EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Yellow n° 2

COLIPA n° B41

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

The SCCS adopted this opinion at its 8th plenary meeting

of 21 September 2010

About the Scientific Committees

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

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation 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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1. BACKGROUND

Submission I for HC Yellow n° 2 with the chemical name 2-Nitro-N-(2-hydroxyethyl)aniline was submitted in May 1983 by COLIPA [1] according to COLIPA.

Submission II was submitted in July 2005 by COLIPA. According to this submission HC Yellow n° 2 is used both as an ingredient alone and as a component in non-oxidative hair dye formulations at a maximum concentration of 1.0%.

An additional submission III was submitted in May 2008 applying for extension of the scope for this substance in order to allow it to be used in oxidative hair dyes with a concentration up to 0.75% on the scalp.

2. TERMS OF REFERENCE

1. *Does SCCS consider HC Yellow n° 2 safe for use as an ingredient in oxidative hair dye formulations with a maximum concentration of up to 0.75% on the scalp taken into account the scientific data provided?*

2. *Does SCCS consider HC Yellow n° 2 safe for use as a non-oxidative hair dye with a maximum concentration of 1.0% in the finished cosmetic product taken into account the scientific data provided?*

3. *And/or does the SCCS have any further scientific concerns with regard to the use of HC Yellow n° 2 in hair dye formulations?*

[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 Yellow n° 2 (INCI name)

3.1.1.2. Chemical names

2-Nitro-N-(2-hydroxyethyl) aniline
Ethanol, 2-(o-nitroanilino)-
N-(2-Hydroxyethyl)-2-nitroaniline
Ethanol, 2-[(2-nitrophenyl) amino
2-[(2-Nitrophenyl) amino] ethanol

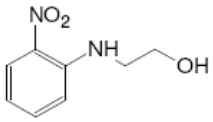
3.1.1.3. Trade names and abbreviations

Colorex HCY2
Covariane Jaune W 1122
Jarocol Yellow 2
Velsol Yellow 2

3.1.1.4. CAS / EC number

CAS: 4926-55-0
EC: 225-555-8

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_8H_{10}O_3N_2$

3.1.2. Physical form

Fine orange powder

3.1.3. Molecular weight

Molecular weight: 182.179 g/mol

3.1.4. Purity, composition and substance codes

GTS03975 Lot #17 was analysed by HPLC-UV, NMR, FT-IR, GC-TEA, CHN, thermogravimetric analysis, melting point and residue of ignition for purity and identity. An aliquot of this lot was used as a reference standard for further HPLC analysis.

Purity:

- HPLC/UV
  Purity of HC Yellow n° 2 was determined by HPLC-UV (254 nm). The peak area of the GTS03975 peak was compared to the total peak area of the chromatogram. The purity was determined to 99.9% at the beginning and at the end of the study. The content of o-chloronitrobenzene, a suspected impurity, was 61 ppm according to an appropriate reference standard.
- GC-TEA
  GC-TEA (Thermal Energy Analyzer) was used for analysis of N-Nitrosodiethanolamine (NDELA) content according to method MP-NIDY-MA. NDELA Lot 316-41C was used as reference standard. The limit of detection was 20 ppb.

Identity:

- 1H-NMR spectrum of GS03975 was in accordance with the structure.
- -FT-IR spectrum of GS03975 was consistent with the structure.
- CHN analysis of GTS03975 showed mean carbon, hydrogen and nitrogen contents of 52.6%, 5.59% and 15.37%. Those results are consistent with the structure (C = 52.74%, H = 5.53% and N = 15.38%).

Analysis	Mean Results
Purity	99.88%
o-chloronitrobenzene	61 ppm
Carbon	52.6%
Hydrogen	5.59%
Nitrogen	15.37%
Residue on Ignition	< 0.10%
Water Content	0.733%
NDELA by GC/TEA	< 20.0 ppb (LOD)

Ref.: 1a

[^{14}C]-HC Yellow n° 2, lot R06-099-054, radiochemical purity 99.9% determined by HPLC (no further information available).

Ref.: 10

Comment

- There is no information on the purity of B41 used for several studies. Especially not for Lot C1R2004005.62, for which stability data were provided.
- No chromatograms or raw data were provided for NDELA analysis.

3.1.5. Impurities / accompanying contaminants

See point 3.1.4. Purity, composition and substance codes

3.1.6. Solubility

Solubility*: Water 0.6-0.9 mg/ml
Ethanol 202-304 mg/ml
DMSO > 796 mg/ml

* solubility measured after sonication for 15 minutes.

Ref.: 33

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: no data supplied

3.1.8. Additional physical and chemical specifications

Melting point:	73.3 °C (ref. 1a)
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	/

3.1.9. Homogeneity and Stability

PEG 400

Formulations of HC Yellow n° 2 in PEG were analysed for homogeneity and stability at 0.29 and 213 mg/ml by HPLC-UV at 254 nm. For homogeneity testing samples of top, middle and bottom of the formulation were analysed. Mean values of each concentration were within 0.4 and 0.3% RSD. Stability tests were performed immediately after preparation, after storage at room temperature for 24 h and after refrigeration for 5, 8 and 15 days. All concentrations were within +-5% of the nominal concentrations.

Ref.: 1b, 12

DMSO

Formulations of HC Yellow n° 2 in DMSO were analysed for stability at 0.05 mg/ml, 100 mg/ml and 500 mg/ml by HPLC-UV at 254 nm. Stability tests were performed immediately after preparation in quadruplicate and after refrigeration for 12/14 days in duplicate. All concentrations were within +-7% of the nominal concentrations.

Ref.: 1b

Oxidative conditions

A formulation of HC Yellow n° 2 in alkaline solution was mixed 1:1 with H_2O_2 lotion (6% H2O2). The mixture was analysed for stability by HPLC-UV (TOSHO TSKgel ODS-80Ts column) at 430 nm. The mixture is analysed directly after preparation and after storage for 60 min. The chromatograms showed that it is stable under oxidative conditions.

Ref.: 25

Saline solution:

Lot C1R2004005.62 HPLC-UV in saline (0.9% sodium chloride)

Stability of formulations of Lot C1R2004005.62 in saline was tested at two concentration levels (1503 and 4 ng/mL) in triplicate analysis by HPLC-UV. The following storing conditions were examined:

- at room temperature for 24h
- one freezing and thawing to room temperature
- 24h at room temperature, one freeze-thaw cycle after 1 day and 4 days at room temperature

Lot C1R2004005.62 was stable in saline according to the previously described stability test within 90-110% of the nominal value.

Ref.: Analytical data

Comment
- No information on the stability and homogeneity in acacia (ref. 2) is available
- No stability data for Lot 2041 in DMSO is available

General Comments to physico-chemical characterisation

- The stability in typical hair dye formulations was not reported.
- HC Yellow n° 2 is a secondary amine, and thus is prone to nitrosation and formation of nitrosamines. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

3.2. Function and uses

HC Yellow n° 2 is used up to 1% in non-oxidative hair dye-formulation.

HC Yellow n° 2, a non reactive dye, is used up to on head concentration of 0.75% in oxidative formulation. HC Yellow n° 2 is shown to be stable under conditions used in oxidative formulations and does not take part in the oxidative colouring forming mechanism.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	/
Species/strain:	Rats, Sprague-Dawley
Group size:	5 males and 5 females per dose group
Test substance:	HC Yellow n° 2
Batch:	/
Purity:	unknown
Dose:	625, 1250, and 2500 mg/kg bw in 3% acacia
Route:	Oral by gavage
Exposure:	single oral dosing
GLP:	/
Date:	February 1987

A 10% suspension of the test substance in 3% acacia was administered once via oral gavage to groups of 5 male and 5 female rats at the dose levels 625, 1250, and 2500 mg/kg bw. At the low dose no mortalities were observed, at the mid dose no deaths were seen in the males while 3 of 5 females died within 8 h after dosing. At the high dose all animals died.

Ref.: 2

Comment
The study did not follow modern standards. No data on the test substance were provided.

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

Guideline: /
Species/strain: New Zealand white rabbits
Group size: 6
Test substance: HC Yellow n° 2
Batch: /
Purity: /
Dose: 500 mg, as aqueous slurry non-occluded
Observ. period: 72 hours
GLP: /
Study period: 1987

Five hundred milligrams of HC Yellow n° 2 were applied as aqueous slurry to the non-occluded, intact skin of the backs of six New Zealand White rabbits and left in contact for 24 hours. The sites were scored for dermal irritation at 24 and 72 hours post test material application according to the Draize Method.

Results
No apparent dermal irritation was produced by HC Yellow n° 2.

Ref.: 5

Comment
Study was not performed according to a guideline and not in compliance with GLP.

3.3.2.2. Mucous membrane irritation

Guideline: /
Species/strain: New Zealand white rabbits
Group size: 4+4 (two experiments)
Test substance: HC Yellow n° 2
Batch: /
Purity: /
Dose: 0.1g neat substance (experiment 1), 0.1 ml of a 10% suspension in 3% acacia
Observ. period: 3 days
GLP: /
Study period: 1987

Two ocular irritation studies were conducted. In the first test, one hundred milligrams of HC Yellow n° 2 were instilled in the conjunctival sac of the left eye of four New Zealand White rabbits. The right eye was untreated. In the second test, 0.1 ml of a 10% suspension of HC Yellow n° 2 in 3% acacia was instilled by syringe into the conjunctival sac of the right eye of four rabbits of the same strain. The left eye was untreated. In both studies, the eyes of one-half of the rabbits were rinsed with 20 ml distilled water 20 seconds after treatment. All

eyes were examined and scored 1 hour and 1, 2 and 3 days post test material instillation according to the Draize Method.
In the first study, HC Yellow n° 2, administered as a powder, elicited conjunctival redness, swelling, and discharge in the eyes of all animals when examined 1 hour post treatment. Low grade corneal opacity with ulcerations was noted in 3 of 4 rabbits at day 1; one animal exhibited irritation of the iris. By Day 2, most conjunctival effects were reduced or absent and the corneal opacity of 1 rabbit cleared. The single incidence of iritis was also absent. No signs of irritation were apparent in any animal 3 days after test material instillation. Overall, the eye rinsed with distilled water after treatment appeared to be less irritated than those not rinsed.
In the second study, HC Yellow n° 2, administered as a 10% aqueous suspension, elicited slight conjunctival responses in the eyes of all animals (rinsed and unrinsed) when examined 1 hour after instillation. Most effects were absent by Day 1 and all eyes were clear 2 days after treatment.

Conclusions
B41 applied directly to the rabbit eye is irritant and diluted at 10% is slightly irritant.
Ref.: 3, 4

Comment
The studies were not performed according to a guideline and not in compliance with GLP. Submission III (2008) contained no new data on eye irritation, only a discussion of the previous tests with the neat and diluted (10% suspension) substance.

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline: /
Species/strain: CBA/CaJ female mice
Group size: 5 animals/group
Test substance: HC Yellow n° 2
Batch: /
Purity: /
Vehicle: DMSO
Concentration: 0, 0.25, 0.5, 1.0, 2.0% w/v
Positive control: p-phenylenediamine
GLP: /
Study period: 1999-2000

25 µL test preparation applied to dorsal aspect of ear lobe daily for 3 days. 5 days after the first application, 250 µL of a solution containing 20 µCi/mL ^{3}H-thymidine was injected intravenously. 5 hours later the animals were euthanized and the draining auricular lymph nodes were removed. Incorporation of ^{3}H-thymidine was measured by β-scintillation counting.

Concentration	Stimulation Index
Test item	
0.25%	1.19
0.5%	0.79
1.0%	1.03
2.0%	1.28
p-phenylenediamine	
0.25%	1.35
0.5%	1.59
1%	5.06
2%	13.79

Results

The compound is not positive (>3) at any concentration and it is not sensitizer in this test.
Ref.: 7

Comment
The concentrations used are too low and a sensitising potential cannot be excluded. The study was not performed according a guideline and not in compliance with GLP.

Human occlusive repeated Patch test

Guideline:	/
Species/strain:	Women (18-65 years old)
Group size:	104
Test substance:	HC Yellow n° 2
Batch:	/
Purity:	/
Vehicle:	12% isopropanol, 2% Tween-80, 2% Natrosol, 0.05% sodium sulfite, water
Concentration:	3% in vehicle
Positive control:	
GLP:	/
Study period:	November 1984

The entire study extended over a six weeks period with three phases: induction, rest and challenge.
The induction phase consisted of 9 consecutive applications of the test material. The patches were removed 24 hours after application and evaluated each 48 hours.
The challenge phase was initiated during the sixth week of the study, with identical patches applied to sites on the opposite arm previously unexposed to the test material. Patches were removed after 24 hours. The sites were grades 24 and 48 hours after removal.

Results
One panellist developed reactions during induction indicative of possible pre-sensitization. This individual was challenged on the back and forearm. She developed positive reactions to the test material and to the vehicle. These responses were interpreted as evidence of pre-sensitization probably to the vehicle

Conclusion
There was no evidence of sensitisation to HC Yellow 2 under the conditions employed in this study.
Ref.: 8

Comment
The SCCS considers HRIPT studies as unethical.

Human occlusive repeated Patch test

Guideline:	/
Species/strain:	Men and women (18-79 years old)
Group size:	98
Test substance:	HC Yellow n° 2
Batch:	/
Purity:	/
Vehicle:	12% isopropanol, 2% Tween-80, 2% Natrosol, 0.05% sodium sulfite, water
Concentration:	3% in vehicle

Positive control:
GLP: /
Study period: June 1984

The entire study extended over a six weeks period with three phases: induction, rest and challenge.
The induction phase consisted of 9 consecutive applications of the test material. The patches were removed 24 hours after application and evaluated each 48 hours.
The challenge phase was initiated during the sixth week of the study, with identical patches applied to sites on the opposite arm previously unexposed to the test material. Patches were removed after 24 hours. The sites were grades 24 and 48 hours after removal.

Results
Two subjects developed reactions on challenge and were re-challenged. The results indicate irritation but not sensitization.

Conclusion
There was no evidence of sensitisation to HC Yellow 2 under the conditions employed in this study.

Ref.: 9

Comment
The SCCS considers HRIPT studies as unethical.

Guinea pig sensitisation, Magnusson–Kligman Maximisation test

Guideline:	/
Species/strain:	Female Hartley albino guinea pigs
Group size:	10 animals/group
Test substance:	HC Yellow n° 2
Batch:	423126
Purity:	/
Vehicle:	propylene glycol
Induction:	site 2: 0.1% (w/v) in water site 3: 0.1% (w/v) RM148 in water with Complete Freund's adjuvant (1:1)
Topical:	25% in propylene glycol
Challenge:	Topical application of 3% in propylene glycol /
Positive control:	/
GLP:	/
Study period:	1979

Induction
The induction was performed by three pairs of intradermal injections made simultaneously with Freund complete adjuvant 1:1 in water, (0.05 ml) of 0.1% solution of hair dye in water and in a 0.1% solution of hair dye in Freund's adjuvant.

Topical patch
One week after the induction, topical application (48 hours under occlusive patch) of a 25% solution of the dyestuff in propylene glycol on the shaved interscapular area.

Challenge
Two weeks later the animals were challenged by a single topical application of the 3% solution of dyestuff in propylene glycol in the shaved flank.

Reading times: 24, 48 and 72 hours post patch removal. Evaluation of skin reaction (erythema and oedema) was on a scale of 1-4.

Results
No sensitization was observed.

Ref.: 6

Comments
The study is not in compliance to GLP and not performed according to the guidelines.

3.3.4. Dermal / percutaneous absorption

Human skin, non-oxidative conditions

Guideline:	OECD 428
Test substance:	HC Yellow n° 2 [^{14}C]-HC Yellow n° 2; 54 mCi/mmol; 1.998 GBq/mmol
Batch:	I06570 Y13170/002 (radiolabelled)
Purity:	101.3% 99.9% (HPLC) (radiolabelled)
Tissue:	dermatomed human skin, 400 µm thick
Skin integrity:	electrical resistance, > 10 kΩ
Method:	glass diffusion cells (2.54 cm²), 12 membranes from 5 subjects
Receptor fluid:	4% polyoxyethylene-20-oleyl ether solution in phosphate buffered saline (PBS/A)
Solubility in the receptor:	> 0.1 mg/ml
Formulation tested:	hair dye cream formulation containing 1% HC Yellow n° 2
Blank formulation:	Loving Care Tint
Dose formulation applied:	20 mg/cm²
Duration of the contact:	30 minutes
Duration of the diffusion:	48 hours
Analytical method:	liquid scintillation counting
GLP:	in compliance
Study period:	24 November – 15 December 2004

The penetration and distribution of HC Yellow n° 2 from a nominal 1% w/w formulation, has been measured *in vitro* through human skin, following the incorporation of [^{14}C]-HC Yellow n° 2. The mixed formulation was applied to 12 human dermatomed skin membranes (nominally 400 µm thick), mounted in glass diffusion cells, at a nominal rate of 20mg/cm^2. After a contact period of 30 minutes, the dose was washed from the surface of the skin using natural sponges soaked in 3% Teepol.

Results

Test Compartment	Amount Recovered (µg/cm²)															
	Cell 30	Cell 31	Cell 32	Cell 33	Cell 34	Cell 35	Cell 36	Cell 37	Cell 38	Cell 41	Cell 42	Cell 45	Mean	SD	SEM	n
Flange	0.040	0.076	0.062	0.086	0.109	0.151	0.024	0.036	0.048	0.075	0.044	0.036	0.066	0.037	0.011	12
Donor Chamber	0.012	0.016	0.012	0.257	0.058	0.040	0.068	0.087	0.032	0.131	0.037	0.135	0.074	0.072	0.021	12
Skin Wash @ 0.5h	180	204	194	209	190	195	200	198	190	197	193	189	195	7.54	2.18	12
Skin Wash @ 48h	0.664	0.717	0.665	0.81	1.76	1.83	0.257	0.330	0.501	0.669	0.609	0.801	0.800	0.493	0.142	12
Stratum Corneum	0.072	0.108	0.185	0.100	0.782	0.414	0.068	0.113	0.131	0.180	0.072	0.131	0.196	0.207	0.060	12
Remaining Epidermis/Dermis	0.194	0.153	0.088	0.179	0.215	0.277	0.075	0.098	0.050	0.296	0.201	0.127	0.163	0.079	0.023	12
Receptor Fluid	6.72	4.36	2.99	5.04	3.47	3.39	2.55	3.79	3.66	2.96	2.59	4.80	3.86	1.21	0.348	12
Systemically Available*	**6.91**	**4.51**	**3.08**	**5.22**	**3.69**	**3.67**	**2.62**	**3.89**	**3.71**	**3.25**	**2.79**	**4.92**	**4.02**	**1.29**	**0.371**	**12**
TOTAL	188	210	198	215	197	201	203	203	195	202	197	195	200	6.93	2.00	12

Test Compartment	Percent of Dose Recovered (%)															
	Cell 30	Cell 31	Cell 32	Cell 33	Cell 34	Cell 35	Cell 36	Cell 37	Cell 38	Cell 41	Cell 42	Cell 45	Mean	SD	SEM	n
Flange	0.020	0.038	0.031	0.043	0.055	0.076	0.012	0.018	0.024	0.038	0.022	0.018	0.033	0.018	0.005	12
Donor Chamber	0.006	0.008	0.006	0.129	0.029	0.020	0.034	0.044	0.016	0.066	0.019	0.068	0.037	0.036	0.010	12
Skin Wash @ 0.5h	90.3	102	97.1	105	95.2	97.7	100	99.3	95.3	98.8	96.7	94.8	97.7	3.78	1.09	12
Skin Wash @ 48h	0.333	0.359	0.333	0.405	0.880	0.915	0.129	0.165	0.251	0.335	0.305	0.401	0.401	0.247	0.071	12
Stratum Corneum	0.036	0.054	0.093	0.050	0.392	0.207	0.034	0.056	0.066	0.090	0.036	0.066	0.098	0.104	0.030	12
Remaining Epidermis/Dermis	0.097	0.077	0.044	0.090	0.108	0.139	0.038	0.049	0.025	0.148	0.101	0.063	0.082	0.039	0.011	12
Receptor Fluid	3.36	2.18	1.50	2.52	1.74	1.70	1.28	1.90	1.83	1.48	1.30	2.40	1.93	0.604	0.174	12
Systemically Available *	**3.46**	**2.26**	**1.54**	**2.61**	**1.85**	**1.84**	**1.31**	**1.95**	**1.86**	**1.63**	**1.40**	**2.47**	**2.01**	**0.644**	**0.186**	**12**
TOTAL	100	103	99.5	102	102	99.8	97.3	103	99.9	106	91.0	98.7	100	3.46	0.997	12

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

The results obtained in this study indicate that the penetration of HC Yellow n° 2 through human skin is very slow. Under non-oxidative conditions the dermal absorption of HC Yellow n° 2 was 4.02 ± 1.29 µg/cm² (2.01 ± 0.64%).

Ref.: 10

Comment
The mean + 1 SD equals 5.31 (4.02 + 1.29) µg/cm² can be used to calculate the MOS under non-oxidative conditions.

Pig skin, oxidative conditions

Guideline:	OECD 428
Test substance:	HC Yellow n° 2
Batch:	C1R2004005.62
Purity:	not indicated by the sponsor
Tissue:	ear porcine skin
Skin integrity:	by conductivity. Intact skin has a conductivity of <900 µS
Method:	glass Static diffusion chambers
Receptor fluid:	Saline (0.9% NaCl)
Formulation tested:	alkaline cream emulsion
Dose formulation applied:	20 µL/cm² (corresponding to 150 µg/cm² a.i.)
Concentration of ingredient:	1.5% (0.75% after mixing with H_2O_2)
Replicate cells:	2 experiments (6 cells/experiment) (only 10 cells used for calculation) 1 cell/donor
Duration of the contact:	30 min
Duration of the diffusion:	24 hours
Analytical method:	HPLC and UV/VIS detection
Validation:	Limit of detection about 1.8 ng/mL
Solubility in the receptor:	up to 2 µg/mL.
GLP:	in compliance
Study period:	2007

Porcine ear obtained from the slaughter house immediately after slaughter and before steam cleaning was used for this experiment. The outer ear region was washed, carefully shaved and the skin was removed by dissection. Thickness of the dissected skin was

approximately 300-400 µm. The surface of the skin which was in contact with the test substance during permeation-assay was 1 cm².

Test procedure
Two independent experiments were performed on fresh pig ear skin to mimic realistic use condition:
Experiment 1+2: A standard hair dye formulation containing 1.5% HC Yellow n° 2 (C1/R/2004005/62) was mixed with the Developer Lotion 6% (1/1) before application to the skin. The skin was mounted in static glass diffusion chamber with diameter of 1.135 cm. In each experiment the test item was analyzed in 6 replicates. Per diffusion cell the ear of one animal was used. Each donor chamber was filled with 20 µL of the test item prepared as described above.
Saline (0.9% NaCl), was used as receptor solution. Solubility and stability of HC Yellow n° 2 in PBS was shown in the implementation study of the analytical method (RCC_2000801). Buffer solution of the acceptor chamber was collected in plastic vials which were replaced according to the sampling times and stored at – 20 °C. The whole test system was set up in an incubator adjusted to 32 °C. After 30 min of incubation the test item was removed from skin with deionised water and shampoo solution. Following the washing procedure the donor chambers were filled with 1ml of Saline to determine the impedance of the skin.
The stratum corneum was separated from the living skin by tape strip method (2x5 Tesa film pieces) since the amount of the dye found in the upper skin is not considered to be bioavailable. The remaining skin membranes after stripping were extracted and determined by HPLC and expected as penetrated respectively absorbed.

Results
HC Yellow n° 2 was detected in all samples relevant for dermal absorption, i.e. in the skin extracts and in the receptor fluid samples after 24 hours. The limit of detection (LOD) under the conditions reported is about 1.8 ng/ml.
The amount of penetrated test item found in the receptor fluid plus that found in the skin extract are considered as penetrated respectively absorbed.
The mean recovery of the test item was 98.2% in the first and 100.4% in the second experiment.

Amount of HC Yellow n° 2 (C.I. 4926-55-0) in	**Expressed as µg/cm² of skin surface mean ± S.D. (n = 6)**			**Expressed as % of dose mean ± S.D. (n = 6)**		
Receptor fluid	5.85	±	2.19	4.55	±	1.69
Stratum corneum (isolated by tape stripping)	0.0354	±	0.0202	0.0276	±	0.0156
Epidermis + Upper dermis (after 24 hours)	0.210	±	0.086	0.1640	±	0.0699
Washing solution (after 30 minutes)	113	±	8.31	87.6	±	5.82
Total balance (recovery)	119	±	8.40	92.4	±	5.93
Dermal absorption	6.06	±	2.21	4.71	±	1.70

Under the reported conditions, the dermal absorption of HC Yellow n° 2 is 6.06 ± 2.21 µg/cm² or 4.71 ± 1.7% (mean value of 10 diffusion cells (10 donors)). For the calculation of the MOS in oxidative conditions, 6.06 + 2.21 equals 8.27 µg/cm² (mean + 1 SD) will be used.

Ref.: 23

Comment

The dermal absorption rate under oxidative conditions was slightly higher than under non-oxidative conditions, even though a lower concentration was applied. However, skin from different species was used in both tests.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral / dermal / inhalation toxicity

No data submitted

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

14 day dose range finding study

Guideline:	/
Species/strain:	Rats, Sprague-Dawley, Crl:CD®(SD)IGS BR
Group size:	10/group (5 males and 5 females)
Test substance:	HC Yellow n° 2
Batch:	GTS03975 commercial material, lot code #17
Purity:	99.9%
Dose:	0, 50, 150, 300, 600 mg/kg bw/d in PEG 400
Route:	oral gavage
Exposure:	once a day for 14 days
GLP:	in compliance
Date:	May 2005

The test article and vehicle were administered once daily, by oral gavage, for 14 consecutive days. General health/mortality and moribundity checks were performed twice daily, in the morning and afternoon. Detailed clinical observations were performed on day -1, prior to dosing on days 0, 3, 7, 10 and 13, and on the day of scheduled euthanasia. Cage-side observations were performed daily approximately 30 to 90 minutes after dose administration. Individual body weights were recorded prior to in-life initiation on day -1 and on days 0, 3, 7, 10 and 13. A final body weight was obtained prior to scheduled euthanasia. Food consumption was measured on days 0, 3, 7, 10 and 13. Blood and urine samples were collected from all animals on the day of scheduled euthanasia for evaluation of selected clinical pathology parameters. All animals were subjected to a complete gross necropsy examination upon death or scheduled euthanasia. Fresh organ weights were obtained for each surviving animal and selected tissues/organs were retained from all animals for possible future histopathological examination.

Results
There were no treatment-related mortalities noted during this study. One control male was found dead on study day 2. However, gross necropsy revealed that this death was due to mechanical injury. All remaining animals survived to scheduled euthanasia. Test article-related clinical signs included abnormal coloured dark yellow or orange urine and yellow or orange staining of the tail and hair coat. There were no statistically significant or toxicologically meaningful differences in mean body weights, body weight changes, food consumption or organ weight data. There were statistically significant changes in a few clinical chemistry and haematology parameters; however, these changes were not considered to be toxicologically meaningful. Gross changes observed at necropsy were unremarkable.

Conclusion
Based on the results of this study, oral administration of HC Yellow n° 2 in the rat for 14 consecutive days at up to 600 mg/kg bw/day did not produce any treatment-related mortality or clinical signs of toxicity. In addition, there were no remarkable changes in the

clinical pathology data, gross necropsy data, or organ weight data to suggest a treatment-related effect.

Ref.: 11

Main study

Guideline:	OECD 408
Species/strain:	Rats, Sprague-Dawley, Crl:CD®(SD)IGS BR
Group size:	30/group (15 males and 15 females); 5/sex recovery animals (control and high dose group)
Test substance:	HC Yellow n° 2
Batch:	GTS03975 commercial material, lot code #17
Purity:	99.9%
Dose:	0, 5, 20, 50 mg/kg bw/d in PEG 400
Route:	oral gavage
Exposure:	once a day for 91 days
GLP:	in compliance
Date:	May 2005

Dosage levels were selected based on the results of a 14-day range-finding study. The test article and vehicle control material were administered once daily, by oral gavage, for 91 consecutive days. Control animals received the vehicle control material at a dosage volume comparable to that received by the test animals. The last five animals in the control group and the high dose group remained on study following the dosing phase for a 28-day recovery phase. General health/mortality and moribundity checks were performed twice daily, in the morning and afternoon. Cage-side observations were performed on all animals approximately 30 to 90 minutes after dosing. Detailed clinical observations were performed weekly on all animals. Functional observation battery (FOB) observations were conducted on 10 rats/sex/group on days -1, 26, 40 and 89. Individual body weights and food consumption were recorded weekly. Ophthalmological examinations were performed on all animals once prior to treatment (days -6/-7) and on days 84 (females) and 85 (males). Recovery animals received ophthalmological examinations on days 114 (females) and 115 (males). Blood and urine samples were collected from all animals for analysis of selected clinical pathology parameters on the day of scheduled euthanasia. All animals were subjected to a complete gross necropsy examination upon death or scheduled euthanasia. Sperm was collected from each male rat following euthanasia for assessment of sperm enumeration, motility and morphology. Fresh organ weights were obtained at scheduled euthanasia from selected organs. A standardized list of tissues were obtained and preserved in 10% neutral buffered formalin for histopathological examination.

Results
Oral administration of the test article preparation was not associated with any test-article related mortalities. In addition, test article treatment did not produce toxicologically meaningful clinical abnormalities, neurological changes, variations in oestrous cyclicity, body weight effects or food consumption changes during this study. There were a variety of statistically significant changes in the female clinical chemistry parameters examined on this study at the end of the treatment phase. These included decreased chloride and GGT (5 mg/kg bw group), decreased creatinine and increased potassium (5 and 20 mg/kg bw group), increased calcium and phosphorus (5, 20 and 50 mg/kg bw group), decreased urea nitrogen (5 and 50 mg/kg bw group), and increased albumin (50 mg/kg bw group). Decreased GGT in group 5 mg/kg bw animals was not considered biologically relevant since there were no correlative findings and no changes in animals from groups 20 and 50 mg/kg bw receiving higher doses. Decreased BUN and creatinine were also not considered to be toxicologically meaningful since the magnitude of the change was small, there was no abnormal renal histopathology and the changes were not dose related. The toxicological significance of the increased albumin, calcium, phosphorus and the decreased chloride in

females is unclear, but in the absence of any notable changes in kidney weights, renal histopathology or urinalysis parameters, these findings are not considered adverse. Statistically decreased MCHC along with statistically increased haematocrit and MCV were observed in 4 females of the 50 mg/kg bw group on day 92. In addition, statistically decreased RBC's were noted in this group with 4 females on day 119. These changes were not considered to be toxicologically significant due to the absence of microscopic changes in the bone marrow or spleen, as well as the lack of correlative changes in the other haematology parameters. There were no toxicologically meaningful changes in the ophthalmology, gross necropsy or organ weight data collected on this study. In addition, there were no test article-related microscopic findings in this study. All microscopic findings were considered incidental and consistent with spontaneous changes in this species. The most notable change on this study was a statistically significant reduction in the percent motility of the sperm in groups 20 and 50 mg/kg bw at the end of the treatment period. However, the change was not dose related and no effects were noted on the percent motile sperm at the end of the recovery period. In addition, these changes did not appear to impact sperm production or sperm morphology as the number of sperm per gram of cauda epididymis and the general appearance of the sperm at the end of the treatment or recovery periods was similar among control and treated groups.

Conclusion
Based on the results of this study, oral administration of HC Yellow n° 2 up to 50 mg/kg bw/d in the rat for 91 consecutive days produced no mortality or notable signs of systemic toxicity. In addition, there were no significant microscopic lesions observed following histopathological evaluation of selected organs/tissues. A variety of statistically significant changes were observed in the 5, 20 and 50 mg/kg bw/d animals, but these were deemed of no toxicological significance. Based on the findings in this study, the no-observed-adverse-effect level (NOAEL) for oral administration of HC Yellow n° 2 to rats for at least 91 consecutive days is 50 mg/kg bw/d.

Ref.: 12

Comment
According to OECD guideline 408 the highest dose level should be chosen with the aim to induce toxicity.

3.3.5.3. Chronic (> 12 months) toxicity

20 month study in mice, topical application of a hair dye formulation

0.05 ml per cm^2 of a hair dye formulation containing 0.5% HC Yellow n° 2 was applied without occlusion three times weekly for twenty months to 60 male and 60 female Eppley Swiss mice. Treatment initiated when mice were eight weeks old. 10 mice/sex/group were sacrificed after 9 months. No adverse effect on average body weight and on survival rate was observed. Chronic inflammation of the skin was observed in the control and treated mice. No observations in the haematological values indicative of a toxic effect due to the hair dye treatments and no observations in the urinary values indicated a toxic effect due to the hair dye.

Ref.: 20

Comment
This was a combined toxicity and carcinogenicity study with 2 oxidative and 12 non-oxidative hair dyes which were part of 14 commercial formulations. HC Yellow n° 2 was part of formulation 7601 which contained further hair dye substances. The carcinogenicity data are discussed in section 3.3.7. The study is of limited value.

Two year dietary study in dogs using a formulation

A hair dye composite material containing 15 hair dye substances was incorporated in the diet and provided *ad libitum* for two years to purebred beagle dogs (6-8 months of age, 6 males and 6 females per group). The concentration of HC Yellow n° 2 in the composite material was 0.28%. The average doses were calculated as 0, 19.5, 97.5 mg/kg bw/d which corresponds to doses of 0, 0.05 and 0.27 mg/kg bw/d HC Yellow n° 2.

All animals survived the 104 week test period. All dogs gained body normal weight. The haematology values were within normal limits. Blood values for serum glucose, uric acid, and creatinine concentrations, and for alkaline phosphatase and serum glutamic-pyruvic transaminase activities were within normal ranges for dogs of all groups. The concentrations of blood urea nitrogen (BUN) usually were within normal ranges except for two control female dogs and one low dosage male at the twelve month test interval. At this time, these dogs showed BUN values of 40-45 mg/100ml. However, at 18 months the BUN for these three dogs were in the range of 15-25 mg/100ml. All animals in both test groups excreted blue-brown coloured urine daily. However, urine analyses showed no remarkable findings at any examination period. Colour was normal in the urines collected following overnight fasting and was probably an indication of clearance. No noteworthy differences between control and test animal organ weights were seen for any of the organ-to-body-weight ratios. 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 from all dogs sacrificed at 18 months.

Ref.: 21

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)	
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA (pKM101)	
Replicates:	duplicate in the 1st /triplicates in 2nd of 2 independent experiments	
Test substance:	GTS03975	
Batch:	17	
Purity:	99.9%	
Vehicle:	DMSO	
Concentration:	initial experiment:	2.5, 7.5, 25, 75, 200, 600, 1800 and 5000 µg/plate without and with S9-mix
	confirmatory experiment:	25, 75, 200, 600, 1200, 1800 and 5000 µg/plate without and with S9-mix
Treatment:	pre-incubation method with 20 ± 2 minutes pre-incubation and 48 – 72 h incubation without and with S9-mix.	
GLP:	in compliance	
Study period:	14 July 2004- 19 August 2004	

GTS03975 was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. The initial mutagenicity assay both without and with S9-mix was used to establish the dose range for the confirmatory assay and to provide a preliminary mutagenicity evaluation. The condition of the bacterial background lawn was evaluated for evidence of toxicity of GTS03975 by using a dissecting microscope; precipitation by visual examination without magnification, both relative to the vehicle

control plates. Both experiments were performed without and with S9-mix using the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
In both the initial and the confirmatory experiment without and with S9-mix no precipitation occurred whereas toxicity was seen at the highest dose tested, 5000 µg/plate.
A biologically relevant and more or less dose dependent increase in the number of revertant colonies was observed in strain TA98 after exposure to GTS03975 in the presence of S9-mix. No mutagenic response was observed in the other tester strains in the presence of S9-mix and with any of the tester strains in the absence of S9-mix.

Conclusion
Under the experimental conditions used GTS03975 was mutagenic in this gene mutation tests in bacteria.

Ref.: 13

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476	
Species/strain:	mouse lymphoma cell line L5178Y ($tk^{+/-}$)	
Replicates:	single cultures in two independent experiments	
Test substance:	GTS039751	
Batch:	17	
Purity:	99.9%	
Vehicle:	DMSO	
Concentrations:	initial assay:	25, 50, 75, 100, 200, 300, 400, 500, 600 and 700 µg/ml without S9-mix 1.5, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 50 µg/ml with S9-mix
	extended treatment assay:	50, 75, 100, 125, 150, 200, 300, 400, 500 and 600 µg/ml without S9-mix
	independent repeat assay:	1, 2.5, 7.5, 10, 12.5, 60 and 75 µg/ml with S9-mix
	confirmatory extended treatment assay:	25, 50, 100, 150, 200, 300, 400, 500, 600 and 650 µg/ml without S9-mix
Treatment	initial assay:	4 h both without and with S9-mix, expression period 48 h, selection growth 10-14 days.
	extended treatment assay:	24 h both without S9-mix, expression period 48h, selection growth 10-14 days.
	independent repeat assay:	4 h both with S9-mix, expression period 48 h, selection growth 10-14 days.
	confirmatory extended treatment assay:	24 h without S9-mix, expression period 48 h, selection growth 10-14 days
GLP:	in compliance	
Study period:	22 July 2004 – 11 October 2004	

GTS039751 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a preliminary test on toxicity measuring suspension growth of the treated cultures relative to the growth of the solvent control cultures after 48 h. In the main test, cells were treated for 4 h (initial assay: both without and with S9-mix, independent repeat assay with S9-mix) or 24 h (extended treatment assay and confirmatory extended treatment assay, without S9-mix), followed by an expression period of 48 h to fix the DNA

damage into a stable *tk* mutation and a selection growth of 10-14 days. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Toxicity was measured in the main experiments as percentage relative total growth of the treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
Precipitation of GTS039751 did not occur in any of the assays. The appropriate level of toxicity (10-20% survival after the highest dose) was reached in the assays both without and with S9-mix pointing to sufficient exposure of the cells.
A dose related increase in mutant frequency was observed in the initial assay (4 h exposure) without S9-mix and at the second highest dose only with S9-mix. In the extended treatment assay (24 h treatment) a mid dose (200 µg/ml) and the highest dose showed an increase in mutant frequency. The independent repeat assay for the 4 h treatment with S9-mix again demonstrated a dose dependent increase in the mutant frequency. Finally a dose dependent increase in mutant frequency was also found in the confirmatory extended treatment assay (24 h treatment) without S9-mix. The data on colony size distribution pointed to an increase in the frequency of small colonies when the treated cultured were compared to the solvent control cultures indicating to a clastogenic rather then a mutagenic potential of GTS039751.

Conclusion
Under the experimental conditions used, GTS03975 was mutagenic in this gene mutation tests at the *tk* locus of mouse lymphoma cells. The data on colony size distribution pointed to a clastogenic rather than a mutagenic potential of GTS039751

Ref.: 15

In vitro Chromosome Aberration Test

Guideline:	OECD 473	
Cells:	Chinese Hamster Ovary (CHO-K_1) cells	
Replicates:	duplicate cultures in one test	
Test substance:	GTS03975	
Solvent:	DMSO	
Batch:	17	
Purity:	99.9 %	
Concentrations:	4 h treatment:	250, 500 and 1000 µg/ml without S9-mix
		500, 875 and 1000 µg/ml with S9-mix
	20 h treatment:	180, 375 and 750 µg/ml without S9-mix
Treatment:	4 h treatment and harvest time 20 after start of treatment both in the absence and presence of S9-mix or 20 h treatment and harvest immediately after the end of treatment.	
GLP:	in compliance	
Study period:	19 July 2004 – 7 September 2004	

GTS03975 has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. Test concentrations were based on the results of a preliminary toxicity assay on cell count, cell viability, cell growth index and cell growth inhibition with 4 h and 24 h treatment. The highest dose in the pre-test with 4 h treatment was the prescribed maximum concentration (1820 µg/ml ≈ 10 mM). The highest dose selected for the main experiment was the lowest dose which induced substantial toxicity (at least 50% cell growth inhibition relative to the solvent control). In the main test cells were treated for 4 h and harvested 20 h after the start of treatment or treated for 20 h and harvested immediately after the end of treatment. Two h before harvest, colcemid (final

concentration 0.1 µg/ml) was added to each culture to block cells at metaphase of mitosis. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring relative cell growth inhibition and by the decrease in the mitotic index. Chromosome (metaphase) preparations were stained with 5% Giemsa and examined microscopically for chromosomal aberrations and the mitotic index. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test, precipitation was not observed up to the prescribed maximum concentration (1820 µg/ml ≈ 10 mM). In the main test the highest doses evaluated showed around 60% cell growth inhibition. No relevant influence of GTS03975 on the osmolarity or on pH was observed.
Four h treatment with GTS03975 did not result in a biological relevant increase in the number of cells with chromosome aberrations. Biological relevant and dose-dependent increases in the number of cells with chromosome aberrations were found after 4 h treatment with GTS03975 with S9-mix and after 20 h treatment without S9-mix.

Conclusion
Under the experimental conditions used the increase in cells with structural chromosomal aberrations indicates a genotoxic (clastogenic) activity of GTS03975 in CHO cells *in vitro*.
Ref: 14

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	OECD 474 (1998)
Species/strain:	ICR mice
Group size:	5 mice/sex/group
Test substance:	GTS03975
Batch no:	17
Purity:	99.9%
Dose level:	0, 250, 500 and 1000 mg/kg bw
Route:	oral gavage
Vehicle:	polyethylene glycol 400
Sacrifice times:	24 h and 48 h (for the control and high dose only) after treatment.
GLP:	in compliance
Study period:	18 October 2004 – 2 December 2004

GTS03975 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the findings of two dose range finding. In both the pilot toxicity study (3 mice/sex/group treated with 250, 500, 1000 and 2000 mg/kg bw), the toxicity study (3 mice/sex/group treated with 1200, 1500 and 1800 mg/kg bw) and the definitive micronucleus test the study animals were observed for clinical signs of toxicity and mortality immediately after dose administration, approximately 1 h and 4 h post dosing and daily during the course of the experiments. In the definitive experiment mice were exposed by oral gavage to doses of 0, 250, 500 and 1000 mg/kg bw. Bone marrow cells were collected 24 h or 48 h (control and high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/EC).
Satellite groups of 3 mice/sex/time point were treated with the highest dose of GTS03975 (1000 mg/kg bw) for the determination of plasma concentrations of GTS03975. Satellite animals were bled at 1, 2, 4, 6 and 8 h post-dose. In addition 3 mice treated with the solvent and designed for bone marrow collection at 24 h post-dose and 3 mice treated with the highest dose (1000 mg/kg bw) and designed for bone marrow collection at 24 h and 48 h post-dose were bled at the time of bone marrow collection.

Bone marrow preparations were stained with May-Grünwald-Giemsa and examined microscopically for the PCE/EC ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pilot toxicity study, all animals (treatments of 250, 500, 1000 and 2000 mg/kg bw) showed orange urine, suggesting bioavailability of each applied dose. From the 2000 mg/kg bw group 1 of the 3 males and all 3 females died. From the toxicity study (treatments of 1200, 1500 and 1800 mg/kg bw) 1 of the 3 females of the 1200 mg/kg bw group, 1 male and 1 female of the 1500 mg/kg bw group and all animals of the 1800 mg/kg bw group died. All surviving animals showed orange coloured urine, lethargy, piloerection, orange skin tone, a.o.
In the main study, reductions up to 18% in the ratio of PCEs to total erythrocytes were observed in the GTS03975-treated groups relative to the vehicle control. Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 or 48 h after treatment and neither for male and females.

Conclusion
Under the experimental conditions used GTS03975 did not induce an increase in bone marrow cells with micronuclei in treated mice and, consequently, GTS03975 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 16

In vivo Unscheduled DNA Synthesis (UDS) test

Guideline:	OECD 486 (1998)
Species/strain:	male Sprague Dawley rats
Group size:	3 rats per dose
Test substance:	GTS03975
Batch:	17
Purity:	99.9%
Dose level:	0, 750 and 1500 mg/kg bw
Route:	oral gavage
Vehicle:	polyethylene glycol 400
Sacrifice times:	2-4 h and 12-16 h after dosing
GLP:	in compliance
Study period:	2 February 2005 – 28 April 2005

GTS03975 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Doses were selected on the basis of the results from the pilot toxicity assay. The high dose level was selected as the maximum tolerated dose level producing signs of toxicity such that a higher dose level would be expected to produce lethality. Animals were observed after dose administration and at time of harvest for clinical signs of chemical effect. Hepatocytes for UDS analysis were collected 2-4 h and 12-16 h after administration of GTS03975. Ninety to 180 minutes after plating the cells were incubated for 4 h with 10 µCi/ml ^{3}H-thymidine followed by 17-20 h incubation with unlabelled thymidine. Evaluation of autoradiography was done after 3 days.
UDS was reported as net nuclear grain: a net nuclear grain count was calculated for each nucleus by subtracting the mean cytoplasmic area count from the nuclear area count. Moreover, the percentage of cells in repair (defined as cells with a net grain count of at least +5) was calculated for each animal. Unscheduled synthesis was determined in 50 randomly selected hepatocytes per 3 replicate slides per rat. Negative and positive controls were in accordance with the OECD guideline.

Results

In the pilot toxicity assay (treatments up to 2000 mg/kg bw) one rat from the highest dose (2000 mg/kg bw) died. All rats treated with 250 mg/kg bw and above showed yellow fur and yellow genital area; all rats treated with 500 mg/kg bw and above showed piloerection on day 1.
In the main study, neither a biological relevant increase in mean net nuclear grain count nor in the percentage of cells in repair as compared to the untreated control was found in hepatocytes of any treated animal both for the 2-4 h and the 12-16 h treatment time.

Conclusion
Under the experimental conditions used GTS03975 did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 17

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 Yellow n° 2 among other hair dyes
Batch:	/
Purity:	/
Dose level:	pure dye
Vehicle:	/
Route:	topical, dermal, 0.5 ml to an area of approximately 1 square inch on the back of each animal. The areas were shaved and the sites of application were switched between adjacent areas to
Treatment:	twice weekly for 10 weeks
GLP:	/
Study period:	/

A permanent hair dye containing 0.01% HC Yellow n° 2 and some other hair dyes was 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.

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 Yellow n° 2 and some other hair dyes did not induce heritable translocations and, consequently, is not genotoxic in rats in this heritable translocation test.

Ref.: 22

Comment
Reference 22 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 semi-permanent hair dye contains next to HC Yellow n° 2 (0.01%) also Disperse Blue (0.12%), Disperse Black (0.04%), HC Red #3 (0.01%), HC Yellow #3 (0.21%), HC Blue #1 (0.30%), Acid Orange #3 (0.06%) and Disperse Violet #11 (0.07%). HC Yellow n° 2 was not present in the oxidative hair dye.

3.3.7. Carcinogenicity

Oral administration

Dogs

Guideline:	/
Species/strain:	Beagles
Group size:	6 Animals per sex and dose
Test substance:	A semipermanent hair dye formulation containing 0.28% HC Yellow n° 2
Batch:	/
Purity:	not stated
Dose:	0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation (0, 54.6 and 273 µg/kg bw/day of HC Yellow n° 2)
Route:	Oral - diet
Exposure period:	24 months
GLP:	/
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 0.28% HC Yellow n° 2 in formulations up to 97.5 mg/kg bw/day did not result in any signs of toxicity.

Ref.: 21

Comment
No conclusions concerning potential carcinogenic effects can be made from the study with dogs due to the low concentration of HC Yellow n° 2. Moreover, it should be noted that the hair dye formulation contained 0.61% Disperse Blue 1 (EU carcinogen category 2) 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:	semipermanent hair dye formulation (7601) containing 0.5% HC Yellow n° 2
Batch:	/
Purity:	/
Dose level:	0.05 ml of a solution containing 0.5% HC Yellow n° 2
Route:	Topical, 3 application weekly
Exposure period:	20 months
GLP:	/
Study period:	Before 1984

2 oxidative and 12 non-oxidative hair dye formulations were tested. Two of the non-oxidative hair dye formulation including the one with HC Yellow n° 2 contained 0.3% Disperse Blue 1 (EU carcinogen category 2).

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 cm^2) 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% H_2O_2 just prior to application. One of the non-oxidative hair dye formulations contained 0.5% HC Yellow n° 2. 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 Yellow n° 2.

Ref.: 20

Comments
A number of different hair dye formulations were tested in the same study. Although some of the formulations contained Disperse Blue 1 (EU carcinogen category 2) none of the formulations induced tumours. Thus, no conclusion with regard to carcinogenicity can be made from the studies.

Conclusion on carcinogenicity
No conclusion concerning potential carcinogenic effects can be made from an oral study with dogs and a skin painting study with mice. HC Yellow n° 2 was present in low concentrations (0.5% 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

Fertility and reproductive performance in rats using a formulation

A fertility and reproductive performance study was performed on Sprague-Dawley CD rats. Sixty males and 120 females were divided into six groups. Levels of 0, 0.195 and 0.78% of a hair dye composite material containing 15 hair dye substances which were incorporated in the diet and provided *ad libitum.* The concentration of HC Yellow n° 2 in the composite material was 0.28%. The average doses were calculated as 0, 86 and 351 mg/kg bw/d which corresponds to doses of 0, 0.24 and 0.98 mg/kg bw/d HC Yellow n° 2. In part I of the study the females received the basal diet from week 8 prior to mating through the weaning of their litters. The males siring these litters were fed the test diets from week 8 prior to mating and during the mating period. In part II of the study males received the basal diet from week 8 prior to mating and during mating while the females received the test diets from week 8 prior to mating and during gestation and lactation.

There were no dose related significant differences in any of the maternal parameters examined, which included female fertility, length of gestation and number of females with resorption sites. The female fertility index in the high dosage group in part I of the study was lower than the control values, but the differences were not statistically significant. At the dietary concentrations fed, there were no effects on food consumption and body weight gain.
There were no dose related significant differences in male fertility. No effects on food consumption and body weight gain were observed.

There were no dose related significant differences in any of the foetal parameters examined which included number of live pups per litter, body weights and survival. The average pup weight in the high dosage group in part II of the study was lower than the control values but the differences were not statistically significant. No abnormal pups were seen upon dissection of embryos after thirteen days of gestation or upon gross examination at weaning after 21 days.

Ref.: 21

Comment
The study is of limited value since only low concentrations were tested and several hair dyes were applied.

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Dose range finding developmental toxicity study

Guideline:	/
Species/strain:	Rats, Sprague-Dawley, Crl:CD®(SD)IGS BR
Group size:	8 females/group
Test substance:	HC Yellow n° 2
Batch:	GTS03975 commercial material, lot code #17
Purity:	99.9 %
Dose:	0, 25, 75, 200 and 500 mg/kg bw/d in Polyethylene Glycol 400
Route:	oral
Exposure:	once daily via gavage from GD 6-19
GLP:	in compliance
Date:	June 2005

Forty presumed pregnant Crl:CD®(SD)IGS BR VAF/Plus® rats were randomly assigned to five dosage groups, eight rats per group. Solutions of the test substance or the vehicle, PEG 400, were administered orally (gavage) once daily to these naturally bred female rats on days 6 through 20 of presumed gestation (GD 6 - 20) at dosages of 0, 25, 75, 200 and 500 mg/kg bw/d. Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on GD 21. The gravid uterus was excised, weighed and subsequently examined for the number and distribution of corpora lutea, implantation sites and uterine contents. A gross necropsy of the thoracic, abdominal and pelvic viscera was performed. Foetuses were weighed and examined for gross external alterations and sex.

Result
One rat in the 500 mg/kg bw/d dosage group was sacrificed due to moribund condition on GD 18 and had orange discoloured urine, fur and skin, excess salivation, gasping and dyspnea. The rat lost body weight from GD 17 - 18; feed consumption values for this rat were generally comparable to other rats in this dosage group. Necropsy revealed yellow discoloration of the intestines, pancreas, stomach, mesentery and abdominal adipose tissue; all other tissues appeared normal. The litter consisted of 12 foetuses and one early resorption. Based on the veterinarian examination that indicated open-mouthed and increased breathing and the rat maintaining an extended neck, the moribund condition was considered the result of an intubation accident. All other rats survived to scheduled sacrifice. Clinical observations considered to be related to the test substance included discoloured (orange) skin, fur and/or urine in all groups administered the test substance and excess salivation in the 500 mg/kg bw/d dosage group. No additional gross lesions were revealed by necropsy examination. There were no test substance related effects on body weights, body weight gains, gravid uterine weights, corrected maternal body weights or corrected maternal body weight gains or absolute or relative feed consumption values. No Caesarean-sectioning or litter parameters were affected by dosages of the test substance as high as 500 mg/kg bw/day. All foetuses appeared normal at gross external examination. Based on these data, dosages of 50, 200 and 500 mg/kg bw/d were selected for the main study.

Ref.: 18

Main study

Guideline:	OECD 414
Species/strain:	Rats, Sprague-Dawley, Crl:CD®(SD)IGS BR
Group size:	25 females/group
Test substance:	HC Yellow n° 2

Batch:	GTS03975 commercial material, lot code #17
Purity:	99.9%
Dose:	0, 50, 200 and 500 mg/kg bw/d in polyethylene glycol 400
Route:	oral
Exposure:	once daily via gavage from GD 6-19
GLP:	in compliance
Date:	July 2005

One hundred presumed pregnant Crl:CD®(SD)IGS BR rats (25/group) were randomly assigned to four dosage groups. Solutions of the test substance or the vehicle, 100 % polyethylene glycol 400 (PEG 400), were administered orally once daily to rats on days 6 - 20 of gestation (GD 6 - 20) at dosages of 0, 50, 200 and 500 mg/kg bw/d. Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on GD 21. The gravid uterus was excised, weighed and subsequently examined for the number and distribution of corpora lutea, implantation sites and uterine contents. A gross necropsy was performed. Foetuses were weighed and examined for gross external, soft tissue and skeletal alterations and sex.

Results
One 200 and two 500 mg/kg bw/d dosage group rats were found dead on GD 17, 19 or 20. These deaths were considered related to aspiration of the test substance. Significantly increased incidences of yellow and/or orange urine and discoloration and yellow perioral substance occurred in the 50, 200 and 500 mg/kg bw/d dosage groups. Discoloration of internal organs was also observed. All of these colour changes were not considered to be adverse. Body weights, body weight gains and uterine weights of dams were not affected by doses as high as 500 mg/kg bw/d. Absolute and relative feed consumption values were significantly reduced in the 500 mg/kg bw/d dosage group on GD 15 - 18, as compared to the vehicle control group value. No other effects of the test substance occurred on absolute (g/day) and relative (g/kg bw/day) feed consumption values at dosages as high as 500 mg/kg bw/d. Although within the historical control range for the testing facility, the female foetal body weights were significantly reduced compared to the vehicle control group value. The 50, 200 or 500 mg/kg bw/d dosages of HC Yellow n° 2 were associated with significant reductions in the average number of ossified fore- and/or hind- limb phalanges that were also all within historical control range. Administration of dosages of HC Yellow n° 2 up to and including 500 mg/kg bw/d did not affect any other Caesarean-sectioning, litter observations or cause any gross external, soft tissue or skeletal alterations.

Conclusion
On the basis of these data, the maternal no-observable-adverse-effect- level (NOAEL) is 500 mg/kg bw/d. The developmental NOAEL is also 500 mg/kg bw/d. The small reduction in foetal body weights and reduced ossification site averages for phalanges were not considered adverse.

Ref.: 19

Developmental toxicity study in rats using a formulation

A teratology study was performed on Virgin CFE-S rats. Levels of 0, 0.195 and 0.78% of a hair dye composite material containing 15 hair dye substances were incorporated in the diet and provided *ad libitum* from GD 6 to GD 15 to 3 groups of each 20 pregnant animals. The concentration of HC Yellow n° 2 in the composite material was 0.28%. The average doses were calculated as 0, 124 and 554 mg/kg bw/d which corresponds to doses of 0, 0.35 and 1.55 mg/kg bw/d HC Yellow n° 2.

The animals excreted blue-brown coloured urine. No evidence was found to indicate that the composite material has any adverse effect on the pregnant rat or any of her pups.

Ref.: 21

Comment
The study is of limited value.

Developmental toxicity study in rabbits using a formulation

A teratology study was performed on New Zealand White rabbits. The animals were artificially inseminated and allotted to four groups of 12 rabbits each (vehicle 0.5% aqueous methylcellulose, vehicle + composite material without dyes, composite material with dyes resulting in doses of 19.5 and 97.5 mg/kg bw/d). A hair dye composite material containing 15 hair dyes was used. The concentration of HC Yellow n° 2 in the composite material was 0.28%. The material was applied by oral gavage to the rabbits at the doses 0, 19.5, 97.5 mg/kg bw/d which corresponds to doses of 0, 0.05 and 0.27 mg/kg bw/d HC Yellow n° 2. The animals were dosed daily from GD 6 to GD 18.

There was no evidence of a teratologic effect in any group. Foetal survival was not adversely affected. The variations in the degree of ossification showed no relationship to treatment.

Ref.: 21

Comment
The study is of limited value.

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 Yellow n° 2
(Non-oxidative formulation)

Absorption through the skin	**A (mean + 1 SD)**	**=**	**5.31 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**3.08 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.05 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**50 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**974**

(Oxidative formulation)

Absorption through the skin	**A (mean + 1 SD)**	**=**	**8.27 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**4.80 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose	**SAS x A x 0.001/60**	**=**	**0.08 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**50 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**625**

3.3.14. Discussion

Physico-chemical properties
In only 50% of the toxicological studies batches of HC Yellow n° 2 have been used which had been characterized according to identity, purity, stability and homogeneity.
The chemical identity of HC Yellow n° 2 which has been used for the NOAEL setting sub chronic 91 day study, is well documented with respect to identity, purity (99.88%), stability and homogeneity.
The stability in a typical hair dye formulation was not reported.
HC Yellow n° 2 is a secondary amine, and thus is prone to nitrosation and formation of nitrosamines. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

Toxicity
HC Yellow n° 2 was toxic after oral gavage dosing of 1250 mg/kg bw, not at 625 mg/kg bw.
In a subchronic toxicity study in rats the no-observed-adverse-effect level (NOAEL) for oral administration of HC Yellow n° 2 is 50 mg/kg bw/d (the highest dose tested). In a developmental toxicity study in rats the maternal no-observable-adverse-effect- level (NOAEL) is 500 mg/kg bw/d. The developmental NOAEL is also 500 mg/kg bw/d. The study on reproductive toxicity was performed only with a formulation containing HC Yellow n° 2 which corresponds to a dose of 0.98 mg/kg bw/d.

Skin/eye irritation and sensitisation
No apparent dermal irritation was produced under the test conditions by HC Yellow n° 2.
Undiluted HC Yellow n° 2 is irritant to the rabbit eye. A 10% dilution is slightly irritant to the eyes.
The concentrations used in a LLNA study were too low. Thus, a sensitisation potential cannot no excluded. No sensitisation was observed in a Magnusson-Kligman maximisation test. No sensitisation was observed in human occlusive repeated patch tests. The SCCS considers the HRIPT studies as unethical.

Percutaneous absorption
The percutaneous absorption of HC Yellow nº 2 under oxidative conditions was 6.06 ± 2.21 = 8.27 µg/cm²and 4.02 ± 1.29 = 5.31 µg/cm² under non-oxidative conditions.

Mutagenicity/genotoxicity
Overall, the genotoxicity of HC Yellow n° 2 is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. HC Yellow n° 2 induced gene mutations both in bacteria and in mammalian cells. The increase in mutant frequency in mammalian cells appeared due to an increase in small colonies which is an indication for a clastogenic effect. The latter was confirmed in the in vitro chromosome aberration test which showed an increase in cells with chromosomal aberrations.

The positive results in the *in vitro* tests for both gene mutations and chromosome aberrations after treatment with HC Yellow n° 2 could not be confirmed *in vivo*. Both a mouse bone marrow micronucleus tests and an *in vivo* UDS test were negative. A permanent hair dye containing 0.01% HC Yellow n° 2 next to some other hair dyes did not induce heritable translocations.
As the genotoxic effects found *in vitro* were not confirmed in *in vivo* tests, HC Yellow n° 2 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No conclusion concerning potential carcinogenic effects can be made from an oral study with dogs and a skin painting study with mice. HC Yellow n° 2 was present in low concentrations (0.5% 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.

4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of HC Yellow n° 2 with a maximum on-head concentration of 0.75% in oxidative and of 1.0% in non-oxidative hair dye formulations does not pose a risk to the health of the consumer.

A possible sensitising potential of HC Yellow n° 2 cannot be excluded.

HC Yellow n° 2 is a secondary amine, and thus is prone to nitrosation and formation of nitrosamines. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submission II, 2005

1a. Test Article Bulk Characterisation for Procter & Gamble Study 2747-53903, VKA00014AX, P&G study # 2747-53903, June 10, 2005
1b. Method Validation and Stability Analysis for Charles River Labs Discovery and Development Services Worcester Division Study VKA-AC00-04-205, P&G study # 2747-53903, June 10, 2005
2. Acute Toxicity of HC Yellow #2 in Male and Female Rats, Clairol Report C6674-004, February 12, 1987
3. Rabbit Eye Irritation with HC Yellow #2, Clairol Report C6676-020, January 16, 1987
4. Rabbit Eye Irritation with HC Yellow #2, Clairol Report C6826-06, February 20, 1987
5. Primary Skin Irritation with HC Yellow #2, Clairol Report C6675-08, January 8, 1987
6. Studies in Guinea Pigs to Determine the Potential of Hair Dyes to Induce Allergic Contact Dermatitis, Clairol Report, April 4, 1979
7. Chapdelaine, J., Local Lymph Node Assay # 0787XB05.007, Study # 99012, Report to Clairol, Chrysalis Preclinical Services, June 30, 2000
8. M.L. Reed, J. Anderson, D. Napoli, I. Goldberg, M. Roche, and D. Sullivan, Repeated Insult Patch Test: Study #841012, Report to Clairol, TKL Research Inc., June 29, 1984
9. M.L. Reed, J. Anderson, D. Napoli, I. and S. Mruczek, Repeated Insult Patch Test: Study #841015, Report to Clairol, TKL Research Inc., November 27, 1984
10. HC Yellow 2: In Vitro Penetration from a Formulation through Human Skin, P&G Study #2747-53910, June 6, 2005
11. A 14-Day Range-Finding Oral Toxicity Study in Rats with GTS03975, P&G Study # 2747-53906, May 6, 2005
12. A 91-Day Oral Gavage Toxicity Study With GTS03975 in Rats with a 28-Day Recovery Phase, P&G study # 2747-53907, July 12, 2005
13. Bacterial Mutation Test (Ames), P&G Study # 2747-53912, June 20, 2005
14. In Vitro Chromosome Aberration Assay, P&G Study # 2747-53908, June 20, 2005
15. In Vitro Mouse Lymphoma (L5178Y/TK+/-) Mutation Assay, P&G Study # 2747-53909, June 28, 2005
16. In Vivo Micronucleus Assay, P&G Study # 2747-53905, June 27, 2005
17. In Vivo Unscheduled DNA Synthesis (UDS) Test in Rats, P&G Study # 2747-54629, July 19, 2005
18. Oral (Gavage) Dosage-Range Developmental Toxicity Study of GTS03975 in Rats, P&G Study # 2747-53983, June 2, 2005
19. Oral (Gavage) Developmental Toxicity Study Of GTS03975 in Rats, P&G Study # 2747-53904, July 5, 2005
20. M.M. Jacobs, C.M. Burnett, A.J. Penicnak, J.A. Herrera, W.E. Morris, P. Shubik, M. Apaja, and G. Granroth, Evaluation of the Toxicity and Carcinogenicity of Hair Dyes in Swiss Mice, 19 Drug and Chemical Toxicology, 7: 573-586, 1984
21. T. Wernick, B.M. Lanman, and J.L. Fraux, Chronic Toxicity, Teratologic and Reproduction Studies with Hair Dyes, Toxicol. Appl. Pharmacol., 32: 450-460, 1975
22. C. Burnett, R.F. Loehr, and J.F. Corbett, Heritable Translocation Study on Two Hair Dye Formulations, Fund. App. Toxicol., 1: 325-328, 1981

Submission II, addendum 2008

23. M. Jäger, (2007). Skin permeability in vitro absorption through porcine ear skin with HC Yellow 2 in alkaline cream emulsion; RCC Project No. 2000803; Test Report, Rossdorf, July 4, 2007
24. M. Jäger; (2007). Quantification of HC Yellow 2; RCC_ 2000801

25. Colipa (2008). Submission summary, attachment 1: Stability of HC Yellow 2 to peroxide over the dyeing time under alkaline conditions