Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Red n° 3

COLIPA n° B50

The SCCS adopted this opinion at its 16th plenary meeting

of 18 September 2012

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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Explanation after commenting period:

This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged. Revised opinions carry the date of revision.

TABLE OF CONTENTS

1. BACKGROUND

Submission I for HC Red n° 3 chemical name 2-[(4-Amino-2-nitrophenyl) amino]ethanol has been submitted in March 2003 by COLIPA[1].

Submission II for HC Red n° 3 was submitted by COLIPA in July 2005.

The Scientific Committee on Consumer Safety expressed its opinion (SCCS/1293/10) with the following conclusions:

Based on the data provided, the SCCS is of the opinion that the use of HC Red n° 3 as a non-oxidative hair dye with a maximum on-head concentration of 3.0% does not pose a risk to the health of the consumer, apart from its sensitising potential.

HC Red n° 3 is an extreme contact sensitiser in the GPMT and strong sensitiser in the LLNA. HC Red n° 3 is a secondary amine, and thus prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

Industry now also requests the possibility to use this ingredient in oxidative colouring products.

The information, including data on dermal penetration and stability under oxidative use conditions, are subject of the submission III.

2. TERMS OF REFERENCE

1. *Does the SCCS consider HC Red n° 3 safe for use as oxidative hair dye with a concentration on-head of maximum 0.45% taking into account the scientific data provided?*

2. *And/or does the SCCS recommend any further restrictions with regard to the use of HC Red n° 3 in oxidative hair dye formulations?*

3. *Does the SCCS consider the conclusion from the previous opinion (SCCS/1293/10) still valid?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Red n° 3 (INCI)

3.1.1.2. Chemical names

Ethanol, 2-((4-amino-2-nitrophenyl)amino)- (CAS)
1N-(2-Hydroxyethyl)-2-nitro-p-phenylenediamine
1-(ß-hydroxyethyl)-amino-2-nitro-4-aminobenzene
4-(2-Hydroxyethyl)amino-3-nitroaniline

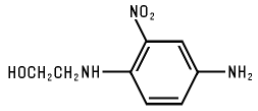
3.1.1.3. Trade names and abbreviations

COLIPA n° B50

3.1.1.4. CAS / EC number

CAS: 2871-01-4
EC: 220-701-7

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_8H_{11}N_3O_3$

3.1.2. Physical form

Submission 1: fine dark red crystals
Submission 2: dark green-brown fine powder

3.1.3. Molecular weight

Molecular weight: 197.2 g/mol

3.1.4. Purity, composition and substance codes

HC Red n° 3 (GTS 03959) Lot 35939

Chemical identification was performed by NMR- and FTIR spectroscopy and elemental analysis

Purity: 97.6 ± 2.2% (w/w), by HPLC using a reference standard of 99.1% HPLC purity
Moisture: 0.403%
Ignition residue: <0.001%

Impurities:

1,4-diamino-2-nitrobenzene:	177 ± 6.7 ppm
6-aminoquinoxaline:	470 ± 14.7 ppm
4-fluoro-3-nitroaniline:	<20 ppm
4-(hydroxyethyl)amino-4'-amino-2',3'-dinitrodiphenylamine:	0.66 ± 0.07% (HPLC peak area)
4-(hydroxyethyl)amino-3-nitrophenyl-formamide:	0.53 ± 0.06% (HPLC peak area)
N-Nitrosodiethanolamine (NDELA):	<20 ppb

Ref.: 1a

HC Red n° 3, Lot 5890377

Analytical characterisation by elemental analysis and NMR and IR spectroscopy
UV spectra shows λmax at 245nm, 298 nm and 506 nm
Purity as amino function titrated using perchloric acid: 98.1 ± 0.7%
Water content (Karl Fischer analysis): 0.22 ± 0.03%

Impurities by HPLC (peak area %, detection at 254 nm):
2 impurities, each 1.2%
5 trace impurities

Impurities by TLC:
1 major and 3 minor/trace impurities

HC Red n° 3, Lot C080480
Analytical characterisation by elemental analysis and NMR and IR spectroscopy
UV spectra shows λmax at 245nm, 298 nm and 506 nm
Purity as amino function titrated using perchloric acid: 97.0 ± 0.5%
Water content (Karl Fischer analysis): 0.21 ± 0.02%

Impurities by HPLC (peak area %, detection at 254 nm):
3 impurities (2 impurities, combined 3.2%; 1 impurity 0.2%)
Impurities by TLC:
1 major and 4 minor/trace impurities

Ref.: 17

3.1.5. Impurities / accompanying contaminants

See 3.1.4.

3.1.6. Solubility

Solubility after sonication for 15 min

Water: 1.15-2.50 mg/ml
(The same solubility range was determined after stirring for 24 hours)
Ethanol: 10.7-16.1 mg/ml
DMSO: 216-324 mg/ml

Comment:
The water solubility is not determined by EC method A.6.

3.1.7. Partition coefficient (Log P_{ow})

Log Po/w: 0.29 (Submission 1)
Log Po/w: 0.426 ± 0.530 (calculated) (Submission 2)

Comment
In submission I, Log Po/w is described without the description of the methodology used. Log Po/w is not determined by EC method A.8

3.1.8. Additional physical and chemical specifications

Melting point:	121.8 – 124.4 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm):	/

3.1.9. Homogeneity and Stability

HC Red n° 3 was stable protected from light at room temperature for 65 days.

The solutions of HC Red n° 3 (1 mg/mL and 200 mg/mL) in aqueous PEG, stored refrigerated, were stable (variation up to 6%) up to 15 days.

The aqueous solutions of HC Red n° 3 (0.05 mg/mL and 50 mg/mL), stored frozen, were stable (variation up to 6%) up to 7 days.

Ref.: 1a, 1b

HC Red n° 3 was shown to be stable up to 30 min (study period) in a hair dye formulation after mixing with peroxide based developer (1:1). The maximum deviation from the initial concentration was ≤4% (w/w).

Ref.: Sub III, ref. 2

General Comments to physico-chemical characterisation

- Chemical characterisation as well as purity and impurities of 3 batches of HC Red n° 3 were provided, while many other batches were used in the submitted dossier. In addition, test materials are not identified in some cases.
- The Log Pow strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log Pow, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.
- The water solubility of HC Red n° 3 was not determined by EC method A.6.
- HC Red n° 3 is a secondary amine, and thus it is prone to nitrosation. It should not be used together with nitrosating agents. The nitrosamine content should be <50 ppb.

3.2. Function and uses

HC Red n° 3 is used in semi-permanent hair dye formulations at a maximum concentration of 3.0% and in oxidative hair colouring formulations at on-head concentrations of up to 0.45%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCS/1293/10

Guideline:	/
Species/strain:	rat, BOR: WISW, SPF TNO
Group size:	40 (5 males and 5 females per doses)
Test substance:	HC Red n° 3
Batch:	not specified
Purity:	not specified
Vehicle:	25% suspension in 0.5% carboxymethylcellulose
Dose:	1000, 2500, 3500 and 5000 mg/kg bw
Route:	oral gavage
GLP statement:	not specified
Study period:	August 1983

Male and females Wistar rats of the BOR: WISW strain, in the weight range 160 to 199 g (male) and 140-174 g (female) were administered HC Red n° 3 at the doses of 1, 2.5, 3.5 and 5 g/kg bw by oral intubation (5 males and 5 females per dose). During the observation period of 14 days, a record was kept for mortalities and signs of toxicity. Body weights were recorded on day 0 and day 14 for the surviving animals. All rats that died were investigated macroscopically to identify organ changes in the skull, thorax and abdomen and surviving animals were similarly examined at the end of the 14-day post-observation.

Results
At the dose of 5 g/kg bw, 9 of 10 rats died within 24h. At the dose of 3.5 g/kg bw, 4 of 10 rats died within 24h and a total of 6 after 48h. 1 of 10 rats died within 24h at the dose of 2.5 g/kg bw. No mortalities were observed at 1g/kg bw.
Red-blue colorations of mucosae and urine were observed in all rats. At the tested doses, reduced activity was observed during the first 30 minutes and continued in the surviving rats up to 72h. After that and during the rest of the observation period, these animals had normal appearance.

No effect on the body weight was recorded in the surviving rats of all groups.

The results of the test indicated that the lethal oral dose (LD5O) was 3940 mg/kg bw in males and 2950 mg/kg bw in females.

Ref.: 2

Guideline:	not specified
Species/strain:	rat, Sprague Dawley
Group size:	25 (2 groups of 5 males and 3 groups of 5 females)
Test substance:	HC Red n° 3

Batch:	not specified
Purity:	not specified
Vehicle:	10% suspension in 3% aqueous acacia
Dose:	1250, and 5000 mg/kg bw in males and 1250, 2500 and 5000 mg/kg bw in females
Route:	oral gavage
GLP statement:	not specified
Study period:	14 January – 5 February 1987

Male and females Sprague-Dawley Rats, in the weight range 190 to 240 g were treated *via* oral gavage with HC Red n° 3 in a 10 % suspension in 3% acacia in water at the doses of 1250 and 5000 mg/kg bw for male rats and 1250, 2500 and 5000 mg/kg bw for female rats (5 rats per dose). Animals were observed during 14 days after treatment.

Results
In male rats, no death was observed at the dose of 1250 mg/kg bw and 5/5 deaths at the dose of 5000 mg/kg bw. In female rats, no death was observed at the dose of 1250 mg/kg bw, 1/5 death at the dose of 2500 mg/kg bw and 5/5 deaths at the dose of 5000 mg/kg bw. The results of this study indicated that the median lethal oral dose (LD5O), was in the region of 1250 to 5000 mg/kg bw in the male rats and in the region of 2500 and 5000 mg/kg bw in females. Signs of reaction to treatment were not recorded.

Ref.: 3

Comment
The experiments did not conform to a guideline and the experimental descriptions are incomplete.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Taken from SCCS/1293/10

Guideline:	/
Species/strain:	New Zealand white rabbits
Group size:	6 total (4 males, 2 females)
Test substance:	HC Red #3
Batch:	/
Purity:	/
Vehicle:	
Dose volume:	500 mg applied as aqueous slurry
GLP:	/
Study period:	1987

500 mg applied as an aqueous slurry of HC Red n° 3 was applied without occlusion to 1 square inch of shaved intact skin (contact time not specified in report).
Reading times: 24 and 72 hours post application.
No evidence of oedema or erythema was observed at the scoring intervals.

Conclusion
HC Red #3 was non-irritating under these test conditions.

Ref.: 6

Guideline:	/
Species/strain:	New Zealand white rabbits
Group size:	6 total (sex not specified in report)
Test substance:	HC Red #3
Batch:	Geb.3
Purity:	/
Vehicle:	water
Dose volume:	0.5 ml as 3% solution
GLP:	/
Study period:	1983

0.5 ml applied as 3% solution in distilled water was applied for 4 hours to shaved intact skin (right side) and to scarified skin (left side) with occlusion (size of test site not specified in report).
Reading times: 30, 60 minutes and 24, 48 and 72 hours post application.
Main findings: Slight erythema of the scarified and intact skin 30 minutes after application.

Conclusion
The test authors considered HC Red #3 non-irritating under these test conditions. Skin alterations were not observed after 24 hours.

Ref.: 7

Guideline:	/
Species/strain:	New Zealand white rabbit
Group size:	6 total (2 males, 4 females)
Test substance:	RM148 (not identified as HC Red n° 3 in the study)
Batch:	/
Purity:	/
Vehicle:	
Dose volume:	0.5 ml of 3% RM148 in 'Schultz vehicle'
GLP:	/
Study period:	1983

0.5 ml of 3% RM148 in 'Schultz vehicle' was applied every 24 hours for 3 consecutive days to a single test site of 1 square inch of shaved intact skin without occlusion.
Reading times: 24 hours after each application, each test site was rinsed and scored for irritation and the test material reapplied. Irritation was scored at 24, 48 and 72 hours following the first application.
Main findings: No evidence of oedema or erythema at the scoring intervals.

Conclusion
HC Red #3 was non-irritating under these test conditions.

Ref.: 8

Comment skin irritation
None of the experiments followed a guideline and the specifications of the test substance were not described. Curiously, the 3% aqueous solution caused some irritation in one experiment while the slurry caused no irritation to rabbit skin. A 3% preparation in 'Schultz Vehicle' caused no irritation.

3.3.2.2. Mucous membrane irritation

Taken from SCCS/1293/10

Guideline: /
Species/strain: New Zealand white rabbits
Group size: 4 total (sex not specified in report)
Test substance: HC Red 3
Batch: /
Purity: /
Vehicle: neat
Dose level: /
Dosing volume: 100 mg
GLP: /
Study period: 1987

100 mg of undiluted material were instilled into the left eye per animal. The eyes of two animals were rinsed with 20 ml distilled water 20 seconds post instillation.
Negative control: Untreated right eye.
Reading times: 1 hour, 1, 2 and 3 days post instillation.
Main findings: Conjunctival redness, swelling, and discharge in treated, rinsed eyes after 1 hour. Similar irritation, with the addition of low grade corneal opacity with ulceration in one eye, occurred in the treated non-rinsed eyes.
Recovery: After one day, treated, rinsed eyes displayed slight redness which resolved within one or two days. Treated, non-rinsed eyes appeared normal by the third day.

Ref.: 4

Guideline: /
Species/strain: New Zealand white rabbits
Group size: 9 total (sex not specified in report)
Test substance: HC Red #3
Batch: /
Purity: /
Vehicle: water
Dose level: 3%
Dosing volume: 0.1 ml
GLP: /
Study period: 1983

0.1 ml of a distilled water solution of 3% test substance was instilled into left eye per animal. The eyes of three animals were washed with 20 ml distilled water 4 seconds post instillation and the eyes of three animals were washed with 20 ml distilled water 30 seconds post instillation. The eyes of the remaining three animals were not washed. Negative control: Untreated right eye.
Reading times: 1, 2, 8, 24 hours and 1, 2, 3, 4, 5, 6 and 7 days post instillation.
Main findings: Slight redness of the conjunctiva was observed in unwashed eyes with no corneal or iridial involvement. No ocular irritation appeared in eyes washed out 4 or 30 seconds after administration throughout the observation period. Unwashed eyes appeared normal 8 hours post administration

Ref.: 5

Comment Mucous Membrane Irritation
Neither of the experiments followed a guideline and the specifications of the test substance were not described. Both the neat and a 3% aqueous solution caused irritation to rabbit eyes.

3.3.3. Skin sensitisation

Taken from SCCS/1293/10

Guinea pig sensitisation – Kligman-Magnusson Maximisation test

Guideline:	/
Species/strain:	Hartley albino guinea pigs
Group size:	10 total (all female)
Test substance:	RM148 (not identified as HC Red n° 3 in the study)
Batch:	5300378
Purity:	/
Induction:	site 1: adjuvant only site 2: 0.1% (w/v) RM148 in propylene glycol site 3: 0.1% (w/v) RM148 in propylene glycol with Complete Freund's adjuvant (1:1)
Challenge:	Topical application of 5% RM148in propylene glycol and 25% HC Red #3 in propylene glycol to a naive 2 x 2 cm2 skin site
Positive control:	/
GLP:	/
Study period:	1979

Induction
Intradermal injection: 2 rows of 3 injections (each 0.05 ml):
site 1: adjuvant only
site 2: 0.1% (w/v) RM148 in propylene glycol
site 3: 0.1% (w/v) RM148 in propylene glycol with Complete Freund's adjuvant (1:1)

Topical patch
One week after the injections, a topical occlusive patch with 25% (w/v) RM148 in propylene glycol was applied for 48 hours to a 4 x 2 cm² area over the injection site that had been pretreated 24 hours earlier with 10% sodium lauryl sulfate in petrolatum.

Interval between induction and challenge application: Two weeks

Challenge
Topical application of 5% RM148 in propylene glycol and 25% RM148 in propylene glycol to a naive 2 x 2 cm2 skin site. Applied under occlusive patch for 24 hours.

Reading times: 24, 48 and 72 hours post patch removal

Main findings
Redness was noted during the range finding study, likely due to staining. To avoid confounding by staining, animals were challenged with a 5% as well as a 25% dose.
Intradermal injection with adjuvant caused dermal irritation. Evidence of contact sensitisation was observed in 9/10 animals at 5% levels and in 10/10 animals at 25% levels with erythema and oedema being observed at the challenge sites.

Sensitisation rate:
5% challenge concentration – 90% sensitisation (9/10 animals);
25% challenge concentration – 100% sensitisation (10/10 animals).

Ref.: 9

Comment
Under the condition of this experiment, the test substance was an extreme skin sensitiser.

Guinea pig sensitisation – (Buehler test)

Guideline:	/
Species/strain:	Pirbright albino guinea pigs
Group size:	30 total (all female) – 20 test material, 10 negative controls
Test substance:	HC Red #3
Batch:	Geb.3
Purity:	/
Induction:	0.5 ml of 3% HC Red #3 in water once weekly for 3 weeks.
Challenge:	0.5 ml of 3% HC Red #3 in water
Vehicle:	water
Concentration:	3%
Positive control:	/
GLP:	/
Study period:	1983

Induction
0.5 ml of 3% HC Red #3 in distilled water for was applied for 6 hours, once a week for three weeks (size of test site not specified in report) under occlusive patch.

Interval between induction and challenge application: 14 days.

Challenge route
Single topical application under occlusive patch.

Challenge
0.5 ml of 3% HC Red #3 in distilled water was applied to a naive skin site (size of test site not specified in report).

Reading times
24 and 48 hours post patch removal

Results
3% concentration of HC Red n° 3 in aqueous vehicle was non-irritating throughout the study. Following challenge, no evidence of sensitisation was observed in any control or test group animals.

Conclusion
3% challenge concentration – No allergic contact sensitisation (0/10 animals).

Ref.: 10

Comment
A 3% solution of the test substance was not a skin sensitiser under the condition of this experiment.

Local Lymph Node Assay (LLNA)

Guideline:	/
Species/strain:	CBA/CaJ mice
Group size:	45 total (including vehicle control and positive control) - 5 per group all female
Test substance:	TM#2045 (not identified as HC Red n° 3 in the study)
Batch:	/
Purity:	/
Vehicle:	dimethylsulfoxide (DMSO)
Concentration:	0, 0.25, 0.5, 1.0 and 2.0% (w/v)

Positive control: p-phenylenediamine
GLP: /
Study period: March 1999

Concentrations of 0, 0.25, 0.5, 1.0 and 2.0% (w/v) TM#2045 in dimethylsulfoxide (DMSO) were applied to the dorsal surfaces of both ears (25 µl per ear) once per day, on 3 consecutive days.
On day 5, the mice were injected intravenously with 250 µl of 3H-thymidine (20 µCuries). Five hours later, mice were killed and the draining auricular lymph nodes removed. A cell suspension was prepared from the lymph nodes of each animal. After two phosphate buffered saline washes, the cells were precipitated in 1 ml of 5% trichloroacetic acid (TCA) in distilled water at 2-8 °C overnight. Samples were centrifuged and re-suspended in 1 ml of 5% TCA and the pellets transferred to scintillation vials containing 10 ml of scintillation fluid and analysed.
Mean stimulation indices are presented below:

Concentration % (w/v)	Mean Stimulation Index (SI)	
	TM#2045	Positive control (p-phenylenediamine)
0.25	0.84	0.58
0.5	0.66	1.66
1.0	0.83	2.21
2.0	0.86	7.10

Results
No evidence of stimulating allergic contact sensitisation as the mean stimulation index did not exceed a value of 3 at any dose. The positive control, para-phenylenediamine, at 2.0% resulted in test/control ratios greater than 3.0 indicating a positive response.

Conclusion
Non-sensitising under the conditions of this study.

Ref.: 11

Comment
The concentrations used were not high enough and a sensitizing potential cannot be excluded.

Local Lymph Node Assay (LLNA)

Guideline: OECD 406, 429
Species/strain: CBA/CaOlaHsd mice
Group size: 30 total (including vehicle control and positive control) - 3 females per group for test material and vehicle control, 4 females per group for positive control
Test substance: HC Red 3
Batch: L-28231
Purity: /
Vehicle: acetone:olive oil, 4:1 (v/v)
Concentration: 0, 0.1, 0.25, 0.5, 1.0 and 2.5%
Positive control: α-hexylcinnamaldehyde 5, 10, 20% (performed at later date)
GLP: yes
Study period: July 2001

Induction
Concentrations of 0, 0.1, 0.25, 0.5, 1.0 and 2.5% HC Red 3 (w/v) in acetone:olive oil, 4:1 (v/v) applied topically to the dorsum of each ear lobe (left and right) (25 µl per ear) once daily on 3 consecutive days.

Reading times
On day 5, mice were injected intravenously with 250 µl of 3H-methyl thymidine (21.44 µCi). Five hours later (six hours with positive control), mice were euthanized and the draining auricular lymph nodes removed. Lymph nodes from each animal within a dose group were pooled and a cell suspension prepared. After three phosphate buffered saline washes, cells were precipitated in 3 ml of 5% trichloroacetic acid (TCA) at 4°C overnight. Samples were re-suspended in 1 ml of 5% TCA and transferred to scintillation vials containing 10 ml of scintillation fluid and analysed.

Main findings
Mean stimulation indices are presented below

Concentration % (w/v)	Mean Stimulation Index (SI)	
	HC Red N) 3	Alpha hexylcinnamaldehyde
0.1	0.5	-
0.25	1.2	-
0.5	1.9	-
1.0	1.8	-
2.5	3.3	-
5.0	-	2.4
10.0	-	3.7
20.0	-	7.0

Results
No evidence of stimulating allergic contact sensitisation when HC Red n° 3 was tested at 0.1%, 0.25%, 0.5% and 1.0% (w/v) as the mean stimulation index did not exceed a value of 3, but HC Red n° 3 showed a positive lymphocyte proliferative increase when tested at 2.5% (w/v).

Conclusion
HC Red n° 3 was sensitising when tested at 2.5%.

Ref.: 12

Comment
No EC3 value was calculated by the study authors for the experiment but an EC3 of 2.2 can be derived. The highest concentration tested was too low. HC Red n° 3 is a moderate skin sensitiser.

(Standard equation: EC3=c+[(a-c)*(3-d)]/(b-d) with corresponding SIs and the concentrations lying immediately above and below the stimulation index of 3 taken from the LLNA study report: (a = 1, b = 1.8) and (c =2.5, d = 3.3))

Comments on skin sensitisation
HC Red n° 3 is a skin sensitiser, being an extreme contact sensitiser in a GPMT and a moderate sensitiser in a LLNA.

Repeat insult patch test (Shelanski and Shelanski as modified by Ludwig)

Guideline: /
Species/strain: Human
Group size: 21 total
Test substance: HC Red #3
Batch: /
Purity: /
Induction: 1% solution of HC Red # 3 for three weeks
Challenge: Single application of 1% solution of HC Red #3
Positive control: /

GLP: /
Study period: 1982

Induction
Topical application (patch type not specified in report). 1% solution of HC Red #3 for three weeks. Interval between induction and challenge application: 10 days

Challenge route: Topical application (patch type not specified in report)
Reading times: Not specified in the report
Main findings: There were neither irritation nor sensitisation reactions in the study

Results
Non-irritating and non-sensitising

Ref.: 13

Comment
Only a brief summary is available. HRIPT experiments are not considered ethical by the SCCS

Repeat insult patch test

Guideline: /
Species/strain: human
Group size: 98 total (male and female)
Test substance: #1431282
Batch: /
Purity: /
Induction: 0.1 ml of a hair dye base, containing 3% #1431282
Challenge: 0.1 ml of a hair dye base, containing 3% #1431282
Positive control: /
GLP: /
Study period: 1983

Induction
Topical application under semi-occlusion of 0.1 ml of a hair dye base, containing 3% #1431282 (no additional dyes), to 1.4 cm2 for 24 hours, three times a week for three weeks (ten applications)
Interval between induction and challenge application: 11 days

Challenge route
Topical application under semi-occlusion. Single application of 0.1 ml of a hair dye base, containing 3% #1431282and no additional dyes, was applied to a naïve 1.4 cm2 site for 48 hours

Reading times: 48 and 72 hours after patch application

Main findings
There were neither irritation nor sensitisation reactions in the study

Conclusion
Non-irritating and non-sensitising

Ref.: 14

Comment
HRIPT experiments are not considered ethical by the SCCS.

Repeat insult patch test

Guideline:	/
Species/strain:	Human
Group size:	100 total (male and female)
Test substance:	HC Red 3 - #1431282
Batch:	/
Purity:	/
Induction:	0.1 ml of 3% HC Red #3 in a hair dye base
Challenge:	0.1 ml of 3% HC Red #3 in a hair dye base
Positive control:	/
GLP:	/
Study period:	1984

Induction: Topical application under semi-occlusion. 0.1 ml of 3% HC Red 3 in a hair dye base which contained no additional dyes was applied to a 1.4 cm2 for 24 hours, three times a week for three weeks (ten applications).
Interval between induction and challenge application: 11 days
Challenge: Topical application under semi-occlusion. Single application of 0.1 ml of 3% HC Red 3 in a hair dye base which contained no additional dyes was applied to a 1.4 cm² for 48 hours

Reading times: 48 and 72 hours after patch application

Main findings: There were neither irritation nor sensitisation reactions in the study

Conclusion
Non-irritating and non-sensitising.

Ref.: 15

Comment
HRIPT experiments are not considered ethical by the SCCS.

3.3.4. Dermal / percutaneous absorption

3.3.4. Dermal / percutaneous absorption

Submission III, 2011

Guideline:	OECD 428
Tissue:	Human skin, dermatomed to 400µm
Group size:	12 membranes from 6 donors
Skin integrity:	Electrical resistance
Diffusion cell:	Static glass; 2.54 cm^2 exposed
Test substance:	HC Red No 3
Batch:	L60357
Purity:	99.4%
Radiochemical:	[^{14}C]-HC Red No 3; batch CFQ40855; purity 99.6%
Test item:	Typical oxidative hair dye formulation mixed 1:1 with peroxide developer to yield 0.45% HC Red No 3
Dose volume:	20.0 mg/cm^2 ≡ 90.0 µg HC Red No.3/cm^2
Receptor fluid:	4% polyoxyethylene 20 oleyl ether in PBS
Solubility receptor fluid:	0.75 mg/ml (from reference 16)
Stability receptor fluid:	/
Method of Analysis:	liquid scintillation counting
GLP:	in compliance

Study period: September 2010

Membrane integrity was checked by measurement of electrical resistance. The doses were applied to the surface of 12 intact skin membranes (from 6 human donors) at a rate of 20 mg/cm^2, corresponding to a nominal 90 µg/cm^2 of HC Red No.3. At the end of the 30 minute exposure period, the skin surface was washed with water (10 x 1270 µL) followed by 2% sodium dodecyl sulphate in water (1 x 1270 µL).

The skin surface was washed with sponges soaked in 3% Teepol®L in water and further sponges pre-wetted with water. The stratum corneum was removed by a tape stripping process removing a maximum of 20 strips from each skin membrane. The flange skin was cut away from the dermis and the epidermis on the remaining skin disc was separated from the dermis using a heat separation technique.

The distribution of HC Red No.3 within the test system was measured and a 24 hour penetration profile was determined by collecting receptor fluid samples 0.5, 1, 2, 4, 8, 12, 16, 20 and 24 hours following application.

Results

Individual Distribution of HC Red No.3 through Human Dermatomed Skin (µg/cm^2)

Test Compartment	Amount Recovered (µg/cm²)													
	Cell 3	Cell 4	Cell 5	Cell 6	Cell 18	Cell 22	Cell 24	Cell 27	Cell 30	Cell 31	Cell 32	Cell 35	Mean	SD
Donor chamber	0.010	0.004*	<LOD	0.003*	0.002*	0.0003*	0.019	0.004*	0.001*	0.008	0.020	0.003*	0.007	0.007
Skin wash at 0.5 hours	85.0	83.6	85.1	84.8	84.9	84.5	84.3	87.8	84.0	84.8	83.5	83.8	84.7	1.14
Skin wash at 24 hours	0.290	0.334	0.255	0.400	0.155	0.119	0.150	0.076	0.168	0.244	0.279	0.235	0.225	0.095
Stratum corneum	0.050	0.060	0.039	0.068	0.041	0.074	0.071	0.117	0.008	0.060	0.008	NS	0.054	0.031
Remaining epidermis	0.137	0.050	0.054	0.145	0.069	0.134	0.070	0.011	0.031	0.031	0.053	0.064	0.071	0.044
Dermis	0.013	0.027	0.011	0.025	0.013	0.018	0.009	0.002	0.003	0.009	0.023	0.015	0.014	0.008
Flange	0.001*	<LOD	0.002*	0.009	0.005	0.003	0.001*	<LOD	<LOD	0.007	0.008	0.012	0.005	0.004
Receptor fluid	0.048	0.050	0.035	0.041	0.069	0.266	0.219	0.054	0.039	0.072	0.333	0.240	0.122	0.109
Total non-absorbed	85.4	84.0	85.4	85.3	85.1	84.7	84.5	88.0	84.2	85.1	83.8	84.1	85.0	1.12
Systemically available	0.198	0.127	0.100	0.211	0.151	0.418	0.298	0.067	0.074	0.112	0.409	0.319	0.207	0.126
TOTAL	85.6	84.1	85.5	85.5	85.2	85.1	84.8	88.1	84.3	85.2	84.2	84.4	85.2	1.07

Key to terminology:

Where values were below the mean LOD the value has been reported as <LOD in the table above and not included in the means, SDs or totals.
Values between the mean LOD and mean LOQ have been flagged with * and used as positive values in the means, SDs or totals.
Systemically available = Sum of remaining epidermis, dermis and receptor fluid.
Total non-absorbed = Sum of donor chamber, skin wash, flange and *stratum corneum*.
Stratum corneum = Amount in tape strips.
Remaining epidermis = Epidermal tissue after tape stripping.
NS = Tape stripping was not possible.

Individual Distribution of HC Red No.3 through Human Dermatomed Skin (% of Applied Dose)

Test Compartment	Percent of Dose Recovered (%)													
	Cell 3	Cell 4	Cell 5	Cell 6	Cell 18	Cell 22	Cell 24	Cell 27	Cell 30	Cell 31	Cell 32	Cell 35	Mean	SD
Donor chamber	0.011	0.004*	<LOD	0.004*	0.003*	0.0003*	0.021	0.004*	0.001*	0.008	0.022	0.003*	0.007	0.008
Skin wash at 0.5 hours	93.1	91.6	93.2	92.8	92.9	92.5	92.3	96.2	92.0	92.8	91.4	91.8	92.7	1.25
Skin wash at 24 hours	0.318	0.366	0.279	0.438	0.169	0.131	0.164	0.083	0.184	0.267	0.305	0.257	0.247	0.104
Stratum corneum	0.054	0.066	0.042	0.074	0.045	0.081	0.077	0.128	0.008	0.066	0.008	NS	0.059	0.034
Remaining epidermis	0.150	0.055	0.059	0.159	0.076	0.147	0.076	0.012	0.034	0.034	0.058	0.070	0.077	0.049
Dermis	0.015	0.029	0.012	0.027	0.014	0.020	0.010	0.002	0.003	0.010	0.025	0.017	0.015	0.009
Flange	0.001*	<LOD	0.002*	0.010	0.006	0.003	0.001*	<LOD	<LOD	0.007	0.009	0.013	0.006	0.004
Receptor fluid	0.053	0.055	0.039	0.045	0.076	0.291	0.240	0.059	0.043	0.079	0.365	0.262	0.134	0.119
Total non-absorbed	93.5	92.0	93.5	93.4	93.2	92.7	92.5	96.4	92.2	93.2	91.8	92.1	93.0	1.23
Systemically available	0.217	0.139	0.109	0.231	0.165	0.458	0.326	0.074	0.081	0.123	0.448	0.349	0.227	0.138
TOTAL	93.7	92.1	93.6	93.6	93.3	93.2	92.9	96.5	92.3	93.3	92.2	92.4	93.3	1.17

The mean total systemically available dose of HC Red No.3 (remaining epidermis plus dermis and receptor fluid) was 0.227 ± 0.138% of the applied dose (corresponding to 0.207 ± 0.126 µg/cm^2).

Ref.: 1 (subm III)

Comments
This was a well performed study conforming to guidelines. An adequate number of chambers and donors were used. Dosing was appropriate. Accordingly, under oxidative conditions the amount of HC Red No 3 in a typical hair dye formulation containing 0.45% HC Red No 3 on hair, the amount considered as absorbed is (mean +1SD) 0.365% or 0.333 µg/cm^2

Taken from SCCS/1293/10

Guideline:	OECD n° 428
Tissue:	Human dermatomed skin membranes (400 µm thick); 5 donors
Group size:	12 chambers
Skin integrity:	electrical resistance (<10 kΩ rejected)
Diffusion cell:	glass cells; 2.54 cm²
Test substance:	HC Red 3
Batch:	L35939
Purity:	97.9%
Radiochemical:	14C Red 3; lot 530-044-076; 99.5%; 2.109 GBq/mmol (57.0 mCi/mmol)
Test item:	3% HC Red #3 in a non-oxidative hair dye base containing no other dye precursors.
Dose volume:	20mg/ml (600 µg/cm2 HC Red 3)
Receptor fluid:	4% polyoxyethylene 20 oleyl ether in PBS
Solubility receptor fluid:	0.75 mg/ml
Stability receptor fluid:	/
Method of Analysis:	liquid scintillation counting
GLP:	yes
Study period:	Nov-Dec 2004

[14C]-radiolabelled HC Red 3 was incorporated into the dose to give 1x108 to 1x109 dpm/ml.

Duration of contact: 30 minutes followed by rinsing.
Sampling: 0.5, 1, 2, 4, 6, 24, 29, and 48 hours after application.

Results of the experiment are tabulated below

Hours	Amount penetrated	
	µg/cm²	% of applied dose
0.5	0.001	0.000
1	0.006	0.001
2	0.012	0.002
4	0.022	0.004
6	0.036	0.006
24	0.218	0.036
29	0.269	0.044
48	0.436	0.072

Test Compartment	Amount Recovered (µg/cm²)															
	Cell 2	Cell 5	Cell 6	Cell 7	Cell 8	Cell 10	Cell 11	Cell 12	Cell 13	Cell 15	Cell 16	Cell 21	Mean	SD	SEM	n
Flange	0.068	0.075	0.133	0.047	0.080	0.031	0.095	1.36	0.266	0.084	0.090	0.111	0.203	0.369	0.107	12
Donor Chamber	0.111	0.313	0.290	0.239	0.363	0.107	0.252	0.938	0.239	0.325	0.398	0.182	0.313	0.217	0.063	12
Skin Wash @ 0.5h	616	593	590	597	594	589	597	585	593	584	586	601	594	8.58	2.48	12
Skin Wash @ 48h	0.556	1.83	1.70	0.641	2.22	0.674	2.81	4.11	1.57	2.07	2.01	1.31	1.79	1.01	0.290	12
Stratum Corneum	0.069	0.100	0.335	0.195	0.158	0.239	0.607	1.40	0.630	0.524	0.450	0.434	0.480	0.404	0.116	12
Remaining Epidermis/Dermis	0.208	0.466	0.603	0.257	0.518	0.223	1.15	1.39	0.548	0.684	0.705	0.590	0.612	0.355	0.103	12
Receptor Fluid	0.234	0.426	0.571	0.499	0.711	0.332	0.443	0.403	0.280	0.271	0.283	0.782	0.436	0.177	0.051	12
Systemically Available*	**0.442**	**0.892**	**1.17**	**0.756**	**1.23**	**0.555**	**1.59**	**1.80**	**0.828**	**0.955**	**0.989**	**1.37**	**1.05**	**0.532**	**0.154**	**12**
TOTAL	617	596	594	599	598	591	602	595	596	588	590	605	598	7.50	2.17	12

Test Compartment	Percent of Dose Recovered (%)															
	Cell 2	Cell 5	Cell 6	Cell 7	Cell 8	Cell 10	Cell 11	Cell 12	Cell 13	Cell 15	Cell 16	Cell 21	Mean	SD	SEM	n
Flange	0.011	0.012	0.022	0.008	0.013	0.005	0.016	0.224	0.044	0.014	0.015	0.018	0.034	0.061	0.018	12
Donor Chamber	0.018	0.052	0.048	0.039	0.060	0.018	0.042	0.155	0.039	0.054	0.066	0.030	0.052	0.036	0.010	12
Skin Wash @ 0.5h	102	97.9	97.4	98.5	98.0	97.3	98.6	96.7	97.9	96.4	96.8	99.3	98.0	1.42	0.409	12
Skin Wash @ 48h	0.092	0.302	0.281	0.106	0.366	0.111	0.463	0.679	0.259	0.342	0.332	0.216	0.296	0.166	0.048	12
Stratum Corneum	0.011	0.017	0.055	0.032	0.026	0.040	0.100	0.231	0.104	0.087	0.074	0.072	0.079	0.067	0.019	12
Remaining Epidermis/Dermis	0.034	0.077	0.100	0.042	0.085	0.037	0.189	0.230	0.090	0.113	0.116	0.097	0.101	0.059	0.017	12
Receptor Fluid	0.039	0.070	0.094	0.082	0.117	0.055	0.073	0.067	0.046	0.045	0.047	0.129	0.072	0.029	0.008	12
Systemically Available *	**0.073**	**0.147**	**0.194**	**0.125**	**0.203**	**0.092**	**0.262**	**0.297**	**0.137**	**0.158**	**0.163**	**0.226**	**0.173**	**0.088**	**0.025**	**12**
TOTAL	102	98.0	98.0	98.8	98.7	97.5	99.5	98.2	98.4	97.0	97.4	99.8	98.7	1.24	0.36	12

* Systemically Available = Sum of Remaining Epidermis and Receptor Fluid data

- 48 hour receptor fluid value = 0.436 µg/cm2 (0.072% of applied dose)
- Amount remaining in epidermis/dermis after 48 hours = 0.612 µg/cm2 (0.101% of applied dose)
- Systemically available material = 1.05 µg HC Red 3/cm2 skin (0.173% of applied dose)
- Total recovery = 98.7%

Ref.: 16

Comment
5 donors were used with at least 2 cells from a donor. The absorption of a HC Red 3 from a non-oxidative hair dye formulation was 1.05 (+ 0.53) µg/cm² or 0.17 (+ 0.09) % of the applied dose. A figure of 1.58 µg/cm² (mean + 1SD) may be used for calculating the MOS.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral toxicity

Taken from SCCS/1293/10

Guideline:	/
Species/strain:	F344/N rat, B6C3F1 mice
Group size:	5 male and 5 female rats, and 5 male and 5 female mice per dose
Test substance:	HC Red n° 3
Batch:	Lot 5890377
Purity:	>97%
Dose levels:	rats: 0, 62, 125, 250, 500 and 1000 mg/kg bw/d mice: 0, 31, 62, 125, 250 and 500 mg/kg bw/d
Vehicle:	1% carboxymethylcellulose in water
Route:	oral
Administration:	oral gavage, for 14 days
GLP statement:	not specified but performed following NTP practices
Study period:	20 September- 3 October 1978

The study followed the NTP guidelines.
In this 14-day Oral Toxicity Study in F344/N rats and B6C3F1 mice, HC Red n°3 was administered daily in diet in both sexes at dose levels of 0, 62, 125, 250, 500 and 1000 mg/kg bw/d in male and female rats (5 animals per dose), and 0, 31, 62, 125, 250 and 500 mg/kg bw/d in male and female mice (5 animals per dose). Animals were observed twice daily for signs of toxicity. Clinical observations were recorded on the day of necropsy.
Animals were weighed at the start of the study, and on day 15. Necropsies were performed on all animals. No histopathology examination was performed.

Results
Rats
All animals survived to the end of the studies. The urine of all dosed animals was maroon to orange throughout the studies. Differences in mean body weight gains were not dose related. Dark thyroid glands were observed in 5/5 male rats that received 1000 mg/kg bw/d, 2/5 males that received 500 mg/kg bw/d and 2/5 males that received 250 mg/kg bw/d.

Mice
All animals survived to the end of the studies. The urine of all dosed animals was maroon to orange throughout the studies. Mean body weight gains were comparable between dosed or control groups.

Conclusion
In the 14-day gavage rat study, the NOAEL was 1000 mg/kg bw/d.
In the 14-day gavage mice study, the NOAEL was 500 mg/kg bw/d.

Ref.: 17

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

Taken from SCCS/1293/10

Guideline:	/
Species/strain:	F344/N rat, B6C3F1 mice
Group size:	10 male and 10 female rats, and 10 male and 10 female mice per dose
Test substance:	HC Red n° 3
Batch:	Lot 5890377
Purity:	>97%
Dose levels:	Rats: 0, 62, 125, 250, 500 and 1000 mg/kg bw/d Mice: 0, 15, 31, 62, 125 and 250 mg/kg bw/d
Vehicle:	corn oil
Route:	oral

Administration: oral gavage, 5 day/week for 13 weeks
GLP statement: not specified but performed following NTP practices
Study period: January- April 1979

The study followed the NTP guidelines.
In this 13-week oral Toxicity Study in F344/N rats and B6C3F1 mice, HC Red n° 3 was administered by gavage at dose levels of 0, 62, 125, 250, 500 and 1000 mg/kg bw/d to male and female rats (10 animals per dose), and 0, 15, 31, 62, 125 and 250 mg/kg bw/d to male and female mice (10 animals per dose). Animals were observed twice daily for signs of toxicity. Clinical observations were recorded daily. Animals were weighed at the start of the study and weekly thereafter. Feed consumption per cage was determined weekly. Complete necropsies and histopathological exam were performed on all animals. The skin, mandibular lymph node, mammary gland, salivary gland, thigh muscle, femur including marrow, thymus, trachea, lungs and bronchi, heart, thyroid gland, stomach, parathyroids, oesophagus, small intestine, colon, mesenteric lymph node, liver, pancreas, spleen, kidneys, adrenal glands, urinary bladder, vesicular gland/prostate/testis or ovary/uterus, brain, pituitary gland. Kidneys and thyroid gland of rats administered 250 or 500 mg/kg bw/d were also examined microscopically.

Results
Rats
All animals survived to the end of the studies.
The final mean body weights of males that received doses of 1000 mg/kg bw/d and 500 mg/kg bw/d were respectively 7% and 5% lower than those of controls. Final body weights of dosed female were greater than those of controls.
The urine of all dosed animals was orange to purple throughout the studies.
Granules of brown to golden-brown pigment were found in the cytoplasm of the thyroid gland follicular epithelial cells in 10/10 males and 10/10 females that received 1000 mg/kg bw/d and in 10/10 males and 7/10 females that received 500 mg/kg bw but not in any of the rats that received 250 mg/kg bw/d. Similar pigment was found in the cytoplasm of convoluted tubular epithelial cells in the kidneys of all rats that received 1000 mg/kg bw/d and in 7/10 males and 10/10 females that received 500 mg/kg bw and in 6/10 males and 7/10 females that received 250 mg/kg bw/d.

Mice
All deaths that occurred were related to gavage technique.
The final mean body weights of males that received doses of 250 mg/kg bw/d were 7% lower than those of controls. Final body weights of dosed female were comparable to those of controls.
The urine of all dosed animals was red throughout the studies.
No compound-related gross or microscopic pathologic effects were recorded.

Conclusion
Pigmentation was not considered by the applicant as adverse effects and based on the body weight decrease observed at 1000 mg/kg bw/d and 500 mg/kg bw/d, the NOAEL is 250 mg/kg bw/d in rats while the NOEL is 125 mg/kg bw/d (adjusted for 5 days treatment per week: 180 and 90 mg/kg bw/d, respectively). In mice, based on the body weight decrease observed at 250 mg/kg bw/d the NOAEL is 125 mg/kg bw/d (adjusted to 90 mg/kg bw/d).
Ref.: 17

3.3.5.3. Chronic (> 12 months) toxicity

Taken from SCCS/1293/10

Guideline: Not specified
Species/strain: Eppley Swiss mice
Group size: 60 male and 60 female mice per dose

Test substance:	HC Red n° 3
Batch:	/
Purity:	/
Dose levels:	0.05 ml of formulation applied to area approximately 1 cm2, three times weekly
Vehicle:	0.3% in hair dye formulation (formula 7601)
Route:	topical
Administration:	lifetime (20 months) in formulation
GLP statement:	Not specified
Study period:	not specified, before 1984

A hair dye formulation containing 0.3% of HC red n°3 was topically applied three times a week during 20 months on an area approximately 1 cm2 to 120 mice (60 males and 60 females). Mortality, behaviour and physical appearance, especially dermal changes were observed daily. 10 mice per sex per group were sacrificed nine months after treatment for clinical tests, haematology and necropsy. A gross necropsy was performed on all mice found dead or sacrificed in moribund condition and on those sacrificed after 9 months and at termination of the study.

Results
Body weights and survival differed little between treated and control groups. Differences between treated and control groups in absolute and relative liver and kidneys weights and in haematological and urinary values were not considered to be indicative of toxicological effects. Microscopic examinations of the skin revealed occasional hyperplasia, necrosis, ulceration and other lesions not significantly increased by dye treatment. Chronic inflammation of the skin was observed in the control and treated mice.

Ref.: 27

Comments
The experiment did not conform to a guideline and was not performed according to GLP. The purity and specifications of test article are not known. Different hair dye formulations were tested in this study. The results are sparsely reported. No NOAEL can be derived from this study.

Guideline:	/
Species/strain:	Beagles
Group size:	36 (6 Animals per sex and dose)
Test substance:	Hair dye formulation containing 0.02% HC Red n° 3.
Batch:	/
Purity:	/
Dose:	0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation
Route:	Oral in diet
Exposure period:	24 months
GLP:	not in compliance
Study period:	Before 1975

36 beagle dogs were orally exposed daily during 2 years with an hair dye formulation containing 0.02% HC Red n° 3 at the doses of 0, 19.5 and 97.5 mg/kg bw/d of hair dye formulation (6 dogs per sex and per dose). Each animal was observed daily for signs of toxic or pharmacologic effects. Individual records of body weight and food consumption were kept on a weekly and daily basis.
Necropsy was performed on one male and one female from each group at 6, 12 and 18 months. Individual organ weights and organ to body weight ratios of the major organs were recorded. Sections from 30 tissues or organs were prepared and examined microscopically. Electron microscopic evaluation of the livers and urinary bladder from all 18 dogs at 24 months was performed.

Results
No noteworthy differences were seen in any of the parameters studied between the controls and the animals receiving 19.5 or 97.5 mg/kg bw/d. All dogs gained weight normally and survived to end of the 104 weeks. All dogs in the two test groups excreted urine of a blue-brown colour on a daily basis. However urine analysis showed no remarkable findings.
Colour was normal in urine collected after overnight fasting.
No gross or microscopic changes were seen in the various tissues and organs that could be attributed to the test material. No ultra-structural changes were observed in the electron microscopic studies conducted on sections of liver and urinary bladder.

Conclusion
The authors concluded that oral dosing exposure of a hair dye formulation containing 0.02% HC Red n° 3 in formulations up to 97.5 mg/kg bw/day did not result in any signs of toxicity.
Ref.: 28

Comments
The experiment did not conform to a guideline and was not performed according to GLP. The purity and specifications of test article are not known. Different hair dyes were tested in this study. The concentration of HC Red n° 3 tested was too low compared to commercial formulations. The results are sparsely reported. No conclusions concerning long term toxic effects can then be made from this study.

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Taken from SCCS/1293/10

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium*: TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA (pKM101)
Replicates:	initial trial duplicates; confirmatory trial triplicates per test concentration
Test substance:	H.C. Red #3
Batch:	L-35939
Purity:	97.9%
Vehicle:	deionised water
Concentration:	0, 2.5, 5, 20, 50, 200, 500, 2000 and 5000 µg/plate with and without S9-mix
Treatment:	pre-incubation method was used with 20 ± 2 minutes pre-incubation and at least 60 ± 12 h incubation time both without and with S9-mix in both experiments.
GLP:	in compliance
Study period:	28 February – 8 July 2005

GTS03959 was investigated for the induction of gene mutations in bacteria (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity and mutagenicity in a concentration-selection assay with all *Salmonella* and *Escherichia* strains with concentrations up to the prescribed maximum concentration of 5000 µg/plate both without and with S9-mix. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and a qualitative evaluation of the bacterial lawn. The concentrations tested in both mutagenicity assays were 2.50, 5.00, 20.0, 50.0, 200, 500, 2000 and 5000 µg/plate in both the presence and absence of S9-mix. Both experiments were performed

with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline

Results
In both experiments precipitation was observed at 5000 µg/plate, the highest concentration tested. In both experiments a biologically relevant and statistically significant concentration dependent increase in the number of revertants was observed in strain TA98 both without and with S9-mix and in strain TA1537 without S9-mix only. No increases in the mean number of revertants per plate were observed with any of the other tester strains in either the presence or absence of S9-mix.

Conclusion
Under the experimental conditions used GTS03959 was mutagenic in this gene mutation tests in bacteria.

Ref.: 18

In Submission I (March 2003) and in Submission II (July 2005), 4 other old gene mutation tests in bacteria are mentioned. These tests all confirm the positive result obtained in the tests performed recently according to the existing guideline. However, 3 of the 4 tests only mentioned "a mutagenic response" whereas 1 performed by the NTP discriminated between the *Salmonella* strains reporting that HC Red n° 3 was mutagenic in strains TA97, TA98 and TA100, but not in TA1535, both in the presence or absence of S9-mix when tested according to the preincubation protocol.

In vitro **Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1997)	
Species/strain:	mouse lymphoma cell line L5178Y+/-	
Replicates:	single cultures per concentration	
Test substance:	HC Red #3	
Batch:	L-35939	
Purity:	97.9%	
Vehicle:	cell culture grade water	
Concentrations:	initial assay:	7.85, 15.7, 31.3, 62.5, 125, 250, 500 and 1000 µg/ml without S9-mix 50, 75, 100, 200, 400, 600, 800 and 1000 µg/ml with S9-mix
	repeat initial assay:	50, 75, 100, 200, 400, 600, 800 and 1000 µg/ml without S9-mix
	confirmatory assay:	50, 60, 70, 80, 90, 100 and 150 µg/ml without S9-mix 75, 100, 200, 400, 600, 800, 900 and 1000 µg/ml with S9-mix
Treatment:	initial assay:	4 h both without and with S9-mix; expression period 48 h, selection growth 12 days
	repeat initial assay:	4 h both without S9-mix; expression period 48 h, selection growth 12 days
	confirmatory assay:	24 h without S9-mix expression period 48 h, selection growth 13 days. 4 h both without S9-mix; expression period 48 h, selection growth 13 days.
GLP:	in compliance	
Study period:	5 August – 8 July 2005	

HC Red #3 was assayed for gene mutations at the *tk*-locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9 fraction from Aroclor

1254-induced rats was used as exogenous metabolic activation system. H.C. Red #3 test concentrations were based on the results of a concentration range-finding cytotoxicity assay with treatment up to the prescribed maximum concentration of 5000 µg/ml both in the presence and absence of S9-mix measuring cell growth relative to the concurrent vehicle control cell cultures. In the three independent experiments (initial, repeat initial and confirmatory assays), cells were treated for 4 h or 24 h (confirmatory assay without S9-mix only) followed by an expression period of 48 h to fix the DNA damage into a stable *tk* mutation. Toxicity was measured as percentage relative total growth of the treated cultures relative to the survival of the solvent control cultures; each assay must have at least one concentration which results in 10-20% relative total growth. In addition to the numbers of mutant colonies, the size of the colonies was determined and the ratio of small versus large colonies was calculated. According to modern standards, the test article was evaluated positive if there is a positive concentration response and one or more of the concentrations exhibit a mutant frequency which is greater than or equal to 90 mutants per 10^6 clonable cells over the concurrent background mutant frequency (ICH S2B Guideline, 1996). Negative and positive controls were in accordance with the OECD guideline.

Results
In general, precipitation was observed at dosing and again at termination at concentrations ≥ 500 µg/ml. Only in the confirmatory experiment with S9-mix precipitation was observed at concentrations of 200 µg/ml and above.
In all experiments in the absence of S9-mix the appropriate level of toxicity (about 10-20% survival after the highest concentration) was reached; however, in the confirmatory assay in the presence of S9-mix the appropriate level of toxicity was not reached.

In the initial assay performed in the absence of metabolic activation (treatment of 4 h), a more or less concentration dependent increase in the mutant frequency was found. Treatment at 1000 µg/ml induced a mutant frequency that met criteria for a positive response. However, the presence of precipitation at cloning may have interfered with the assay. This response in the initial assay was not confirmed in the repeat initial assay under more controlled conditions. In the repeat initial assay none of the analyzed treatments induced a mutant frequency that met the criteria for a positive response. In the confirmatory assay without metabolic activation with 24 h treatment none of the analyzed treatments induced an increase in the mutant frequency that met the criteria for a positive response.

In the initial assay performed in the presence of metabolic activation (treatment of 4 h), a biologically relevant and concentration dependent increase in the mutant frequency was not found. At the highest concentration 1000 µg/ml an increase in the mutant frequency was observed; however, the value did not meet the criteria for a positive response. In the confirmatory mutation assay with a 4-hour treatment period a biologically relevant and concentration dependent increase in the mutant frequency was again not found. Treatment at 1000 µg/ml induced an increase in the mutant frequency that met the criteria for a positive response but was not observed in the initial trial. The increase was therefore not reproducible and considered irrelevant.

Colony sizing was performed on all cultures. Treatments which induced a positive result (e.g. initial test without S9-mix and the highest concentration in the tests with S9-mix) exhibited a preferential increase in small colonies indicating to a clastogenic rather than a mutagenic effect

Conclusion
Under the experimental conditions used, H.C. Red #3 was not mutagenic in the mouse lymphoma assay at the *tk*-locus.

Ref.: 20

Comment

Sporadic increases in mutant frequencies were observed but were not repeatable, exhibited a preferential increase in small colonies indicating to a clastogenic rather then a mutagenic effect and may have been related to the heavy precipitation observed.

***In vitro* unscheduled DNA Synthesis Test**

Guideline:	/
Cells:	Primary hepatocytes of Fischer 344 rats
Replicates:	triplicate cultures
Test substance:	C7634/99
Solvent:	DMSO
Batch:	987134
Purity:	/
Concentrations:	10, 25, 50 and 100 µg/ml.
Treatment:	18 - 20 h
GLP:	in compliance
Date:	10 February - 19 May 1993

C7634/99 was investigated for the induction of unscheduled DNA synthesis (UDS) in primary hepatocytes obtained from rats. To isolate the primary hepatocytes, the liver was perfused with collagenase. Viability of the hepatocytes was measured by trypan blue exclusion. C7634/99 was assayed at concentrations of 0.1 up to 3000 µg/ml. Due to toxicity at concentrations greater than 500 µg/ml evidenced by a low grain incorporation, the highest concentration scored for the assay was 100 with three lower concentrations of 10, 25 and 50 µg/ml. Hepatocytes for UDS analysis were allowed to attach for approximately 2 h and exposed for 18 to 20 h to C7634/99 in serum free medium together with 10 µCi/ml ^{3}H-thymidine (specific activity 50 - 80 Ci/mM). Evaluation of autoradiography was done after 7 days. UDS was reported as net nuclear grain: the nuclear grain count subtracted with the highest of 3 cytoplasmic grain counts from 3 nuclear sized areas adjacent to each nucleus. A total of 150 hepatocytes/concentration (50 per triplicate culture) was scored. 2-Acetamidofluorene (2-AAF) was included as a positive control and DMSO as a negative control.

Results
C7634/99 did not produce mean net nuclear grain counts > 5 at any of the concentrations scored. The percentage of hepatocytes in repair showed slightly elevated values for 25 and 100 µg/ml. Since there was not a concentration related response, these increases were considered not biologically relevant.

Conclusion
Under the experimental conditions reported C7634/99 did not induce DNA-damage leading to unscheduled DNA synthesis in rat primary hepatocytes and, consequently, C7634/99 is not genotoxic in this *in vitro* UDS test.

Ref.: 21, submission I

Comment
The test was not performed according to the OECD guideline. Purity of C7634/99 was not given.

***In vitro* Mammalian Chromosome Aberration Test**

Guideline:	OECD 473 (1997)
Species/strain:	Chinese hamster ovary cells (CHO-WBL)
Replicates:	duplicate cultures per concentration
Test substance:	H.C. Red #3
Batch:	L-35939

Purity:	97.9%
Vehicle:	cell culture grade water
Concentrations:	4h treatment: 250, 750 and 1500 µg/ml without S9-mix 250, 1000, 2000 and 3250 µg/ml with S9-mix 18 h treatment: 125, 250 and 375 µg/ml without S9-mix
Treatment:	4 h both in absence and presence of S9-mix; harvest time 20 h after start of treatment. 20 h in absence of S9-mix; harvest immediately after start of treatment.
GLP:	in compliance
Study period:	8 September 2004 – 7 July 2005

H.C. Red #3 and its metabolites have been investigated in the absence and presence of metabolic activation for their potential to induce chromosome aberrations in cultured Chinese hamster ovary (CHO) cells with and without S9-mix. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Suitable ranges of test concentrations for the main experiment were based on the results of an initial toxicity assay measuring replication index (RI) measuring cell growth (mean cells per treatment group/mean cell per control group x 100) and cell growth inhibition (100% - % cell growth). The highest concentration tested in this toxicity assay was 5000 µg/ml, a concentration above the solubility limit of H.C. Red #3, being the prescribed maximum concentration by the OECD Test Guidelines both in the presence and absence of S9-mix.
In the confirmatory assay, the treatment period was for 4 h with and without S9-mix or 18h without S9-mix; cultures were harvested 20 h after the start of treatment. The final 2 h before harvest cells were cultured in the presence of Colcemid (at a final concentration of 0.1 µg/ml) to block cells at metaphase of mitosis. Toxicity was determined by measuring the reduction in mitotic index (MI) and the inhibition of cell growth. Chromosome (metaphase) preparations were stained with 5% Giemsa and examined microscopically for chromosomal aberrations. Negative and positive controls were in accordance with the OECD guideline.

Results
Treatment with H.C. Red #3 for four hours resulted in an inhibition of cell growth of 52% (without S9-mix) and 42% (with S9-mix); 18 h treatment resulted in an inhibition of 46%. Mitotic index was reduced with 8% (4 h treatment without S9-mix), 0% (4 h treatment with S9-mix) and 55% (18 h treatment without S9-mix).
Biologically relevant increases in the number of cells with chromosome aberrations, polyploidy, or endoreduplication were not observed after treatment with H.C. Red #3 in any of the cultures analysed both with and without metabolic activation.

Conclusion
Under the experimental conditions used H.C. Red #3 did not induce an increase in cells with chromosomal aberrations and, consequently, is not genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 19

In vitro **Mammalian Chromosome Aberration Test**

Guideline:	/
Species/strain:	Chinese hamster ovary cells (CHO-WBL)
Replicates:	duplicate cultures per concentration
Test substance:	C7634/99
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentrations:	125, 250, 375, 500, 625 and 750 µg/mL without and with S9-mix

Treatment:	4 h in the presence of S9-mix; harvest time 20 h after start of treatment. 20 h in absence of S9-mix; harvest immediately after start of treatment.
GLP:	in compliance
Study period:	22 December 1992 – 30 April 1993

C7634/99 has been investigated in the absence and presence of metabolic activation for its potential to induce chromosome aberrations in cultured Chinese hamster ovary (CHO) cells with and without S9-mix. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Selection of the concentration levels was based on the results of a preliminary toxicity test with concentrations ranging from 10 - 3000 µg/ml as indicated by a decline in cell growth potential.
In the chromosomal aberration test, the treatment period was 4 h with S9-mix and 20 h without S9-mix; cultures were harvested 20 h after the start of treatment. 2 h before harvest, Colcemid was added to the culture at a final concentration of 0.1 µg/ml to block cells at metaphase of mitosis. Toxicity was determined by measuring the reduction in mitotic index (MI). Cyclophosphamide was included as a positive control in the S9 activated part, mitomycin C in the nonactivated part of the experiment.

Results
Due to excessive cytotoxicity cells treated with 750 µg/ml without S9-mix and 125, 250 and 750 µg/ml with S9-mix could not be evaluated. Cells treated with 625 µg/ml without and with S9-mix had a reduction in the mitotic index >50%. Both without and with S9-mix a biologically relevant, statistically significant concentration dependent increase in the % cells with chromosomal aberrations was found. Both without and with S9-mix C7634/99 exposure of CHO cells did not result in an increase in polyploidy cells.

Conclusion
Under the experimental conditions used C7634/99 did induce an increase in cells with chromosomal aberrations and, consequently, is genotoxic (clastogenic) in this chromosomal aberration test in CHO cells *in vitro*.

Ref.: 20, submission I

Comment
The test was not performed according to the OECD guideline. Batch number and purity of C7634/99 were not mentioned. Consequently the value of the test is limited

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Taken from SCCS/1293/10

Gene mutation assay with transgenic mice

Guideline:	as an OECD guideline for this assay was not available at the time of performance, OECD 474 (1997) was used.
Species/strain:	mouse, Big Blue® B6C3F1
Group size:	6 mice/treatment group
Test substance:	HC Red no 3
Batch:	71, barrel 922169
Purity:	97.9 (area % HPLC)
Vehicle:	carboxymethylcellulose 1% in n-saline
Dose level:	0, 500 and 1000 mg/kg bw
Dosing volume:	20 ml/kg bw
Route:	gavage, daily for 8 weeks
GLP:	in compliance
Study period:	4 June 2003 – 5 July 2005

HC Red n° 3 was evaluated for its mutagenic potential in Big Blue® heterozygous *lacI/cII* mice using the *cII*-locus as reporter gene. Male Big Blue® B6C3F1 mice were treated daily for 8 weeks. The decision of the dose levels was based on a dose-range finding study oriented at the recommended maximum dose for sub-chronic studies. Based on the results of this dose-range finding study the mice were treated with 500 and 1000 mg/kg bw/day in 1% carboxymethylcellulose in n-saline.
Each mouse was observed at least once daily for mortality and morbidity. Each mouse was also observed before the start of treatment, prior to treatment on day 1 and weekly thereafter for clinical signs. Each mouse was weighed at least once before the start of treatment, prior to treatment on day 1, weekly thereafter and at termination of the study. Peripheral blood smears were prepared from each mouse in all treatment groups on day 1, after 4 weeks of treatment and at sacrifice for evaluation of micronucleus induction.
All animals of all treatment groups were sacrificed after 8 weeks of treatment 24 ± 4 h after the last dose by CO_2 asphyxiation. Necropsy included an examination of the external features of the carcass, including all external orifices, the abdominal, thoracic, and cranial cavities and all organ/tissues. Sections of all tissues from each animal were preserved in 10% NBF. Liver, urinary bladder and kidney were observed for gross or macroscopic lesions and examined microscopically. Tissue from the left lobe of the liver and the entire urinary bladder were collected from each mouse and evaluated for mutation on the *cII*-locus. The mutant frequency was determined by dividing the number of mutant plaques divided by the number of plaques evaluated from each tissue.
N-ethyl-nitrosourea (ENU) was used as positive control. Five male Big Blue® B6C3F1 mice were treated intraperitoneally with 50 mg/kg bw with 5 daily doses during week 1 only.

Results
Dose analysis demonstrated that animals received the intended target doses. All subjects were successfully administered the test article and control treatments daily for 8 weeks.
Survival to the terminal sacrifice was 100% for all treatment groups. There were no significant treatment-related clinical observations noted during this study. The bedding of the HC Red n° 3 treated groups was stained red during the treatment period which is considered evidence of systemic availability.
The group mean body weights for all groups initially decreased for all treatment groups from week 1 to 2, but thereafter increased slightly over the course of the study. The largest increase in main group body weight was seen in the vehicle treated control group. There were no statistical differences found in the group mean body weight gains between the treatment groups.
At necropsy there were enlarged and discoloured lymph nodes in 1/6 mice from the 500 mg/kg bw/day group in 2/6 mice of the 1000 mg/kg bw/day group and 2/5 mice of the ENU treated group. In 1/6 mice from the 500 mg/kg bw/day group a discoloured spleen was noted. In 1/6 mice from the 1000 mg/kg bw/day group an atrophied seminal vesicle was found. In 1/5 mice of the ENU treated group an enlarged thymus was found and in 2/5 mice testicular atrophy.
There was no statistically significant or biologically relevant increase in mutant frequency in the 500 mg/kg bw or 1000 mg/kg bw HC Red n° 3 treated groups compared to the control for either the liver or urinary bladder tissues. The positive control ENU induced large increases in mutant frequencies in both liver and urinary bladder which were statistically highly significant, demonstrating the ability of the test system to detect mutagens under the applied test conditions and in the selected target organs.

Conclusion
Under the experimental conditions used HC Red n° 3 did not induce an increase in the mutant frequency in the *cII*-locus in cells from the liver and urinary bladder and, consequently, HC Red n° 3 was not mutagenic in this gene mutation test with transgenic mice.

Ref.: 24

***In vivo* Mammalian Erythrocytes Micronucleus Test**

Guideline:	OECD 474 (1997, draft updated)
Species/strain:	male B6C3F1 mice
Group size:	6 animals per dose group
Test substance:	HC Red no 3
Batch:	71 barrel 922169
Purity:	97.9 area% (HPLC)
Vehicle:	1% carboxymethylcellulose (CMC) dissolved in n-saline
Route:	oral gavage, once daily on 3 consecutive days 24 h apart and on day 4 20 h after the dose received on day 3.
Dose level:	0, 250, 500 and 1000 mg/kg bw/day
Sampling:	4 h ± 19 min after the final treatment on day 4
GLP:	in compliance
Study period:	10 June – 30 September 2003

HC Red n° 3 was evaluated for its potential to induce micronuclei in bone marrow after oral administration of the test article to male mice. Doses for the test were selected based on historical data provided by previous toxicology and carcinogenesis studies in the literature. Groups of 6 mice were treated with test material dissolved in 1% CMC in normal saline, orally by gavage once daily (24 hr apart) for three consecutive days, and then were treated with a fourth dose 20 hr after the previous dose. Doses of 0, 250, 500, and 1000 mg/kg bw/day were used. During the acclimatization period each animal was observed at least once daily for mortality and morbidity. Prior to dosing, within 1 h of dosing and at the end of each workday each animal was observed for health concerns. Tissue sampling occurred 4 hours ± 19 minutes after the final treatment on day 4.
At the time of necropsy, blood samples were collected when the *vena cava* was cut to exsanguinate the animals. Following exsanguinations duplicate bone marrow slides were prepared for micronucleus analysis. Bone marrow slides were fixed with methanol and stained with acridine orange. For each animal, the number of PCEs among a total of 200 erythrocytes was determined to assess if HC Red no 3 inhibits erythropoesis. For micronuclei evaluation, 2000 PCEs per animal were evaluated.
The statistical analysis was based on the number of micronucleated PCEs. Negative and positive controls were in accordance with the OECD guideline.

Results
During treatment, one animal in the 1000 mg/kg bw/day dose group displayed lethargy, hunched posture, a rough coat, and sunken eyes following dosing on day 3 and before being found dead on day 4. Although no necropsy was performed on this animal, the absence of any clinical signs of stress in any of the other animals in the study appeared to indicate that the death of the animal was most likely not associated with chemical treatment.
Analysis of micronuclei did not sustain an increase in the number of micronucleated PCEs. The % PCE present in bone marrow was not substantially changed in the treated animals indicating that HC Red n° 3 did not have cytotoxic properties in the bone marrow and that systemic distribution and thus bioavailability of HC Red n° 3 in bone marrow is not confirmed.

Conclusion
Under the experimental conditions used HC Red n° 3 did not induce an increase in the number of bone marrow cells with micronuclei in treated mice and, consequently, HC Red n° 3 was not genotoxic (clastogenic and/or aneugenic) in these micronucleus test with mice.
Ref.: 22

Comment
Bone marrow was collected and analysed for micronuclei from the same treated animals which were used for the comet assay. Since the % PCE present in bone marrow was not

substantially changed systemic distribution and thus bioavailability of HC Red n° 3 in bone marrow is not confirmed.
However, the doses used in the present test caused in the gene mutation test with transgenic mice (ref. 24) red stained beddings during the treatment period which is considered evidence of systemic availability. Moreover, the doses used were more then 4 times higher then those used in an NTP rodent bioassay for carcinogenesis [125 and 250 mg/kg bw/d, see Technical Report 281, NTP], which caused pigmentation of epithelial cells of the thyroid gland and kidney in studies lasting 13 wk or longer.

***In vivo* Mammalian Erythrocytes Micronucleus Test**

Guideline:	OECD 474 (1981)
Species/strain:	albino CD1 mice
Group size:	5 mice/sex/group
Test substance:	HC Red 3
Batch:	H230692
Purity:	/
Vehicle:	arachis oil B.P.
Dose level:	3000 mg/kg bw
Sacrifice times:	24, 48 and 72h after dosing
GLP:	in compliance
Study period:	13 august - 7 October 1992

HC Red 3 was investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the results of a range-finding toxicity study with doses up to the recommended dose of 5000 mg/kg bw. All animals (2 males and 2 females per group) in this range-finding study were dosed once by gavage or intraperitoneally. The mice were observed 1 h after dosing and subsequently once daily for 3 days. Any deaths and evidence for overt toxicity were recorded at each observation. In the micronucleus study mice were treated intraperitoneally with the maximum tolerated dose level of 3000 mg/kg bw. The mice were observed 1 h after dosing and subsequently once daily as applicable.
Bone marrow cells of both treated and untreated mice were collected 24, 48 and 72 h after dosing. Thousand polychromatic erythrocytes were scored for the presence of micronuclei. Toxicity and thus exposure of the target cells was determined by measuring the NCE/PCE ratio. Cyclophosphamide (50 mg/kg bw) was used as positive control.

Results
In the range-finding toxicity study, there was one premature death at 5000 mg/kg bw in the animals treated orally, in the animals treated intraperitoneally there were 3 premature deaths at both 4000 and 5000 mg/kg bw. After both treatments clinical signs observed were red extremities, red/purple coloured urine, ptosis, lethargy, hunched posture, gasping respiration, decreased respiratory rate, laboured respiration and piloerection in all doses treated with HC Red 3.
With no premature deaths and clinical signs of red extremities and red coloured urine and only 2 animals with lethargy the maximum tolerated dose selected for the micronucleus assay was 3000 mg/kg bw via the intraperitoneal route.
Clinical signs in the main micronucleus study were hunched posture, lethargy, ptosis, ataxia, red/purple coloured urine and purple extremities in all treated animals. Several premature deaths were recorded after 24, 48 and 72 h exposure in all groups. However they were considered not to affect the integrity of the study.
No relevant change in the NCE/PCE ratio was observed after dosing with HC Red 3.
However, the clinical signs observed indicated bioavailability of the test compound.
Particularly, colouration of urine is considered evidence of systemic exposure to the test article. There was no evidence for a biologically relevant increase in the number of polychromatic erythrocytes with micronuclei in animals dosed with HC Red 3 when compared to the concurrent vehicle control groups.

Conclusion
Under the experimental conditions used HC Red 3 did not induce an increase in the number of polychromatic erythrocytes with micronuclei in treated mice and, consequently, HC Red 3 was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 23

Comment
The purity of the test substance was not reported. A minimum of four animals of each sex was analyzed at the 24 and 48 hour time points, meeting current OECD guidelines (72 hours is no longer required). Therefore, the lethality that occurred in this study does not compromise the integrity of the results.
The clinical signs (e.g. coloured urine) and lethality observed in this study indicate systemic availability of the test compound.
The highest dose exceeds the current OECD maximum dose of 2000 mg/kg bw. One thousand PCEs per animal were analyzed in this study which is not in agreement with the current guideline. However, combining the results of the males and females yielded a minimum of 8000 PCE per group at 24 hours and 9000 PCE per group at 48 hours when compared to the concurrent vehicle control groups.

***In vivo* Comet assay**

Guideline:	The test methodology for the Comet assay is in accordance with current literature and accepted scientific and regulatory principles.
Species/strain:	male B6C3F1 mice
Group size:	6 animals per dose group
Test substance:	HC Red no 3
Batch:	71 barrel 922169
Purity:	97.9 area% (HPLC)
Vehicle:	1% carboxymethylcellulose (CMC) dissolved in n-saline
Route:	oral gavage, once daily on 3 consecutive days 24 h apart and on day 4 20 h after the dose received on day 3.
Dose level:	0, 250, 500 and 1000 mg/kg bw/day
Sampling:	4 h ± 19 min after the final treatment on day 4
GLP:	in compliance
Study period:	10 June – 30 September 2003

HC Red n° 3 was evaluated for its potential to induce DNA damage in cells of the liver, urinary bladder and peripheral blood after oral administration of the test article to male mice. Doses for the test were selected based on historical data provided by previous toxicology and carcinogenesis studies in the literature. Groups of 6 mice were treated with test material dissolved in 1% CMC in normal saline, orally by gavage once daily (24 hr apart) for three consecutive days, and then were treated with a fourth dose 20 hr after the previous dose
The test article was administered by oral gavage at 0, 250, 500, and 1000 mg/kg bw/day.
During the acclimatization period each animal was observed at least once daily for mortality and morbidity. Prior to dosing, within one h of dosing and at the end of each workday each animal was observed for health concerns. Tissue sampling occurred 4 hours ± 19 minutes after the final treatment on day 4.
At the time of necropsy, blood samples were collected when the *vena cava* was cut to exsanguinate the animals. Following exsanguinations the liver and urinary bladder were removed for comet analysis. Additional samples of each tissue were fixed in 10% Neutral Buffered Formalin (NBF) and embedded for potential future histopathological analysis.
Comet slides were prepared from the fresh liver, urinary bladder and blood samples. Two slides per animal and per blood or tissue sample were exposed to alkali (pH>13) for 20 minutes, followed by electrophoresis for 20 minutes at 0.9 V/cm and 300 mA, stained with

ethidium bromide and scored for comets. The extent of DNA migration (measured as tail length, the percentage of migrated DNA, and Olive tail moment) was determined by scoring 100 cells (50 cells per each of two slides, if possible)
One slide per tissue was analyzed in the absence of electrophoresis for the frequency of cells with extremely low molecular weight (LMW) DNA as an indication of the presence of necrosis or apoptosis. The frequency of cells with extremely LMW DNA was determined by scoring 100 cells for levels of diffusion ranging from I (condensed DNA) to II (diffused DNA).
The statistical analysis was based on the extent of DNA migration as measured by the Olive tail moment and on the frequency of cells with LMW DNA. Negative and positive controls were incorporated in the study.

Results
During treatment, one animal in the 1000 mg/kg bw/day dose group displayed lethargy, hunched posture, a rough coat, and sunken eyes following dosing on day 3 and before being found dead on day 4. Although no necropsy was performed on this animal, the absence of any clinical signs of stress in any of the other animals in the study appeared to indicate that the death of the animal was most likely not associated with chemical treatment.
Following oral administration of dose levels up to 1000 mg/kg bw/day administered on four consecutive days, HC Red n° 3 did not induce a biologically relevant increase in DNA migration in the liver, urinary bladder or blood. No increase in the percentage of cells with LMW DNA was found in the liver, urinary bladder or blood of treated animals. For liver and urinary bladder exposure was not demonstrated.

Conclusion
Under the experimental conditions used HC Red n° 3 did not induce DNA damage in cells from the liver, urinary bladder or blood of treated mice and, consequently, HC Red n° 3 was not genotoxic (clastogenic and/or mutagenic) in this mouse study.

Ref.: 22

Comment
Samples were collected and analysed from the same treated animals which were used for bone marrow collection for the *in vivo* micronucleus assay. For liver and urinary bladder exposure is not demonstrated. However, the doses used in the present test caused in the gene mutation test with transgenic mice (ref. 24) red stained beddings during the treatment period which is considered evidence of systemic availability. Moreover, the doses used were more then 4 times higher then those used in an NTP rodent bioassay for carcinogenesis [125 and 250 mg/kg bw/d, see Technical Report 281, NTP], which caused pigmentation of (epithelial cells of the thyroid gland and kidney) in studies lasting 13 wk or longer.

Heritable translocation test in rats

Guideline:	/
Species/strain:	male Sprague Dawley Charles River CD rats
Group size:	50 rats per dose
Test substance:	semi permanent dye containing 0.01% HC Red #3 among other hair dyes
Batch:	/
Purity:	/
Dose level:	pure dye
Vehicle:	/
Route:	topical, dermal,
Treatment:	twice weekly for 10 weeks. 0.5 ml to an area of approximately 1 square inch on the back of each animal. The areas were shaved
GLP:	/
Study period:	/

A permanent hair dye containing 0.01% HC Red #3 and some other hair dyes were investigated for the induction of heritable translocations in rats. Male Sprague Dawley rats were treated topically twice weekly for 10 weeks. Every treated male was then mated for 7 days to 3 sexually mature 10 week old female rats (1 per week for 3 weeks). At birth the number and sex of live and dead pups of the F1 were recorded and after 4 days each litter was culled to a maximum of 6 males. After weaning at 21 days, 2 healthy males of each litter were raised to maturity. At week 12, 100 of the F1 males from each group were selected for mating, each with 3 sexually mature females of the same strain (1 per week for 3 weeks). Pregnancies were timed in order to do caesarean sections on days 14-16 of gestation. At that time the females were killed and the uterus examined for the number of implants, resorptions and live and dead foetuses. The F1 males were retained for possible cytological analysis of germ cells pending analysis of the litter data.

Results
Fertility rates were high for both generations. Average litter sizes for the hair dye group and the control in the F1 generation were nearly identical and were in excess of 11 live pups per litter. There was no evidence from the present results that frequent topical application of the hair dye caused stable chromosome rearrangements such as translocations which may result in reduced fertility of the offspring of the treated subject.

Conclusion
Under the experimental conditions used a permanent hair dye containing 0.01% HC Red #3 and some other hair dyes did not induce heritable translocations and, consequently, are not genotoxic in rats in this heritable translocation test.

Ref.: 29

Comment
Reference 29 is a paper from the open literature describing a heritable translocation study with 2 hair dye formulations, a semi-permanent dye and an oxidation dye. The semipermanent hair dye contains, in addition to HC Red #3 (0.01%), also Disperse Blue (0.12%), Disperse Black (0.04%), HC Yellow #2 (0.01%), HC Yellow #3 (0.21%), HC Blue #1 (0.30%), Acid Orange #3 (0.06%) and Disperse Violet #11 (0.07%). HC Red #3 was not present in the oxidative hair dye.
Consequently, due to these confounding 7 other hair dyes as well as due to the low concentration in the formulation, no conclusion can be made concerning the potential of HC Red #3 to induce heritable chromosomal mutations by topical application in a hair dye formulation.

3.3.7. Carcinogenicity

3.3.7.1 Carcinogenicity in vitro

Taken from SCCS/1293/10

Syrian Hamster Embryo Cell (SHE) Assay

Guideline:	/
Species/strain:	Cryopreserved Syrian Hamster Embryo Cells isolated on gestation day 13
Replicates:	300 target cells on 6x104 feeder cells.
Test substance:	HC Red n° 3
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentrations:	16 – 600 µg/ml

Treatment: Assay terminated after 7 days. The test was performed in two different laboratories.
Positive control: Benzo(a)pyrene. 18 chemicals were tested
GLP: /
Study period: Before 1988

Test System
Fibroblasts isolated from Syrian golden hamster embryos on gestational day 13 were used in a cellular transformation assay.

Test Procedure
HC Red n° 3 was evaluated for cellular transformation capability using the Syrian Hamster Embryo cell (SHE) assay. Two independent laboratories tested HC Red n° 3 at five dosage levels up to 500 and 600 µg/ml, respectively. Duplicate assays utilizing a pH 7.4 culture protocol were performed for a duration of 7 days. Benzo[a]pyrene (B[a]P) was included as a positive control. A positive transformation response was determined using strict morphological guidelines. The transformation activity was determined by pooling the data for the same dose level in both assays. The response was first determined within each laboratory, then between the laboratories. A positive assay response was defined as a three-fold elevation over the control level of transformation at two or more doses in at least two assays.

Results
HC Red n° 3 did not induce transformation at any dosage level in the high pH SHE assay in either laboratory.

Ref.: 23, subm I

BALB/C-3T3 Transformation Assay

Guideline: /
Species/strain: Immortalized cells from A13-1-13 clone of the BALB/c-3T3 mouse
Replicates: 18-20 vessels/dose, 3.2 x 104 cells/vessel
Test substance: HC Red n° 3
Batch: /
Purity: /
Vehicle: /
Concentrations: 0.75 – 7.89 mM
Treatment: test material added on day 2 after plating and the cultures continued for a total of 28 days. Tested both coded and non-coded.
Positive control: Benzo(a)pyrene
GLP: /
Study period: Before 1993

Test Procedure
HC Red n° 3 was among 168 chemicals evaluated for cellular transformation capability using the BALB/c-3T3 assay in a blinded (coded) and non-blinded (noncoded) protocol. The experimental design incorporated several modifications from established protocols, involving selection of dosage levels, solubilization of compounds, and cell plating densities. Four dosage levels were selected, ranging from 0.75 to 7.89 mM (1.55 mg/ml) in 2-fold increments, which resulted in a cell survival range of 10-100%. The test material was included in the cell culture medium beginning on day 2 after plating, and the culture was continued for a total of 28 days with biweekly replenishment of medium. Duplicate assays were performed. Negative controls included either untreated cultures or solvent controls.
Benzo[a]pyrene (B[a]P) was included as a positive control. A positive transformation response was scored according to strict morphological guidelines. The data were log-transformed and rank-ordered to account for non-normal distribution of biological effects.

For each assay, the activity of a test chemical was determined as: sufficient positive (SP), limited activity (LA), sufficient negative (SN) and limited negative (LN), depending on factors including the statistical significance of deviations from control values in one or several doses, dose-related behaviour, and the occurrence of cytotoxicity of the compound and the positive control. For the final call on the compound, which assessed results of both assays, a chemical was considered active, weakly active, inactive or indeterminant.

Results
For HC Red No. 3, the cytotoxic LD50 was 3.72 mM (734 µg/ml) and 4.50 mM (887 µg/ml) respectively, for the uncoded and coded samples. In duplicate transformation assays, the uncoded HC Red No. 3 had two SP (positive) responses. The coded sample had LA and SP calls.
Overall, the chemical was evaluated to be active (positive) in the BALB/c-3T3 transformation assay.

Ref.: 24, subm. I

3.3.7.2 Carcinogenicity in vivo

Taken from SCCS/1293/10

Oral administration

Rats

Guideline:	/
Species/strain:	F344/N rats
Group size:	50 animals per sex and dose
Test substance:	HC Red n° 3
Batch:	Clairol Research Laboratories (Stamford, Connecticut)
Purity:	Lot No. 5890377 and CO80480; purity > 97% Nitrosamine (not identified) content < 50 ppm
Dose level:	0, 250, or 500 mg/kg bw HC Red n° 3 in corn oil, 5 days per week
Route:	Oral, gavage
Exposure period:	105 weeks
GLP:	In compliance
Study period:	27 November 1979 – 27 November 1981

The study was carried out by the US National Toxicology Program.
F344/N rats, groups of 50 males and 50 females (7-8 weeks old), were exposed to HC Red n° 3 by oral gavage 5 days per week for 105 weeks. The rats received 0 (control), 250 (low dose), and 500 mg/kg bw (high dose). The animals were observed twice daily. No significant differences in survival were observed between any groups of either sex. Neither did the treatment affect body weight or body weight gains. No compound-related clinical signs were observed.

Pigmentation of various tissues was a common observation. The pigment was not identified but was presumed to be a derivative of HC Red n° 3. Very minimal nephropathy was found in dosed female rats, but its relationship to HC Red n° 3 was considered equivocal.
There was an increase in the incidence of mammary gland fibroadenomas or cystadenomas in low dose female rats. The incidence of this lesion in high dose female rats was not increased (vehicle control, 14/50, 28%; low dose, 25/50, 50%; high dose, 11/50, 22%).
Largely because of the lack of a dose response, the increased incidence in the low dose females was not considered to be due to HC Red n° 3. No increased incidences of neoplasms were seen in male rats.

Transitional cell papillomas of the urinary bladder were detected in one high dose male rat, two low dose female rats, and one high dose female rat; none was observed in the vehicle

controls. These uncommon neoplasms were found in animals that survived to the termination of the study and were not accompanied by other proliferative lesions.

It was concluded that under the conditions of this 2-year gavage study of HC Red n° 3, there was no evidence of carcinogenicity for male or female F344/N rats given 250 or 500 mg/kg bw/day. Both sexes of the rats may have been able to tolerate higher doses of HC Red n° 3. Therefore, the sensitivity of this study for detecting carcinogenesis may have been limited.

Ref.: 17

Mice

Guideline:	/
Species/strain:	B6C3F1mice
Group size:	50 animals per sex and dose
Test substance:	HC Red n° 3
Batch:	Clairol Research Laboratories (Stamford, Connecticut)
Purity:	Lot No. 5890377 and CO80480; purity > 97% Nitrosamine (not identified) content < 50 ppm
Dose level:	0, 125, or 250 mg/kg bw HC Red n° 3 in corn oil, 5 days per week
Route:	Oral, gavage
Exposure period:	104 weeks
GLP:	In compliance
Study period:	27 November 1979 – 20 November 1981

The study was carried out by the US National Toxicology Program.

B6C3F1 mice, groups of 50 males and 50 females (8 weeks old), were exposed to HC Red n° 3 by oral gavage 5 days per week for 104 weeks. The mice received 0 (control), 125 (low dose), and 250 mg/kg bw (high dose). The animals were observed twice daily. Mean body weights of high dose mice of each sex were comparable to or greater than those of the vehicle controls throughout most of the study. The body weights of both male and female low dose mice tended to be lower than those of other groups throughout the study. No compound-related clinical signs were observed. The survival of the low dose male mice was significantly greater than of the vehicle control group. The survival of female mice, including vehicle controls, was reduced relative to historical survival rates due to a reproductive tract infection. The infection, accompanied by weight loss, high mortality, and inflammation of multiple organs, was found in 36/50 vehicle control, 32/50 low dose, and 29/50 high dose female mice. *Klebsiella pneumoniae* was isolated from infected tissues.

Pigmentation of various tissues was a common observation. The pigment was not identified but was presumed to be a derivative of HC Red n° 3. Mild nephrosis was found in dosed female mice, but this effect may have been secondary to the infection of the genital tract.

The incidence of hepatocellular adenomas or carcinomas (combined) was increased in high dose male mice, whereas the incidence of these neoplasms in low dose male mice was significantly lower than that in the vehicle controls (25/50, 50%; 15/50, 30%; 35/50, 70%). Hepatocellular carcinomas in three vehicle control, one low dose, and five high dose male mice metastasized to the lung. The incidences of liver neoplasms in dosed female mice were not significantly different from those in the vehicle control group.

It was concluded that under the conditions of this 2-year gavage study of HC Red n° 3, there was equivocal evidence of carcinogenicity for male B6C3F1 mice as indicated by an increased incidence of hepatocellular adenomas or carcinomas (combined) in the high dose group. Poor survival coupled with lack of significant findings rendered the study in female B6C3F1 mice an inadequate study of carcinogenicity. Both sexes of the mice may have been

able to tolerate higher doses of HC Red n° 3. Therefore, the sensitivity of this study for detecting carcinogenesis may have been limited.

Ref.: 17

Comment
The carcinogenicity of HC Red n° 3 has been studied under the US National Toxicology Program in rats and mice after oral gavage administration for 2 years. No evidence of carcinogenicity was found in rats. There was equivocal evidence of carcinogenicity for male mice as indicated by an increased incidence of hepatocellular adenomas or carcinomas (combined) in the high dose group. Poor survival coupled with lack of significant findings rendered the study in female mice an inadequate study of carcinogenicity. Both sexes and species may have been able to tolerate higher doses of HC Red n° 3. Therefore, the sensitivity of these studies for detecting carcinogenesis may have been limited.

Dogs

Guideline:	/
Species/strain:	Beagles
Group size:	6 Animals per sex and dose
Test substance:	A semipermanent hair dye formulation containing 0.02% HC Red No. 3
Batch:	/
Purity:	not stated
Dose:	0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation (0, 3.9 and 19.5 µg/kg bw/day of HC Red No. 3)
Route:	oral - diet
Exposure period:	24 months
GLP:	not in compliance
Study period:	Before 1975

Diets were prepared daily with the incorporation of the hair dye formulation which contained 15 hair dye substances to give doses of 0, 19.5 and 97.5 mg/kg bw/day to the beagles dogs. The dogs were 7 – 9 month of age when the study was started. Adjustments of concentrations in the diet were made weekly according to body weight changes. Each animal was observed daily for signs of toxic or pharmacologic effects. Individual records of body weight and food consumption were kept on a weekly and daily basis. No positive control group was used.
Physical examinations including funduscopic, EKG, blood pressure, pulse rate and body temperature were conducted initially and at 3, 6, 12, 18 and 24 months. Haematological, blood chemical and urinalysis parameters were determined on all high dose and control dogs and on 3 males and 3 females from the low dose group. Haematologic studies included determination of total and differential leucocyte counts, haematocrit, haemoglobin concentration, erythrocyte sedimentation rate and prothrombin time. Clinical chemistry determinations were conducted on animals that had been fasted for 18 hours. These included serum glucose, blood urea nitrogen, creatinine and uric acid concentrations and alkaline phosphatase and serum glutamic pyruvic transaminase activities. Urinalysis included detection of occult blood, albumin, glucose, pH and microscopic examination of urinary sediment.
Necropsy was performed on one male and one female from each group at 6, 12 and 18 months. Individual organ weights and organ to body weight ratios of the major organs were recorded. Sections from 30 tissues or organs were prepared and examined microscopically.
Electron microscopic evaluation of the livers and urinary bladder from all 18 dogs at 24 months was performed.
No noteworthy differences were seen in any of the parameters studied between the controls and the animals receiving 19.5 or 97.5 mg/kg bw/day. All dogs gained weight normally and survived to end of the 104 weeks. All dogs in the two test groups excreted urine of a blue brown colour on a daily basis. However urine analysis showed no remarkable findings.

Colour was normal in urine collected after overnight fasting.
No gross or microscopic changes were seen in the various tissues and organs that could be attributed to the test material. No ultra-structural changes were observed in the electron microscopic studies conducted on sections of liver and urinary bladder.
The authors concluded that oral dosing exposure of a hair dye formulation containing 0.02% HC Red No. 3 in formulations up to 97.5 mg/kg bw/day did not result in any signs of toxicity.

Ref.: 28

Comment
No conclusions concerning potential carcinogenic effects can be made from the study with dogs due to the low concentration of HC Red No. 3. Moreover, it should be noted that the hair dye formulation contained 0.61% Disperse Blue 1 (GHS carcinogen category 1B) and 1.54% HC Blue I (evaluated by IARC, sufficient evidence for carcinogenicity in animals).

Topical application

Mice

Guideline:	/
Species/strain:	Eppley Swiss mice
Group size:	60 animals per sex
Test substance:	A semipermanent hair dye formulation (7601) containing 0.3% HC Red No. 3
Batch:	/
Purity:	/
Dose level:	0.05 ml of a solution containing 0.3% HC Red No. 3
Route:	Topical, 3 application weekly
Exposure period:	20 months
GLP:	not in compliance
Study period:	Before 1984

2 oxidative and 12 non-oxidative hair dye formulations were tested. Two of the nonoxidative hair dye formulation including the one with HC Red No. 3 contained 0.3% Disperse Blue 1 (GHS carcinogen category 1B).
Swiss mice (8 weeks old), groups of 60 males and 60 females, were painted three times weekly with a hair dye formulation for 20 months. Aliquots of 0.05 ml were delivered to an area of skin (1 cm2) in the interscapular region. The mice were shaved 24 hours before treatment as needed. Two control groups of were shaved only and received no treatments.
The oxidative dye solutions were mixed with an equal volume of 6% H2O2 just prior to application. One of the non-oxidative hair dye formulations contained 0.3% HC Red No. 3. A gross necropsy was performed on all mice.
The application of hair dyes did not have an adverse effect on average body weight gains or survival of any group. Body weights were not depressed more than 10% in any group compared to the controls. The predominant tumours seen were those that occur commonly in the Eppley Swiss mouse, namely lung adenomas, liver haemangiomas, and malignant lymphomas. No unusual tumours developed in any of the groups.
The authors concluded that no toxic or carcinogenic effects were induced by HC Red No. 3.

Ref.: 27

Comments
One study with HC Red No. 3 in a semipermanent hair dye formulation (7601) involving topical application of mice has been submitted. The concentration of HC Red No.3 was 0.3%. A number of different hair dye formulations were tested in the same study. Although some of the formulations contained Disperse Blue 1 (GHS carcinogen category 1B) none of

the formulations induced tumours. Thus, no conclusion with regard to carcinogenicity can be made from the studies.

Conclusion
No conclusions concerning potential carcinogenic effects can be made from an oral study with dogs and a skin painting study with mice. HC Red No. 3 was present in low concentrations (0.3% or less) in semipermanent hair dye formulations. Moreover, although substances classified as carcinogens were present in the formulations studied, no carcinogenic effects were found in any of the studies indicating low sensitivity.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Taken from SCCS/1293/10

2-Generation reproduction toxicity testing with formulation

Guideline:	/
Species/strain:	Sprague-Dawley CD rats
Group size:	180 (10 males and 20 females per dose)
Test substance:	Hair dye formulation containing 0.02% HC Red n° 3
Batch:	/
Purity:	/
Dose:	0, 1950 and 7800 ppm of hair dye formulation in the diet
Route:	Oral diet
Exposure period:	Part 1: males rats fed with the test diet from 8 weeks prior to mating and during the mating period and females were not exposed Part 2: females fed with the test diet from 8 weeks prior to mating and during gestation and 21 days of lactation and males were not exposed
GLP:	not in compliance
Study period:	Before 1975

In this oral dosage fertility and reproductive study, a formulation containing 0.02% of HC Red n° 3 was administered in the diet once daily to 180 Sprague Dawley rats at the doses of 0, 1950 and 7800 ppm (10 males and 20 females per dose). In the first part of the study, only male rats were fed with the test diet from 8 weeks prior to mating and during the mating period and females were not exposed and in the second part of the study, females were fed with the test diet from 8 weeks prior to mating and during gestation and 21 days of lactation and males were not exposed.
One female pregnant by each male was killed on day 13 to obtain information regarding the early stages of gestation. The uterus was examined for the number and distribution of embryos, the presence of empty implantation sites, and the number of embryos undergoing resorption. Each embryo was microscopically examined.
All remaining dams were allowed to deliver normally. A necropsy was performed on all females that did not deliver a litter to determine whether pregnancy has occurred. The duration of gestation was reported and the litters were examined. The pups were weighed at birth, and at 4 and 21 days. At 21 days, all surviving pups were killed and macroscopically examined for abnormalities.

Results

Maternal parameters
No effects on body weight gains and food consumption were observed. No significant differences were observed in any of the parameters examined including fertility, length of gestation, and numbers of females with resorption sites. The female fertility index in the

high dosage group in part one of the study was lower than the control values, but the differences were not statistically significant.

Paternal parameters
No dose related significant differences in male fertility were observed. No effects on body weight gains and food consumption were observed.

Foetal parameters
No significant differences were observed in any of the parameters examined including live pups per litter, pup body weights and pup survival. No abnormal pups were observed upon dissection of embryos after 13 days of gestation or upon gross examination at weaning after 21 days.

Conclusion
Based on the results of this study, a maternal, paternal and foetal NOAEL of 7800 ppm of the test material/ kg bw/d was proposed by the applicant.

Ref.: 28

Comments
The experiment did not conform to a guideline and was not performed according to GLP. The purity and specifications of test article is not known. Different hair dyes were tested in this study. The concentration of HC Red n° 3 tested was too low compared to commercial formulations. The results are sparsely reported. No conclusions concerning long term toxic effects can then be made from this study. The highest dose corresponds to 23 mg/kg bw/day.

3.3.8.2. Teratogenicity

Taken from SCCS/1293/10

Prenatal developmental toxicity study, range finding study

Guideline:	OECD 414 (2001)
Species/strain:	rat, Crl:CD® (SD)IGS BR VAF/Plus®
Group size:	40 (8 presumed-pregnant females per group)
Test substance:	HC Red n° 3
Batch:	L 35939
Purity:	97.9%
Vehicle:	100% polyethylene glycol 400
Dose levels:	0, 50, 200, 500 and 1000 mg/kg bw
Dose volume:	5 ml/kg bw
Route:	oral, gavage
Administration:	once daily on Day 6 through Day 20
GLP statement:	in compliance
Study period:	August -September 2004

In this oral dosage range finding developmental toxicity study, HC Red n° 3 was administered orally once daily to 40 presumed-pregnant Crl:CD® (SD)IGS BR VAF/Plus® from day 6 to day 20 of gestation at the doses of 0, 50, 200, 500 and 1000 mg/kg bw/d in 100% polyethylene glycol 400 (8 rats per dose). The dosage volume was 5 ml/kg bw.
Viabilities, clinical observations, body weights and feed consumption values were recorded.
All surviving rats were sacrificed on day 21. The gravid uterus was weighed and examined for gross external alterations and sex. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results

Maternal parameters:
All dams survived to GD 21.
Urine discoloration, purple or orange fur and purple, red or orange skin were observed in all of the groups treated with HC Red n° 3. A significant increase in the incidence of purple, red or orange perivaginal substance occurred in the 1000 mg/kg bw/dosage group. Purple or red perinasal substance was observed in on rat at the dose of 1000 mg/kg bw/d. Orange amniotic fluid, and purple, red or orange viscera were observed in the 1000 mg/kg bw/dosage group.
Mean body weights were significantly reduced in the rats treated at the dose of 200, 500 and 1000 mg/kg bw/d on GDs 6 to 9 as well as on gestation days 15 to 18. However the reductions were not dosage dependent. Mean body weight gains were comparable among the dosage groups throughout the dosage and gestation period. HC Red n° 3 did not affect mean body weights, gravid uterine weights or corrected maternal body weights at any dose.
No significant differences in food consumption were observed. No significant differences were observed in the number of corpora lutea in all treated rats when comparing with the control rats. No significant differences in the number of implantations or resorptions were observed in the treated rats when comparing with the control rats. Clinical signs were limited to discoloration of skin, urine and fur observed in all treated groups.

Foetal parameters
No significant differences in body weight were observed between treated and control animals. No significant differences the litter sizes were observed between treated and control animals. No significant differences in the number of live or resorbed foetuses were observed between treated and control animals. No differences in foetal abnormalities were observed between treated and control groups.

Conclusion
Based on the results of this range-finding study, the doses selected for the main study were 0, 50, 200 and 1000 mg/kg bw/d.

Ref.: 25

Prenatal developmental toxicity, main study

Guideline:	OECD 414 (2001)
Species/strain:	rat, Crl:CD® (SD)IGS BR VAF/Plus®
Group size:	100 (25 presumed-pregnant females per group)
Test substance:	HC Red n° 3
Batch:	L 35939
Purity:	97.9%
Vehicle:	100% polyethylene glycol 400
Dose levels:	0, 50, 200, and 1000 mg/kg bw
Dose volume:	5 ml/kg bw
Route:	oral, gavage
Administration:	once daily on Day 6 through Day 20
GLP statement:	in compliance
Study period:	October 2004

In this oral dosage developmental toxicity study, HC Red n° 3 was administered orally once daily to 100 presumed-pregnant Crl:CD® (SD)IGS BR VAF/Plus® female rats from day 6 to day 20 of gestation at the doses of 0, 50, 200 and 1000 mg/kg bw/d in 100% polyethylene glycol 400 (8 rats per dose). The dosage volume was 5 ml/kg bw.
Viabilities, clinical observations, body weights and feed consumption values were recorded.
All surviving rats were sacrificed on day 21. The gravid uterus was weighed and examined for gross external alterations and sex. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results

Maternal parameters
All dams survived to GD 21. One female rat in the 1000 mg/kg bw/d dosage group delivered one pup and was sacrificed on GD 21.
Urine discoloration, purple or orange fur and purple, red or orange skin were observed in all of the groups treated with HC Red n° 3. A significant increase in the incidence of purple, red or orange perivaginal substance occurred in the 1000 mg/kg bw/dosage group. Purple or red perinasal substance was observed in on rat at the dose of 1000 mg/kg bw/d. Orange amniotic fluid, and purple, red or orange viscera were observed in the 1000 mg/kg bw/dosage group.
Mean body weights were significantly reduced in the rats treated at the dose of 1000 mg/kg bw/d on GDs 6 to 9 and reduced throughout the dosage and gestation period. Corrected maternal body weights for the weight of the gravid uterus and body weight gains were significantly reduced in the 1000 mg/kg bw/d dosage group on GDs throughout the dosage and gestation period. Gravid uterine were comparable among the four dosage group.
Mean absolute and relative feed consumptions were significantly reduced in the rats treated at the dose of 1000 mg/kg bw/d on GDs 6 to 9 and 18 to 21 and reduced in the rats treated at the dose of 1000 mg/kg bw/d for the entire dosage and gestation periods.
No significant differences were observed in the number of corpora lutea in all treated rats when comparing with the control rats. No significant differences in the number of implantations were observed in the treated rats when comparing with the control rats.

Foetal parameters:
No significant differences in body weight were observed between treated and control animals. No significant differences the litter sizes were observed between treated and control animals. No significant differences in the number of live foetuses were observed between treated and control animals.
Reversible delays in ossification, i.e. reduction in the number of ossified hindlimbs metatarsals and phalanges were observed in the 1000 mg/kg bw/d dosage group and were considered to be related to the test substance. However these delays were minor in nature, not associated with decreased foetal body weights and are considered to be non-adverse variations.

Conclusion
Based on the results of this study, the maternal NOAEL was 200 mg/kg bw/d and the developmental NOAEL was 1000 mg/kg bw/d.

Ref.: 26

Comments
Since the delayed ossification observed in the foetus at 1000 mg/kg bw/d are considered to be due to maternal toxicity, the developmental NOAEL is 1000 mg/kg bw/d

Guideline:	/
Species/strain:	New Zealand white rabbits
Group size:	48 (12 female per sex and dose)
Test substance:	Hair dye formulation containing 0.02% HC Red n° 3.
Batch:	/
Purity:	/
Dose:	0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation
Route:	Oral, gavage
Exposure period:	once daily on Day 6 through Day 18
GLP:	not in compliance
Study period:	before 1975

In this oral dosage developmental toxicity study, a formulation containing 0.02% of HC Red n° 3 was administered orally once daily to 48 artificially inseminated New Zealand white rabbit females from day 6 to day 18 of gestation at the doses of 0, 19.5 and 97.5 mg/kg bw/d of the total formulation (12 females per dose). The dosage volume was 1 ml/kg bw.
All rabbits were sacrificed on day 30 of gestation. The gravid uterus was examined for gross external alterations and sex. Foetal observations were conducted.

Results
The number of pregnancies, maternal weight gains and mean values per pregnant female for numbers of corpora lutea, implantation and resorptions were measured but not reported. There was no evidence of a teratologic effect in any group. Foetal survival was not adversely affected by the dye formulation. No grossly abnormal foetuses and no soft tissues defects were seen. The principle findings of the skeletal examination were variations in the degree of ossification and in the number of ribs in this species. The distribution of these changes showed no relationship to treatment. Animals receiving the high dose excreted blue-brown coloured urine within an hour after dosing. Urine colour was normal the next day prior to dosing which indicates a rapid elimination.

Conclusion
Based on the results of this study, a maternal and foetal NOAEL of 97.5 mg test material/ kg bw/d corresponding to 19.5 µg/kg bw/d of HC Red n° 3 was proposed by the applicant.
Ref.: 28

Comment
The experiment did not conform to a guideline and was not performed according to GLP.
The purity and specifications of test article is not known. Different hair dyes were tested in this study. The concentration of HC Red n° 3 tested was too low compared to commercial formulations. The results are sparsely reported. No conclusions concerning long term toxic effects can therefore be made from this study.

Guideline:	/
Species/strain:	Virgin CFE-S rats
Group size:	120 (20 males – not treated with the formulation and 20 females per dose)
Test substance:	Hair dye formulation containing 0.02% HC Red n° 3.
Batch:	/
Purity:	/
Dose:	0, 1950 and 7800 ppm of hair dye formulation in the diet
Route:	oral diet
Exposure period:	once daily on Day 6 through Day 15
GLP:	not in compliance
Study period:	before 1975

In this oral dosage developmental toxicity study, a formulation containing 0.02% of HC Red n° 3 was administered in the diet once daily to 120 presumed-pregnant CFE-S female rats from day 6 to day 15 of gestation at the doses of 0, 1950 and 7800 ppm (20 rats per dose). The dosage volume was 1 ml/kg bw.
All surviving rats were sacrificed on day 19. The gravid uterus was weighed and examined for gross external alterations and foetuses were sexed. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results
No effects on body weight gains and food consumption were observed. No significant differences were observed in any of the parameters examined including numbers of implantation sites, live pups and early or late resorption per litter or in the number of

females with one or more resorption sites. No grossly abnormal pups were observed in the treated rats when comparing with the control rats except one pup at the high dosage group.
Ref.: 28

Comments
The experiment did not conform to a guideline and was not performed according to GLP.
The purity and specifications of test article is not known. Different hair dyes were tested in this study. The concentration of HC Red n° 3 tested was too low compared to commercial formulations. The results are sparsely reported. No conclusions concerning long term toxic effects can then be made from this study.

3.3.9. Toxicokinetics

No data submitted

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)

CALCULATION OF THE MARGIN OF SAFETY

HC Red n° 3

(non-oxidative conditions)

Absorption through the skin	**A (mean + SD)**	**=**	**1.58 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.92 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED) bw/d	**SAS x A x 0.001/60**	**=**	**0.015 mg/kg bw**
No observed adverse effect level (90-day, oral, mice)	**NOAEL**	**=**	**90 mg/kg bw/d**
50% bioavailability*		**=**	**45 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**3000**

(oxidative conditions)

Absorption through the skin	**A (mean + SD)**	**=**	**0.333 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.19mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED) bw/d	**SAS x A x 0.001/60**	**=**	**0.003 mg/kg bw**
No observed adverse effect level (90-day, oral, mice)	**NOAEL**	**=**	**90 mg/kg bw/d**
50% bioavailability*		**=**	**45 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**14000**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation

3.3.14. Discussion

Physico-chemical properties
HC Red n° 3 is used in semi-permanent hair dye formulations at a maximum concentration of 3.0%.
Chemical characterisation as well as purity and impurities of three batches of HC Red n° 3 is provided, while the characterisation and purity of several other test batches is not described. In addition, the test materials were not identified in some cases. The Log Pow strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log Pow, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.
The water solubility of HC Red n° 3 is not determined by EC method A.6.
HC Red n° 3 was stable protected from light at room temperature for 65 days.

HC Red n° 3 is a secondary amine, and thus it is prone to nitrosation. It should not be used together with nitrosating agents. The nitrosamine content should be <50 ppb.
HC Red n° 3 was shown to be stable up to 30 min (study period) in a hair dye formulation after mixing with peroxide based developer (1:1).

Toxicity
Based on two acute oral toxicity studies in rats, HC Red n°3 is considered as having a low acute oral toxicity.
In a 14-day gavage rat and mice studies, doses up to 1000 mg/kg bw/d and 500 mg/kg bw/d respectively caused no toxic effects.
After 90 days of exposure by oral route in rats and mice, a slight decrease in body weights of male rats and mice was observed. A pigmentation of epithelial cells in the thyroid gland and in the kidney of rats was also observed but not considered as an adverse effect.
NOAELs of 250 mg/kg bw/d in rats and 125 mg/kg bw/d in mice (adjusted to 180 and 90 mg/kg bw/d, respectively for 5 days/week treatment) may be derived from this 90 days oral toxicity study.
The provided long term toxicity studies by dermal routes with hair dye formulations did not follow OECD guidelines and were not performed under GLP conditions; they are not considered appropriate to derive a NOAEL for HC Red n° 3.
In oral developmental toxicity studies performed in rats, a decrease in body weight and food consumption of female rats was observed at 1000 mg/kg bw/d. On foetuses, the only toxic effect related to HC Red n° 3 observed was a reduction in the numbers of ossified hindlimbs metatarsals and phalanges in the 1000 mg/kg bw/d dosage group, which was considered to be due to maternal toxicity. Based on the results of this study, a maternal NOAEL of 200 mg/kg bw/d and a developmental NOAEL of 1000 mg/kg bw/d were derived.
A two-generation toxicity study with oral administration of a hair dye formulation was provided but not considered suitable for safety evaluation.

Skin/eye irritation and sensitisation
None of the skin irritation experiments followed a guideline and the specifications of the test substance were not described. Curiously, the 3% aqueous solution caused some irritation in one experiment while the slurry caused no irritation to rabbit skin. A 3% preparation in 'Schultz Vehicle' caused no irritation.
None of the mucous membrane irritation experiments followed a guideline and the specifications of the test substance were not described. Both the neat and a 3% aqueous solution caused irritation to rabbit eyes.
In the Kligman-Magnusson Maximisation test, the test substance was an extreme skin sensitiser. 3% test substance was not a skin sensitiser under the condition of this Buehler test.

Local Lymph Node Assay: In a first experiment, the concentrations used were too low and a sensitizing potential cannot be excluded. In a second Local Lymph Node Assay, no EC3 value was calculated by the study authors, but an EC3 of 2.2 can be derived. The concentrations tested were too low. HC Red n° 3 is a moderate skin sensitiser in the LLNA.
Overall, HC Red n° 3 is a skin sensitiser, being an extreme contact sensitiser in a GPMT and moderate sensitiser in a LLNA.
Three human repeat insult patch tests have been performed (only a brief summary is available for the first experiment). In the three tests HC Red n°3 was non-irritating and non-sensitising. Such experiments are not considered ethical by the SCCS.

Percutaneous absorption
In an in vitro dermal absorption assay with human skin, 5 donors were used with at least 2 cells from a donor. The absorption of a HC Red n° 3 from a non-oxidative hair dye

formulation was 1.05 (+ 0.53) µg/cm² or 0.17 (+ 0.09) % of the applied dose. A figure of 1.58 µg/cm² (mean + 1SD) was used for calculating the MOS.
Under oxidative conditions the amount of HC Red No 3 in a typical hair dye formulation containing 0.45% HC Red No 3 on hair, the amount considered as absorbed is (mean +1SD) 0.365% or 0.333 µg/cm^2 which was used for calculating the MOS.

Mutagenicity/genotoxicity
Overall, the genotoxicity of HC Red n° 3 is sufficiently investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. HC Red n° 3 treatment resulted in the induction of gene mutations in bacteria. A significant and dose related increase in the number of revertant colonies was induced in a recent test in strain TA98 both without and with S9-mix and in strain TA1537 without S9-mix only. Four older gene mutation tests in bacteria confirm this positive result. In the gene mutation assay in mammalian cells at the *tk*-locus of mouse lymphoma cells, this mutagenic result could not be confirmed as an induction of gene mutations was not found. In a recently performed *in vitro* chromosome aberration test, HC Red n° 3 did not induce an increase in the number of cells with chromosomal aberrations, whereas an older test was positive. Since the latter test was not performed according to the OECD guideline and batch number and purity were not reported, this test has only limited value. In an *in vitro* UDS test, unscheduled synthesis was not observed.
HC Red n° 3 did not induce gene mutations at the *cII*-locus in the liver and bladder of Big Blue® heterozygous *lacI/cII* mice overruling the positive result from the *in vitro* gene mutation assay in bacteria and confirming the negative result found in an *in vitro* gene mutation assay in mammalian cells. The negative Comet assay also indicated the absence of a mutagenic potential of HC Red n° 3. The clastogenicity found under *in vitro* conditions was overruled by 2 negative *in vivo* micronucleus tests and a Comet assay measuring chromosomal breaks in liver, urinary bladder and blood, although target cell exposure was exclusively demonstrated in the oldest micronucleus test (ref. 23). In the other tests, the doses used caused in the gene mutation test with transgenic mice (ref. 24) red stained beddings during the treatment period which is considered evidence of systemic availability. Moreover, the doses were also considerably higher then those used in an NTP rodent carcinogenicity assay in which pigmentation of epithelial cells of the thyroid gland and kidney were observed. The results of a heritable translocation assay in rats were not suitable to conclude on the potential of HC Red n° 3 to induce heritable chromosomal mutations.
As positive effects found *in vitro* were not confirmed in *in vivo* tests, HC Red n° 3 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
The carcinogenicity of HC Red n° 3 has been studied under the US National Toxicology Program in rats and mice after oral gavage administration for 2 years. No evidence of carcinogenicity was found in rats. There was equivocal evidence of carcinogenicity for male mice as indicated by an increased incidence of hepatocellular adenomas or carcinomas (combined) in the high dose group. Poor survival coupled with lack of significant findings rendered the study in female mice an inadequate study of carcinogenicity. Both sexes and species may have been able to tolerate higher doses of HC Red n° 3. Therefore, the sensitivity of these studies for detecting carcinogenesis may have been limited.
No conclusions concerning potential carcinogenic effects can be made from an oral study with dogs and a skin painting study with mice. HC Red n° 3 was present in low concentrations (0.3% or less) in semi-permanent hair dye formulations. Moreover, although substances classified as carcinogens were present in the formulations studied, no carcinogenic effects were found in any of the studies indication low sensitivity. HC Red n°3 induced transformation in the BALB/C-3T3 transformation assay, but not in the Syrian Hamster Embryo Cell (SHE) Assay.

4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of HC Red n° 3 in oxidative hair dye formulations at a maximum on-head concentration of 0.45% and in non-oxidative (see SCCS/1293/10) hair dye formulations at a maximum on-head concentration of 3.0% does not pose a risk to the health of the consumer, apart from its sensitisation potential.

HC Red n° 3 is a skin sensitiser, being an extreme contact sensitiser in a GPMT and a moderate sensitiser in a LLNA.

HC Red n° 3 is a secondary amine, and thus prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

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