EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

MEMORANDUM (addendum) on the *in vitro* test EPISKIN™ for skin irritation testing

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 9th plenary meeting on 14 December 2010

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

Scientific Committee members

Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Qasim Chaudhry, Gisela Degen, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Kai Savolainen, Jacqueline Van Engelen, Maria Pilar Vinardell, Rosemary Waring, Ian R. White

Contact
European Commission
Health & Consumers
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-Sc6-Secretariat@ec.europa.eu

ISSN 1831-4767 ISBN 978-92-79-12748-9

Doi:10.2772/25967 ND-AQ-09-020-EN-N

ACKNOWLEDGMENTS

Prof. G. Degen
Prof. T. Platzek
Dr. S.C. Rastogi
Prof. V. Rogiers (chairman)
Prof. T. Sanner
Dr. J. van Benthem (associate scientific advisor)
Dr. J. van Engelen
Prof. P. Vinardell

Keywords: SCCS, scientific memorandum, Episkin, skin irritation, *in vitro* test, alternative methods, directive 76/768/ECC

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), memorandum (addendum) on the *in vitro* test EPISKIN™ for skin irritation testing, 14 December 2010

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1. BACKGROUND

In 2007, the ECVAM Scientific Advisory Committee (ESAC) declared the EPISKIN™ method a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. It was considered adequate as a replacement for the Draize skin irritation test (OECD TG 404 & Method B.4 of Regulation N° 440/2008) for the purposes of distinguishing between R38 skin irritating and non-skin irritating substances (ESAC, 2007).

As a follow-up of that ESAC statement, the SCCP issued a Memorandum on the application of the EPISKIN™ method for skin irritation testing of cosmetic ingredients (SCCP/1145/07). Therein the SCCP welcomed the availability of the validated EPISKIN™ method as a replacement alternative highly needed for the animal-free assessment of skin irritation of cosmetic ingredients, but still some important points of concern were identified. In first instance, it was noted that the set of compounds used to validate the in vitro assay contained only one substance present on the Annexes to Dir. 76/768/EEC. In addition, no information was available to the SCCP with respect to the testing of coloured substances, whereas the problem of dyes interfering with the MTT colorimetric method was already mentioned in a study assessing the applicability of the EPISKIN™ model for phototoxicity testing (Lelièvre et al. 2007). As this aspect is potentially of high importance for colour ingredients and hair dyes, the SCCP was of the opinion that additional data were necessary to fully support the EPISKIN™ method for the safety assessment of cosmetic ingredients present on the Annexes of Directive 76/768/EEC.

As a response to these concerns stated by the SCCP in December 2007, additional data was received in three stages:

Submission 1 (December 2007):

Episkin™ test results on 15 substances (12 non-irritants and 3 irritants in vivo):
- 6 UV-filters
- 3 preservatives
- 6 other cosmetic ingredients

Submission 2 (July 2008):

Episkin™ test results on 22 hair dye substances (20 non-irritants and 2 irritants in vivo):
- 7 oxidative hair dye substances
- 10 direct (semi-permanent) hair dye substances
- 5 oxidative hair dye substances, also used as semi-permanent hair dyes

Submission 3 (March 2009):

Episkin™ test results on 4 colour ingredients (all irritants in vivo).

2. OVERVIEW OF THE NEWLY INTRODUCED DATA

SUBMISSION 1 (UV-FILTERS, PRESERVATIVES, OTHERS)

The firstly introduced data set consists of a detailed description of the Episkin™ method and the results for 15 compounds, of which 6 UV-filters, 3 preservatives and 6 'other function' cosmetic ingredients (See Table 1 for more detail). For all these substances, the *in vivo* skin irritation data have been previously evaluated by the SCC(NF)P.

The Episkin™ skin irritation assay was performed according to the validated and published ECVAM Standard Operating Procedure, also included in the submission.

The main steps in the procedure consist of:

- topical application of the test substance to the epidermis model (3 epidermis units per test material, positive and negative control) and exposure for 15 minutes;
- termination of exposure by rinsing with Phosphate Buffer Saline (using a cotton bud if necessary);
- further incubation of the epidermis at 37°C for 42 ± 1 hours (37°C, 5% CO_2, 95% humidity);
- assessment of cell viability by incubating the tissues for 3 hours with MTT solution, after which the precipitated formazan is extracted and quantified spectrophotometrically at 570 nm.

For each test material, three independent tests with three different batches of Episkin™ samples have been carried out. For each treated tissue the viability is expressed as a percentage of the mean of the negative control tissues' values.
In case viability of the tissues is > 50% after treatment, a second endpoint, namely interleukin-1α (IL-1 α) release into the culture medium, is measured. To that end, samples are taken after the 42 hours of incubation and frozen until subjected to an ELISA (enzyme-linked immunosorbant assay) test.

The accompanying prediction model combines cell viability and IL-1 α release as follows:

Viability level and IL-1 α concentrations	Classification of the test material
Mean viability value ≤ 50% OR [IL-1 α RM release] ≥ 50pg/ml	Potentially **irritant** test material
Mean viability value > 50% AND [IL-1 α RM release] < 50pg/ml	Potentially **non-irritant** test material

In the following table, the testing results of the 15 substances are summarized, together with the original results from the *in vivo* assays as reported in the individual SCC(NF)P opinions.

Table 1: Summary of Episkin™ results for the 15 substances of Submission 1

Substance name *(function)*	Sample (S)	Mean viability	Calculated IL- α release (pg/ml)	Result Episkin™	Result *in vivo* according to SCC(NF)P opinions
Zinc oxide - S76 *(UV-filter)*	pos ctrl S1 S2	13-14 106 97	n.a. -14 -2	**Non-irritant** Neat substance	**Non-irritant** 50% in water
Benzophenone-3 - S38 *(UV-filter)*	pos ctrl S1 S2 S3	7-31 64 110 119	134-143 -24 -7 -22	**Non-irritant** Neat substance	**Non-irritant** Neat substance
Camphor Benzalkonium Methosulfate - S57 *(UV-filter)*	pos ctrl S1 S2 S3	5-15 131 147 116	165-194 -11 -10 37	**Non-irritant** Neat substance	**Non-irritant** 30% aqueous solution
Phenylbenzimidazole Sulfonic Acid - S45 *(UV-filter)*	pos ctrl S1 S2	8-17 86 82	n.a. 0 4	**Non-irritant** Neat substance	**Non-irritant** Neat substance
4-Methylbenzylidene Camphor - S60 *(UV-filter)*	pos ctrl S1 S2	8-17 119 130	n.a. -9 -11	**Non-irritant** Neat substance	**Non-irritant** Neat substance
Diethylamino Hydroxybenzoyl Hexyl Benzoate - S83 *(UV-filter)*	pos ctrl S1 S2	9-11 117 109	n.a. -6 29	**Non-irritant** 35% in ethyl hexyl methoxy-cinnamate	**Non-irritant** Neat substance
Benzoic Acid *(preservative)*	pos ctrl S1 S2 S3	10-43 98 97 99	n.a. no data -19 1	**Non-irritant** Neat substance	**Non-irritant** Neat substance
Sodium Benzoate *(preservative)*	pos ctrl S1 S2 S3	4-23 98 91 100	150-169 27 no data 9	**Non-irritant** Neat substance	**Non-irritant** Neat substance
Methylisothiazolinone *(preservative)*	pos ctrl S1 S2	5 106 106	128-204 11 19	**Non-irritant** 9.5 ppm aqueous solution	**Corrosive** Neat substance **Non-irritant** 100 ppm solution
Piroctone Olamine *(preservative)*	pos ctrl S1 S2	9-11 11 7	n.a. n.a. n.a.	**Irritant** Neat substance	**Irritant** Neat substance **Non-irritant** 3% in propylene glycol
Cyclomethicone *(skin/hair conditioning agent, emollient)*	pos ctrl S1 S2 S3	5-48 102 116 102	100 -32 no data 14	**Non-irritant** 37% in siloxane mixture	**Non-irritant** Neat substance
Ethoxydiglycol *(solvent, humectant, viscosity decreasing agent)*	pos ctrl S1 S2	6-17 110 79	189-204 11 18	**Non-irritant** 23% in root extract	**Non-irritant** Neat substance
Cetrimonium Chloride (*preservative)*	pos ctrl S1 S2	4-18 71 75	141 101 75	**Irritant** 25% aqueous solution	**Irritant** 25% aqueous solution
Diethyl Phthalate (*solvent, denaturant)*	No detailed report available. Reference to validation study (Spielmann et al. 2007)			**Non-irritant** Neat substance	**Non-irritant** Neat substance
Salicylic Acid *(preservative)*	pos ctrl S1 S2	8-14 94 77	n.a. -20 -40	**Non-irritant** Neat substance	**Irritant** Neat substance **Non-irritant** 2% alcoholic solution

n.a. = not analysed

Please note that in Table 1, negative control values are not displayed since:
- the negative control viability is *a priori* arbitrarily set to 100%;
- the IL-1 α release values are *a priori* arbitrarily set to 0 pg/ml, as all IL-1 α release results are expressed as $(\text{IL-1 }\alpha \text{ release})_{\text{sample}}$ - $(\text{IL-1 }\alpha \text{ release})_{\text{neg ctrl}}$.

Comments on Submission 1:

Whereas **colour ingredients were specifically stated as potential problematic compounds in this assay, no such substances were included** in this first submission. Moreover, **only three irritating substances *in vivo* were included** in the selected set of compounds. Of these three, **salicylic acid was not identified as irritant by the Episkin™ method.**

In certain cases, the tested *in vitro* **concentrations** diverge from the ones tested *in vivo*. The applicant considers the discrepancies as slight and not impacting the overall evaluation, but in reality the **differences are sometimes as high as a factor 10**.

Finally, some significant shortcomings were noted when assessing the individual test descriptions and the presented results. They are listed in detail in the appendix to this opinion.

SUBMISSION 2 (22 HAIR DYE SUBSTANCES)

The second submission consisted of a detailed updated SOP for the Episkin™ method and the results for 22 hair dye substances, of which 2 irritants and 20 non irritants *in vivo* (according to the SCC(NF)P opinions).

1) New features of the updated SOP for the Episkin™ skin irritation assay

Based upon the finding that a number of colour ingredients showed to interfere with the viability measurement in the MTT assay, leading to potentially misleading results (Lelièvre et al., 2007, Faller et al, 2002), the *in vitro* skin irritancy protocol was adapted to include an additional control. More specifically an extra epidermis was treated with the test substance but not incubated with MTT. In this way the optical density (OD) due to the residual test substance colour (OD_{TCC}) (unrelated to viability), could be quantified.

By subtracting this OD_{TCC} from the OD of the epidermis treated with the test substance and incubated with MTT, the applicant claims that the true MTT metabolic conversion OD (TOD_{TT}), i.e. the real viability, can be determined.
The relative viability is calculated applying the following formula $[TOD_{TT}/OD_{NC}] \times 100$.
OD_{NC} corresponds to the optical density of the negative control incubated with MTT.
The main steps of the protocol and the prediction model are the same as in the first submission. The main difference is the inclusion of the above-mentioned extra control epidermis.

Finally, the renewed SOP states that *'for each test substance, a minimum of two independent assays with preferably two different batches of EPISKIN-SM™ must be performed. If the results of the two independent assays (2 epidermis batches) are discordant, a third assay is necessary. For each test substance, at least 2 tissues should be tested concurrently in each assay'*. The protocol, however, does not give information on the threshold of discordance triggering the requirement of a third sample.

2) Results for 22 hair dye substances

The individual results are summarized in Table 2, together with the original results from the *in vivo* assays as reported previously in the individual SCC(NF)P opinions.

Table 2: Summary of Episkin™ results for the 22 hair dye substances of Submission 2

Substance name (*type of hair dye*)	Sample (S)	Mean viability	Calculated IL-α release (pg/ml)	Additional 'MTT' control (OD)	Result Episkin™	Result *in vivo* according to SCC(NF)P opinions
p-Phenylenediamine - A007 (*oxidative hair dye*)	pos ctrl S1 S2	10-15 110 117	n.d. 2 -3	0.013-0.017	**Non-irritant** 2.5% in water	**Slightly irritant** 2.5% in water
Lawsone - C146 (*semi-permanent hair dye*)	pos ctrl S1 S2	10-15 78 77	n.d. 26 -3	0.024-0.025	**Non-irritant** Neat substance	**Non-irritant*** Neat substance
1,2,4,-Trihydroxybenzene - A033 (*semi-permanent hair dye*)	pos ctrl S1 S2	24-30 106 93	n.d. -10 2	0.030**	**Non-irritant** 3% in water	**Slightly irritant*** 3% in water
Phenyl methyl pyrazolone - A039 (*oxidative hair dye*)	pos ctrl S1 S2	8 89 106	n.d. 3 -3	0.014-0.019	**Non-irritant** 1% in 1,2-propanediol	**Slightly irritant** 1% in propylene glycol
2-Methyl-5-hydroxyethylaminophenol - A031 (*oxidative hair dye*)	pos ctrl S1 S2	20-24 89 92	n.d. -7 6	0.016**	**Non-irritant** Neat substance	**Non-irritant** Neat substance
2,4.Diaminophenoxyethanol HCl - A042 (*oxidative hair dye*)	pos ctrl S1 S2	8 84 109	n.d. 22 2	0.009-0.014	**Non-irritant** Neat substance	**Non-irritant** Neat substance
3-Nitro-p-hydroethylaminophenol - B054 (*semi-permanent hair dye*)	pos ctrl S1 S2	5-7 96 96	n.d. -2 8	0.012-0.014	**Non-irritant** 6% in water	**Non-irritant*** 6% in CMC 0.5%
2-Methylresorcinol - A044 (*oxidative & semi-permanent hair dye*)	pos ctrl S1 S2	1-10 104 95	n.d. 21 8	0.010-0.011	**Non-irritant** Neat substance	**Slightly irritant** Neat substance
Basic Brown 17 - B007 (*semi-permanent hair dye*)	pos ctrl S1 S2	2-5 84 93	n.d. 6 -0.5	0.032-0.089	**Non-irritant** Neat substance	**Non-irritant*** Neat substance
Basic Red 76 - C008 (*semi-permanent hair dye*)	pos ctrl S1 S2	5-7 84 72	n.d. 2 16	0.021-0.048	**Non-irritant** Neat substance	**Non-irritant** Neat substance
Basic Brown 16 - C009 (*semi-permanent hair dye*)	pos ctrl S1 S2	7-15 88 85	n.d. 2 30	0.021-0.043	**Non-irritant** Neat substance	**Non-irritant*** Neat substance
2-Nitro-5-glyceryl methylaniline - B060 (*semi-permanent hair dye*)	pos ctrl S1 S2	8 96 107	n.d. 10 2	0.002-0.009	**Non-irritant** 1% in 1-2-propanediol	**Non-irritant*** 1% suspension in 1,2-propanediol
Basic Yellow 57 - C010 (*semi-permanent hair dye*)	pos ctrl S1 S2	10-15 105 108	n.d. 5 5	0.021-0.030	**Non-irritant** Neat substance	**Non-irritant** Neat substance

Substance name (*type of hair dye*)	Sample (S)	Mean viability	Calculated IL-α release (pg/ml)	Additional 'MTT' control (OD)	Result Episkin™	Result *in vivo* according to SCC(NF)P opinions
HC Violet Nº 1 - B066 (*semi-permanent hair dye*)	pos ctrl S1 S2	5-7 88 91	n.d. -4 4	0.038-0.053	**Non-irritant** Neat substance	**Slightly irritant*** Neat substance
HC Blue Nº 2 - B037 (*semi-permanent hair dye*)	pos ctrl S1 S2	17-20 90 92	n.d. -7 6	0.015**	**Non-irritant** 3% in PEG300	**Non-irritant*** 3% in CMC 0.5%
HC Yellow Nº7 - B080 (*semi-permanent hair dye*)	pos ctrl S1 S2	5-7 91 96	n.d. -2 7	0.049-0.135	**Non-irritant** Neat substance	**Non-irritant** Neat substance
HC Yellow Nº 9 - B069 (*semi-permanent hair dye*)	pos ctrl S1 S2	20-24 79 61	n.d. -7 3	0.023**	**Non-irritant** Neat substance	**Non-irritant** Neat substance
6-Hydroxyindole - A128 (*oxidative hair dye*)	pos ctrl S1 S2	2-8 114 112	n.d. -1 0.3	0.023-0.028	**Non-irritant** Neat substance	**Non-irritant** Neat substance
Hydroxypropyl bis (N-hydroxyethyl-p-phenylenediamine HCl - A121 (*oxidative hair dye*)	pos ctrl S1 S2 S3 S4	8-35 59 37 79 79	n.d. 34 n.d. 30 38	0.019***	**Non-irritant** Neat substance	**Irritant** Neat substance
Basic Red 51 - B116 (*semi-permanent hair dye*)	pos ctrl S1 S2	7-10 77 88	n.d. -4 -5	0.094-0.103	**Non-irritant** Neat substance	**Non-irritant** Neat substance
Basic Yellow 87 - B117 (*semi-permanent hair dye*)	pos ctrl S1 S2	8 90 120	n.d. 14 -8	0.008	**Non-irritant** Neat substance	**Non-irritant** Neat substance
1-Hydroxyethyl-4,5-diamino pyrazole sulphate - A154 (*oxidative hair dye*)	pos ctrl S1 S2	7-15 92 82	n.d. -2 11	0.014-0.011	**Non-irritant** Neat substance	**Irritant** Neat substance

n.d. not determined
* Discoloration of skin noticed in *in vivo* assay
** Exactly the same OD (optical density) measurements for **2** different skin samples
*** Exactly the same OD measurements for **4** different skin samples

Comments on Submission 2:

In order to demonstrate the validity of the Episkin™ irritation assays for colouring substances, Industry performed the test with 22 hair dye substances. The protocol was adapted by the introduction of additional control tissues, treated with the test substance but not incubated with MTT. These controls allow the quantification of the Optical Density (OD) due to the residual test substance colour that is unrelated to cell viability.
The obtained *in vitro* results show all 22 substances to be classified as non-irritative, as none of the compounds displayed a viability of $\leq$ 50% or an IL-1α $\geq$ 50 pg/ml. This means that **Hydroxypropyl-bis-(N-hydroxyethyl-p-phenylenediamine) HCl**, identified by the applicant as an **irritant *in vivo***, was **not identified as a skin irritant in the *in vitro* assay**. The applicant argued that the unusually high and low values obtained with this compound for IL-1α release and cell viability, respectively, may be indicative of a possible skin irritation potential.

The authors of the study also indicate that for all tested substances, including direct and indirect hair dye substances, there were no interferences of the test material with the read-out system.

The SCCS points out that **1-Hydroxyethyl-4,5-diamino pyrazole sulphate was also designated as a skin irritant in SCCP/0990/06,** although the individual scores of the assay were not provided in that opinion. The statement made by **Industry that it is a non-irritant *in vivo*,** however, is not fully accepted and could mean that in this Submission 2, there is a second compound for which the *in vitro* method failed to predict the irritative potential. In any case, the Committee is of the opinion that **more irritating compounds need to be included** in the list of test compounds.

Finally, some significant shortcomings were noted when assessing the individual test descriptions and the presented results. They are listed in detail in the appendix to this opinion.

SUBMISSION 3 (ADDITIONAL COLOR INGREDIENTS)

Following criticism that few irritant substances were contained in submission 2, Industry submitted data on 4 additional irritant colour ingredients in March 2009.
The applicant stated that a thorough review of the scientific literature and of databases present within cosmetic companies only identified 4 irritant colouring materials: Copper Sulphate, Basazol C Violet Pr 8055, Basazol C Blue Pr 8056, Basazol Orange 52 L. They were all tested in the adapted Episkin™ skin irritation assay as described in detail in Submission 2.

The results reveal a significant interference of Basazol C Violet and Basazol C Blue PR with the read-out system when tested in undiluted form. They were consequently tested in dilution and the interference disappeared. No such interference had been observed for the substances contained in earlier submissions.
Copper Sulphate and Basazol Orange 52 were tested undiluted.

Table 3: Summary of Episkin™ results for the 4 colour ingredients of Submission 3

Substance name	Sample (S)	Mean viability	Calculated IL-α release (pg/ml)	Result Episkin™	Result *in vivo*
Copper Sulphate	pos ctrl S1 S2	26-31 4 26	n.d. n.d. n.d.	**Irritant** Neat substance	**Irritant** Neat substance
Basazol Orange 52L	pos ctrl S1 S2 S3	4-31 49 42 30	n.d. n.d. n.d. n.d.	**Irritant** Neat substance	**Irritant** Neat substance
Basazol C Violet	pos ctrl S1 S2	4-31 17 16	n.d. n.d. n.d.	**Irritant** 50% in water	**Irritant** Neat substance
	pos ctrl S1 S2	4-31 82 71	n.d. -19 -12	**Non irritant** 25% in water	**No data** 25% in water
Basazol C Blue	pos ctrl S1 S2	8-30 10 4	n.d. n.d. n.d.	**Irritant** 35% in water	**Irritant** Neat substance
	pos ctrl S1 S2	8-30 63 58	n.d. -6 6	**Non irritant** 25% in water	**No data** 25% in water

n.d. =not determined

Comments on Submission 3:

The SCCS acknowledges the problem of the non-availability of irritating colouring substances and welcomes the presented results.

Nevertheless, the **interference of two colour ingredients** (Basazol C Violet and Basazol C Blue) with the test system and the **subsequent testing of a dilution** (50% and 35**%), hamper the *in vivo* / *in vitro* comparison** of the obtained results. No data is available on the *in vivo* irritant potential of the applied dilutions. The applicant provides no discussion on how the discrepancies in the concentrations tested *in vivo* and *in vitro* in submission 3 might have influenced the overall outcome.

As far as the *in vivo* data on **the two remaining substances** are concerned, the International Chemical Safety Card (ICSC) confirms the skin irritative potential of Copper Sulphate, but no study reports on the evaluation of its *in vivo* skin irritation properties could be identified. The same is true for Basazol Orange 52L, of which the Safety Data Sheet states that it is a skin irritant according to EU classification criteria, but **no raw data nor an *in vivo* study** are available.

Some additional shortcomings noted when assessing the individual test descriptions and the presented results, are listed in detail in the appendix to this opinion.

3. OVERALL DISCUSSION ON THE INFORMATION SUBMITTED

Overall, this assessment is undertaken to create trust that irritative colour ingredients can be detected reliably using the Episkin™ *in vitro* model with MTT as an endpoint. Therefore, the applicant provided information on 41 substances in total, of which 26 hair dye substances and/or colour ingredients.

In the first submission of cosmetic ingredients, the reported 'concordance' between the *in vivo* and *in vitro* results showed to be high (93%, 14/15). Some discrepancies, however, between the concentrations used in *in vitro* and *in vivo* experiments, were observed with the lower concentration being tested in the Episkin™ assay in 4 of the 6 cases. It was also noted that **out of the 3 *in vivo* irritating substances, one was not correctly classified through the Episkin™ assay.**
The major remark of the SCCP, however, was that **no coloured substances** were investigated in this submission, whereas these compounds were expected to interfere with the colorimetric MTT-determination of the assay.

The subsequently submitted data contained 22 hair dyes. It was noted that **only 2 irritating compounds were included and that the adapted Episkin™ assay failed to identify them as irritant.** The reasoning that '*diverging results would point towards potential irritating properties* of the substance' was not considered acceptable. Compounds that in earlier SCC(NF)P opinions were identified as 'slightly' or 'mildly' irritating did not display a decreased viability or increased IL-α release as could be reasonably expected. 1-Hydroxyethyl-4,5-diamino pyrazole sulphate, identified in the study as an *in vivo* non-irritant, but considered as an *in vivo* irritant by the SCCP, showed to be negative in the submitted results.

Independent of the above made remarks, **the applicant proposes to pool the data** for the 4 colour ingredients of Submission 3 and the 22 hair dye substances of submission 2 to evaluate the overall predictive performance of the EPISKIN-SM™ assay that was specifically adapted for colour ingredients (see Table 4).

Table 4: Data pooling according to the applicant of 4 colour ingredients and 22 hair dye substances

		In vitro Episkin^{SM} test result		
		Irritant	Non-irritant	TOTAL
In vivo study result	Irritant	4	1	5
	Non-irritant	0	21	21
	TOTAL	4	22	26

- Overall concordance = 96% (25/26)
- Sensitivity = 80% (4/5)
- Specificity = 100% (21/21)

The SCCS is of the opinion that **this way of assessing the results is not appropriate** for the following reasons:

- Due to interference with the test system observed for 2 out of the 5 irritating substances that were analysed, these 2 compounds could only be tested in dilution. No discussion on a potential effect of concentration differences between *in vivo* and *in vitro* testing was present. Under these conditions, it is difficult to assess the value of the method. Indeed, from the data submitted, one cannot conclude that diluting Basazol C Violet from 50% to 25% renders the diluted compound non-irritating. The same is true for the Basazol C

Blue dilution from 35% to 25%. **Considering the (worst) case that both dilutions would still be irritating, the sensitivity would decrease from 80% (4/5) to 40% (2/5).**
- In case 1-Hydroxyethyl-4,5-diamino pyrazole sulphate is considered to be irritating (as evaluated by the SCCP), the sensitivity would even decrease to 33% (2/6).
- Although it is understood that *in vivo* irritant colour ingredients/hair dye substances cannot easily be identified, the **unbalanced distribution of irritants/non-irritants reduces the scientific value of the overall 'concordance'** given by Industry.

4. CONCLUSION

The current Memorandum reflects the opinion of the SCCS on the ability of the Episkin™ skin irritation assay to adequately replace the Draize skin irritation test (OECD TG 404 & Method B.4 of Regulation N° 440/2008) for the purpose of distinguishing between skin irritating and non-irritating substances, the only distinction to be made in the European classification[1].
It must, however, be noted that for cosmetic ingredients, in order to assess the risk in terms of skin contact, exposure time, frequency of use, etc., it is also important to obtain information on possible irritative properties below this initial threshold for classification.

In order to provide trust that irritative cosmetic ingredients can be detected reliably using the Episkin™ skin irritation assay with MTT as an endpoint, Industry submitted test results for 2 distinct groups of substances, namely (i) a set of 15 UV-filters/ preservatives/skin conditioning agents and (ii) a set of 26 hair dye substances/colour ingredients. The second set was specifically requested by the SCCP as there was a suspicion that colour ingredients might interfere with the colorimetric determination of the Episkin™ assay.

The results for the first set of 15 compounds show that there was a relatively high correlation between *in vivo* and *in vitro* data, although only 2 of the 3 irritating substances *in vivo*, could be identified as irritants by the Episkin™ method. A number of methodological remarks were formulated and are taken up in appendix.

As far as the hair dye substances/colour ingredients are concerned, a modified Episkin™ assay was developed to ensure a good correlation between *in vivo* and *in vitro* data. The sensitivity of this assay, based upon the results from the 26 coloured substances was reported to be 80%. As a number of serious shortcomings, however, were noted with respect to colour interference with the test system, classification of the test substances, and differences in dilutions tested *in vivo* and *in vitro*, the SCCS is of the opinion that this high value is not supported by the data provided.
In addition, a number of remarks on the raw data and the reporting in general are provided in the appendix.

Overall, the SCCS is of the opinion that the results obtained in the two submissions that cover 26 hair dye substances/colour ingredients, do not provide sufficient proof that the MTT test can be used as a suitable endpoint when colour ingredients/hair dye substances are tested for their potential skin irritative properties. The additional control tissue does provide slightly elevated OD values for a number of coloured compounds, but the overall results do not generate the required *in vivo* / *in vitro* correlation needed for this class of chemicals.
The SCCS is therefore of the opinion that for coloured substances, a different endpoint, not involving optical density quantification, should be envisaged. Analytical methods such as

[1] According to UN GHS: Mild irritant: mean *in vivo* oedema/erythema score of ≥ 1.5 and < 2.3
Irritant: mean *in vivo* oedema/erythema score of ≥ 2.3
The EU CLP took over the classification as 'Irritant', but did not include the 'Mild Irritant' category.

HPLC/UPLC might be more appropriate to detect formazan in the *in vitro* assay (McNamee et al. 2009).

5. REFERENCES

ESAC, 2007 Statement on the validity of in-vitro tests for skin irritation. http://ecvam.jrc.ec.europa.eu [accessed June 15, 2010]

ECVAM 2007 Skin Irritation Validation Study Phase II: Analysis of the primary endpoint MTT and the secondary endpoint IL-1α. Online: http://ecvam.jrc.it/ [accessed September 20, 2010]

ESAC 2008 Statement on the scientific validity of in vitro tests for skin irritation testing. ESAC 29th meeting Online: http://ecvam.jrc.it/ [accessed September 20, 2010]

Faller C, Bracher M 2002 Reconstructed skin kits: reproducibility of cutaneous irritancy testing. Skin Pharmacol Appl Skin Physiol. 15 Suppl 1:74-91.

Faller C, Bracher M, Dami N, Roguet R. 2002. Predictive ability of reconstructed human epidermis equivalents for the assessment of skin irritation of cosmetics. Toxicol in vitro 16, 557-572.

Lelièvre D, Justine P, Chirstiaens F, Bonaventure N, Coutet J, Marrot L, Cotovio H, 2007. The episkin phototoxicity assay (EPA): Development of an in vitro tiered strategy using 17 reference chemicals to predict phototoxic potency. Toxicol in vitro 21, 977-995.

Mc Namee P, Faller IC, Goebel C, Pfuhler S, Tieber T, Egham I. Use of HPLC/UPLC instead of photometry for evaluation of MTT in in vitro RhT assays for irritation; assessment of colored materials, in Altex, volume 26, special issue, Abstracts 7th World Congress, Rome, 2009, p281.

OECD TG 404 OECD Guideline for the testing of chemicals: Acute dermal irritation/corrosion, 2002.

SCCP/1145/07 Memorandum on the in vitro test EPISKIN™ for skin irritation testing, adopted by the SCCP during the 14th plenary meeting on 18 December 2007.

Spielmann H, Hoffmann S, Liebsch M, Botham P, Fentem JH, Eskes C, Roguet R, Cotovio J, Cole T, Worth A, Heylings J, Jones P, Robles C, Kandárová H, Gamer A, Remmele M, Curren R, Raabe H, Cockshott A, Gerner I, Zuang. V. 2007. The ECVAM international validation study on in vitro tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test. Altern Lab Anim. 35,559-601.

Test Method B.4 Acute toxicity: dermal irritation/corrosion. Commission Regulation (EC) No440/2008/EC

Test Method B.46 In vitro skin irritation: reconstructed human epidermis model test. Annex to 440/2008/EC. Commission Regulation (EC) No 761/2009/EC.

6. APPENDIX

General comments on all submissions

- The results were changed to 100% when the measured viability was higher than 100%. It is quite uncommon to replace a measured result by 100% when the original values are still used to calculate a mean ± SD.
- In case the calculation of (IL-1α release)$_{sample}$ - (IL-1 α release)$_{neg\ ctrl}$ yields a negative result, this result is set at 0 pg/ml because a negative IL-1 α release is considered non-biological. Nevertheless, the result is derived from a calculation and not directly measured, meaning that a negative value can be acceptable.
- Calculating a standard deviation (SD) for 2 values is quite unusual.

Specific comments with regard to individual test reports and the summary report on Submission 1

- For Camphor Benzalkonium Methosulfate, the third sample yields a mean IL-1 α release of 49 pg/ml, whereas the study report indicates 21.9 pg/ml as the mean of 46.4, 67.5 and 33.0 pg/ml. In the last column, the mean IL-1 α values are copied, instead of subtracting the negative control values.
- For Camphor Benzalkonium Methosulfate, it is surprising that two different batches (07-EKIN-003 and 07-EKIN-007) of Episkin show exactly the same values for IL-1 α for the positive and negative controls.
- In the summary report for benzoic acid, the IL-1 α values of experiments 2 and 3 are interchanged.
- With respect to cyclomethicone it is concluded that the substance is non-irritant based on the results of the MTT-test and that therefore it was not necessary to perform the IL-1 α test. This is the opposite of the initial reasoning, as the protocol states that the classification should be based on the two endpoints when the cell viability is higher than 50%.
- For Methylisothiazolinone, the final report mentions 3 samples with accompanying results, but the detailed report only presents data of 2 samples.
- In some cases both the viability of "dead epidermis" and the viability of "living epidermis" are measured. The relevance and meaning of this second set of data (dead epidermis) is, however, not documented.
- Only for 6 of the 15 substances, 3 samples have been measured. However, the SOP mentions that three independent tests with three different batches of Episkin™ samples are to be systematically undertaken.

Specific comments with regard to individual test reports and the summary report on Submission 2

- For 1,2,4,-Trihydroxybenzene, 2 different batches show exactly the same individual OD values for each MTT control. The same is observed for 2-Methyl-5-hydroxyethylaminophenol, HC Blue n° 2, HC Yellow n° 9, and Hydroxypropyl bis (N-hydroxyethyl-p-phenylenediamine HCl. For the latter, the MTT control values are exactly the same for 4 different tissues. An explanation for these results should be provided.
- According to the reports, p-Phenylenediamine and Lawsone generate exactly the same IL- α release values when applied to the Episkin batch 08-EKIN-015. This probably is a copy-paste error, as the first Lawsone sample generates a higher IL-1 α release value.
- There is an inconsistency in the Hydroxypropyl bis (N-hydroxyethyl-p-phenylenediamine HCl IL- α release values. More specifically, the mean IL-1 α value for the negative control and for the test substance are reported to be 35.2 pg/ml and 48.0 pg/ml, respectively. This means that the IL-1 α values for treated tissues - the negative control values would

yield 0.0 pg/ml and 12.8 pg/ml for negative control and test item, respectively. The results' table, however, mentions 17.8 pg/ml and 30.5 pg/ml instead.
- Only one of the 22 compounds was tested on 4 different tissues; all other compounds were only tested on 2 tissues. In none of the cases, an explanation is given for the redundancy of a third skin sample.

Specific comments with regard to individual test reports and the summary report on Submission 3

- Results show a high variability for Copper sulphate (4.0% and 26.3%): mean 15.2% and SD 15.8%.
- The table of the summary report corresponding to Basazol C Blue shows dilutions of the compounds to be 35% in both cases instead of 25% and 35%.
- Only one of the 4 compounds was tested on 3 different tissues; the other compounds were tested on 2 tissues only. In none of the cases, an explanation is given for the redundancy of a third skin sample.