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First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (4) Evaluation of Hemoglobin Denaturation (HD) Test

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SUMMARY

The hemoglobin denaturation (HD) test to detect eye irritation potential of cosmetic ingredients was primarily validated by eight laboratories using nine surfactants and physiological saline as test substances. The test procedures were controlled under the common standard operating procedure (SOP) in which the denaturation was measured spectrophotometrically using microplate reader and the hemoglobin denaturation ratio (HDR) for each concentration level of test chemicals was calculated. The rank order of test chemicals among laboratories was similar with respect to the concentration for 10% HDR. However, HDR values themselves varied widely among laboratories. This seemed to be caused by the differences in the model of microplate reader and its filter. Multiple linear regression analysis proved that HDRs calculated in individual laboratories were highly correlated to *in vivo* maximal average Draize total scores (MAS). The mean correlation coefficient was 0.846. From these results, we concluded that the protein denaturation test using hemoglobin is a promising alternative method to the Draize rabbit eye irritation test (Draize test). Further validation of this method using a wider range of cosmetic ingredients is under way.

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INTRODUCTION

Protein denaturation has been considered to be one of the mechanisms of eye irritation by chemicals. Hemoglobin, a metalloprotein

containing heme, exhibits an absorption maximum at about 418 nm (the visible region) at pH 6.86, and the intensity of this absorption is decreased by protein denaturation. Hayashi et al.[1-4], using a commercial grade of hemoglobin and a microplate reader, measured the decrease in the absorbance at around this absorption maximum as an endpoint of protein denaturation. They compared the hemoglobin denaturation ratio (HDR) calculated from these results with the results of the Draize rabbit eye irritation test (Draize test) by multiple regression analysis, and indicated that HD test might be available as an alternative to the Draize test.

We have conducted a first-phase interlaboratory validation of the hemoglobin denaturation test using nine surfactants and physiological saline as a negative control in eight independent laboratories under the same standard operating procedure (SOP). The results are presented and discussed in this report. This forms a part of the Ministry of Health and Welfare (MHW) project entitled "Studies on the test methods to evaluate the safety of new ingredients of cosmetics"[5].

MATERIALS AND METHODS

Materials.

The 10 test substances used in this project are listed in Table I. They comprise one cationic surfactant, 4 anionic surfactants, 4 nonionic surfactants and isotonic sodium chloride solution (physiological saline)[6]. They complied with the Japanese standards of cosmetic ingredients[7,8] and were supplied by the Japan Cosmetic Industry Association (JCIA) to the national Institute of Health Sciences (NIHS). The substances were coded by the Test Substance Control Committee and supplied to all participating laboratories, which were blinded as to the nature of the test materials. Bovine hemoglobin and standard phosphate buffer (pH 6.86) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Test procedures

The method was as described by Hayashi et al[1-3]. Briefly, hemoglobin (0.5 g/liter) was dissolved in the standard phosphate buffer (pH 6.86) at 0.05% (w/v) concentration. Surfactants were diluted with ion-exchanged water to make a 2.0% (w/v) solution. In a 96-well microplate (Becton Dickinson, NJ, USA), 100 μl aliquots of surfactant solution at 11 concentration levels, prepared by the serial two-fold dilution method, were distributed. The same operations were carried out for 8 rows. Equal volumes of hemoglobin/buffer solution were added to each well of 4 rows. The other 4 rows were filled with equal amounts of buffer solution. The microplate was then incubated for 5 minutes at room temperature and the absorbance at about 418 nm was measured with a microplate reader. The obtained data (n=4) were processed in accordance with the following equation (Equation-1) and the hemoglobin denaturation ratio (HDR%) for each concentration level was calculated.

Table I. List of the test substances

No.	Test substances	Abbreviation	Classification
S-1	Isotonic Sodium Chloride Solution	Physiological saline	-
S-2	Polyoxyethylene Hydrogenated Castor Oil (60E.O.)	POE hydrogenated castor oil	Nonionic
S-3	Polyoxyethylene Sorbitan Monolaurate (20E.O.)	Tween 20	Nonionic
S-4	Polyethyleneglycol Monolaurate (10E.O.)	PEG monolaurate	Nonionic
S-5	Sodium N-Lauroyl Sarcosinate (30% solution)	Lauroyl sarcosinate	Anionic
S-6	Sodium Hydrogenated Tallow L-Glutamate	HT-glutamate	Anionic
S-7	Sodium Lauryl Sulfate	SLS	Anionic
S-8	Sodium Polyoxyethylene Laurylether Sulfate (2E.O.) (27% solution)	POE laurylether sulfate	Anionic
S-9	Polyoxyethylene Octylphenylether (10E.O.)	Triton X-100	Nonionic
S-10	Benzalkonium Chloride	Benzalkonium chloride	Cationic

$$HDR\% = 100 - \{Abs(SHB) - Abs(SB)\}/\{Abs(WHB) - Abs(WB)\} \times 100(\%) \text{(Equation-1)}$$

where Abs (SHB); absorbance of hemoglobin/buffer solution containing surfactants; Abs(SB); absorbance of buffer solution containing surfactants; Abs (WHB); absorbance of hemoglobin/buffer solution diluted with ion-exchanged water; Abs (WB); absorbance of buffer solution diluted with ion-exchanged water.

In vivo test

In vivo testing was performed by the conventional Draize eye irritation test method[9] and the results have been separately reported by Ohno et al[10].

Statistical Analysis

Satistical calculations were done by both an IBM5550 system and by using the Lotus 1-2-3 Multi-Variate Analysis Program provided by Audemain, Tokyo.

RESULTS AND DISCUSSION

Hemoglobin denaturation (HD) test

Typical dose-response relationships of the ten test chemicals with respect to the HDR obtained in laboratory A are shown in Fig. 1. The HDR of five chemicals (lauroyl sarcosinate: S5, HT-glutamate: S6, SLS:S7, POE laurylether sulfate: S8 and benzalkonium chloride: S10) increased with increasing concentration of the test compounds. Hemoglobin denaturation by the other five chemicals was not observed even at maximum concentration (1%) tested according to the SOP.

As shown in Tables II and III, the eight laboratories that participated in this program used different types of filters. Six laboratories used 415 nm, one used 418 nm and one used 420 nm filters. Laboratory A performed the hemoglobin denaturation (HD) test using filters of both 418 nm and 415 nm. The results of the HD test for 1.0%, 0.125% and 0.016% test chemicals (final concentrations) are

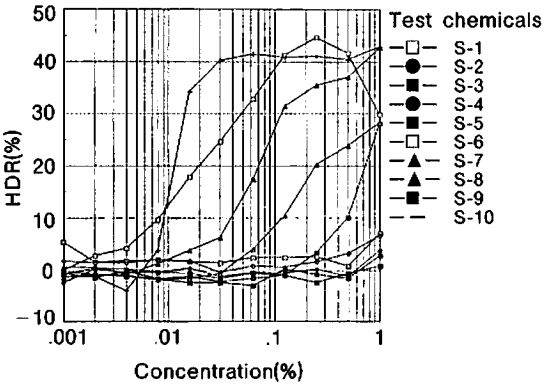

Figure 1. The dose-response relationships for ten test chemicals.
*The values of hemoglobin denaturation ratio (HDR) were obtained from laboratory A using a filter of 418 nm.

shown in Table II. Similar results were obtained in all laboratories even though the filter wavelengths were slightly different among laboratories.

HDR was measured twice for each chemical and the values of the ratio of the results, indicating intra-laboratory variance, are shown in Table III. Although two-fold serial dilutions of test chemicals were used in this SOP, the ratio was less than 2.0 when HDR was higher than 10%. It was notable that the intra-laboratory variances were small in the case of high HDR.

In order to assess inter-laboratory reproducibility, the means of HDR results in each laboratory were calculated, and the results are shown in Table IV. The coefficient of variance (CV) for the mean HDR was high, especially, for low HDR. The high CV value for HT-glutamate (S6) may be a consequence of its low solubility in water.

Because of low inter-laboratory reproducibility, we could not use the mean HDR of all laboratories, results as the parameter to be correlated with the *in vivo* test. Therefore, we tried to identify the cause of the low inter-laboratory reproducibility.

Studies on the inter-laboratory variance

We searched for the cause of the rather large inter-laboratory variance in the obtained

Table II. HDR values obtained from each laboratory

Laboratory		A				B		C		D		E		F		G		H	
wavelength(filter)		418nm		415nm		415nm		420nm		414nm		415nm		415nm		415nm		415nm	
Sample No	Conc.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
S-1	1.000%	7.03	1.58	7.30	4.39	1.44	-3.41	-2.29	3.75	2.90	-10.65	-9.0	-0.3	-5.8	-6.3	0.707		1.0	-6.6
	0.125%	2.33	1.47	2.64	1.22	-0.86	-3.60	-0.15	-0.45	2.90	-10.91	-14.3	-2.7	4.1	-0.9	-0.133		-0.8	-6.2
	0.016%	1.58	0.98	1.87	0.93	-1.05	-2.67	-1.38	1.50	6.00	-7.24	-12.9	-1.8	-2.2	-3.9	1.502		-1.2	-5.8
S-2	1.000%	3.75	7.56	3.70	7.83	-2.66	-4.88	2.56	-0.16	0.77	0.65	0.5	2.5	1.4	-0.7	4.569	5.551	-2.7	-1.3
	0.125%	-0.67	1.52	-0.88	1.38	-4.84	-5.63	-1.21	-2.34	-0.87	-5.18	-1.0	-0.1	-0.7	-3.5	2.336	2.003	-3.3	0.1
	0.016%	0.40	0.65	0.15	0.47	-5.24	-7.15	-2.41	-1.09	-0.14	-2.76	-1.4	-1.6	0.7	-4.0	1.298	1.127	-4.5	-1.5
S-3	1.000%	0.84	1.43	0.49	0.93	-4.57	-6.01	-2.37	-2.53	-7.71	-1.58	0.2	-1.6	2.3	0.5	-2.264	-0.850	0.3	3.9
	0.125%	-0.98	-1.39	-1.12	-1.81	-6.56	-6.44	-8.04	-4.80	-8.01	-2.35	-1.3	-4.4	1.8	-2.7	0.924	-0.085	-2.9	0.5
	0.016%	-1.29	-0.36	-1.36	-0.78	-5.48	-6.20	-4.42	-0.31	-4.64	-0.73	-1.3	-3.5	1.1	-3.4	-2.421	-2.083	-1.0	3.2
S-4	1.000%	6.57	7.79	6.81	8.08	1.04	3.05	5.99	4.50	3.42	2.69	-0.3	0.5	6.9	8.0	3.648	9.583	14.7	12.0
	0.125%	0.25	0.75	0.38	0.58	-5.66	-7.30	-2.69	-7.13	-1.54	-2.35	-1.7	-3.2	-1.4	-5.5	-0.657	4.000	2.8	2.6
	0.016%	1.78	4.22	2.48	4.33	-5.46	-5.98	-1.20	-5.27	-1.50	-0.54	-1.3	-3.0	2.0	-3.1	-3.826	4.292	10.5	4.2
S-5	1.000%	27.91	27.24	16.68	16.24	35.13	33.95	38.62	40.03	25.59	20.69	34.9	29.1	32.0	28.4	56.676	15.221	4.8	7.3
	0.125%	-1.06	-5.49	-2.13	-1.41	-6.38	-5.25	-3.69	-0.75	-2.37	-7.51	-2.8	0.6	1.2	-1.9	-0.986	-0.085	-2.2	0.7
	0.016%	-0.66	-0.71	-0.97	-0.73	-4.51	-5.25	-1.54	-1.35	-1.16	-4.58	-2.0	1.1	-1.2	-0.8	-2.106	-1.828	2.1	8.8
S-6	1.000%	29.83	15.70	5.94	-13.57	59.47	59.35	53.17	61.75	27.36	24.85	29.9	56.3	42.7	52.6	-6.439	6.792	9.1	11.2
	0.125%	41.22	25.91	31.23	16.27	38.82	38.53	50.60	61.99	37.67	25.70	26.6	16.5	40.1	36.6	21.226	25.333	81.6	91.4
	0.015%	17.83	10.99	10.60	3.59	24.07	24.20	30.66	31.25	14.60	8.93	13.7	12.4	17.3	15.4	7.238	9.583	38.6	58.3
S-7	1.000%	42.55	43.10	27.60	27.87	54.31	53.34	60.06	60.53	43.02	40.54	52.4	45.0	49.8	49.5	28.289	28.199	33.5	26.9
	0.125%	31.35	32.26	19.76	20.43	42.23	44.01	47.87	48.96	28.01	31.30	34.2	34.7	42.9	38.7	49.123	33.544	11.1	0.7
	0.016%	3.79	5.91	-4.86	-2.80	17.68	13.92	22.83	25.96	3.60	-0.08	1.3	3.9	11.7	7.0	-3.904	-3.030	4.1	-4.0
S-8	1.000%	28.19	31.37	16.83	20.47	42.56	42.29	43.34	43.69	29.38	26.36	27.1	27.0	33.1	34.5	31.532	17.645	37.3	35.1
	0.125%	10.50	10.40	0.88	0.96	19.30	19.04	25.27	27.23	8.67	5.64	0.6	1.9	15.3	15.1	-6.485	0.340	9.1	1.0
	0.016%	-1.65	-0.13	-2.15	-0.58	-4.53	-4.74	-0.15	1.23	-1.99	-3.88	-1.9	-2.9	2.4	-1.4	-4.417	-1.701	1.1	-4.6
S-9	1.000%	2.83	3.32	2.61	2.80	-3.78	-6.01	1.17	2.25	-4.33	-1.36	-0.3	0.2	-4.3	-2.6	3.047	-1.284	1.3	-1.6
	0.125%	0.27	-0.93	0.14	-0.92	-6.01	-7.05	3.07	-3.00	-5.13	-2.50	-3.6	-3.9	-1.7	-1.6	1.927	0.985	-0.3	-0.2
	0.015%	-2.35	-0.84	-2.27	-0.97	-7.84	-5.62	1.17	-2.10	-3.54	-2.18	-3.1	-2.8	-2.1	-8.1	0.179	-0.214	0.2	1.3
S-10	1.000%	42.77	43.11	31.30	31.54	51.21	49.82	53.12	53.79	43.28	41.08	44.9	45.8	49.6	65.6	31.360	33.087	44.4	44.2
	0.125%	40.74	40.54	29.75	29.34	48.91	48.97	50.31	51.74	43.59	39.80	44.4	46.7	49.3	45.9	32.149	34.031	25.9	22.6
	0.016%	34.26	33.44	24.67	24.00	44.78	46.32	37.97	49.84	35.38	39.48	56.9	38.0	46.5	43.7	30.439	52.217	-5.3	-7.5

*The results are presented as HDR(%).
**The MD tests were performed two times.

Table III. The ratio of two HDR results obtained from each laboratory

Laboratory		A		B	C	D	E	F	G	H	Mean
Filter		418nm	415nm	415nm	420nm	414nm	415nm	415nm	415nm	415nm	
Sample No.	Conc.	Ratio of two HDR results									
S-1	1.000%	4.449	1.663	2.368	1.538	5.672	30.000	1.086	—	6.600	8.435
	0.125%	1.585	2.164	4.186	3.000	3.762	5.296	4.556	—	7.752	4.337
	0.016%	1.612	2.011	2.543	1.087	1.207	7.167	1.713	—	4.833	2.179
S-2	1.000%	2.016	2.116	1.835	16.000	1.185	5.000	2.000	1.271	2.077	3.722
	0.125%	2.269	1.586	1.163	1.934	5.954	2.400	5.000	1.156	33.000	6.250
	0.016%	1.625	3.133	1.365	2.211	19.714	1.143	5.714	1.152	3.000	4.340
S-3	1.000%	1.702	1.898	1.315	1.110	4.880	8.000	4.600	3.226	9.565	4.044
	0.125%	1.418	1.616	1.019	1.675	3.409	3.384	1.500	10.871	5.800	3.410
	0.015%	3.583	1.744	1.496	14.258	6.356	2.769	2.182	1.152	3.200	4.083
S-4	1.000%	1.186	1.186	2.933	1.331	1.271	1.667	1.159	2.627	1.225	1.621
	0.125%	3.000	1.526	1.290	2.651	1.372	1.582	3.929	5.997	1.077	2.525
	0.016%	2.371	1.746	1.095	4.392	2.953	2.308	1.550	1.122	2.500	2.227
S-5	1.000%	1.025	1.027	1.035	1.037	1.225	1.199	1.127	3.734	1.717	1.458
	0.125%	2.163	1.511	1.213	4.920	3.169	4.667	1.583	1.625	3.143	2.667
	0.016%	1.075	1.329	1.167	1.141	3.948	1.818	1.500	1.152	4.190	1.925
S-6	1.000%	1.785	2.285	1.002	1.162	1.101	1.883	1.232	1.055	1.231	1.415
	0.125%	1.591	1.919	1.008	1.223	1.466	1.512	1.056	1.193	1.120	1.359
	0.016%	1.622	2.952	1.005	1.019	1.635	1.105	1.123	1.324	1.534	1.480
S-7	1.000%	1.013	1.010	1.018	1.008	1.061	1.164	1.056	1.003	1.245	1.059
	0.125%	1.029	1.034	1.042	1.023	1.117	1.015	1.109	1.454	15.857	2.743
	0.016%	1.559	1.736	1.270	1.137	45.000	3.000	1.667	1.288	1.025	6.409
S-8	1.000%	1.128	1.216	1.006	1.008	1.115	1.004	1.042	1.787	1.063	1.152
	0.125%	1.010	1.091	1.014	1.078	1.537	3.167	1.013	19.074	8.100	4.232
	0.016%	12.692	3.707	1.046	8.200	1.437	1.526	1.714	2.597	4.182	4.122
S-9	1.000%	1.173	1.073	1.590	1.923	3.184	1.500	1.654	2.373	1.231	1.746
	0.125%	3.444	6.571	1.175	1.023	2.052	1.083	1.063	1.956	1.500	2.207
	0.016%	2.798	2.340	1.395	1.795	1.570	1.107	3.857	1.196	6.500	2.518
S-10	1.000%	1.008	1.008	1.028	1.013	1.071	1.020	1.323	1.055	1.005	1.059
	0.125%	1.005	1.014	1.001	1.028	1.098	1.052	1.074	1.059	1.146	1.053
	0.016%	1.025	1.028	1.034	1.313	1.116	1.497	1.064	1.715	1.415	1.245
Mean	1.000%	1.649	1.448	1.513	2.723	1.977	5.244	1.623	2.013	2.706	2.322
	0.125%	1.851	2.001	1.411	1.956	2.494	2.556	2.192	4.934	7.949	3.038
	0.016%	2.996	2.173	1.342	3.655	8.505	2.344	2.214	1.412	3.238	3.098

data. Since we did not use the same lot of hemoglobin in all the laboratories, we first studied the effect of hemoglobin lot on inter-laboratory reproducibility by using SLS (S7) as a test chemical with the same model of microplate reader (Bio-Rad Model 3550, Laboratory A). With a filter of 418 nm, the mean and SD of HDR at 1.0% SLS (S7) as a final concentration were 42.7 and 1.3 (CV: 0.032), respectively (Table V). With a 415 nm filter, they were 27.8 and 1.6 (CV: 0.060), respectively (Table VI). These results suggest that HDR is not influenced by hemoglobin lot.

Then, we studied effects of the model of microplate reader and its filter on inter-laboratory reproducibility, also using SLS (S7) as a test chemical. The HDRs of SLS (S7)

Table IV. The mean of the two HDR results in each laboratory

Laboratory		A		B	C	D	E	F	G	H	Mean	SD	CV
Filter		418nm	415nm	415nm	420nm	414nm	415nm	415nm	415nm	415nm			
Sample No.	Conc.	Mean of the two HDR results											
S-1	1.000%	4.305	5.845	-0.985	0.730	-3.875	-4.650	-6.050	0.707	-2.800	-0.753	4.042	—
	0.125%	1.900	1.930	-2.230	-0.300	-4.005	-8.500	1.600	-0.133	-3.569	-1.471	3.465	—
	0.016%	1.280	1.400	-1.860	0.060	-0.620	-7.350	-3.050	1.502	-3.500	-1.349	2.935	—
S-2	1.000%	5.655	5.765	-3.770	1.200	0.710	1.500	0.350	4.960	-2.000	1.597	3.343	2.094
	0.125%	0.425	0.250	-5.235	-1.775	-3.025	-1.700	-2.100	2.170	-1.700	-1.410	2.149	—
	0.016%	0.525	0.310	-6.195	-1.750	-1.450	-1.500	-1.650	1.213	-3.000	-1.500	2.211	—
S-3	1.000%	1.135	0.710	-5.290	-2.500	-4.645	-0.700	1.400	-0.557	1.600	-0.983	2.604	—
	0.125%	-1.185	-1.465	-6.500	-6.420	-5.180	-2.850	-0.450	0.420	-1.200	-2.759	2.629	—
	0.016%	-0.825	-1.070	-6.840	-2.365	-2.685	-2.450	-0.650	-2.252	1.100	-2.004	2.179	—
S-4	1.000%	7.180	7.445	2.045	5.245	3.055	0.100	7.450	6.616	13.350	5.832	3.864	0.663
	0.125%	0.500	0.480	-6.480	-4.910	-1.945	-2.450	-3.450	1.667	2.760	-1.543	3.100	—
	0.016%	3.000	3.405	-5.720	-3.235	-1.070	-2.150	-0.550	0.233	7.350	0.140	3.933	28.026
S-5	1.000%	27.575	16.450	34.540	39.325	23.240	32.000	30.200	35.949	6.250	27.281	10.486	0.384
	0.125%	-0.775	-1.770	-5.820	-2.220	-4.940	-1.100	-0.350	-0.775	-0.750	5.832	3.864	0.603
	0.016%	-0.685	-0.850	-4.885	-1.445	-2.870	-0.450	-1.000	-1.967	5.450	-0.967	2.778	—
S-6	1.000%	23.265	-3.815	59.415	57.465	26.105	43.100	47.650	0.177	10.150	29.279	23.941	0.818
	0.125%	33.565	23.750	38.675	56.250	31.685	21.550	38.350	23.280	86.500	39.289	20.619	0.526
	0.016%	14.410	7.095	24.135	30.955	11.765	13.050	16.350	8.411	48.150	19.369	13.170	0.680
S-7	1.000%	42.825	27.735	53.825	60.295	41.780	48.700	49.650	28.244	30.200	42.584	11.766	0.276
	0.125%	31.805	20.095	43.120	48.415	29.655	34.450	40.800	41.334	5.900	32.842	13.161	0.401
	0.016%	4.850	-3.830	15.800	24.395	1.760	2.600	9.350	-3.467	0.050	5.723	9.337	1.631
S-8	1.000%	29.780	18.650	42.425	43.515	27.870	27.050	33.850	24.589	36.200	31.542	8.215	0.260
	0.125%	10.450	0.920	19.170	26.250	7.155	1.250	15.200	-3.073	5.050	9.153	9.562	1.045
	0.016%	-0.890	-1.365	-4.635	0.540	-2.425	-2.400	0.500	-3.059	-1.750	-1.720	1.662	—
S-9	1.000%	3.075	2.705	-4.495	1.710	-2.845	-0.050	-3.450	0.882	-0.150	-0.335	2.816	—
	0.125%	-0.330	-0.390	-6.535	0.035	-3.815	-3.750	-1.650	1.456	-0.250	-1.692	2.518	—
	0.016%	-1.595	-1.620	-6.730	-0.465	-2.910	-2.950	-5.100	-0.018	0.750	-2.293	2.426	—
S-10	1.000%	42.940	31.420	50.515	53.455	42.530	45.350	57.600	32.224	44.300	44.482	8.781	0.197
	0.125%	40.640	29.545	48.940	51.025	41.745	45.550	47.600	33.090	24.250	40.265	9.341	0.232
	0.016%	33.850	24.335	45.550	43.905	37.430	47.450	45.100	41.328	-6.400	34.728	17.036	0.491

Table V. Effect of hemoglobin lot on HDRs of S7 (418 nm)

Laboratory	A		B		C		D		E		F		G		H		Mean	SD	CV
Hemoglobin lot	SAL8361		WDH8059		WDH8059		LKN8361		WDN9745		WDH8056		WDL9380		WDH9056				
Tested No.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2			
HDRs of 1.000% S7	39.86	42.20	44.10	44.11	43.11	42.10	42.96	45.45	41.12	42.0	42.75	43.28	42.83	41.99	44.72	42.16	42.798	1.378	0.032
0.125% S7	30.63	33.80	35.33	36.12	35.37	34.94	35.43	35.42	32.57	32.31	36.16	34.68	33.81	33.63	36.52	34.85	34.477	1.593	0.046
0.016% S7	5.08	7.24	7.56	8.81	6.75	6.29	7.60	7.91	5.61	5.41	7.34	7.37	6.52	5.13	9.15	6.22	6.937	1.153	0.166
Absorbance of control	0.536	0.559	0.661	0.667	0.630	0.628	0.632	0.635	0.570	0.578	0.667	0.568	0.629	0.624	0.656	0.639	0.624	0.041	0.066

*The HDR of S7 was measured with a Bio-Rad Model 3550 reader (Laboratory A:418 nm).

Table VI. Effect of hemoglobin lot on HDRs of S7 (415 nm)

Laboratory	A		B		C		D		E		F		G		H		Mean	SD	CV
Hemoglobin lot	SAL8361		WDH8059		WDH8059		LKN8361		WDN9745		WDH8059		WDL9380		WDH8059				
Tested No.	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2			
HDRs of 1.000% S7	31.33	27.18	29.38	29.32	28.10	25.81	29.27	31.19	25.05	26.60	27.89	28.38	27.99	26.93	30.29	27.02	27.858	1.681	0.060
0.125% S7	18.73	22.45	23.83	24.68	23.75	22.97	23.81	24.26	20.62	20.25	24.82	23.14	22.16	22.14	25.25	23.09	22.872	1.784	0.078
0.016% S7	-3.25	-0.93	-0.91	0.29	-1.96	-2.58	-0.78	-0.39	-3.08	-3.13	-1.15	-1.06	-2.05	-2.23	0.83	-2.09	-1.553	1.218	—
Absorbance of control	0.491	0.512	0.604	0.609	0.575	0.573	0.577	0.580	0.520	0.527	0.611	0.611	0.574	0.569	0.600	0.586	0.578	0.038	0.066

*The HDR of S7 was measured with a Bio-Rad Model 3550 reader (Laboratory A:415 nm).

Table VII. The HDR of S7 using same hemoglobin lot (No. SAL8361)

Laboratory	A		B	C	D	E	F	G	H	Mean	SD	CV
Microplate reader,	BIO-RAD		Corona E.	Inter Med	Krabo	Nolte. Div.	Tosoh	BIO-RAD	Corona E.			
Maker and model No.	Model 3550		MTP-32	MJ-2000	NP-500	Enax	MPR-A4i	Model 3550	MTP-100			
Wavelength (filter:nm)	418	415	415	420	414	415	415	415	415			
HDRs of 1.000% S7	42.55	27.60	52.54	57.85	42.40	25.73	45.30	24.63	45.30	40.430 (36.945)	11.952 (12.233)	0.295 (0.332)
0.125% S7	31.35	19.76	42.89	44.20	29.34	-20.50	40.80	20.15	36.10	27.088 (25.150)	20.094 (23.753)	0.742 (1.026)
0.016% S7	3.79	-4.86	13.69	20.31	3.56	-31.50	5.90	-3.30	4.50	1.036 (-2.542)	14.462 (15.692)	13.832 (—)
Absorbance of control	0.567	0.523	0.588	0.586	0.584	0.319	0.578	0.474	0.619	0.523 (0.503)	0.091 (0.104)	0.173 (0.207)

*The HDR of S7 was obtained from each laboratory.

**The results with a filter of 415 nm are shown in parentheses.

measured using the same hemoglobin lot (Lot. SAL8361), are shown in Table VII. The mean and SD of HDR at 1.0% SLS (S7) as a final concentration were 40.4 and 11.9 (CV: 0.295), respectively, and these values were almost the same as those in Table II. Particu-

Table VIII. The rank order of test chemicals

Sample No. \ Laboratory	A		B	C	D	E	F	G	H
wavelength (filter:nm)	4 1 8	4 1 5	4 1 5	4 2 0	4 1 4	4 1 5	4 1 5	4 1 5	4 1 5
S − 1	−	−	−	−	−	−	−	−	−
S − 2	−	−	−	−	−	−	−	−	−
S − 3	−	−	−	−	−	−	−	−	−
S − 4	−	−	−	−	−	−	−	−	I
S − 5	I	I	I	I	I	I	I	I	−
S − 6	IV	IV	V	V	IV	IV	IV	IV	V
S − 7	III	III	III	III	III	III	III	III	III
S − 8	II	II	II	II	II	II	II	II	II
S − 9	−	−	−	−	−	−	−	−	−
S − 10	V	V	IV	IV	V	V	V	V	IV

* "I" indicates that 10% HDR was obtained at the highest concentration.

** "−" indicates that 10% HDR was not observed at the maximum concentration (1% final conc.).

Table IX. Results of the Draize eye irritation test on the ten samples

Sample No.	Maximum score				24 hr score				Area ratio under the curve*			
	Total	Cornea	Iris	Conjunctiva	Total	Cornea	Iris	Conjunctiva	Total	Cornea	Iris	Conjunctiva
S-1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S-3	0.7 (1)**	0.0	0.0	0.7 (1)	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1
S-4	3.3 (1)	0.0	0.0	3.3 (1)	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2
S-5	10.3 (48)	8.3 (48)	0.0	8.0 (1,4)	8.3	5.0	0.0	3.3	3.4	1.9	0.0	1.5
S-6	26.7 (24)	16.7 (24,48,72)	1.7 (72)	12.0 (4)	26.7	16.7	0.0	10.0	14.9	10.7	0.8	3.5
S-7	15.0 (4)	8.3 (48,72)	0.0	10.0 (4)	14.7	6.7	0.0	8.0	7.1	4.2	0.0	3.0
S-8	10.0 (4)	3.3 (48)	0.0	10.0 (4)	2.7	0.0	0.0	2.7	2.0	0.7	0.0	1.4
S-9	41.3 (72)	30.0	5.0 (168)	10.0 (48)	24.7	15.0	1.7	8.0	26.9	18.4	2.3	6.3
S-10	78.0 (24)	66.7 (24)	5.0 (96-168)	14.7 (96)	78.0	66.7	0.0	11.3	57.3	43.9	2.5	10.9

*: The area ratio under the curve means the ratio (%) of the area under the line connecting scores at each observation period to those based on the theoretical maximum Draize total score until 7 days after treatment.

**: The values in parenthesis are the time (hour) at which the scores became maximum.

larly when the results with a filter of 415 nm were compared, there was a large inter-laboratory variance (the mean and SD were 36.845 and 12.233 (CV: 0.332), respectively). Thus, we concluded that, even when the filters of the same wavelength were used, there were differences in the filter effects among laboratories, leading to the low inter-laboratory reproducibility.

Inter-laboratory reproducibility at 10% HDR

We tried to rank the chemicals in terms of the concentration of test chemicals at which 10% of hemoglobin was denatured. The rank order of test chemicals in all labortories agreed well (Table VIII). These results suggest that, even though HDR differs among laboratories depending on the filters we used, the rank order of may be useful to rank the potency of eye irritation.

Correlation between in vivo and *in vitro* test results

Since the rank order of test chemicals in all the laboratories agreed well, even though HDR values differed among laboratories, the correlation coefficients between the maximal average Draize total scores (MAS) quoted from Ohno et al.[10] (Table IX) and the HDRs from each laboratory were calculated individually by multiple linear regression analysis (Table X). Correlation coefficients were in the range of 0.625 to 0.980 (mean±SD: 0.846±0.099). These high correlations suggest the availability of the HD test for the evaluation of eye irritancy of cosmetic ingredients. But, because of divergences between laboratories, which might be caused by variations in the filters of microplate readers, a data-base on *in vivo-in vitro* relationships should be constructed by using HDR of each laboratory.

Table X. Multiple linear regression analysis between the Draize total scores (maximum) and HDRs obtained at each laboratory

Laboratory	Filter	Multiple linear regression formula	Correlation coefficient
A	418nm	y= 9.241 +1.483*HDR(0.031)	0.836
A	415nm	y=13.626 -16.104*HDR(0.004)	0.884
B	415nm	y=13.230 +1.088*HDR(0.016)	0.787
C	420nm	y= 3.513 +0.942*HDR(0.016) -5.697*HDR(0.001)	0.861
D	414nm	y=12.681 +1.396*HDR(0.031)	0.861
E	415nm	y=12.622 +1.345*HDR(0.016)	0.873
F	415nm	y=-2.252 +2.165*HDR(0.031) -2.966*HDR(0.008) -6.740*HDR(0.004)	0.980
G	415nm	y=17.104 +1.913*HDR(0.031) -8.177*HDR(0.002)	0.915
H	415nm	y= 6.472 +0.835*HDR(0.500)	0.625

*HDR(0.031) means the HDR with 0.031% test chemicals.

Table XI. Correlation between *in vivo* and *in vitro* results

Draize scores		Filter	Multiple linear regression formula	Correlation coefficient
Total scores	(maximum)	418nm	y= 9.241 +1.483*HDR(0.031)	0.836
	(24hr)	418nm	y= 7.484 +1.429*HDR(0.031) -5.184*HDR(0.004)	0.956
	(AUC)	418nm	y= 6.319 +0.929*HDR(0.031) -5.346*HDR(0.004)	0.905
Cornea scores	(maximum)	418nm	y= 7.832 +1.057*HDR(0.031) -6.336*HDR(0.004)	0.910
	(24hr)	418nm	y= 3.661 +1.518*HDR(0.016) -4.747*HDR(0.004)	0.967
	(AUC)	418nm	y= 3.725 +0.912*HDR(0.016) -3.884*HDR(0.004)	0.915
Iris scores	(maximum)	418nm	y= 0.605 +0.090*HDR(0.031)	0.503
	(24hr)	418nm	no multiple linear regression formula	—
	(AUC)	418nm	y= 0.266 +0.046*HDR(0.031)	0.640
Conjunctivae scores	(maximum)	418nm	y= 3.140 +0.253*HDR(0.500)	0.808
	(24hr)	418nm	y= 1.717 +0.223*HDR(0.125)	0.822
	(AUC)	418nm	y= 1.394 -0.206*HDR(0.031)	0.807

*HDRs of laboratory A were used as *in vitro* data.

**HDR(0.031) means the HDR for 0.031% test chemicals.

Table XII. Compatibility between *in vivo* and *in vitro* results

		Calculated scores from *in vitro* results	
		0−15	15−110
In vivo results	0 1 15	S−1, S−2, S−3 S−4, S−5, S−8	—
	15 1 110	S−9 (false negative)	S−6, S−7, S−10

*The cut-off point was set at a maximum total score of 15.

separately.

The MAS are calculated from basic scores of changes in cornea, iris, and conjunctivae. Thus, we compared the HDRs obtained from laboratory A with those basic scores to find out which changes are best correlated with the HD test results (Table XI). The HDRs obtained by using the 418 nm filter showed the best correlation with cornea scores (0.927). The correlation with conjunctivae scores was also good (0.811). On the other hand, the iris data were insufficient to make a proper comparison. Similar results to those described above were obtained by using a 415 nm filter in laboratory A.

Compatibility with in vivo test results

Predictability of irritation potential was

assessed by multiple linear regression with the MAS for ten test chemicals (Table XII). The cut-off point was set at a maximum total score of 15. Good compatibility between *in vivo* and *in vitro* tests results was found for all these test chemicals except Triton X-100 (S9), which was considered to be false-negative in the *in vitro* test. Since Triton X-100 is commonly used for extraction and purification of enzymes because of its low protein denaturation potential, these results indicate that, even though there might be false-negative cases, this HD test will be appropriate for most eye irritants, providing basic data on the mechanism of eye irritation.

Towards further validation

Eye irritation by chemicals may be caused by many mechanisms depending on the chemical, physical, biochemical, or pharmacological properties of the chemicals and most of the *in vitro* alternative methods have been designed to cover only one or a few of these mechanisms. Thus, it seems necessary to use a battery of test methods to decrease the chance of obtaining false-negative data. For that purpose it is desirable that each of the *in vitro* methods should be based on a specific, scientifically proven mechanism. The HD method is classified as a protein denaturation test in terms of the reaction mechanism. The results obtaind in each laboratory showed good reproducibility of the method and a good correlation with the scores in the Draize test. In addition, the reason for false-negativity seems to be clear. These data are favorable from the viewpoint of predicting eye irritation of surfactants. However, the inter-laboratory reproducibility was relatively low. This was shown to be a consequence of differences in the filters, depending on the maker and lot of the filter. This result suggests that the filter must be coordinated with respect to its product lot in further validation studies. If this is not done, the microplate reader should be used only for a preliminary test and should thereafter be replaced by a spectrophotometer.

The second-phase validation of this method using a wider range of cosmetic ingredients is planned.

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