EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Red n° 10 + HC Red n° 11

COLIPA n° B71

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

The SCCS adopted this opinion at its 12th plenary meeting

of 20 September 2011

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.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

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.).

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Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on HC Red n° 10 + HC Red n° 11, 20 September 2011

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1. BACKGROUND

Submission I for HC Red n° 10 + HC Red n° 11 with the chemical name for HC Red n° 10: 1-Amino-5-chloro-4-(2,3-dihydroxypropyl)amino-2-nitrobenzene (CAS 95576-89-9) and for HC Red n° 11 1-chloro-2,5-di((2,3-dihydroxypropyl)amino)-4-nitrobenzene (CAS 95576-92-4) was submitted in March 1992 by COLIPA[1] according to COLIPA.

The first scientific opinion (SPC/1076/93) was adopted the 25 of June 1993.

Submission II was submitted in July 2005 by COLIPA.

The second scientific opinion (SCCP/1134/07) was adopted at the 15 of April 2008 with the following conclusion: *"The mixture of HC Red n° 10 + HC Red n° 11 (COLIPA B71) is highly variable with the two compounds having different physico-chemical properties which possibly also affect toxicological properties.*
Consequently, the SCCP is not in a position to perform a risk assessment of HC Red n° 10 +HC Red n° 11 because of the absence of a proper chemical characterisation of the test substance."

According to this submission HC Red n° 10 + HC Red n° 11 is used as:

a) a non-reactive hair colouring agent ("direct dye") in semi-permanent hair dye formulations at a maximum on-head concentration of 2.0%.

b) a non-reactive hair colouring agent ("direct dye") in oxidative hair dye formulations at a maximum on-head concentration of 1.0%.

The "substance" is currently regulated in Annex 3, part 2 under entry 50 on the list of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down until 31.12.2010.

The current submission III on HC Red n° 10 and n° 11 is a response to the SCCS opinion (1134/07) and consist of comments and an in vitro dermal absorption study under oxidative and non-oxidative conditions.

2. TERMS OF REFERENCE

1. *Does SCCS consider HC Red n° 10 + HC Red n° 11 safe for use as a non-oxidative hair dye with an on-head concentration of maximum 2.0 % taken into account the scientific data provided?*

2. *Does SCCS consider HC Red n° 10 + HC Red n° 11 safe for use in oxidative hair dye formulations with an on-head concentration of maximum 1.0% taken into account the scientific data provided?*

3. *And/or does the SCCS have any further concerns with regard to the use of HC Red n° 10 + HC Red n° 11 in any hair dye formulations?*

[1] COLIPA – The European Cosmetics Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Mixture of HC Red n° 10 (INCI) and HC Red n° 11 (INCI)

3.1.1.2. Chemical names

HC Red n° 10
- 1-amino-2-nitro-4-(2',3'-dihydroxypropyl)amino-5-chlorobenzene
- 1,2-Propanediol, 3-[(4-amino-2-chloro-5-nitrophenyl)amino]-, (CA, Index name, 9CI)
- 3-(4-Amino-2-chloro-nitroanilino)-1,2-propanediol (IUPAC)
- 1-Amino-5-chloro-4-(2,3-dihydroxypropyl)amino-2-nitrobenzene

HC Red n° 11
- 1,4-bis-(2',3'-dihydroxypropyl)amino-2-nitro-5-chlorobenzene
- 1,2-Propanediol, 3,3'-[(2-chloro-5-nitro-1,4-phenylene)diimino]bis-, (CA, Index name, 9CI)
- 1,2-Propanediol, 3,3'-[(4-amino-2-chloro-5-nitrophenyl)amino]bis-,
- 1-Chloro-2,5-di[(2,3-dihydroxypropyl)amino]-4-nitrobenzene

3.1.1.3. Trade names and abbreviations

Rubinrot Y
COLIPA B71

3.1.1.4. CAS / EC number

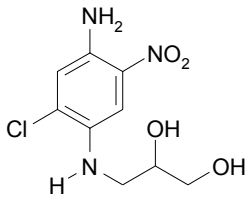
HC Red n° 10
CAS: 95576-89-9
EC: /

HC Red n° 11
CAS: 95576-92-4
EC: /

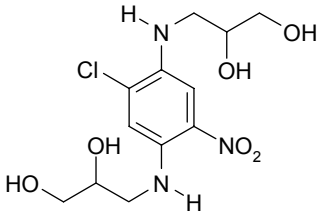
Rot Y or Rubinrot Y
CAS: /
EC: 408-240-1 (1-amino-2-nitro-4-(2',3'-dihydroxypropyl)amino-5-chlorobenzene and 1,4-bis-(2',3'-dihydroxypropyl)amino-2-nitro-5-chlorobenzene)

3.1.1.5. Structural formula

HC Red n° 10



HC Red n° 11



3.1.1.6. Empirical formula

HC Red n° 10: $C_9H_{12}ClN_3O_4$
HC Red n° 11: $C_{12}H_{18}ClN_3O_6$

3.1.2. Physical form

Red brown powder

3.1.3. Molecular weight

HC Red n° 10: 261.67
HC Red n° 11: 335.74

3.1.4. Purity, composition and substance codes

HC Red n° 10 and HC Red n° 11 were chemically characterised by NMR spectroscopy.

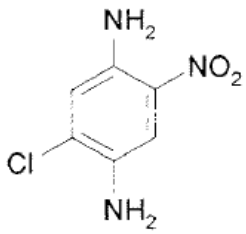
Table 1: comparison of different batches

Description of sample:	**L4/141 (V07/600pf)**		**9375 (R00058409)**		**228-41185**		**LEH5/1**	
Analysis reference	G 2005/008		A 2001/038 G 2005/008		A 2002/247 G 2005/008		G 2005/008	
	HC Red n° 10	HC Red n° 11	HC Red n° 10	HC Red n° 11	HC Red n° 10	HC Red n° 11	HC Red n° 10	HC Red n° 11
NMR content / %w/w	55.0	40.2	57.2	32.8	53.0	38.6	43.6	21.3
HPLC purity/area % (parameters same as for determination of impurity A)								

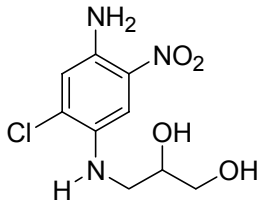
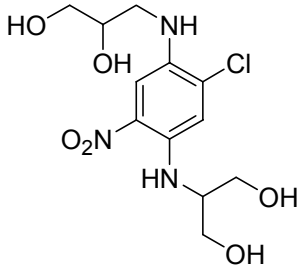
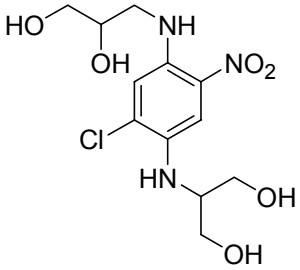
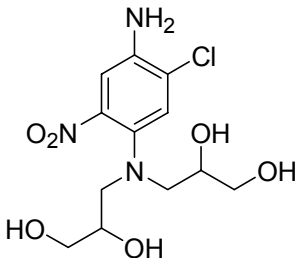
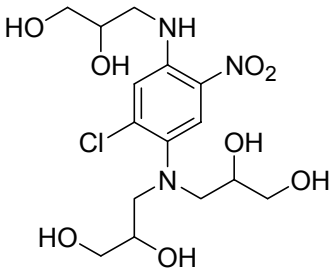
Description of sample:	**L4/141 (V07/600pf)**		**9375 (R00058409)**		**228-41185**		**LEH5/1**	
Analysis reference	G 2005/008		A 2001/038 G 2005/008		A 2002/247 G 2005/008		G 2005/008	
210 nm	60.0	33.6	62.7	33.6	60.9	36.5	65.0	24.2
254 nm	58.4	37.0	62.5	34.9	59.2	38.7	64.5	27.4
500 nm	58.0	38.5	62.2	35.9	59.3	39.5	65.2	29.1
HPLC content / %, w/w	51.8	35.4	53.6	32.3	53.2	38.4	40.3	19.3
(5-Chloro-2-nitro-p-phenylene-diamine), Rot C, %w/w	0.24		0.14		0.35		0.48	
Impurity A, %w/w	0.21		0.16		0.21		0.15	
Impurity B, %w/w Sum of 2 isomers	0.65		0.7		0.91		0.2	
Impurity C, %w/w	1.2		1.8		1.2		2.2	
Impurity D, %w/w	0.19		0.64		0.19		0.43	
Impurity E, %w/w	0.35		0.2		0.44		0.67	
2-Chloro-1,4-diaminobenzene / ppm	Not detected, LOD = 10ppm		Not detected, LOD = 10ppm		Not detected, LOD = 10ppm		Not detected, LOD = 10ppm	
Content of Chloride/ %w/w	0.07		0		*		6.28	
Water content / %, w/w	0.94		1.66		*		1.69	
Loss on drying / %, w/w	0.37		2.1		*		1.34	
Sulphated ash / %, w/w	0.15		2.7		*		5.95	
Element analysis / ppm	Na 369 Mg 25 Si 110 Ca 174 Fe 13 Br 28		Not analysed		Na 400 Mg 23 Si 100 Ca 150 Br 21		Na 896 Si 98 K 25600 Ca 50 Fe 492 Pb 25	
Mass Balance	**98.6**		**98.4**		**94.9**		**76.3**	

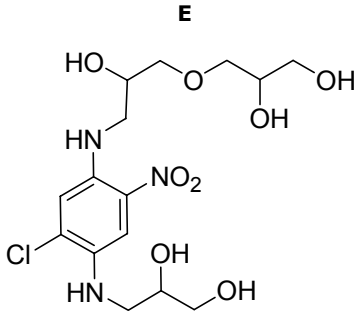
* Not determined because lack of substance

Chemical structure of impurity 5-Chloro-2-nitro-p-phenylenediamine



Impurities A-E

<table>
<tr><th colspan="2">Impurity structure</th></tr>
<tr><td colspan="2">A
 
3-(4-Amino-2-chloro-5-nitro-phenylamino)-propane-1,2-diol</td></tr>
<tr><td colspan="2">B consisted of 2 isomers that could not be separated.</td></tr>
<tr><td>B: Isomer A
 
3-[2-Chloro-4-(2-hydroxy-1-hydroxymethyl-ethylamino)-5-nitro-phenylamino]-propane-1,2-diol</td><td>B: Isomer B
 
3-[5-Chloro-4-(2-hydroxy-1-hydroxymethyl-ethylamino)-2-nitro-phenylamino]-propane-1,2-diolmethane;</td></tr>
<tr><td colspan="2">C
 
3-[(4-Amino-5-chloro-2-nitro-phenyl)-(2,3-dihydroxy-propyl)-amino]-propane-1,2-diol</td></tr>
<tr><td colspan="2">D
 
3-[[2-Chloro-4-(2,3-dihydroxy-propylamino)-5-nitro-phenyl]-(2,3-dihydroxy-propyl)-amino]-propane-1,2-diol</td></tr>
</table>

Impurity structure
E  3-{2-Chloro-4-[3-(2,3-dihydroxy-propoxy)-2-hydroxy-propylamino]-5-nitro-phenylamino}-propane-1,2-diol

Declaration by the applicant

"……batch LEH5/1 does not represent the market quality. It was however included in the batch comparison as it was used as undiluted test material to determine skin and eye irritation potential, and was found to be non irritant for both endpoints. Although the content of batch LEH5/1 deviates from the current market specification, the results obtained with this batch are considered sufficiently reliable to evaluate the skin and eye irritation potential of B071 under use conditions. A maximum use concentration of 2 % is unlikely to cause skin and eye irritation……"

Comments
- All six impurities identified and quantified in HC Red n° 10 and HC Red n° 11 are derivatives of aromatic amines and they contain primary/secondary and tertiary amino groups.
- An additional impurity has been characterised as starting material, but the starting material is not reported. Its content has also not been reported.-The water content in the batch L4/141(0.94%) was surprisingly higher than the loss on drying (0.37%)

3.1.5. Impurities / accompanying contaminants

See point 3.1.4. Purity, composition and substance codes

3.1.6. Solubility

Water solubility of HC Red n° 10 and HC Red n° 11 determined by EC Method A.6 was:

1.734 g/L for HC Red n°10
3.058g/L for HC Red n° 11

Solubility of Mixture of HC Red n° 10 and HC Red n° 11

DMSO: > 100 g/l
Ethanol: 40 < S < 80 g/l

3.1.7. Partition coefficient (Log P_{ow})

P_{ow} determined by EC Method A.8

HC Red n° 10
P_{ow}: 10.23
Log P_{ow}: 1.01

HC Red n° 11
P_{ow}: 1.028
Log P_{ow}: 0.012

pH: 7.5 at 25 °C

Ref.: 1

3.1.8. Additional physical and chemical specifications

pH:	7.3 (saturated aqueous solution, 22 °C)		(ref. 2)
Melting range:	109.8 - 111.8 °C	(OECD 102)	(ref. 3)
Boiling point:	252.9 °C (decomposition)	(OECD 103)	(ref. 4)
Density:	1.53 g/cm³ (25 °C)	(OECD 109)	(ref. 5)
Vapour pressure:	13.9 hPa (25 °C)	(OECD 104)	(ref. 6)
Surface tension (in water):	72.7 mN/m (26.2 °C)	(EU – A.5)	(ref. 7)
Water solubility:	2175.6 mg/l (22 °C)	(EU – A.6)	(ref. 2)
Flammability (solids):	not highly flammable	(EU – A.10)	(ref. 8)
Explosive properties:	not explosive	(EU – A.14)	(ref. 9)
Relative self-ignition temp.:	> 400 °C	(EU – A.16)	(ref. 10)
Oxidising properties:	not oxidising	(EU – A.17)	(ref. 11)

3.1.9. Stability

In the dermal absorption studies, it was mentioned that the test substance was stable for 167 hours in receptor fluid (phosphate buffered saline) and 286 hors in skin wash (soap solution). However, no specific data on stability was provided.

Stability of the test material in solutions, stored at room temperature in the absence of light, was reported in a certificate of analysis attached to the teratogenicity study, but no specific data was provided.

DMSO (approx. 10% w/v):	100% at t = 0, 101.4% at t = 6h, 100.2% at t = 2 days, 99.4% at t = 7 days
Acetone/Water 1:1 (approx. 8% w/v):	100% at t = 0, 99.7% at t = 6h, 99.4% at t = 2 days, 85.5% at t = 7 days
Water (Approx.0.1% w/v) pH 7.5:	100% at t = 0, 102.3% at t = 6h, 96.3% at t = 2 days, 101.7% at t = 7 days

Homogeneity and stability of HC Red n° 10/HC Red n° 11 [WR23532, batch 9375 (Fa. OSL)] suspensions were tested in 0.5% aqueous CMC. The test suspensions (3-30 mg/ml) after 7 days storage at -20°C were shown to be homogeneous (variation -5% to +9% among top, middle and bottom layers). The test suspensions (3-30 mg/ml) after 7 days storage in a refrigerator were shown to be stable (variation <10%).

The stability of HC Red n° 10 and HC Red n° 11 under oxidative conditions was examined in a 1:1 mixture of the cream formulation and Welloxon Perfect 6% H_2O_2 at ambient temperature for a total time period of 45 min. The recovery of the dye at 15 min, 30 min and 45 min were 96%, 96% and 94% respectively.

General Comments to physico-chemical characterisation

- All six impurities identified and quantified in HC Red n° 10 and HC Red n° 11 are derivatives of aromatic amines and they contain primary/secondary and tertiary amino groups. Such compounds are well known hazardous substances. Five of these impurities can form nitrosamines, which may be carcinogenic.
- An additional impurity has been characterised as starting material, but the starting material is not reported. Its content has also not been reported.
- HC Red n° 10 and HC Red n° 11 are secondary amines and therefore prone to nitrosation. The nitrosamine content in the hair dye HC Red n° 10 and HC Red n° 11 is not reported.
- The stability of the mixture of HC Red n° 10 and HC Red n° 11 in typical hair dye formulations is not reported.

3.2. Function and uses

a) Semipermanent Hair Colorants

HC Red n° 10 + HC Red n° 11 is used as a direct dye in semipermanent hair dye formulations at a maximum on-head concentration of 2%.

b) Oxidative Hair Colorants

HC Red n° 10 + HC Red n° 11 is used as a direct dye in oxidative hair dye formulations at a maximum on-head concentration of 1%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.2. Acute oral toxicity

Guideline:	/	
Species/strain:	Wistar rat; CF 1 mice	
Group size:	6 rats per sex and dose; 10 mice per sex and dose	
Test substance:	Rot Y	
Batch:	L4/126	
Purity:	/	
Vehicle:	aqua dest.	
Dose levels:	mouse, male:	1.20, 1.80, 2.40 and 3.00 g/kg bw
	mouse, female:	1.05, 1.80, 2.55 and 3.30 g/kg bw
	rat, male:	1.20, 2.40 and 3.60 g/kg bw
	rat, female:	1.00, 2.00 and 3.00 g/kg bw
Route:	oral, gavage	
GLP:	in compliance	
Study period:	25 November 1983 – 1 February 1984	

According to the applicant the test substance Rot Y consisted of 51 % HC Red n° 10, 46 % HC Red n° 11, and 2 % 5-chloro-2-nitro-p-phenylenediamine (Rot C). The test substance was suspended in distilled water and administered by oral gavage to groups of CF 1 mice and Wistar rats at 4 and 3 dose levels, respectively. Mortality and clinical signs were recorded daily and body weights weekly. At the end of the 2 weeks observation period the animals were subjected to gross necropsy.

Results
Reduced activity, side position and clonic-tonic spasms were noted. Based on the observed mortality rates the following LD_{50} figures were calculated:

	LD_{50} (mg/kg bw)
rat (female)	1830
rat (male)	2196
mouse (female)	1875
Mouse (male)	1860

Ref.: 13

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402
Species/strain:	rabbit, White New Zealand
Group size:	5 males and 5 females
Test substance:	Rot Y
Batch:	LEH5/1
Purity:	/
Vehicle:	/
Test sample:	undiluted, moistened with water
Dose levels:	2000 mg/kg bw
Route:	dermal
Administration:	directly to intact skin
GLP:	in compliance
Study period:	15 – 30 September 1988

The test substance moistened with water was applied to the dorsal skin of 5 male and 5 female rabbits at 2000 mg/kg bw and the area was covered. 24 h later the patches were removed. And the animals were observed for clinical signs at 1, 2, 3, 6, 24 and 48 h and later daily for 2 weeks. The study was terminated by necropsy.

Results
No mortality, no clinical signs and changes in body weight gain were observed. Due to red discolouration no skin reactions could be evaluated. No gross pathological findings were seen.

Ref.: 14

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404 (1981)
Species/strain:	New Zealand White rabbit
Group size:	3 females
Test substance:	Rubinrot Y
Batch:	LE H5/1
Purity:	/
Vehicle:	aqua dest.
Dose level:	0.5 g, soaked with 1 ml aqua dest.
GLP:	in compliance
Study period:	1990

Over a cellulose patch (5x4cm), 0.5g Rubinrot Y was applied over 6cm^2 and moistened with 1 ml of water. The patch was applied to the shaved dorsal skin and taped down for 4 hours.

After 4 hours, no signs of irritation were observed.

Ref.: 15

Comment
Under the conditions of this experiment, Rubinrot Y was not irritating to rabbit skin.

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405 (1987)
Species/strain:	New Zealand White rabbit
Group size:	3 females
Test substance:	Rubinrot Y
Batch:	LE H5/1
Purity:	/
Vehicle:	/
Dose volume:	0.1 ml (57 – 62 mg test substance)
GLP:	in compliance
Study period:	1990

Approximately 60 mg of Rubinrot Y was placed into the conjunctival sac of the right eye of each animal. The left eyes served as controls. Observations were made at 1, 24 and 72 hours.

Conjunctival redness was observed in all animals at the three time points. Oedema was present in 2/3 at 1 and 24 hours. Chemosis was present in 1 animal at 1 hour.

Ref.: 16

Comment
Under the conditions of this experiment, Rubinrot Y was irritating to rabbit eyes.

3.3.3. Skin sensitisation

Guideline:	OECD 429 (2002)
Species/strain:	mouse CBA/J
Group size:	50 females (5 per group, including vehicle controls)
Test substance:	HC Red n° 10 + HC Red n° 11, WR 23532
Batch:	9375
Purity:	considered as 100% for dose calculation HC Red n° 10 66.0%; HC Red n° 11 32.6% by area at 254nm
Vehicle:	vehicle 1: DMSO vehicle 2: acetone/water (1:1) mixed with olive oil (3:1)
Concentration:	0.5, 1.5, 5.0 and 10.0% in DMSO 0.5, 1.5, 5.0 and 9.5% in acetone/water (1:1) mixed with olive oil (3:1)
Positive control:	p-phenylenediamine 1%
GLP:	in compliance
Study period:	15 – 20 January 2004

The skin sensitising potential for WR 23532 was studied with the LLNA where two vehicles were employed: DMSO and acetone/water/olive oil.

On days 0, 1 and 2, 25 µl of the test substance was applied to the dorsal surface of each ear. On day 5, 250 µl of PBS containing 24.4 µCi [^{3}H]-methyl thymidine was injected into each animal which were killed by CO_2 inhalation 5 hours later. The draining lymph nodes were dissected out and weighed. Single cell suspensions were prepared, washed with PBS and precipitated with 5% trichloroacetic acid. After re-suspension in trichloroacetic acid, liquid scintillation counting was used to measure incorporation.

Vehicle DMSO	Stimulation Index (SI)		Vehicle acetone/water/olive oil	Stimulation Index (SI)
0.5%	1.4		0.5%	1.1
1.5%	1.4		1.5%	0.9
5.0%	1.4		5.0%	1.8
10.0%	2.3		9.5%	1.3

As no Stimulation Index exceeded 3.0, it was not possible to calculate an EC3 value. The positive control, p-phenylenediamine, produced a Stimulation Index of 7.7 at the 1% test concentration.

Under the conditions of this study, WR 23532 was not shown to be a sensitizer when tested at up to 10% in DMSO or 9.5% in acetone/water/olive oil as vehicles.

Ref.: 17

Comment
The highest concentration tested (10%) was too low for hazard identification. Therefore, a sensitising potential cannot be excluded.

3.3.4. Dermal / percutaneous absorption

Percutaneous absorption in vitro **(non-oxidative conditions)**

Guideline: OECD 428 (2004)

Tissue:	Human skin (abdomen or breast; thickness: 200-400 µm)
Membrane integrity	Tritiated water
Method:	Automated PTFE flow-through chambers
No. of chambers:	12 (from 6 donors)
Test substance:	B71 tested at a concentration of 2.0% (HC Red n° 10 1.336%; HC Red n° 11 0.627% w/w) in a typical hair dye formulation (DTF 0488085AF) under non-oxidative conditions.
Batch:	R0035285 (identical with batch R00058409)
Purity:	/
Area Dose:	20 mg formulation/cm²
Receptor fluid	PBS with 0.01% sodium azide
Solubility in receptor	500mg/L
Stability	/
Analyses	liquid chromatography – MS/MS for HC Red n° 10 and HC Red n° 11
Date	Sept-Dec 2009
GLP:	In compliance

Skin absorption of B71 was investigated with human skin (abdomen and breast (400 µm). An area dose of 20 mg/cm² of the final formulation (representing 0.4 mg/cm² of the dye) was applied once to the skin (0.64 cm²) in a commercial non-oxidative hair dye formulation in the absence of hydrogen peroxide.

The integrity of the skin was monitored at the beginning of the experiment using tritiated water.

Automated PTFE flow-through chambers were used. The receptor solution (phosphate buffered saline containing sodium acid (ca. 0.1% w/v)) was pumped through the receptor chamber at a rate of 1.5 ml/h. Twelve chambers were investigated.

Thirty minutes after substance application, the test item was removed by washing the skin three times with 0.32 ml water, then once with 0.32 ml washing solution (2% (v/v) sodium dodecyl sulphate) and again ten times with water. The water and sodium dodecyl sulphate solution were pooled in one pre weighed skin wash vial per skin sample and methanol (10 ml) was added. Duplicate weighed aliquots (1 ml) were removed from each skin wash vial, and measured by LC-MS/MS.

Receptor fluid was collected in 30 min fractions from 0 to 1 h post dose and hourly fractions from 1 to 6 h post dose and then in 2 hourly fractions from 6 to 72 h post dose. All receptor fluid samples were analysed by LC-MS/MS for each sub-component of the dye. At termination of the experiment, the skin was washed as described above. The stratum corneum was removed with 20 successive tape strips. Each tape was placed into a separate vial and the sample analysed by LC-MS/MS for each sub-component of the dye. The remaining epidermis and dermis were heat-treated and the epidermis was mechanically separated from the dermis. The dye content was determined in both skin compartments after extraction with methanol and analysed by LC-MS/MS for each sub-component of the dye.

Results
The integrity of each skin preparation used was demonstrated by examination of penetration characteristics with tritiated water. All skin sample/diffusion chamber units were within the limit of acceptance (0.6%).

The total recovery was within the range of 100 ± 15% of the applied dose for 9 skin samples regarding the total dye, and therefore confirmed the validity of the test. Therefore, the following results are based on 9 samples of skin from 6 donors.

Table 1 **Distribution of HC Red No. 10 (% Applied Dose) at 72 h Following Topical Application of Test Preparation 1 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 22 0291	Cell 23 0271	Cell 24 0296	Cell 25 0288	Cell 26 0298	Cell 27 0216	*Cell 29 0291*	*Cell 30 0271*	*Cell 31 0296*	Cell 32 0288	Cell 33 0298	Cell 34 0216	Mean	SD
Skin Wash 30 min	89.69	90.79	88.23	91.89	94.45	92.62	*99.58*	*101.04*	*109.46*	94.82	93.72	92.26	92.05	2.19
Tissue Swab 30 min	0.42	1.92	6.46	0.95	0.15	0.25	*1.05*	*0.75*	*0.20*	0.19	0.15	0.46	1.22	2.05
Pipette Tips 30 min	*0.04	1.15	0.21	0.14	0.37	*0.04	**0.04*	*0.37*	**0.05*	*0.03	0.28	*0.03	0.25	0.36
Dislodgeable Dose 30 min	90.15	93.86	94.89	92.98	94.97	92.91	*100.67*	*102.16*	*109.71*	95.04	94.15	92.74	93.52	1.56
Skin Wash 72 h	0.19	0.11	0.34	0.20	0.08	0.15	*0.12*	*0.12*	*0.11*	0.06	0.06	0.21	0.15	0.09
Tissue Swab 72 h	0.03	*0.02	0.04	0.04	*0.01	0.03	**0.02*	*0.06*	**0.03*	*0.01	*0.01	0.04	0.03	0.01
Pipette Tips 72 h	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*°0.00*	*°0.00*	°0.00	°0.00	°0.00	0.00	0.00
Cell Wash	*0.06	*0.09	*0.07	*0.09	*0.07	*0.06	**0.13*	**0.07*	**0.07*	*0.07	*0.06	0.48	0.12	0.14
Dislodgeable dose 72 h	0.28	0.21	0.45	0.33	0.17	0.23	*0.27*	*0.25*	*0.21*	0.14	0.14	0.73	0.30	0.19
Total Dislodgeable Dose	90.43	94.08	95.34	93.31	95.13	93.14	*100.94*	*102.41*	*109.92*	95.18	94.29	93.47	93.82	1.52
Stratum corneum 1-5	*0.01	0.03	0.04	0.04	*0.01	0.03	**0.02*	*0.04*	**0.02*	*0.02	*0.02	0.03	0.03	0.01
Stratum corneum 6-10	0.03	0.05	0.05	0.04	*0.02	*0.02	**0.03*	*0.03*	**0.02*	*0.02	0.03	0.03	0.03	0.01
Stratum corneum 11-15	0.03	*0.02	*0.01	0.04	*0.01	*0.02	*0.04*	*0.03*	**0.02*	0.04	*0.02	*0.03	0.03	0.01
Stratum corneum 16-20	0.03	*0.03	*0.01	*0.03	*0.01	*0.02	*0.04*	**0.01*	**0.01*	*0.02	*0.01	0.03	0.02	0.01
Stratum Corneum	0.10	0.13	0.11	0.14	0.06	0.10	*0.12*	*0.12*	*0.07*	0.11	0.08	0.12	0.10	0.02
Unexposed Skin	*0.02	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	**0.02*	*°0.00*	°0.00	°0.00	0.28	0.03	0.09
Total Unabsorbed	90.55	94.21	95.45	93.45	95.19	93.24	*101.06*	*102.54*	*109.99*	95.29	94.37	93.86	93.96	1.51
Epidermis	0.07	°0.00	°0.00	0.05	*0.02	0.09	*0.03*	**0.02*	*0.09*	*0.03	*0.02	0.04	0.04	0.03
Dermis	*0.02	*0.02	*0.02	*0.02	°0.00	0.03	**0.02*	**0.02*	**0.02*	*0.02	*0.02	*0.02	0.02	0.01
Receptor Fluid	0.11	0.04	0.11	0.09	0.02	0.11	*0.11*	*0.06*	*0.15*	0.08	0.03	0.13	0.08	0.04
Receptor Rinse	*0.00	*0.00	*0.00	*0.00	*0.00	0.00	**0.00*	**0.00*	**0.00*	*0.00	*0.00	0.00	0.00	0.00
Total Absorbed	0.11	0.04	0.11	0.09	0.02	0.11	*0.11*	*0.06*	*0.15*	0.08	0.03	0.13	0.08	0.04
Dermal Delivery	0.21	0.06	0.13	0.17	0.04	0.23	*0.17*	*0.10*	*0.26*	0.12	0.07	0.20	0.14	0.07
Mass Balance	90.76	94.27	95.58	93.62	95.23	93.47	*101.22*	*102.64*	*110.25*	95.41	94.43	94.06	94.09	1.46

Cells 29, 30 and 31 rejected from mean and SD due to high mass balance for HC Red No. 11 (>115%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Table 3 **Distribution of HC Red No. 10 (µg/cm^2) at 72 h Following Topical Application of Test Preparation 1 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 22 0291	Cell 23 0271	Cell 24 0296	Cell 25 0288	Cell 26 0298	Cell 27 0216	*Cell 29 0291*	*Cell 30 0271*	*Cell 31 0296*	Cell 32 0288	Cell 33 0298	Cell 34 0216	Mean	SD
Skin Wash 30 min	257.25	260.40	253.05	263.55	270.90	265.65	*285.60*	*289.80*	*313.95*	271.95	268.80	264.60	264.02	6.28
Tissue Swab 30 min	1.21	5.52	18.52	2.73	0.43	0.73	*3.01*	*2.16*	*0.58*	0.55	0.42	1.32	3.49	5.87
Pipette Tips 30 min	*0.10	3.30	0.60	0.40	1.05	*0.10	**0.12*	*1.05*	**0.14*	*0.09	0.82	*0.07	0.72	1.03
Dislodgeable Dose 30 min	258.56	269.21	272.17	266.68	272.38	266.48	*288.73*	*293.01*	*314.67*	272.59	270.04	265.99	268.23	4.47
Skin Wash 72 h	0.55	0.31	0.96	0.56	0.23	0.42	*0.35*	*0.35*	*0.32*	0.17	0.17	0.59	0.44	0.26
Tissue Swab 72 h	0.08	*0.05	0.11	0.13	*0.04	0.08	**0.06*	*0.16*	**0.07*	*0.04	*0.04	0.12	0.08	0.04
Pipette Tips 72 h	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*°0.00*	*°0.00*	°0.00	°0.00	°0.00	0.00	0.00
Cell Wash	*0.18	*0.25	*0.21	*0.25	*0.20	*0.16	**0.36*	**0.20*	**0.21*	*0.19	*0.19	1.38	0.33	0.39
Dislodgeable dose 72 h	0.80	0.62	1.28	0.94	0.48	0.66	*0.77*	*0.71*	*0.60*	0.40	0.40	2.09	0.85	0.54
Total Dislodgeable Dose	259.36	269.83	273.45	267.62	272.85	267.15	*289.50*	*293.72*	*315.27*	272.99	270.43	268.08	269.09	4.36
Stratum corneum 1-5	*0.04	0.09	0.13	0.10	*0.03	0.08	**0.04*	*0.11*	**0.05*	*0.05	*0.06	0.13	0.08	0.04
Stratum corneum 6-10	0.08	0.15	0.15	0.11	*0.06	*0.07	**0.08*	*0.09*	**0.06*	*0.07	0.09	0.09	0.09	0.03
Stratum corneum 11-15	0.09	*0.06	*0.02	0.12	*0.04	*0.07	*0.11*	*0.10*	**0.05*	0.12	*0.06	*0.08	0.07	0.03
Stratum corneum 16-20	0.09	*0.07	*0.02	*0.07	*0.03	*0.06	*0.11*	**0.03*	**0.04*	*0.07	*0.02	0.09	0.06	0.03
Stratum Corneum	0.30	0.37	0.32	0.40	0.17	0.28	*0.35*	*0.33*	*0.20*	0.31	0.22	0.38	0.30	0.08
Unexposed Skin	*0.05	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	**0.05*	*°0.00*	°0.00	°0.00	0.79	0.09	0.26
Total Unabsorbed	259.71	270.20	273.77	268.03	273.02	267.42	*289.85*	*294.10*	*315.47*	273.30	270.65	269.25	269.48	4.32
Epidermis	0.20	°0.00	°0.00	0.15	*0.05	0.26	*0.09*	**0.05*	*0.25*	*0.07	*0.05	0.12	0.10	0.09
Dermis	*0.07	*0.04	*0.06	*0.07	°0.00	0.10	**0.07*	**0.05*	**0.07*	*0.05	*0.05	*0.06	0.05	0.03
Receptor Fluid	0.32	0.13	0.31	0.26	0.07	0.31	*0.32*	*0.18*	*0.43*	0.23	0.10	0.37	0.23	0.11
Receptor Rinse	*0.00	*0.00	*0.00	*0.00	*0.00	0.00	**0.00*	**0.00*	**0.00*	*0.00	*0.00	0.00	0.00	0.00
Total Absorbed	0.33	0.13	0.31	0.26	0.07	0.31	*0.32*	*0.19*	*0.43*	0.23	0.10	0.38	0.23	0.11
Dermal Delivery	0.59	0.17	0.37	0.48	0.12	0.67	*0.47*	*0.29*	*0.74*	0.36	0.19	0.56	0.39	0.20
Mass Balance	260.31	270.37	274.14	268.51	273.14	268.09	*290.33*	*294.39*	*316.21*	273.66	270.85	269.81	269.87	4.20

Cells 29, 30 and 31 rejected from mean and SD due to high mass balance for HC Red No. 11 (>115%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Table 6 **Distribution of HC Red No. 11 (% Applied Dose) at 72 h Following Topical Application of Test Preparation 1 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 22 0291	Cell 23 0271	Cell 24 0296	Cell 25 0288	Cell 26 0298	Cell 27 0216	*Cell 29 0291*	*Cell 30 0271*	*Cell 31 0296*	Cell 32 0288	Cell 33 0298	Cell 34 0216	Mean	SD
Skin Wash 30 min	103.10	103.10	102.28	103.10	109.59	109.59	*117.71*	*120.14*	*129.07*	109.59	107.15	108.78	106.25	3.28
Tissue Swab 30 min	0.41	1.84	6.52	1.09	0.12	0.26	*1.00*	*0.66*	*0.27*	0.19	0.13	0.41	1.22	2.07
Pipette Tips 30 min	*0.04	0.94	0.20	0.14	0.28	*0.03	*0.05*	*0.30*	*0.05*	*0.03	*0.03	0.20	0.21	0.29
Dislodgeable Dose 30 min	103.55	105.88	109.00	104.32	110.00	109.88	*118.75*	*121.11*	*129.40*	109.81	107.31	109.38	107.68	2.53
Skin Wash 72 h	0.21	0.11	0.36	0.21	0.06	0.11	*0.12*	*0.14*	*0.12*	0.05	*0.04	0.17	0.15	0.11
Tissue Swab 72 h	*0.02	*0.02	0.04	0.04	*0.02	0.03	*0.02*	*0.09*	*0.04*	*0.01	*0.01	0.04	0.03	0.01
Pipette Tips 72 h	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*°0.00*	*°0.00*	°0.00	°0.00	°0.00	0.00	0.00
Cell Wash	°0.00	*0.10	*0.08	*0.09	*0.08	°0.00	*0.17*	*0.09*	*0.09*	*0.07	*0.08	0.63	0.13	0.19
Dislodgeable dose 72 h	0.23	0.23	0.48	0.35	0.16	0.14	*0.30*	*0.31*	*0.25*	0.14	0.13	0.85	0.30	0.24
Total Dislodgeable Dose	103.77	106.11	109.49	104.67	110.15	110.02	*119.06*	*121.42*	*129.65*	109.95	107.44	110.23	107.98	2.56
Stratum corneum 1-5	*0.02	0.05	0.06	0.05	*0.01	0.05	*0.02*	*0.07*	*0.04*	*0.03	*0.02	0.06	0.04	0.02
Stratum corneum 6-10	0.03	0.05	0.05	0.06	*0.01	0.03	*0.04*	*0.03*	*0.04*	0.03	*0.02	*0.02	0.04	0.02
Stratum corneum 11-15	*0.03	*0.01	°0.00	0.06	*0.01	*0.03	*0.05*	*0.02*	*0.03*	0.04	*0.01	0.03	0.02	0.02
Stratum corneum 16-20	*0.02	*0.02	°0.00	*0.02	°0.00	*0.02	*0.03*	*0.01*	*0.02*	*0.02	°0.00	*0.02	0.01	0.01
Stratum Corneum	0.10	0.14	0.11	0.19	0.03	0.12	*0.14*	*0.13*	*0.12*	0.13	0.05	0.14	0.11	0.05
Unexposed Skin	*0.01	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*0.01*	*°0.00*	°0.00	°0.00	0.26	0.03	0.08
Total Unabsorbed	103.88	106.24	109.60	104.86	110.18	110.14	*119.19*	*121.56*	*129.77*	110.07	107.49	110.63	108.12	2.58
Epidermis	0.03	°0.00	°0.00	*0.02	°0.00	0.04	*0.01*	*°0.00*	*0.07*	*0.01	°0.00	*0.02	0.01	0.02
Dermis	*0.01	°0.00	°0.00	*0.01	°0.00	*0.02	*0.01*	*°0.00*	*0.01*	°0.00	°0.00	*0.01	0.01	0.01
Receptor Fluid	0.03	0.00	0.00	0.02	0.00	0.00	*0.00*	*0.00*	*0.00*	0.02	0.00	0.00	0.01	0.01
Receptor Rinse	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*°0.00*	*°0.00*	°0.00	°0.00	°0.00	0.00	0.00
Total Absorbed	0.03	0.00	0.00	0.02	0.00	0.00	*0.00*	*0.00*	*0.00*	0.02	0.00	0.00	0.01	0.01
Dermal Delivery	0.07	0.00	0.00	0.06	0.00	0.06	*0.03*	*0.00*	*0.08*	0.03	0.00	0.03	0.03	0.03
Mass Balance	103.96	106.25	109.60	104.92	110.18	110.20	*119.22*	*121.56*	*129.85*	110.11	107.49	110.66	108.15	2.57

Cells 29, 30 and 31 rejected from mean and SD due to high mass balance (>115%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Table 8 **Distribution of HC Red No. 11 (µg/cm^2) at 72 h Following Topical Application of Test Preparation 1 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 22 0291	Cell 23 0271	Cell 24 0296	Cell 25 0288	Cell 26 0298	Cell 27 0216	*Cell 29 0291*	*Cell 30 0271*	*Cell 31 0296*	Cell 32 0288	Cell 33 0298	Cell 34 0216	Mean	SD
Skin Wash 30 min	133.35	133.35	132.30	133.35	141.75	141.75	*152.25*	*155.40*	*166.95*	141.75	138.60	140.70	137.43	4.25
Tissue Swab 30 min	0.53	2.38	8.44	1.41	0.16	0.33	*1.29*	*0.85*	*0.35*	0.24	0.16	0.53	1.58	2.68
Pipette Tips 30 min	*0.05	1.22	0.25	0.18	0.36	*0.04	*0.06*	*0.39*	*0.07*	*0.04	*0.04	0.26	0.27	0.38
Dislodgeable Dose 30 min	133.93	136.95	140.99	134.94	142.28	142.12	*153.60*	*156.65*	*167.37*	142.04	138.80	141.48	139.28	3.27
Skin Wash 72 h	0.27	0.14	0.47	0.28	0.08	0.14	*0.15*	*0.18*	*0.16*	0.06	*0.05	0.22	0.19	0.14
Tissue Swab 72 h	*0.03	*0.03	0.05	0.05	*0.02	0.05	*0.02*	*0.12*	*0.05*	*0.02	*0.02	0.05	0.03	0.01
Pipette Tips 72 h	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*°0.00*	*°0.00*	°0.00	°0.00	°0.00	0.00	0.00
Cell Wash	°0.00	*0.13	*0.11	*0.12	*0.11	°0.00	*0.22*	*0.11*	*0.12*	*0.10	*0.10	0.82	0.16	0.25
Dislodgeable dose 72 h	0.30	0.29	0.63	0.45	0.20	0.18	*0.39*	*0.40*	*0.32*	0.18	0.16	1.09	0.39	0.31
Total Dislodgeable Dose	134.23	137.25	141.62	135.39	142.48	142.30	*153.99*	*157.05*	*167.69*	142.21	138.97	142.58	139.67	3.31
Stratum corneum 1-5	*0.02	0.07	0.07	0.06	*0.01	0.06	*0.03*	*0.09*	*0.05*	*0.04	*0.03	0.08	0.05	0.02
Stratum corneum 6-10	0.04	0.07	0.07	0.08	*0.02	0.04	*0.05*	*0.04*	*0.05*	0.04	*0.02	*0.03	0.05	0.02
Stratum corneum 11-15	*0.04	*0.02	°0.00	0.07	*0.01	*0.04	*0.06*	*0.03*	*0.04*	0.06	*0.02	0.04	0.03	0.02
Stratum corneum 16-20	*0.03	*0.03	°0.00	*0.00	°0.00	*0.03	*0.04*	*0.01*	*0.03*	*0.03	°0.00	*0.03	0.02	0.01
Stratum Corneum	0.13	0.17	0.14	0.22	0.04	0.16	*0.18*	*0.16*	*0.16*	0.16	0.07	0.19	0.14	0.06
Unexposed Skin	*0.01	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*0.02*	*°0.00*	°0.00	°0.00	0.33	0.04	0.11
Total Unabsorbed	134.37	137.42	141.76	135.61	142.52	142.46	*154.17*	*157.23*	*167.85*	142.38	139.03	143.09	139.85	3.34
Epidermis	0.04	°0.00	°0.00	*0.03	°0.00	0.06	*0.02*	*°0.00*	*0.09*	*0.02	°0.00	*0.02	0.02	0.02
Dermis	*0.02	°0.00	°0.00	*0.02	°0.00	*0.02	*0.01*	*°0.00*	*0.02*	°0.00	°0.00	*0.02	0.01	0.01
Receptor Fluid	0.03	0.00	0.00	0.03	0.00	0.00	*0.00*	*0.01*	*0.00*	0.03	0.00	0.00	0.01	0.01
Receptor Rinse	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	*°0.00*	*°0.00*	°0.00	°0.00	°0.00	0.00	0.00
Total Absorbed	0.03	0.00	0.00	0.03	0.00	0.00	*0.00*	*0.01*	*0.00*	0.03	0.00	0.00	0.01	0.01
Dermal Delivery	0.09	0.00	0.00	0.08	0.00	0.08	*0.04*	*0.01*	*0.11*	0.04	0.00	0.04	0.04	0.04
Mass Balance	134.46	137.43	141.76	135.68	142.52	142.54	*154.21*	*157.23*	*167.96*	142.42	139.04	143.13	139.89	3.32

Cells 29, 30 and 31 rejected from mean and SD due to high mass balance (>115%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Conclusion

Under the described test conditions (non-oxidative hair dye formulation), a total mean amount of 0.43 µg/cm² HC Red n° 10 and HC Red n° 11 is obtained by summing up the amounts present in receptor fluid and in both separated skin compartments.

Ref.: 6 (subm III)

Comment

The experiment was well performed. The mean + 1SD may be used in calculating MOS.

For HC Red n° 10 the amount considered as absorbed is 0.59 µg/cm² and for HC Red n° 11 the amount considered as absorbed is 0.08 µg/cm². Therefore the amount of B71 that may be considered absorbed is 0.67 µg/cm² under non-oxidative conditions in a hair dye formulation containing 2.0% B71.

Percutaneous absorption *in vitro* (oxidative conditions)

Guideline:	OECD 428 (2004)
Tissue:	Human skin (abdomen or breast; thickness: 200- 400 µm)
Membrane integrity	Tritiated water
Method:	Automated PTFE flow-through chambers
No. of chambers:	12 (from 6 donors)
Test substance:	B71 tested at a concentration of 1.0% (HC Red n° 10, 0.668%; HC Red n° 11, 0.314% w/w) in a typical hair dye formulation (DTF 0488085AF) under oxidative conditions.
Batch:	R0035285 (identical with batch R00058409)
Purity:	/
Area Dose:	20 mg formulation/cm²
Receptor fluid	PBS with 0.01% sodium azide
Solubility in receptor	500mg/L
Stability	/
Analyses	liquid chromatography – MS/MS for HC Red n° 10 and HC Red n° 11
Date	Sept-Dec 2009
GLP:	in compliance

The skin absorption of B71 was investigated with human skin (abdomen and breast (400 µm). An area dose of 20 mg/cm² of the final formulation (representing 0.2 mg/cm² of the dye) was applied once to the skin (0.64 cm²) in a commercial oxidative hair dye formulation in the presence of hydrogen peroxide.

The integrity of the skin was monitored at the beginning of the experiment using tritiated water.

Automated PTFE flow-through chambers were used. The receptor solution (phosphate buffered saline containing sodium acid (ca. 0.1% w/v) was pumped through the receptor chamber at a rate of 1.5 ml/h. Twelve chambers were investigated.

Thirty minutes after substance application, the test item was removed by washing the skin three times with 0.32 ml water, then once with 0.32 ml washing solution (2% (v/v) sodium dodecyl sulphate) and again ten times with water. The water and sodium dodecyl sulphate solution were pooled in one pre weighed skin wash vial per skin sample and methanol (10 ml) was added. Duplicate weighed aliquots (1 ml) were removed from each skin wash vial, and measured by LC-MS/MS.

Receptor fluid was collected in 30 min fractions from 0 to 1 h post dose and hourly fractions from 1 to 6 h post dose and then in 2 hourly fractions from 6 to 72 h post dose. All receptor fluid samples were analysed by LC-MS/MS for each sub-component of the dye. At termination of the experiment, the skin was washed as described above. The stratum corneum was removed with 20 successive tape strips. Each tape was placed into a separate vial and the sample analysed by LC-MS/MS for each sub-component of the dye. The remaining epidermis and dermis were heat-treated and the epidermis was mechanically separated from the dermis. The dye content was determined in both skin compartments after extraction with methanol and analysed by LC-MS/MS for each sub-component of the dye.

Results
The integrity of each skin preparation used was demonstrated by examination of penetration characteristics with tritiated water. All skin sample/diffusion chamber units were within the limit of acceptance (0.6 %).

The total recovery was within the range of 100 ± 15 % of the applied dose for 8 skin samples regarding the total dye, and therefore confirmed the validity of the test. Therefore, the following results are based on 8 samples of skin from 6 donors.

Table 11 **Distribution of HC Red No. 10 (% Applied Dose) at 72 h Following Topical Application of Test Preparation 2 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 1 0291	*Cell 3* 0271	Cell 4 0296	*Cell 5* 0288	Cell 6 0298	Cell 7 0216	Cell 8 0291	Cell 10 0271	Cell 11 0296	Cell 12 0288	Cell 13 0298	*Cell 14* 0216	Mean	SD
Skin Wash 30 min	*82.48*	*81.68*	95.29	*80.88*	90.49	84.08	89.69	91.29	90.49	92.09	88.88	*82.48*	90.29	3.17
Tissue Swab 30 min	*0.69*	*0.49*	0.41	*0.38*	0.92	0.39	0.14	0.45	0.23	0.58	0.35	*0.42*	0.43	0.24
Pipette Tips 30 min	**0.03*	**0.02*	*0.04	**0.02*	*0.04	*0.07	*0.03	*0.02	*0.03	*0.03	*0.03	**0.02*	0.04	0.02
Dislodgeable Dose 30 min	*83.19*	*82.18*	95.74	*81.28*	91.44	84.54	89.85	91.76	90.74	92.70	89.27	*82.91*	90.75	3.20
Skin Wash 72 h	*0.18*	*0.26*	0.27	*0.21*	0.20	0.31	0.23	0.22	0.26	0.22	0.34	*0.37*	0.25	0.05
Tissue Swab 72 h	**0.04*	**0.03*	*0.05	**0.04*	*0.06	*0.05	*0.03	*0.04	*0.03	*0.03	*0.04	**0.05*	0.04	0.01
Pipette Tips 72 h	**0.03*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	0.00	0.00
Cell Wash	**0.12*	**0.13*	*0.13	**0.12*	*0.45	*0.12	°0.00	*0.14	°0.00	*0.12	*0.12	**0.12*	0.14	0.14
Dislodgeable dose 72 h	*0.37*	*0.42*	0.45	*0.37*	0.71	0.48	0.26	0.40	0.28	0.37	0.50	*0.53*	0.43	0.14
Total Dislodgeable Dose	*83.56*	*82.60*	96.19	*81.65*	92.15	85.02	90.11	92.16	91.02	93.07	89.77	*83.45*	91.18	3.20
Stratum corneum 1-5	**0.05*	**0.04*	*0.05	**0.08*	*0.04	0.06	*0.03	*0.05	*0.04	0.07	*0.05	*0.13*	0.05	0.01
Stratum corneum 6-10	**0.06*	*0.07	0.06	*0.11*	*0.04	*0.04	0.06	0.13	*0.05	0.11	*0.06	*0.08*	0.07	0.03
Stratum corneum 11-15	*0.08	**0.04*	*0.05	*0.06*	*0.04	*0.05	0.12	*0.05	0.10	*0.04	*0.04	*0.06*	0.06	0.03
Stratum corneum 16-20	**0.04*	**0.03*	°0.00	**0.03*	*0.03	0.04	*0.03	*0.04	0.07	*0.03	*0.03	°0.00	0.03	0.02
Stratum Corneum	*0.23	*0.18	0.16	*0.27	0.14	0.19	0.25	0.28	0.26	0.25	0.18	*0.27	0.21	0.05
Unexposed Skin	°0.00	°0.00	*0.06	°0.00	*0.03	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	0.01	0.02
Total Unabsorbed	*83.79*	*82.78*	96.41	*81.93*	92.32	85.21	90.35	92.44	91.28	93.31	89.94	*83.71*	91.41	3.21
Epidermis	*0.11*	**0.06*	*0.03	**0.03*	0.16	0.17	0.12	0.12	0.13	*0.03	0.19	**0.06*	0.12	0.06
Dermis	**0.04*	**0.03*	*0.05	**0.03*	*0.03	*0.04	*0.04	*0.03	*0.04	°0.00	*0.03	**0.03*	0.03	0.02
Receptor Fluid	*0.10	*0.04	0.48	*0.09	0.08	0.09	0.08	0.10	0.33	0.07	0.06	*0.13	0.16	0.16
Receptor Rinse	**0.00*	°0.00	0.00	**0.00*	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	**0.00*	0.00	0.00
Total Absorbed	*0.10	*0.04	0.48	*0.09	0.08	0.09	0.08	0.10	0.33	0.07	0.06	*0.14	0.16	0.16
Dermal Delivery	*0.24	*0.13	0.57	*0.15	0.27	0.30	0.24	0.25	0.50	0.10	0.27	*0.23	0.31	0.15
Mass Balance	*84.03*	*82.91*	96.98	*82.08*	92.59	85.52	90.60	92.69	91.78	93.41	90.21	*83.94*	91.72	3.26

Cells 1, 3, 5 and 14 rejected from mean and SD due to low mass balance (<85%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Table 13 **Distribution of HC Red No. 10 (µg/cm^2) at 72 h Following Topical Application of Test Preparation 2 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 1 0291	*Cell 3* 0271	Cell 4 0296	*Cell 5* 0288	Cell 6 0298	Cell 7 0216	Cell 8 0291	Cell 10 0271	Cell 11 0296	Cell 12 0288	Cell 13 0298	*Cell 14* 0216	Mean	SD
Skin Wash 30 min	*108.15*	*107.10*	124.95	*106.05*	118.65	110.25	117.60	119.70	118.65	120.75	116.55	*108.15*	118.39	4.15
Tissue Swab 30 min	*0.90*	*0.64*	0.54	*0.50*	1.20	0.51	0.18	0.59	0.30	0.76	0.46	*0.54*	0.57	0.31
Pipette Tips 30 min	**0.03*	**0.02*	*0.05	**0.03*	*0.05	*0.09	*0.04	*0.03	*0.03	*0.03	*0.04	**0.03*	0.05	0.02
Dislodgeable Dose 30 min	*109.08*	*107.76*	125.54	*106.58*	119.91	110.85	117.81	120.32	118.98	121.55	117.05	*108.72*	119.00	4.20
Skin Wash 72 h	*0.23*	*0.34*	0.35	*0.28*	0.26	0.40	0.30	0.29	0.33	0.28	0.45	*0.49*	0.33	0.07
Tissue Swab 72 h	**0.05*	**0.04*	*0.07	**0.05*	*0.07	*0.07	*0.04	*0.05	*0.04	*0.04	*0.05	**0.06*	0.05	0.01
Pipette Tips 72 h	**0.04*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	0.00	0.00
Cell Wash	**0.16*	**0.17*	*0.17	**0.16*	*0.60	*0.16	°0.00	*0.19	°0.00	*0.16	*0.16	**0.16*	0.18	0.19
Dislodgeable dose 72 h	*0.49*	*0.55*	0.59	*0.48*	0.93	0.63	0.34	0.53	0.37	0.48	0.65	*0.70*	0.56	0.18
Total Dislodgeable Dose	*109.57*	*108.31*	126.12	*107.07*	120.83	111.48	118.15	120.85	119.35	122.03	117.70	*109.42*	119.57	4.20
Stratum corneum 1-5	**0.06*	**0.06*	*0.07	**0.11*	*0.05	0.08	*0.04	*0.07	*0.05	0.10	*0.07	*0.17*	0.07	0.02
Stratum corneum 6-10	**0.07*	*0.09	0.08	*0.14*	*0.05	*0.05	0.08	0.18	*0.07	0.14	*0.07	*0.10*	0.09	0.04
Stratum corneum 11-15	*0.10	**0.06*	*0.07	*0.07*	*0.05	*0.06	0.16	*0.07	0.13	*0.05	*0.05	*0.08*	0.08	0.04
Stratum corneum 16-20	**0.06*	**0.04*	°0.00	**0.04*	*0.04	0.06	*0.04	*0.06	0.09	*0.04	*0.04	°0.00	0.05	0.03
Stratum Corneum	*0.30	*0.24	0.22	*0.36	0.19	0.26	0.32	0.37	0.34	0.33	0.23	*0.35	0.28	0.07
Unexposed Skin	°0.00	°0.00	*0.08	°0.00	*0.04	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	0.01	0.03
Total Unabsorbed	*109.87*	*108.55*	126.42	*107.42*	121.06	111.73	118.48	121.22	119.69	122.36	117.94	*109.77*	119.86	4.21
Epidermis	*0.14*	**0.08*	*0.04	**0.04*	0.21	0.22	0.15	0.15	0.17	*0.04	0.24	**0.08*	0.15	0.08
Dermis	**0.05*	**0.04*	*0.07	**0.04*	*0.04	*0.06	*0.05	*0.04	*0.05	°0.00	*0.04	**0.05*	0.04	0.02
Receptor Fluid	*0.13	*0.05	0.63	*0.12	0.10	0.12	0.11	0.13	0.43	0.09	0.07	*0.18	0.21	0.21
Receptor Rinse	**0.00*	°0.00	0.00	**0.00*	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	*0.00	**0.00*	0.00	0.00
Total Absorbed	*0.13	*0.05	0.63	*0.12	0.10	0.12	0.11	0.13	0.43	0.09	0.07	*0.18	0.21	0.21
Dermal Delivery	*0.32	*0.17	0.75	*0.20	0.35	0.40	0.32	0.33	0.65	0.13	0.36	*0.30	0.41	0.20
Mass Balance	*110.19*	*108.72*	127.16	*107.62*	121.41	112.13	118.79	121.54	120.34	122.49	118.29	*110.07*	120.27	4.27

Cells 1, 3, 5 and 14 rejected from mean and SD due to low mass balance (<85%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Table 16 **Distribution of HC Red No. 11 (% Applied Dose) at 72 h Following Topical Application of Test Preparation 2 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 1 0291	*Cell 3* 0271	Cell 4 0296	*Cell 5* 0288	Cell 6 0298	Cell 7 0216	Cell 8 0291	Cell 10 0271	Cell 11 0296	Cell 12 0288	Cell 13 0298	*Cell 14* 0216	Mean	SD
Skin Wash 30 min	*85.92*	*86.08*	100.53	*88.59*	89.85	86.23	94.56	93.30	95.81	97.07	96.29	*87.80*	94.20	4.45
Tissue Swab 30 min	*0.53*	*0.35*	0.41	*0.39*	0.76	0.40	0.13	0.49	0.24	0.51	0.31	*0.42*	0.40	0.19
Pipette Tips 30 min	**0.03*	**0.03*	*0.05	**0.03*	*0.04	*0.08	*0.03	*0.03	*0.03	*0.03	*0.04	**0.03*	0.04	0.02
Dislodgeable Dose 30 min	*86.48*	*86.45*	100.98	*89.01*	90.65	86.71	94.72	93.82	96.08	97.61	96.64	*88.25*	94.65	4.39
Skin Wash 72 h	*0.10*	*0.21*	0.17	*0.15*	0.13	0.21	0.13	0.22	0.14	0.13	0.14	*0.29*	0.16	0.04
Tissue Swab 72 h	**0.03*	**0.02*	*0.04	**0.04*	*0.04	*0.05	*0.02	*0.04	*0.03	*0.04	*0.03	**0.04*	0.04	0.01
Pipette Tips 72 h	*°0.00*	*°0.00*	°0.00	*°0.00*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	0.00	0.00
Cell Wash	*°0.00*	*°0.00*	*0.14	**0.13*	0.58	°0.00	°0.00	*0.17	°0.00	°0.00	°0.00	**0.13*	0.11	0.20
Dislodgeable dose 72 h	*0.13*	*0.23*	0.35	*0.33*	0.75	0.26	0.15	0.43	0.17	0.18	0.17	*0.46*	0.31	0.20
Total Dislodgeable Dose	*86.61*	*86.68*	101.34	*89.34*	91.39	86.97	94.87	94.25	96.25	97.79	96.80	*88.71*	94.96	4.32
Stratum corneum 1-5	**0.03*	**0.04*	*0.05	*0.08*	*0.04	0.07	*0.03	*0.03	*0.05	*0.04	*0.05	**0.04*	0.04	0.02
Stratum corneum 6-10	**0.04*	**0.05*	*0.05	*0.07*	*0.03	*0.04	*0.05	*0.05	0.08	*0.05	*0.05	**0.04*	0.05	0.02
Stratum corneum 11-15	**0.05*	**0.03*	*0.04	**0.04*	*0.03	*0.04	*0.05	*0.05	*0.03	0.09	*0.02	**0.03*	0.05	0.02
Stratum corneum 16-20	**0.02*	**0.02*	°0.00	**0.02*	*0.02	*0.03	*0.03	*0.03	*0.04	*0.06	°0.00	**0.02*	0.03	0.02
Stratum Corneum	*0.15*	*0.14*	0.14	*0.21*	0.12	0.18	0.15	0.15	0.21	0.23	0.12	*0.12*	0.16	0.04
Unexposed Skin	*°0.00*	*°0.00*	*0.04	*°0.00*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	0.01	0.02
Total Unabsorbed	*86.76*	*86.82*	101.53	*89.55*	91.51	87.15	95.02	94.40	96.46	98.02	96.92	*88.83*	95.13	4.33
Epidermis	**0.03*	**0.03*	*0.02	*°0.00*	*0.05	*0.06	*0.04	0.07	0.12	°0.00	0.07	**0.02*	0.05	0.03
Dermis	*°0.00*	*°0.00*	*0.03	*°0.00*	°0.00	*0.02	*0.02	°0.00	*0.02	°0.00	°0.00	*°0.00*	0.01	0.01
Receptor Fluid	*0.00*	*0.00*	0.00	*0.00*	0.00	0.00	0.00	0.01	0.00	0.00	0.00	*0.00*	0.00	0.00
Receptor Rinse	*°0.00*	*°0.00*	°0.00	*°0.00*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	0.00	0.00
Total Absorbed	*0.00*	*0.00*	0.00	*0.00*	0.00	0.00	0.00	0.01	0.00	0.00	0.00	*0.00*	0.00	0.00
Dermal Delivery	*0.03*	*0.03*	0.05	*0.00*	0.05	0.08	0.06	0.07	0.14	0.00	0.07	*0.03*	0.07	0.04
Mass Balance	*86.79*	*86.85*	101.58	*89.55*	91.57	87.23	95.09	94.48	96.60	98.02	96.99	*88.86*	95.19	4.33

Cells 1, 3, 5 and 14 rejected from mean and SD due to low mass balance for HC Red No. 10 (<85%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Table 18 **Distribution of HC Red No. 11 (µg/cm²) at 72 h Following Topical Application of Test Preparation 2 to Human Split-Thickness Skin**

	Cell Number and Donor Number													
	Cell 1 0291	*Cell 3* 0271	Cell 4 0296	*Cell 5* 0288	Cell 6 0298	Cell 7 0216	Cell 8 0291	Cell 10 0271	Cell 11 0296	Cell 12 0288	Cell 13 0298	*Cell 14* 0216	Mean	SD
Skin Wash 30 min	*57.44*	*57.54*	67.20	*59.22*	60.06	57.65	63.21	62.37	64.05	64.89	64.37	*58.70*	62.97	2.97
Tissue Swab 30 min	*0.35*	*0.23*	0.28	*0.26*	0.51	0.26	0.09	0.33	0.16	0.34	0.21	*0.28*	0.27	0.13
Pipette Tips 30 min	**0.02*	**0.02*	*0.03	**0.02*	*0.03	*0.06	*0.02	*0.02	*0.02	*0.02	*0.03	**0.02*	0.03	0.01
Dislodgeable Dose 30 min	*57.81*	*57.79*	67.51	*59.50*	60.59	57.97	63.32	62.72	64.23	65.25	64.60	*58.99*	63.27	2.93
Skin Wash 72 h	*0.07*	*0.14*	0.12	*0.10*	0.08	0.14	0.09	0.15	0.09	0.09	0.09	*0.19*	0.11	0.03
Tissue Swab 72 h	**0.02*	**0.01*	*0.03	**0.03*	*0.03	*0.03	*0.02	*0.03	*0.02	*0.03	*0.02	**0.03*	0.03	0.01
Pipette Tips 72 h	*°0.00*	*°0.00*	°0.00	*°0.00*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	0.00	0.00
Cell Wash	*°0.00*	*°0.00*	*0.09	**0.09*	0.39	°0.00	°0.00	*0.11	°0.00	°0.00	°0.00	**0.09*	0.07	0.13
Dislodgeable dose 72 h	*0.09*	*0.15*	0.24	*0.22*	0.50	0.17	0.10	0.29	0.11	0.12	0.11	*0.31*	0.21	0.14
Total Dislodgeable Dose	*57.90*	*57.94*	67.74	*59.72*	61.09	58.14	63.42	63.01	64.34	65.37	64.71	*59.30*	63.48	2.89
Stratum corneum 1-5	**0.02*	**0.03*	*0.04	*0.05*	*0.02	0.05	*0.02	*0.02	*0.04	*0.03	*0.03	**0.02*	0.03	0.01
Stratum corneum 6-10	**0.03*	**0.03*	*0.03	*0.05*	*0.02	*0.02	*0.03	*0.03	0.05	*0.03	*0.03	**0.02*	0.03	0.01
Stratum corneum 11-15	**0.03*	**0.02*	*0.03	**0.02*	*0.02	*0.03	*0.04	*0.04	*0.02	0.06	*0.02	**0.02*	0.03	0.01
Stratum corneum 16-20	**0.02*	**0.02*	°0.00	**0.02*	*0.02	*0.02	*0.02	*0.02	*0.02	*0.04	°0.00	**0.02*	0.02	0.01
Stratum Corneum	*0.10*	*0.09*	0.10	*0.14*	0.08	0.12	0.10	0.10	0.14	0.16	0.08	*0.08*	0.11	0.03
Unexposed Skin	*°0.00*	*°0.00*	*0.03	*°0.00*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	0.00	0.01
Total Unabsorbed	*58.00*	*58.04*	67.87	*59.86*	61.17	58.26	63.52	63.11	64.48	65.53	64.79	*59.38*	63.59	2.90
Epidermis	**0.02*	**0.02*	*0.01	*°0.00*	*0.03	*0.04	*0.03	0.04	0.08	°0.00	0.05	**0.02*	0.03	0.02
Dermis	*°0.00*	*°0.00*	*0.02	*°0.00*	°0.00	*0.01	*0.01	°0.00	*0.01	°0.00	°0.00	*°0.00*	0.01	0.01
Receptor Fluid	*0.00*	*0.00*	0.01	*0.00*	0.01	0.00	0.00	0.01	0.00	0.00	0.00	*0.00*	0.00	0.00
Receptor Rinse	*°0.00*	*°0.00*	°0.00	*°0.00*	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	°0.00	*°0.00*	0.00	0.00
Total Absorbed	*0.00*	*0.00*	0.01	*0.00*	0.01	0.00	0.00	0.01	0.00	0.00	0.00	*0.00*	0.00	0.00
Dermal Delivery	*0.02*	*0.02*	0.04	*0.00*	0.04	0.05	0.04	0.05	0.09	0.00	0.05	*0.02*	0.05	0.02
Mass Balance	*58.02*	*58.06*	67.90	*59.87*	61.21	58.31	63.57	63.16	64.57	65.53	64.84	*59.40*	63.64	2.89

Cells 1, 3, 5 and 14 rejected from mean and SD due to low mass balance for HC Red No. 10 (<85%)
° = Value not quantifiable, no peak detected for analyte.
* = Value less than the lower limit of quantification

Conclusion
Under the described test conditions (oxidative hair dye formulation), a total mean amount of 0.46 µg/cm² HC Red n° 10 and HC Red n° 11 is obtained by summing up the amounts present in receptor fluid and in both separated skin compartments.

Ref.: 6 (subm III)

Comment
The experiment was well performed. The mean +1SD may be used in calculating MOS.

For HC Red n° 10 the amount considered as absorbed is 0.43 µg/cm² and for HC Red n° 11 the amount considered as absorbed is 0.07 µg/cm². Therefore the amount of B71 that may be considered absorbed is 0.50 µg/cm² under oxidative conditions in a hair dye formulation containing a final concentration of 1.0% B71.

Percutaneous Absorption *in vivo*

Guideline: /
Species/strain: rats, Sprague Dawley (Him: OFA (SPF))
Group size: 3 males and 3 females per group
Test substance: ^{14}C labelled Rot Y
Batch: /
Purity: radiochemical purity 97%
Vehicle: Hair dye formulation without peroxide
Hair dye formulation with peroxide
DMSO/water 3:1
Concentration: Hair dye formulation without peroxide I – 1% Rot Y
Hair dye formulation with peroxide II – 0.5% Rot Y
DMSO/water (3:1) – 3.33% Rot Y
Dose: Hair dye formulation without peroxide I – 1.12 mg/cm² Rot Y
Hair dye formulation with peroxide II – 0.56 mg/cm² Rot Y
DMSO/water (3:1) – 1.14 mg/cm² Rot Y
GLP: in compliance
Study period: 1985

The percutaneous absorption of ^{14}C labelled Rot Y in a hair dye formulation without and with hydrogen peroxide and dissolved in DMSO/water was determined in rats 48 hours after topical application.

The preparations were applied to 3 cm x 3 cm areas of the shaved dorsal skin surface and left in place for 30 minutes. Excess preparation was then scraped off, the area washed with shampoo and then rinsed. Urine and faeces were collected each day for 3 days after which the animals were killed and the amounts of Rot Y remaining at the sites of application and in the carcasses determined.

Approximately 60% of the absorbed dose was excreted in the urine and 40% in the faeces.

	Retained in skin % (SD) of application	**Absorption % (SD) of application**	**Absorption µg/cm² (SD)**
Hair dye formulation without peroxide (containing 1% Rot Y)	0.845 (0.375)	0.037 (0.012)	0.41 (0.13)
Hair dye formulation with peroxide (containing 10.5% Rot Y)	1.68 (0.80)	0.061 (0.037)	0.34 (0.21)
DMSO/water 3:1 (6 animals) (containing 3.33% Rot Y)	0.113 (0.043)	0.286 (0.538)	3.27 (6.16)
DMSO/water 3:1 (without outlier)*	0.097 (0.018)	0.066 (0.023)	0.76 (0.26)

* animal #23 produced results considerably different to the others; this was considered to be due to incomplete removal of the test substance from the skin.

Ref.: 20

Comment
This study did not conform to a guideline and batch specifications were not provided. The mean may be considered as representing the amounts absorbed in this *in vivo* rat model, which was, 0.41 µg/cm^2 for a hair dye formulation without peroxide (containing 1% Rot Y) and 0.34 µg/cm² for a hair dye formulation with peroxide (containing 0.5% Rot Y).

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral toxicity

No data submitted

3.3.5.2. Sub-chronic (90 days) toxicity (oral, dermal)

Guideline:	/
Species/strain:	albino Wistar rat (BOR:WISW (SPF/TNO))
Group size:	20 males and 20 females for low and mid- dose group 25 males and 25 females for high dose group 25 males and 25 females for control group
Test substance:	Rot Y
Batch:	V07/600 pf (corresponding to L4/141)
Purity:	see 3.1.4.
Vehicle:	aqua deion.
Dose levels:	0, 20, 40 and 60 mg/kg bw/d
Dose volume:	1 ml/100 g bw
Route:	oral, gavage
GLP:	in compliance
Study period:	1984

The test substance was administered daily by oral gavage to groups of 20 male and female Wistar rats at the doses 20, 40 and 60 mg/kg bw/d for 13 weeks. The control group received distilled water. The recovery groups consisted of additional 5 males and 5 females (control and high dose) and were observed for 4 weeks after cessation of treatment. Mortality was checked twice daily, clinical observations, body weight and food consumption was recorded daily. Ophthalmoscopy and evaluation of auditory function and reflexes were performed prior to first treatment, at week 13 and week 17 with 10 animals per sex and dose group. Haematology and clinical biochemistry was done before treatment, and after 6 and 13 weeks and the end of the recovery period with 5 animals per sex and dose group. After necropsy a number of organs were weighed, fixed and stored. Organs of the control and high dose animals were examined histopathologically.

Results
One animal died due to false application. All substance-dosed animals showed discoloured urine and perigenital fur staining. With the exception of high dose animals exhibiting piloerection post treatment no clinical signs were observed. Ophthalmoscopy, hearing and neurotoxicity tests as well as body weight gain and feed consumption did not reveal compound related effects. No relevant changes in haematological parameters were recorded. Sporadic statistically significantly changed values (prothrombin time, MCHC, MCH, MCV, hematocrit and erythrocytes numbers) were not dose-related and within the normal values of this rat strain. Potassium levels were increased and concomitantly the sodium / potassium ratio decreased in males after 6 weeks at 40 and 60 mg/kg bw/d while this increase was only slight in females. In females of the dose 60 mg/kg bw/d an increase of the absolute and relative liver weight was noted which was not seen in the recovery group.

While the study authors proposed a 'no effect level' of 40 mg/kg bw/d, the applicant deduced a conservative NOAEL of 20 mg/kg bw/d due to the variations in clinical chemistry.
Ref.: 21

Comment of the SCCS
The SCCS agrees with the conservative conclusion of the applicant and sets the NOAEL at 20 mg/kg bw/d.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

Bacterial gene mutation assay

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, and TA1537.
Replicates:	3 replicates in 2 individual experiments both in the presence and absence of S9-mix.
Test substance:	HC Red n° 10 + HC Red n° 11
Solvent:	DMSO
Batch:	9375 (R00058409)
Purity:	HC Red n° 10: 66% (area) or 54.8% (weight) HC Red n° 11: 32.6% (area) or 29.9% (weight)
Concentrations:	Experiment 1: 0, 1, 10, 100, 1000 and 5000 µg/plate without and with S9-mix Experiment 2: 0, 30, 100, 300, 1000 and 3000 µg/plate for TA98, TA102, TA1535 without and with S9-mix 100, 300, 1000, 3000 and 5000 µg/plate for TA100, TA1537 without and with S9-mix
Treatment:	Direct plate incorporation (48 h treatment) method
GLP:	In compliance

HC Red n° 10 + HC Red n° 11 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using the direct plate incorporation method with exposure of 48 h. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was evaluated as growth inhibition on the basis of a reduction in the number of induced revertant colonies. Negative and positive controls were in accordance with the OECD guideline.

Results
Precipitation of the test compound and toxicity was not observed.
In the presence of S9-mix a slight growth inhibiting effect was seen at 5000 µg/plate in strain TA102. Both in the absence and presence of S9-mix clear dose dependent increases in revertant colonies compared to concurrent vehicle controls were seen in TA98 and TA1537 in both experiments whereas slight increases were found in both experiments in TA100 and the first experiment with TA1535. The test compound did not induce an increase in the number of revertant colonies as compared to concurrent vehicle controls in both experiments with TA102 and the second experiment with TA1535.

Conclusion
Under the experimental conditions used the mixture of HC Red n° 10 and HC Red n° 11 was genotoxic (mutagenic) in the gene mutation tests in bacteria both in the absence and the presence of S9 metabolic activation.

Ref. 22

***In Vitro* Mouse Lymphoma assay (*tk* locus)**

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	duplicate cultures in 2 independent experiments
Test substance:	Rubinrot Y WR23532
Solvent:	DMSO

Batch:	9375	
Purity:	HC RED No. 10: 66% (area) or 54.8% (weight)	
	HC RED No. 11: 32.6% (area) or 29.9% (weight)	
Concentrations:	Experiment 1:	43.8, 87.5, 175, 350, 700 and 1400 µg/ml without S9-mix 21.9, 43.8, 87.5, 175, 350 and 700 µg/ml with S9-mix
	Experiment 2:	87.5, 175, 350, 700, 1050 and 1400 µg/ml without S9-mix
Treatment	Experiment 1:	4 h treatment without and with S9-mix; expression period 72 h; selection period of 10-15 days
	Experiment 2:	24 h treatment without S9-mix only; expression period 48 h; selection period of 10-15 days
GLP:	in compliance	

Rubinrot Y WR23532 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring relative suspension growth. In the main test, cells were treated for 4 h (experiment 1) or 24 h (experiment 2) followed by an expression period of 72 h (experiment 1) or 48 h (experiment 2) to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Toxicity was measured in the main experiments as relative suspension growth and/or relative total growth of the treated cultures relative to that of the solvent control cultures. The numbers of colonies were counted manually; colony size distribution was determined in the controls and in all treated concentrations of Rubinrot Y WR23532. Negative and positive controls were in accordance with the OECD guideline.

Results
Measurements on post treatment media indicated that Rubinrot Y WR23532 had no effect on pH values or on osmolarity. Precipitation was observed at 2800 µg/ml in the absence or presence of S9-mix after 4 h exposure and at 1400 µg/ml after 24 h exposure to Rubinrot Y WR23532.
The appropriate level of toxicity (10-20% survival after the highest dose) was reached in experiment 1 with S9-mix and in experiment 2 without S9-mix. In these cultures a biological relevant, dose-dependent increase in the mutant frequency was observed following treatment with Rubinrot Y WR23532. These increases were clearly due to increases in the number of small colonies. In the cultures of experiment 1 without S9-mix the appropriate level of toxicity was not reached pointing to insufficient exposure of the cells which may explain the negative results in these cultures.

Conclusion
Under the experimental conditions used, Rubinrot Y WR23532 was positive in the mouse lymphoma assay at the *tk* locus. The induced increase in small colonies points to a clastogenic potential of Rubinrot Y WR23532.

Ref.: 23

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	/
Species/strain:	NMRI
Group size:	10, random selected within the sexes
Test substance:	Rot Y
Batch:	228-41185
Purity:	97.5%
Dose level:	200, 670 and 2000 mg/kg bw
Route:	oral gavage, once
Vehicle:	DMSO

Sacrifice times: 24 h for all dose levels, vehicle control and positive control, 48 h and 72 h for the highest dose and vehicle control.
GLP: in compliance

Rot Y has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on a dose range finding assay in which clinical (toxic) signs and mortality were recorded. In the main experiment rats were exposed by gavage to single doses of 0, 200, 670 and 2000 mg/kg bw Rot Y. Bone marrow cells were collected 24 h after dosing; bone marrow cells of the highest dose and vehicle controls were also collected 48 h or 72 h after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE ratio). Negative and positive controls were included.

Results
In the dose range finding study 3 out of 4 animals treated with 2500 mg Rot Y/kg bw died within 6 to 24 hours. The highest non-lethal dose was 2000 mg/kg bw. With this dose the toxic reactions incomplete eyelid closure and reduction of the spontaneous activity were observed already a few minutes after treatment demonstrating systemic exposure. Exposure to Rot Y did not result in a clearly decreased PCE/NCE ratio which indicates that it is not cytotoxic to bone marrow cells at the concentrations tested. Rot Y did not induce a biological relevant increase in micronucleated erythrocytes in any of the groups treated.

Conclusion
Under the experimental conditions used Rot Y did not induce micronuclei in bone marrow cells of treated rats and, consequently, it was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of rats.

Ref. 25

Comment
Sufficient exposure of bone marrow cells to Rot Y has not been shown directly by cytotoxicity which would result in altered PCE/NCE ratios. However, the observed toxic reactions demonstrate systemic exposure.

Mouse spot test

Guideline: /
Species/strain: DBA/2J males and NMRI females
Group size: 45 - 113 treated females;
Test substance: Rot Y
Batch: 228-41185
Purity: 97.5%
Dose level: 250, 870 and 2500 mg/kg bw
Route: oral gavage, once
Day of administration: day nine of pregnancy
Vehicle: DMSO
GLP: in compliance

Rot Y has been investigated for the induction of mutations in the mouse spot test. Test concentrations were based on a pre-experiment with doses up to 2500 mg/kg bw. Since at this dose toxic reactions were found but no mortality occurred, 2500 mg/kg bw was chosen as the highest dose. In the main experiment female NMRI mice were exposed by gavage to single doses of 0, 250, 870 and 2500 mg/kg bw Rot Y at day nine of pregnancy. About 6 weeks after the start of the experiment (pairing) the F_1 animals were analysed for the occurrence of spots. Hairs of the spot region of all spot-carrying animals were microscopically analysed for changes in pigmentation pattern. Negative and positive controls were included.

Results
The was no biologically relevant difference in the percentage F_1 mice after three weeks nor in the mean litter size in all treated group included the positive control compared to the untreated controls. Except for the positive controls, there was no biologically relevant increase in the percentage F_1 mice with genetically relevant spots compared to the untreated controls.

Conclusion
Since under the experimental conditions used an increase in somatic gene mutations in pigment precursor cells in treated mice were not found, Rot Y was not mutagenic in this mouse spot test

Ref. 26

3.3.7. Carcinogenicity

Cell transformation assay with Syrian hamster embryo (SHE) cells

Guideline:	/
Species/strain:	Primary Syrian Hamster Embryo (SHE) cells
Test substance:	HC Red n° 10 + HC Red n° 11
Batch:	228-41185
Purity:	HC Red n° 10: 59.2 area% HC Red n° 11: 38.7 area% (HPLC at 254 nm)
Concentrations:	10.0, 50.0, 250.0 and 500.0 µg/ml in the absence of S9-mix 10.0, 100.0, 500.0 and 1000.0 µg/ml in the presence of S9-mix
Replicates:	10 independent feeder cultures per concentration (in total 1000 colonies evaluated)
Incubation:	6 hours in the presence of S9-mix 6 and 48 hours in the absence of S9-mix
GLP:	in compliance

HC Red n° 10 + HC Red n° 11 dissolved in DMSO (1% final concentration in the medium) was assessed for its potential to transform Syrian hamster embryo (SHE) cells in primary/secondary cultures.

SHE cells (primary culture) were prepared from 12-14 day old embryos of Syrian golden hamster strain HAN:AURA. Embryos were removed from the uteri, decapitated, the tissue minced and twice trypsinised for 10 minutes. Foetal calf serum (FCS) was then added, the obtained cell suspension centrifuged and the supernatant discarded. The pellet was suspended in medium containing 7.5% DMSO and stored at – 196 °C as primary stock culture.

Cell suspensions to be used in the assay were taken from the stock culture after washing with medium to reduce DMSO and re-suspended in the nutrient medium containing 10% FCS. Feeder cells were cultivated for about 2-3 days before irradiation with 5000 rad x-rays was performed in PBS buffer. About 40000 to 60000 of those cells were cultivated in medium containing 10% FCS. 24 h later 500 target cells each were added to 10 feeder cultures per test concentration and treatment conditions. The treatment with the test substance and the concurrent controls (in the presence and absence of S9-mix) was started 24 hours later.

Incubation conditions in the tests without S9-mix were 6 and 48 hours, for the tests in the presence of S9-mix, 6 hours. After those incubation times, the medium was changed to remove test items and S9-mix. In total 7 to 9 days after seeding of the target cells the colonies were counted and scored for cell transformation after fixation and Giemsa staining.

In total 1000 colonies (100 colonies from 10 parallel flasks) are analysed per concentration and test condition (with and without S9-mix)

In a pre-test on toxicity (determined as plating efficiency) concentrations from 5 to 1000 µg/ml were analysed. Higher concentrations precipitated in the medium and were therefore not suitable. In this pre-test, marked toxicity was noted starting at 250 µg/ml and being severe at 1000 µg/ml (plating efficiency 15.8%) in the absence of S9-mix, whereas no toxicity was noted in the presence of S9-mix up to the highest soluble concentration of 1000 µg/ml. Based on those findings, SHE cells were treated with concentrations of the test substance of 10.0, 100.0, 500.0 and 1000.0 µg/ml in the presence of S9-mix for 6 hours and with concentrations of the test substance of 10.0, 100.0, 250.0 and 500.0 µg /ml in the absence of S9-mix for 6 and 48 hours. Untreated cells, solvent control (1% DMSO) and positive controls (in the presence of S9-mix: Benzo(a)pyrene, 5 µg/ml, in the absence of S9-mix: N-methyl-N'-nitro-N-nitrosoguanidine, 0.5 µg/ml) were investigated in parallel.

In the main experiment, toxicity evident as reduced relative survival of cells was noted in the presence and to a more pronounced extent in the absence of S9-mix for the highest test concentrations evaluated, demonstrating the interaction of the test substance with the SHE cells. At 1000 µg/ml in the presence of S9-mix, the relative survival was reduced by about 34 %. In the absence of S9-mix the figures at 500 µg/ml after 6 and 48 hours incubation were 48 and 50 %, respectively. None of the groups treated with HC Red n° 10 + HC Red n° 11 showed a biologically relevant rate of transformed colonies in 1000 colonies randomly scored. Only one transformed colony was found with the lowest test concentration investigated at 6 hours in the absence of S9-mix. As this finding was neither reproducible nor dose dependent, it is evaluated as of no toxicological relevance. In addition, the finding fits well into the range of historical control data, indicating a spontaneous rate for transformed colonies of 0.05 to 0.1%. The sensitivity and validity of the test system was demonstrated by the induction of a significant number of transformed colonies in the concurrent positive control groups: 1.9% after 6 h treatment with benzo(a)pyrene in the presence of S9-mix, 1.4% and 1.5% after treatment for 6 h and 48 h, respectively, with N-methyl-N'-nitro-N-nitrosoguanidine in the absence of S9-mix.

The study authors concluded that under the experimental conditions described the test substance HC Red n° 10 + HC Red n° 11 did not induce cell transformation in the SHE cell system, even if tested at toxic concentrations.

Ref.: 24

Comment
The submitted SHE cell transformation test was carried out using some unusual conditions. It is normally not performed in the presence of S9-mix as the cells are primary cells and contain most enzyme systems required for activation of carcinogens. Benzo(a)pyrene is normally used as positive control (in the absence of S9-mix). Usually, the test chemicals are present for the complete or most of the test period. Incubation for only 6 or 48 hours are short and there is little experience of to what extent the test responds to carcinogens under the conditions used. Thus it is not possible to draw any conclusions from the experiment.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline: OECD 414 (2001)
Species/strain: rat, HanBrl: WIST (SPF quality)

Group size:	4 groups of 22 mated female rats
Test substance:	HC Red n° 10 + HC Red n° 11, WR 23532
Batch:	9375 (Fa. OSL)
Purity:	HC Red n° 10: 54.8 weight% (HPLC) HC Red n° 11: 29.9 weight% (HPLC) Rot C: 0.22 weight% (HPLC) (see point 3.1.4.)
Vehicle:	bi-distilled water containing 0.5% CMC (carboxymethylcellulose)
Dose levels:	0, 30, 100 and 300 mg/kg bw/d
Dose volume:	10 ml/kg bw
Route:	oral, gavage
Administration:	once daily from day 6 through to day 20 *post coitum*
GLP statement:	in compliance
Study period:	20 April – 27 September 2004

The test substance was suspended in bi-distilled water containing 0.5% CMC and administered once daily to groups of 22 pregnant rats at doses of 30, 100 and 300 mg/kg bw/d from day 6 to 20 of gestation. The controls received the vehicle. The animals were checked twice daily for mortality, clinical signs and body weight. Food consumption was recorded on 3-day intervals. On day 21 p.c., the dams were sacrificed, the foetuses taken and the common sectio parameters were recorded. Each half of the foetuses was examined for visceral and skeletal abnormalities.

Results
With the exception of discolouration of the fur and tail and urine in all substance groups no clinical signs were observed. However, in the group 300 mg/kg bw/d food consumption (slightly, day 6-12) and body weight gain were reduced. The sectio data on reproduction and the foetal data were not changed. Evaluation of visceral and skeletal abnormalities revealed no treatment-related findings. The NOAEL for maternal effects was 100 mg/kg bw/d and for embryotoxicity/teratogenicity 300 mg/kg bw/d.

Ref.: 27

3.3.9. Toxicokinetics

Bioavailability across the intestinal barrier

Guideline:	/
Cells:	Human intestinal epithelial cell line TC-7, a sub-clone of the Caco-2 cell line
Test substance:	HC Red n° 10+ HC Red n° 11, WR 23532
Batch:	9375 (Fa. OSL)
Purity:	HC Red n° 10: 54.8 weight% (HPLC) HC Red n° 11: 29.9 weight% (HPLC) Rot C: 0.22 weight% (HPLC)
Concentration:	50 µM in HBSS buffer containing 1 % DMSO
Incubation time	60 min
Number of experiments:	two independent experiments
GLP:	Not in compliance but QAU checked
Study period:	1 – 6 April 2004

The bioavailability of HC Red n° 10 and HC Red n° 11 across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro*. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37° C in 96-well Multiscreen plates with shaking for a 60 min contact time. Analysis of the donor (apical) and receiver (basolateral) samples was done by means of HPLC-MS/MS, and the apparent permeability coefficient (P_{app}) was calculated for two independent experiments. ^{14}C-mannitol (4 µM) was used to demonstrate the integrity of the cell monolayer. Only monolayers with a

mannitol permeability of < 2.5 x 10^{-6} cm/sec were used. Propranolol and ranitidine were used to validate the experimental conditions.
According to the laboratory's classification system, a low permeability is considered for test items revealing a P_{app} < 2 x 10^{-6} cm/sec. A P_{app} of 2 - 20 x 10^{-6} cm/sec and a $P_{app} \geq 20 \times 10^{-6}$ cm/sec classify a substance to have a medium or a high permeability, respectively. Ranitidine, which has a 50 % absorption in humans, was used as low permeability reference compound, as recommended by FDA.

Results
The mean recovery of the reference compounds were 72% (propanolol) and 91% (ranitidine), for HC Red n° 10 and HC Red n° 11 the figures were 81% and 98%.
The permeability figures for the reference substances propranolol (P_{app} = 25.9 x 10^{-6} cm/sec), a high permeability reference compound with about 100 % absorption in humans, and ranitidine (P_{app} = 0.20 x 10^{-6} cm/sec) revealing an absorption of about 50 % in humans, were well within the typical range of 20 – 60 x 10^{-6} cm/sec and < 2 x 10^{-6} cm/sec, respectively.
HC Red n° 10 and HC Red n° 11 revealed P_{app} of 55.5 and 0.30 x 10^{-6} cm/sec, respectively. Thus HC Red n° 10 was classified to be of high permeability, indicating a nearly 100 % absorption from the gastro-intestinal tract whereas HC Red n° 11 was classified to be of low permeability. As the absorption from the gastro-intestinal tract is likely to be permeability limited, the high permeability observed with HC Red n° 10 in this assay indicates a good absorption after oral administration whereas the oral bioavailability of HC Red n° 11 may be limited.

Comment
The method used in this study, is a non validated method.

Ref.: 28

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

For the calculation of the Margin of Safety, it was taken into consideration that the dye is a mixture of mainly two components. In the calculation, it is assumed that the toxicity is triggered either by HC Red n° 10 (content 51.8%) or HC Red n° 11 (content 35.4%) alone. By doing this, the NOAEL of the subchronic toxicity study (20 mg/kg bw/d) is adjusted to 10.4 mg/kg bw/d for HC Red n° 10 and 7.1 mg/kg bw/d for HC Red n° 11. Furthermore, the different oral bioavailabilities found in the *in vitro* study were taken into account. To account for this, the NOAEL figure for HC RED n° 11 was further adjusted to 0.7 mg/kg bw/d assuming a 10% oral absorption rate.

CALCULATION OF THE MARGIN OF SAFETY

HC Red n° 10

Non oxidative conditions

Absorption through the skin	**A**	**=**	**0.59 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.34mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.006 mg/kg bw/d**
No Observed Adverse Effect Level (90 d, oral, rat)	**NOAEL**	**=**	**10.4 mg/kg bw/d**
MOS		**=**	**1820**

HC Red n° 10

Oxidative conditions

Absorption through the skin	**A**	**=**	**0.43 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.25 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.004 mg/kg bw/d**
No Observed Adverse Effect Level (90 d, oral, rat)	**NOAEL**	**=**	**10.4 mg/kg bw/d**
MOS		**=**	**2500**

HC Red n° 11

Non oxidative conditions

Absorption through the skin	**A**	**=**	**0.08 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.05 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.0008 mg/kg bw/d**
No Observed Adverse Effect Level (90 d, oral, rat)	**NOAEL**	**=**	**7.1 mg/kg bw/d**
Adjusted for 10% bio-availability		**=**	**0.7**
MOS		**=**	**900**

HC Red n° 11

Oxidative conditions

Absorption through the skin	**A**	**=**	**0.07 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.04 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.0006 mg/kg bw/d**
No Observed Adverse Effect Level (90 d, oral, rat)	**NOAEL**	**=**	**7.1 mg/kg bw/d**
Adjusted for 10% bio-availability		**=**	**0.7**
MOS		**=**	**1030**

3.3.14. Discussion

Physico-chemical properties
HC Red n° 10 + HC Red n° 11 is used as a direct dye in semipermanent hair dye formulations at a maximum on-head concentration of 2%. HC Red n° 10 + HC Red n° 11 is used in oxidative hair dye formulations at a maximum on-head concentration of 1%.

Six impurities identified and quantified in HC Red n° 10 and HC Red n° 11 are derivatives of aromatic amines and they contain primary/secondary and tertiary amino groups. Such compounds are well known hazardous substances. Five of these impurities can form nitrosamines, which may be carcinogenic.
An additional impurity has been characterised as 'starting material', but its identity was not reported. Its content has also not been reported.
HC Red n° 10 and HC Red n° 11 are secondary amines and therefore prone to nitrosation. The nitrosamine content in the hair dye HC Red n° 10 and HC Red n° 11 is not reported. The substance should not be used in combination with nitrosating agents. The nitrosamine content should be less than 50 ppb.
The stability of the mixture of HC Red n° 10 and HC Red n° 11 in typical hair dye formulations is not reported.

Irritation, sensitisation
HC Red n° 10 + HC Red n° 11 was not irritating to rabbit skin but irritating when applied undiluted to rabbit eyes. It was not a skin sensitiser when tested up to 10% in DMSO or 9.5% in acetone:water:olive oil. However a sensitising potential cannot be excluded.

Dermal absorption
Non-oxidative conditions
For HC Red n° 10 the amount considered as absorbed is 0.59 µg/cm² and for HC Red n° 11 the amount considered as absorbed is 0.08 µg/cm². Therefore the total amount of B71 that may be considered absorbed is 0.67 µg/cm² under non-oxidative conditions in a hair dye formulation containing 2.0% B71.

Oxidative conditions
For HC Red n° 10 the amount considered as absorbed is 0.43 µg/cm² and for HC Red n° 11 the amount considered as absorbed is 0.07 µg/cm². Therefore the total amount of B71 that

may be considered absorbed is 0.50 µg/cm² under oxidative conditions in a hair dye formulation containing a final concentration of 1.0% B71.

General toxicity
From studies on the acute oral toxicity in rats and mice LD_{50}-values of 1830 to 2200 mg/kg bw were calculated.
From a sub-chronic (90 days) oral toxicity study in rats a conservative NOAEL of 20 mg/kg bw/d due to the variations in clinical chemistry was deduced.
In a developmental toxicity study in rats the NOAEL for maternal effects was 100 mg/kg bw/d and for embryotoxicity/teratogenicity 300 mg/kg bw/d.
No data on reproductive toxicity were submitted.

Toxicokinetics in vitro
The bioavailability of HC Red n° 10 and HC Red n° 11 across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro*. HC Red n° 10 was classified to be of high permeability, indicating a nearly 100 % absorption from the gastro-intestinal tract whereas HC Red n° 11 was classified to be of low permeability.
It has to be pointed out that the bioavailability is measured by a non validated method

Margin of Safety calculation
For the calculation of the Margin of Safety, it was taken into consideration that the dye is a mixture of mainly two components. In the calculation, it is assumed that the toxicity is triggered either by HC Red n° 10 (content 51.8%) or HC Red n° 11 (content 35.4%) alone. By doing this, the NOAEL of the subchronic toxicity study (20 mg/kg bw/d) is adjusted to 10.4 mg/kg bw/d for HC Red n° 10 and 7.1 mg/kg bw/d for HC Red n° 11. Furthermore, the different oral bioavailabilities found in the study *in vitro* were taken into account. To account for this, the NOAEL figure for HC RED n° 11 was further adjusted to 0.7 mg/kg bw/d assuming a 10% oral absorption rate.
The MOS values obtained were between 900 and 2500.

Mutagenicity
Overall, the genotoxicity of HC Red n° 10 + HC Red n° 11 is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy.
HC Red n° 10 + HC Red n° 11 induced mutations both in a bacterial gene mutation test as well as in a gene mutation test in mammalian cells. However, in the mammalian gene mutation test the increase in mutated colonies was due to an increase in small colonies which is indicative for a clastogenic effect of the test compound.
These positive *in vitro* results could not be confirmed in *in vivo* tests. An *in vivo* micronucleus tests in bone marrow cells of mice did not show an increase in micronucleated bone marrow cells. A mouse spot test was also negative. Based on the data available, HC Red n° 10 + HC Red n° 11 can be considered to have no *in vivo* genotoxic potential and additional test are unnecessary.

Carcinogenicity
The submitted SHE cell transformation test, which showed negative results, was carried out using unusual conditions. is is not normally performed in the presence of S9-mix as the cells used are primary cells and themselves contain most enzyme systems required for activation of carcinogens. Benzo(a)pyrene is normally used as positive control (in the absence of S9-mix). In addition, the test chemicals usually are present for the complete or most of the test period. Incubation for only 6 or 48 hours is short and there is little experience of to what extent the test responds to carcinogens under the conditions used. Thus it is not possible to draw any conclusions from the experiment.

4. CONCLUSION

The evaluation relates to a mixture of HC Red n° 10 (53 – 57%) and HC Red n° 11 (32 – 40%).

Based on the data provided, the SCCS is of the opinion that the use of HC Red n° 10 + HC Red n° 11 with a maximum on-head concentration of 1.0% in oxidative and 2.0% in non-oxidative hair dye formulations does not pose a risk to the health of the consumer.

A sensitising potential of HC Red n° 10 + HC Red n° 11 cannot be excluded.

HC Red n° 10 and HC Red n° 11 are secondary amines and therefore prone to nitrosation. In addition, 5 of the impurities also contain secondary and tertiary amino-groups. The nitrosamine content in the hair dye HC Red n° 10 and HC Red n° 11 is not reported. These substances should not be used in combination with nitrosating agents. The nitrosamine content should be less than 50 ppb.

5. MINORITY OPINION

Not applicable

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