Category 1	EPA ⁵ Category I	Mean Q-Score	SD ⁶	%CV Values
2,2-Dimethylbutanoic acid	-	-	X	12.78	1.93	15.09
Trichloroacetic acid	30%	X	X	12.32	1.89	15.35
Benzalkonium chloride	1%	X	X	4.18	0.68	16.29
Sodium hydroxide	1%	-	-	5.42	0.99	18.20
Butyl acetate	-	-	-	1.63	0.31	18.95
Methyl cyanoacetate	-	-	-	1.38	0.34	24.84
Sodium lauryl sulfate	-	-	-	2.12	0.53	25.25
Triton X-100	5%	-	-	2.25	0.61	27.14
Octanol	-	-	-	1.67	0.47	28.15
Cyclohexanol	-	X	X	4.91	1.42	29.01
Benzalkonium chloride	10%	X	X	5.59	1.72	30.68
Ethyl-2-methylacetoacetate	-	-	-	2.09	0.66	31.74
Methyl isobutyl ketone	-	-	-	1.67	0.53	31.76
Cetylpyridinium bromide	6%	X	-	2.29	0.75	32.56
Triton X-100	10%	-	-	2.32	0.82	35.62
Hexanol	-	-	-	3.88	1.45	37.40
Methyl ethyl ketone	-	-	-	4.60	1.72	37.45
Toluene	-	-	-	3.73	1.41	37.98
Sodium lauryl sulfate	15%	X	X	2.84	1.11	38.93
Cetylpyridinium bromide	10%	X	X	2.98	1.21	40.60
Parafluoriline	-	-	-	3.55	1.57	44.31
Polyethylene glycol 400	-	-	-	1.03	0.46	44.41
Pyridine	-	X	X	8.74	3.88	44.42
Tween 20	-	X	-	0.58	0.27	45.98
Sodium hydroxide	10%	X	X	13.44	6.74	50.12
Isobutanol	-	-	-	3.82	1.98	51.99
Trichloroacetic acid	3%	-	-	10.79	5.68	52.67
Benzalkonium chloride	5%	X	X	4.76	2.61	54.87
Ethyl acetate	-	-	-	2.52	1.39	55.11
Methyl acetate	-	-	-	3.03	1.70	56.12
Ethanol	-	-	-	6.13	3.75	61.16
Acetone	-	-	-	10.75	7.41	68.95
Glycerol	-	-	-	0.79	0.56	70.83
Isopropanol	-	-	-	5.96	4.23	71.93
2,6-Dichlorobenzoyl chloride	-	-	-	5.85	4.23	72.44
2-Ethyl-1-hexanol	-	-	-	1.49	1.12	74.75
Ethyl trimethyl acetate	-	-	-	0.40	0.41	103.70
Gamma-butyrolactone	-	-	-	8.67	9.12	105.19
Cetylpyridinium bromide	0.1%	-	-	0.86	1.15	134.05
Methylcyclopentane	-	-	-	2.42	3.81	157.25
Mean for All Substances (n=40)	-	-	-	-	-	49.83

Substance ²	Conc. ³	GHS ⁴ Category 1	EPA ⁵ Category I	Mean Q-Score	SD ⁶	%CV Values
Median for All Substances	-	-	-	-	-	42.50
Range for All Substances	-	-	-	-	-	15.09- 157.25
Mean for Severe Irritants (GHS) (n=11)	-	-	-	-	-	36.26
Median for Severe Irritants	-	-	-	-	-	38.93
Range for Severe Irritants	-	-	-	-	-	15.35- 54.87
Mean for Severe Irritants (EPA) (n=8)	-	-	-	-	-	33.54
Median for Severe Irritants	-	-	-	-	-	34.81
Range for Severe Irritants	-	-	-	-	-	15.35- 54.87

¹%CV = percent coefficient of variation.

²Substances organized by increasing %CV values.

³Conc. = concentration tested.

⁴GHS = Globally Harmonized System (UN [2003]).

⁵EPA = U.S. Environmental Protection Agency (EPA [1996]).

⁶SD = standard deviation.

Table IV-20. %CV¹ Values for Substances Evaluated Using the S-Score Analysis Method (from Balls et al. [1995])

Substance ²	GHS ³ Category 1	EPA ⁴ Category I	Mean S- Score	Standard Deviation	%CV
4-Carboxybenzaldehyde	-	-	4	2.83	70.71
Fomasafen	-	-	5.25	3.77	71.90
1-Naphthalene acetic acid	X	X	5.75	5.44	94.59
Sodium oxalate	X	X	8	5.48	68.47
Dibenzyl phosphate	-	-	8.25	9.60	116.42
Mean for All Substances (n=5)	-	-	-	-	84.42
Median for All Substances	-	-	-	-	71.90
Range for All Substances	-	-	-	-	68.47-116.4
Mean for Severe Irritants (GHS) (n=2)	-	-	-	-	81.53
Median for Severe Irritants	-	-	-	-	81.5
Range for Severe Irritants	-	-	-	-	68.47-94.59
Mean for Severe Irritants (EPA) (n=2)	-	-	-	-	81.53
Median for Severe Irritants	-	-	-	-	81.5
Range for Severe Irritants	-	-	-	-	68.47-94.59

¹%CV = percent coefficient of variation.

²Substances organized by increasing %CV values.

³GHS = Globally Harmonized System (UN [2003]).

⁴EPA = U.S. Environmental Protection Agency (EPA [1996]).

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3719
3720**Table IV-21. %CV¹ Values for Substances Evaluated Using IS(B) Analysis Method
(from Spielmann et al. [1996])**

Substance Name ²	CASRN ³	IS(B)-10 Mean	IS(B)- 10 SD	%CV for IS(B)-10	IS(B)-100 Mean	IS(B)- 100 SD	%CV for IS(B)-100
7-Acetoxyheptanal		1.55	2.19	141.42	10.95	8.56	78.14
n-Acetyl-Methionine	1115-47-5	9.85	5.30	53.84	-	-	-
Ambuphylline	5634-34-4	13.25	3.61	27.22	14.85	2.90	19.52
<i>4-Amino-5-methoxy-2-methylbenzenesulfonic acid</i>	6471-78-9	9.80	4.34	44.29	12.17	3.20	26.31
Anisole	100-66-3	3.65	5.16	141.42	18.80	0.42	2.26
B 25		0.00	0.00	-	0.00	0.00	-
n-Butanal	123-72-8	3.95	3.89	98.46	19.20	1.56	8.10
n-Butanol	71-36-3	13.95	6.15	44.10	16.60	5.09	30.67
<i>Butyl carbamate</i>	592-35-8	6.80	5.93	87.21	12.67	1.93	15.27
<i>Caffeine sodium benzoate</i>	8000-95-1	6.37	1.66	26.11	13.10	5.31	40.52
Caffeine sodium salicylate	8002-85-5	8.60	1.70	19.73	17.40	1.98	11.38
Camphen	79-92-5	6.00	5.66	94.28	-	-	-
Cerium-2-ethylhexanoate	24593-34-8	7.40	0.71	9.56	17.18	2.93	17.09
1-Chlorooctane-8-ol		5.55	1.77	31.85	16.50	3.11	18.86
3-Cyclohexene-1-methanol	1679-51-2	10.95	1.20	10.98	18.95	0.07	0.37
DC 8		0.00	0.00	-	2.50	3.54	141.42
1,4-Dibutoxybenzene	104-36-9	2.10	2.97	141.42	-	-	-
<i>Diepoxid 126</i>	2386-87-0	5.50	3.38	61.42	10.53	4.82	45.78
<i>2,5-Dimethylhexanediol</i>	110-03-2	6.65	3.61	54.23	13.85	3.89	28.08
3,6-Dimethyloctanol		0.15	0.21	141.42	4.30	0.00	0.00
4,4-Dimethyl-3-oxo-pentanenitrile	59997-51-2	4.95	0.92	18.57	6.20	0.71	11.40
<i>1-(2,6-dimethylphenoxy)-2-propanone</i>	53012-41-2	7.42	9.99	134.67	11.80	7.60	64.42
Diphocars		14.70	5.09	34.63	15.10	3.96	26.22
<i>1,2-Dodecanediol</i>	1119-87-5	5.48	5.75	104.84	3.20	1.27	39.77
DTPA Pentasodium salt	140-01-2	15.58	0.11	0.73	19.65	0.35	1.80
Ede 140		1.70	2.40	141.42	2.30	3.25	141.42
1,2-Epoxydodecane	2855-19-8	2.05	2.90	141.42	4.95	5.02	101.42
Ethiosan		1.90	2.69	141.42	-	-	-
Ethyl butanal	97-96-1	1.80	2.55	141.42	18.05	0.92	5.09

Substance Name ²	CASRN ³	IS(B)-10 Mean	IS(B)-10 SD	%CV for IS(B)-10	IS(B)-100 Mean	IS(B)-100 SD	%CV for IS(B)-100
Gadopentetic acid dimeglumine salt	86050-77-3	4.70	2.40	51.15	5.70	3.54	62.03
Genomoll	115-96-8	9.30	0.14	1.52	10.75	1.20	11.18
<i>C12/C14-Glucoside</i>		9.57	1.01	10.57	16.50	0.20	1.21
L-Glutamic acid hydrochloride	138-15-8	12.95	1.77	13.65	13.45	2.47	18.40
Glycediol		0.90	1.27	141.42	2.04	2.06	101.21
Granuform	30525-89-4	1.45	2.05	141.42	0.00	0.00	#DIV/0!
Hexahydrofarnesyl-acetone	502-69-2	1.75	0.78	44.45	6.10	2.69	44.05
Hexamethylenetetramine	100-97-0	5.05	1.06	21.00	11.15	0.07	0.63
1,2,6-Hexanetriol	106-69-4	7.90	5.09	64.45	17.05	2.47	14.52
Hnol		0.40	0.57	141.42	4.05	2.76	68.09
Hoe MBF		0.00	0.00	-	0.18	0.25	141.42
Hydo 98		11.65	1.77	15.17	-	-	-
2-Hydroxyethyl imino disodium acetate	135-37-5	11.15	3.18	28.54	13.25	3.18	24.01
2-Hydroxyisobutyric acid	594-61-6	12.85	2.90	22.56	13.45	3.04	22.61
Hypo 20		3.60	5.09	141.42	6.51	3.38	51.92
Hypo 36		4.10	0.14	3.45	12.95	4.17	32.22
<i>Hypo 45</i>		5.17	5.15	99.62	8.33	3.76	45.16
Hypo 54		4.15	0.21	5.11	4.15	0.07	1.70
Hyton		15.25	2.47	16.23	18.40	0.28	1.54
<i>Iminodiacetic acid</i>	142-73-4	8.25	7.43	90.01	6.85	5.98	87.23
Isobornyl acetate	125-12-2	2.90	1.70	58.52	6.35	2.47	38.97
Isobutanal	78-84-2	1.05	1.48	141.42	19.70	0.42	2.15
Isodecylglucoside		13.55	5.16	38.10	14.35	5.16	35.97
Isononylaldehyde	35127-50-5	0.00	0.00	-	7.25	3.89	53.64
alpha-Ketoglutaric acid	328-50-7	18.95	0.21	1.12	19.75	0.07	0.36
<i>alpha-Lactid</i>	4511-42-6	8.60	6.08	70.66	3.90	2.75	70.55
L-Lysine Monohydrate	39665-12-8	9.13	1.24	13.56	13.65	4.60	33.67
3-Mercapto-1,2,4-triazole	3179-31-5	11.30	9.90	87.61	-	-	-
m-Methoxybenzaldehyde	591-31-1	3.15	1.34	42.65	12.65	1.48	11.74
Methyl acetate	79-20-9	4.35	0.07	1.63	17.95	2.62	14.58
Methylpentynol	77-75-8	13.85	2.19	15.83	16.50	5.09	30.86
N-(2-methylphenyl)-Imidodi-carbonimidic diamide	93-69-6	17.40	0.42	2.44	-	-	-

Substance Name ²	CASRN ³	IS(B)-10 Mean	IS(B)-10 SD	%CV for IS(B)-10	IS(B)-100 Mean	IS(B)-100 SD	%CV for IS(B)-100
2-Methyl-1-propanol	78-83-1	17.80	0.14	0.79	19.80	0.85	4.29
Methyltriglycol	112-35-6	4.50	0.57	12.57	14.75	3.18	21.57
Methyltriglycol	112-35-6	7.00	5.66	80.81	16.60	5.37	32.37
Napt		3.10	1.70	54.74	8.00	3.25	40.66
Nitro-bis-octylamide		0.85	1.20	141.42	4.05	3.46	85.55
Olak		17.50	1.98	11.31	18.25	1.77	9.69
Ölesulf		16.85	0.07	0.42	19.25	0.49	2.57
Phenylephrine hydrochloride	61-76-7	9.85	1.77	17.95	19.10	1.13	5.92
Phenylthiourea	103-85-5	2.00	2.83	141.42	1.55	2.19	141.42
Phosphonat A		6.70	0.14	2.11	6.80	4.67	68.63
<i>Acefyllin piperazinate</i>	18833-13-1	7.13	9.95	139.49	12.97	3.45	26.63
PO 2		2.15	3.04	141.42	0.15	0.21	141.42
Polyethylene glycol butyl ether	9004-77-7	13.30	3.39	25.52	19.25	0.07	0.37
Polyethylene glycol dimethyl ether	24991-55-7	2.05	2.90	141.42	13.70	8.63	62.97
Polyethylene glycol	25322-68-3	0.50	0.71	141.42	7.15	0.78	10.88
Polyhexamethylene guanidine		10.10	1.27	12.60	15.05	0.64	4.23
Polysolvan	7397-62-8	16.15	0.49	3.06	17.65	2.47	14.02
Potassium cyanate	590-28-3	17.30	2.12	12.26	17.65	2.47	14.02
Potassium hexacyanoferrate II	14459-95-1	16.50	1.84	11.14	11.75	7.71	65.60
Potassium hexacyanoferrate III	13756-66-2	5.23	1.45	27.74	6.08	0.53	8.73
2-Pseudojonon		5.75	4.17	72.56	5.70	2.26	39.70
RK Blau		2.00	2.83	141.42	-	-	-
Sacyclo		1.70	2.40	141.42	3.85	0.78	20.20
Sept		7.00	4.24	60.61	17.85	2.76	15.45
Trimethoxypropylsilane	1067-25-0	3.80	0.14	3.72	9.10	6.51	71.49
Trimethoxyoctylsilane	3069-40-7	5.00	4.10	82.02	9.20	1.13	12.30
Silan 165	29055-11-6	0.35	0.49	141.42	5.65	2.19	38.80
Silan 167	41453-78-5	1.40	1.84	131.32	3.50	1.70	48.49
Silan 253	18784-74-2	3.00	0.00	0.00	12.30	3.39	27.59
Sodium bisulfite	7631-90-5	13.30	0.85	6.38	18.40	2.26	12.30
Sodium sulfite	7757-83-7	12.25	1.34	10.97	14.20	2.69	18.92
Sodium cyanate	917-61-3	12.65	3.04	24.04	9.45	1.77	18.71

Substance Name ²	CASRN ³	IS(B)-10 Mean	IS(B)-10 SD	%CV for IS(B)-10	IS(B)-100 Mean	IS(B)-100 SD	%CV for IS(B)-100
Sodium disilicate	13870-28-5	20.20	0.71	3.50	17.40	1.13	6.50
Sodium hydrogen sulfate	7681-38-1	17.75	1.48	8.37	18.65	0.78	4.17
Sodium lauryl ether sulfate	3088-31-1	14.10	5.09	36.11	18.45	0.78	4.22
Sodium monochloroacetate	3926-62-3	3.75	5.30	141.42	13.45	3.75	27.86
<i>Sodiumpyrosulfite</i>	7681-57-4	14.87	2.41	16.22	14.60	3.05	20.90
4-((2-Sulfatoethyl)sulfonyl)-aniline	2494-89-5	19.05	1.48	7.79	-	-	-
TA 01946 Alkylsilan		8.80	1.70	19.28	13.10	4.38	33.47
Theophylline sodium acetate	8002-89-9	9.40	5.66	60.18	-	-	-
Tocla		16.30	4.81	29.50	16.95	4.88	28.78
Triisooctylamine	25549-16-0	0.40	0.57	141.42	9.05	7.14	78.91
2,2,3-Trimethyl-3-Cyclopentene-1-acetaldehyde	4501-58-0	2.60	0.42	16.32	12.20	3.54	28.98
Trioxane	110-88-3	11.33	2.93	25.91	17.90	0.14	0.79
Wessalith Slurry		6.57	4.86	74.00	9.90	8.20	82.85
Xanthinol nicotinate	437-74-1	7.65	5.16	67.48	13.20	5.94	45.00
Mean %CV Value				60.17			35.21
Median %CV Value				42.65			26.22
Range %CVs				0-141.42			0-141.42
Mean %CV Value (Minus Substances Tested in 3 Laboratories)				58.07			34.62
Median %CV Value (Minus Substances Tested in 3 Laboratories)				31.85			21.57
Range %CVs (Minus Substances Tested in 3 Laboratories)				0-141.42			0-141.42

3721 ¹CV = coefficient of variation.

3722 ³CASRN = Chemical Abstract Service Registry Number.

3723 ²Italicized substances represent chemicals that were tested in three testing laboratories. Data for these
3724 substances were removed to determine their impact on the calculated %CV values for this data set.

3725

3726

3727 median %CV values were lower: 35.21 and 26.22, respectively. When substances that were
3728 tested in three different testing laboratories were removed from the assessment, little change
3729 was seen in the mean and median %CV values for both concentrations tested (**Table IV-21**).

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3731 3.4.2.4 *Hagino et al. (1999) and Ohno et al. (1999)*

3732 The Japanese Ministry of Health and Welfare evaluated the HET-CAM test method in five
3733 different laboratories as part of a validation effort to assess alternative ocular irritation test
3734 method. Nine, 15, and 14 cosmetic ingredients were evaluated in the first, second, and third
3735 steps of the validation study, respectively. These studies used the IS(A) analysis method to
3736 assess potential irritancy classifications. Average individual laboratory results and standard

3737 deviations for tested substances were reported in Hagino et al. (1999). Additional
3738 information on this evaluation can be obtained from the draft HET-CAM BRD.

3739
3740 The average and median %CV for substances classified as GHS Category 1 (UN [2003]) for
3741 the substances described in Hagino et al. (1999)¹, which described the third validation phase,
3742 were not affected by information received subsequent to release of the draft HET-CAM
3743 BRD. The average and median %CV for substances classified as EPA Category I (EPA
3744 [1996]) were 23.86 and 26.0, respectively (see **Table IV-22**).

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3747 **Table IV-22. %CV¹ Values for Substances Evaluated Using the IS(A) Analysis Method**
3748 **(from Hagino et al. 1999)**

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Substance ²	Conc. ³	GHS ⁴ Category 1	EPA ⁵ Category I	%CV
Acetic acid	10%	X	X	8
Potassium laurate	10%	X	X	12
Stearyltrimethylammonium chloride	10%	X	X	22
Domiphen bromide	10%	X	X	26
Butanol	10%	X		28
Di(2-ethylhexyl) sodium sulfosuccinate	10%	X	X	28
Cetyltrimethylammonium bromide	10%	X	X	32
Lactic acid	100%	X	X	39
Mean for Severe Irritants (GHS) (n=8)				24.4
Median for Severe Irritants				27.0
Range for Severe Irritants				8-39
Mean for Severe Irritants (EPA) (n=6)				23.86
Median for Severe Irritants				26.0
Range for Severe Irritants				8-39

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¹%CV = percent coefficient of variation.

²Substances organized by increasing %CV values.

³Conc. = concentration tested.

⁴GHS = Globally Harmonized System (UN [2003]).

⁵EPA = U.S. Environmental Protection Agency (EPA [1996]).

¹ Percent CV values were not determined for the other phases because average data were not provided in literature references.

3755 3.4.3 Additional Reanalyses of Interlaboratory Reproducibility

3756 No additional analyses of interlaboratory reproducibility were received or reviewed
3757 subsequent to the release of the draft HET-CAM BRD.

3758

3759 **3.5 HET-CAM Test Method Historical Positive and Negative Control Data -**
3760 **Reanalysis**

3761

3762 3.5.1 Data Provided by Dr. Philippe Vanparys

3763 HET-CAM studies using 0.9% NaCl as a negative control were provided by Dr. P. Vanparys
3764 in response to a request from NICEATM. Studies were conducted with and without the use
3765 of a Test Substance Applicator (TSA). The use of a TSA, described in Gilleron et al. (1996,
3766 1997) is a device used to contain solids and/or liquids to a specific location on the CAM.

3767

3768 Over 90 tests with 0.9% sodium chloride (NaCl) using the TSA and three tests with 0.9%
3769 NaCl without using TSA were provided. As shown in **Table IV-23**, time to development of
3770 endpoints and the overall irritation scores calculated were consistent and classified as
3771 nonirritants for all tests. HET-CAM studies using dimethyl formamide (DMF) and imidazole
3772 as positive controls were provided by Dr. P. Vanparys in response to a request from
3773 NICEATM. Studies were conducted with and without the use of a TSA.

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3776 **Table IV-23. Comparison of Means and Standard Deviations of 0.9% NaCl¹ With and**
3777 **Without Use of the Test Substance Applicator**

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0.9% NaCl	N ²	Hemorrhage ³ (mean ± SD ⁵)	Lysis ³ (mean ± SD)	Coagulation ³ (mean ± SD)	<i>In Vitro</i> Score ⁴ (mean ± SD)
With TSA ⁶	92	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Without TSA	3	0 ± 0	0 ± 0	0 ± 0	0 ± 0

3779

¹NaCl = sodium chloride.

3780

²N = number of tests

3781

³Mean values of time until development of identified endpoint.

3782

⁴*In Vitro* irritation score calculated as IS(B).

3783

⁵SD = standard deviation.

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⁶TSA = test substance applicator.

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3787 With the DMF studies that were conducted with the TSA, the hemorrhage endpoint was
3788 evaluated inside the TSA and outside the TSA. Of note, the time of development of the
3789 hemorrhage endpoint inside the TSA was significantly lower than the time to development of
3790 the hemorrhage endpoint outside the TSA (**Table IV-24**). The reason for the difference is
3791 not clear. Two proposed reasons for the difference in time to development, according to Dr.
3792 Vanparys, are (1) the vessels outside the TSA may open more easily than those under the
3793 TSA or (2) once the liquid is applied it the liquid accumulates around the edge of the TSA
3794 rather than between the TSA and CAM.

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3795 **Table IV-24. Comparison of Means and Standard Deviations for Positive Controls**
 3796 **Tested With and Without Test Substance Applicator**
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Positive Control	N ¹	Hemorrhage ² (mean ± SD ⁴)	Lysis ² (mean ± SD)	Coagulation ² (mean ± SD)	<i>In Vitro</i> Score ³ (mean ± SD)
DMF ⁵ : With TSA ⁶	69	0.02 ± 0.17 ⁷	6.93 ± 0.03	8.82 ± 15.77	15.77 ± 0.19
DMF: With TSA ³	10	3.36 ± 0.32	6.54 ± 0.19	8.81 ± 0.04	18.71 ± 0.38
DMF: Without TSA	2	4.00 ± 0.13	6.84 ± 0.05	8.76 ± 0.08	19.60 ± 0.15
Imidazole: Without TSA	15	4.50 ± 0.39	6.84 ± 0.08	8.66 ± 0.17	20.00 ± 0.45

3798

¹N = number of tests.

3799

²Mean values of time until development of identified endpoint.

3800

³*In Vitro* irritation score calculated as IS(B).

3801

⁴SD = standard deviation.

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⁵DMF = dimethylformamide

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⁶TSA = test substance applicator.

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⁷Hemorrhage endpoint in studies described in the first row were evaluated inside the TSA while hemorrhage

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endpoint in studies described in the second row were evaluated outside the TSA.

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Using the data provided by Dr. P. Vanparys, the intralaboratory reproducibility of the positive controls was evaluated. For the positive control imidazole, the %CV values were calculated for each endpoint as well as for the overall IS(B) score. The range of %CV values was 0.12-18.97 for the hemorrhage endpoint, 0.34-1.20 for the lysis endpoint, and 0.20-2.11 for the coagulation endpoint. The range of %CV values for the overall IS(B) score was 0.12-1.58. The average and median %CV values for the overall IS(B) score (last column in **Table IV-25**) were 0.97 and 0.5, respectively.

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For the positive control DMF, the data where hemorrhages develop inside the TSA was evaluated. The range of %CV values was 0.00-1.27 for the lysis endpoint and 0.00-1.76 for the coagulation endpoint. For the hemorrhage endpoint, a single test produced a result other than zero for the mean and the tested eggs and the standard deviation; the %CV value for the single test was 173.94. The range of %CV values for the overall IS(B) score was 0.04-14.07. The average and median %CV values for the overall IS(B) score (last column in **Table IV-26**) were 0.59 and 0.29, respectively.

3822

3823 **Table IV-25. Intralaboratory %CV¹ Evaluation for Imidazole**

3824

Experiment	Hemorrhage		%CV of Exp.	Lysis		%CV of Exp.	Coagulation		%CV of Exp.	Total Score		%CV of Exp.
	Mean of Exp. ²	SD ³ of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.	
186	4.35	0.83	18.97	6.82	0.03	0.42	8.40	0.08	0.94	19.58	0.89	4.54
190	4.91	0.02	0.47	6.91	0.03	0.42	8.74	0.05	0.52	20.56	0.09	0.45
194	4.27	0.22	5.04	6.78	0.07	1.09	8.86	0.02	0.20	19.91	0.29	1.45
214	3.95	0.08	1.90	6.89	0.02	0.25	8.76	0.03	0.34	19.60	0.10	0.50
220	4.34	0.12	2.79	6.87	0.08	1.17	8.71	0.11	1.30	19.93	0.10	0.50
269	3.84	0.32	8.35	6.92	0.02	0.33	8.73	0.03	0.34	19.49	0.31	1.58
270	4.00	0.13	3.12	6.73	0.07	1.05	8.31	0.13	1.57	19.05	0.23	1.18
274	4.25	0.10	2.46	6.83	0.05	0.76	8.54	0.03	0.41	19.62	0.17	0.88
278	4.60	0.13	2.83	6.91	0.03	0.42	8.79	0.03	0.34	20.30	0.10	0.49
281	4.56	0.01	0.25	6.92	0.01	0.17	8.75	0.02	0.20	20.24	0.03	0.12
5A	4.88	0.03	0.52	6.66	0.08	1.20	8.88	0.03	0.34	20.41	0.14	0.66
7A-9A	4.94	0.01	0.23	6.87	0.02	0.34	8.49	0.06	0.71	20.30	0.03	0.17
12A	4.93	0.01	0.12	6.81	0.03	0.37	8.54	0.08	0.88	20.28	0.09	0.43
13	4.93	0.02	0.35	6.85	0.05	0.75	8.57	0.18	2.11	20.35	0.24	1.17
14	4.76	0.03	0.56	6.87	0.02	0.34	8.81	0.07	0.79	20.44	0.09	0.43
Mean (SD)	4.5 (0.39)			6.84 (0.08)			8.66 (0.17)			20.00 (0.45)		
Range of %CV	0.12 – 18.97			0.34-1.20			0.20-2.11			0.12-1.58		
Overall %CV	8.6			1.10			1.99			2.23		
Mean Total Score %CV	0.97											
Median Total Score %CV	0.50											

3825 ¹CV = coefficient of variation.3826 ²Exp. = experiment.3827 ³SD = standard deviation.

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3828 **Table IV-26. Intralaboratory Analyses %CV¹ Evaluation for Dimethylformamide**

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Experiment	Hemorrhage		%CV of Exp.	Lysis		%CV of Exp.	Coagulation		%CV of Exp.	Total Score		%CV of Exp.
	Mean of Exp. ¹	SD ² of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.	
183	0.00	0.00		6.83	0.08	1.11	8.83	0.03	0.39	15.66	0.11	0.70
185	0.00	0.00		6.87	0.04	0.52	8.61	0.03	0.35	15.48	0.04	0.26
186	0.00	0.00		6.85	0.10	1.44	8.52	0.15	1.76	15.37	0.20	1.30
188	0.00	0.00		6.90	0.04	0.59	8.36	0.12	1.45	15.26	0.16	1.04
189	0.00	0.00		6.88	0.05	0.69	8.62	0.10	1.12	15.50	0.14	0.91
190	0.00	0.00		6.92	0.01	0.17	8.78	0.02	0.20	15.70	0.03	0.16
191	0.00	0.00		6.93	0.04	0.58	8.82	0.00	0.00	15.75	0.04	0.26
192	0.00	0.00		6.95	0.03	0.36	8.79	0.05	0.59	15.74	0.08	0.48
193	0.00	0.00		6.91	0.05	0.68	8.91	0.03	0.34	15.82	0.08	0.48
194	0.00	0.00		6.82	0.04	0.59	8.90	0.03	0.39	15.72	0.07	0.41
196	0.00	0.00		6.93	0.02	0.29	8.74	0.06	0.71	15.67	0.07	0.45
198	0.00	0.00		6.91	0.03	0.36	8.72	0.05	0.53	15.63	0.07	0.43
201	0.00	0.00		6.91	0.05	0.68	8.65	0.06	0.72	15.56	0.11	0.70
202	0.00	0.00		6.95	0.03	0.42	8.79	0.06	0.68	15.74	0.09	0.55
203	0.00	0.00		6.92	0.01	0.17	8.77	0.02	0.20	15.69	0.03	0.16
205	0.00	0.00		6.95	0.03	0.36	8.87	0.02	0.20	15.82	0.04	0.26
207	0.00	0.00		6.94	0.02	0.33	8.83	0.06	0.71	15.77	0.09	0.54
208	1.42	2.47	173.94	6.92	0.01	0.17	8.68	0.08	0.87	17.02	2.39	14.07
209	0.00	0.00		6.94	0.01	0.17	8.79	0.03	0.34	15.73	0.04	0.26
211	0.00	0.00		6.94	0.01	0.17	8.84	0.05	0.52	15.78	0.06	0.36
212	0.00	0.00		6.95	0.03	0.42	8.85	0.00	0.00	15.80	0.03	0.18
213	0.00	0.00		6.92	0.01	0.17	8.78	0.05	0.52	15.70	0.05	0.29
215	0.00	0.00		6.83	0.05	0.69	8.71	0.05	0.53	15.54	0.09	0.60
217	0.00	0.00		6.91	0.04	0.51	8.80	0.02	0.20	15.71	0.05	0.33
230	0.00	0.00		6.91	0.03	0.36	8.92	0.02	0.19	15.83	0.04	0.26
231	0.00	0.00		6.98	0.00	0.00	8.87	0.02	0.20	15.85	0.02	0.11
232	0.00	0.00		6.96	0.02	0.25	8.86	0.02	0.20	15.82	0.03	0.22
233	0.00	0.00		6.96	0.03	0.41	8.84	0.03	0.39	15.80	0.06	0.35
234	0.00	0.00		6.94	0.01	0.17	8.84	0.02	0.20	15.80	0.02	0.10
235	0.00	0.00		6.96	0.02	0.25	8.86	0.02	0.20	15.82	0.03	0.22
236	0.00	0.00		6.97	0.02	0.25	8.89	0.02	0.19	15.86	0.03	0.19
237	0.00	0.00		6.97	0.02	0.25	8.77	0.09	1.05	15.74	0.08	0.50

Experiment	Hemorrhage		%CV of Exp.	Lysis		%CV of Exp.	Coagulation		%CV of Exp.	Total Score		%CV of Exp.
	Mean of Exp. ¹	SD ² of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.	
238	0.00	0.00		6.94	0.01	0.17	8.86	0.02	0.20	15.80	0.03	0.16
240	0.00	0.00		6.97	0.02	0.25	8.87	0.02	0.20	15.84	0.03	0.22
241	0.00	0.00		6.97	0.02	0.25	8.89	0.03	0.39	15.86	0.05	0.33
242	0.00	0.00		6.94	0.01	0.17	8.87	0.02	0.20	15.81	0.03	0.18
243	0.00	0.00		6.96	0.02	0.25	8.90	0.02	0.19	15.86	0.03	0.19
244	0.00	0.00		6.95	0.03	0.36	8.90	0.03	0.39	15.85	0.06	0.37
245	0.00	0.00		6.96	0.02	0.25	8.89	0.02	0.19	15.85	0.03	0.22
251	0.00	0.00		6.93	0.06	0.90	8.81	0.07	0.79	15.74	0.13	0.83
252	0.00	0.00		6.94	0.01	0.17	8.86	0.02	0.20	15.80	0.03	0.16
253	0.00	0.00		6.95	0.03	0.36	8.84	0.08	0.85	15.79	0.09	0.57
254	0.00	0.00		6.91	0.04	0.51	8.81	0.08	0.86	15.72	0.11	0.70
255	0.00	0.00		6.93	0.00	0.00	8.81	0.05	0.52	15.74	0.05	0.29
256	0.00	0.00		6.94	0.01	0.17	8.86	0.02	0.20	15.80	0.03	0.16
257	0.00	0.00		6.93	0.02	0.29	8.84	0.02	0.20	15.77	0.04	0.23
258	0.00	0.00		6.96	0.02	0.25	8.85	0.03	0.34	15.81	0.05	0.29
259	0.00	0.00		6.93	0.04	0.58	8.85	0.08	0.90	15.78	0.12	0.76
260	0.00	0.00		6.94	0.01	0.17	8.85	0.03	0.34	15.79	0.04	0.26
261	0.00	0.00		6.95	0.03	0.36	8.86	0.05	0.52	15.81	0.07	0.45
262	0.00	0.00		6.94	0.01	0.17	8.87	0.02	0.20	15.81	0.02	0.10
263	0.00	0.00		6.94	0.02	0.33	8.86	0.02	0.20	15.80	0.04	0.22
264	0.00	0.00		6.97	0.02	0.25	8.87	0.02	0.20	15.84	0.02	0.11
265	0.00	0.00		6.96	0.02	0.25	8.88	0.03	0.34	15.84	0.05	0.29
266	0.00	0.00		6.89	0.09	1.27	8.76	0.13	1.49	15.65	0.22	1.39
267	0.00	0.00		6.94	0.01	0.17	8.84	0.02	0.20	15.78	0.02	0.10
268	0.00	0.00		6.95	0.00	0.00	8.89	0.02	0.19	15.84	0.02	0.11
269	0.00	0.00		6.95	0.00	0.00	8.89	0.02	0.19	15.84	0.02	0.11
270	0.00	0.00		6.94	0.01	0.17	8.88	0.03	0.34	15.82	0.04	0.26
271	0.00	0.00		6.94	0.01	0.17	8.84	0.02	0.20	15.78	0.01	0.04
272	0.00	0.00		6.95	0.04	0.51	8.81	0.07	0.79	15.76	0.10	0.65
273	0.00	0.00		6.95	0.03	0.42	8.85	0.03	0.34	15.80	0.06	0.36
274	0.00	0.00		6.94	0.02	0.33	8.86	0.06	0.70	15.80	0.09	0.54
275	0.00	0.00		6.96	0.02	0.25	8.89	0.02	0.19	15.85	0.02	0.11
277	0.00	0.00		6.90	0.04	0.52	8.80	0.06	0.71	15.70	0.10	0.63
278	0.00	0.00		6.94	0.02	0.33	8.82	0.03	0.34	15.76	0.05	0.33

Experiment	Hemorrhage		%CV of Exp.	Lysis		%CV of Exp.	Coagulation		%CV of Exp.	Total Score		%CV of Exp.
	Mean of Exp. ¹	SD ² of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.		Mean of Exp.	SD of Exp.	
279	0.00	0.00		6.93	0.00	0.00	8.83	0.03	0.39	15.76	0.03	0.22
280	0.00	0.00		6.90	0.08	1.10	8.81	0.10	1.09	15.71	0.17	1.10
282	0.00	0.00		6.92	0.02	0.33	8.85	0.03	0.34	15.77	0.05	0.33
Mean (SD)	0.02 (0.17)			6.93 (0.03)			8.82 (0.09)			15.77 (0.19)		
Range⁴ of %CV values	173.94¹			0.00-1.27			0.00-1.76			0.04-14.07		
Overall %CV	850			0.49			1.05			1.20		
Mean Total Score %CV	0.59											
Median Total Score %CV	0.29											

3830 ¹CV = coefficient of variation.3831 ²Exp. = experiment.3832 ³SD = standard deviation.3833 ⁴Range is representative of a single value since CV values for other experiments could not be calculated since mean and SD values were zero.

3834 3.5.2 Data Provided by Dr. med Horst Spielmann and Dr. Manfred Liebsch
 3835 HET-CAM studies using 1% SDS and 0.1 N NaOH were provided by Dr. med H. Spielmann
 3836 and Dr. M. Liebsch in response to a request from NICEATM. Using the mean values
 3837 determined for these studies, the overall irritation score calculated (according to the method
 3838 of Kalweit et al. [1987, 1990]) for these substances classified them as irritants (**Table IV-27**).
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Table IV-27. Means and Standard Deviations of Positive Control Test Substances

Positive Control	Hemorrhage ¹ (mean ± SD ²)	Lysis ¹ (mean ± SD)	Coagulation ¹ (mean ± SD)
1% SDS ³ (n=377)	14.69 ± 5.36	35.18 ± 17.15	--- ⁴
0.1 N NaOH ⁵ (n=336)	8.96 ± 4.96	35.60 ± 24.71	48.04 ± 34.56

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¹Mean values of time until development of identified endpoint.

²SD = standard deviation.

³SDS = sodium dodecyl sulfate.

⁴It was indicated that 1% SDS does not produce coagulation in the CAM after application. However, in the studies conducted coagulation was identified in a single study. In these evaluations, the non-existing data was calculated with an arbitrary value of "0". Therefore, the calculation of a mean value for the coagulation endpoint was not meaningful.

⁵NaOH = sodium hydroxide.

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3.6 Reliability of the HET-CAM Test Method for Identifying Ocular Corrosives and Severe Irritants – Summary of Reanalysis

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Previously, an evaluation of the intralaboratory repeatability and reproducibility of the HET-CAM test method could not be conducted. However, subsequent to the original reliability analysis (see draft HET-CAM BRD, November 1, 2004), replicate data received allowed for a quantitative analysis of intralaboratory repeatability and reproducibility of HET-CAM test method endpoints.

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The analysis of intralaboratory repeatability was evaluated using data from two different publications (Gilleron et al. [1996, 1997]) that were provided in response to a request from NICEATM. In both studies, the hemorrhage endpoint had a high %CV value (104-117). Additionally, the %CV values for the coagulation endpoint were the lowest of the three endpoints evaluated in the HET-CAM test method. However, the actual values were quite disparate between the two studies (e.g., Gilleron et al. [1996] coagulation %CV = 95.69; Gilleron et al. [1997] coagulation %CV = 41.78). The difference in the numbers may be due to several factors including test substances evaluated and differences in the test method protocols used between the two studies. The overall IS(B) %CV values for the two studies were 41.48 (Gilleron et al. [1996]) and 6.99 (Gilleron et al. [1997]). However, the calculated variability for the endpoints and the overall test method may be exaggerated because of the relatively small values that are obtained from each of the endpoints (5 for hemorrhage, 7 for lysis, and 9 for coagulation).

3875 Similar results were obtained from the analysis of intralaboratory reproducibility. The
3876 overall %CV values were 53 and 17.5 for the two studies evaluated. For the study by
3877 Gilleron et al. (1997), where substances could be classified according to the GHS and EPA
3878 classification systems, %CV values for severe irritants were similar to the values obtained for
3879 the overall database.

3880

3881 The previous analysis also included an evaluation of interlaboratory reproducibility using
3882 both qualitative and quantitative approaches. Additional data received subsequent to the
3883 draft HET-CAM BRD allowed for a more in-depth quantitative and qualitative analysis of
3884 interlaboratory reproducibility. For the qualitative evaluation of data from Spielmann et al.
3885 (1996), 100% agreement between testing laboratories was between 80% and 85% for all the
3886 test substances. Furthermore, quantitative evaluation of the interlaboratory reproducibility
3887 for the Spielmann et al. (1996) data yielded an overall %CV value of about 35.

3888

3889 The previous interlaboratory reproducibility analyses also were modified based on the re-
3890 classification of substances as an ocular corrosive/severe irritant or as a non-corrosive/non-
3891 severe irritant. However, the overall results obtained in the revised analysis were not
3892 different from the original analysis.

3893

3894 Finally, historical positive and negative control data were provided by two different sources.
3895 The negative control substance evaluated was 0.9% NaCl. The positive control substances
3896 were DMF, imidazole, 1% SDS, and 0.1 N NaOH. The studies showed that all control
3897 substances consistently produced appropriate responses (e.g., negative control consistently
3898 produced a response that would be classified as nonirritant and positive controls consistently
3899 produced a response that would be classified as severe irritant).

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