Scientific Committee on Consumer Products

SCCP

OPINION ON

HC Orange n° 1

COLIPA n° B47

The SCCP adopted this opinion at its 17th plenary of 30 September 2008

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 Products (SCCP), 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 (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCP
Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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(ISSN)

http://ec.europa.eu/health/ph_risk/risk_en.htm

ACKNOWLEDGMENTS

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Keywords: SCCP, scientific opinion, hair dye, B47, HC Orange n° 1, directive 76/768/ECC, CAS 54381-08-7, EINECS 259-132-4

Opinion to be cited as: SCCP (Scientific Committee on Consumer Products), Opinion on HC Orange n° 1, 30 September 2008

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1. BACKGROUND

Submission I of HC Orange No. 1, with the chemical name 4-[(2-nitrophenyl)amino]phenol, was submitted in August 1981 by COLIPA [1, 2]. No opinion was issued by a Scientific Committee.

According to the current submission II, submitted by COLIPA in July 2005, HC Orange n° 1 is used as an ingredient in non-oxidative hair dye formulations in a concentration up to 1%.

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider HC Orange n° 1 as safe for use as an ingredient in non-oxidative hair dye formulations with an on-head concentration of 1% taking into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use of HC Orange n° 1 in non-oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According the records of COLIPA

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Orange n° 1 (INCI name)

3.1.1.2. Chemical names

4-[(2-Nitrophenyl)amino]phenol
2-nitro-4'-hydroxydiphenylamine

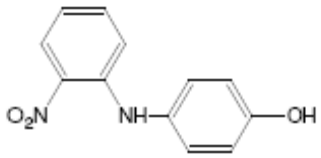
3.1.1.3. Trade names and abbreviations

/

3.1.1.4. CAS / EINECS number

CAS: 54381-08-7
EINECS: 259-132-4

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{12}H_{10}N_2O_3$

3.1.2. Physical form

Orange-red to brown-orange crystalline powder

3.1.3. Molecular weight

Molecular weight: 230.22 g/mol

3.1.4. Purity, composition and substance codes

General Specifications as defined in the Summary Submission (COLIPA, October 2005)

Batch	Lot No. 32	General specification
Reference	*Ref. 1a (analytical data)*	*Summary submission 2005*
Purity by HPLC (area% at 254 nm, corrected for moisture content)	99.8%	> 99% *
Impurities		
p-aminophenol	666 ppm	<2000 ppm
2-nitrofluorobenzene	ND	<100 ppm
Heavy Metals		
Arsenic	-	<5 ppm
Antimony	-	<5 ppm
Lead	-	<20 ppm
Cadmium	-	<10 ppm
Mercury	-	<5 ppm
Moisture (Loss on Drying)	0.101%	<0.5%
Residue on Ignition	<0.100%	<0.5%

* The summary of submission 2005 stated "purity by HPLC versus an external standard". However, the data provided in reference 1a indicates that the above statement is not correct. Thus, absolute content of HC Orange n° 1 in test material (Lot No. 32) is not known. The general specification of purity of HC Orange n° 1 as 99% is based on HPLC peak area at 254 nm.

Chemical identification

Infra Red Spectroscopy: IR spectrum is consistent with structure
NMR Spectroscopy: ^{1}H- and ^{13}C-NMR spectra are consistent with structure
Elemental Analysis: Combustion analysis of C, H and N is consistent with molecular formula (theoretical values C, 62.6%; H, 4.38%; N, 12.17%, observed C, 62.39%; H, 4.59%; N, 12.15%).

Ref.: 16

3.1.5. Impurities / accompanying contaminants

See previous section

3.1.6. Solubility

Solvent	Solubility* (mg/ml)
Water	0.0022 - 0.0034
Ethanol	64.6 - 96.8
DMSO	185 – 277

* Solubility measured after 15 minutes sonication.

3.1.7. Partition coefficient (Log P_{ow})

Log Po/w: 3.25 ± 0.57 (calculated)

3.1.8. Additional physical and chemical specifications

Melting point: 143 - 148 °C (stated in the Summary submission)
146.4-147.5 °C (measured in ref. 1a)

Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	7.2 cs at 96.9 °C
pKa:	/
Refractive index:	/
pH of a solution 5% at 25°C:	6.6
UV_Vis spectrum (200-800 nm):	/

3.1.9. Homogeneity and Stability

Homogeneity and stability were tested in PEG 400 solutions at levels 0.4 mg/ml and 150 mg/ml, and in DMSO solutions at levels 0.005 mg/ml and 100 mg/ml, using samples of Lot 32.
The relative standard deviation for homogeneity was ± 1.6-2.0% in all the cases.
Changes in concentrations were ±0.4-2.0% in all stability measurements covering storage at room temperature for 24 hours and under refrigeration for 7 or 15 days for the DMSO and PEG 400 solutions respectively.

General Comments to physico-chemical characterisation

- The stability of HC Orange n° 1 in marketed products is not described.
- Only one batch of HC Orange n° 1 has been analysed and characterised.
- Log P_{ow}: calculated values cannot be accepted as an estimate of the true physical constant without justification.
- The absolute content of the dye in any of the batches used is not reported.

3.2. Function and uses

HC Orange n° 1 is used as an ingredient in non-oxidative hair dye formulations at levels up to 1%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	/
Species/strain:	Sprague Dawley
Group size:	5
Test substance:	10% suspension HC Orange No 1
Batch:	4440686
Purity:	/
Dose:	males: 1250 and 5000 mg/kg bw, females: 5000 mg/kg bw
Vehicle:	3% aqueous acacia
Route:	oral gavage
Exposure:	Single administration
GLP:	/

Date: 28 January 1987

Animals were observed for 14 days after treatment. One male rat in the 5000 mg/kg dose group died on day 2. No deaths occurred among female rats. No details of clinical reactions to treatment were provided. The acute median lethal oral dose (LD_{50}) of HC Orange n° 1 in Sprague Dawley rat is higher than 5000 mg/kg bw.

Ref.: 2

Comment
No study report was provided. However repetition of the acute oral toxicity test is not scientifically justifiable.

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: New Zealand White rabbit
Group: 4 males and 2 females
Substance: HC Orange n° 1
Batch: 4440686
Purity: /
Dose: 500 mg of an aqueous slurry
Vehicle: water
GLP: /
Study period: May 1987

500 mg of an aqueous slurry was applied to the intact skin of the back of 6 rabbits for 24 hours without occlusion. The sites were scored for dermal irritation at 24 and 72 hours after application.

Conclusion
Under the test conditions, HC Orange n° 1 produced no oedema or erythema and was considered non-irritant.

Ref.: 4

3.3.2.2. Mucous membrane irritation

Guideline: /
Species: rabbit
Group: 4 males
Substance: HC Orange n° 1
Batch: 4440686
Purity: /
Dose: 100 mg, undiluted test material
Vehicle: /
GLP: not in compliance
Study period: June 1987

A single sample of 100 mg of the neat test substance was applied into the conjunctival sac of the eye of 4 male rabbits. The eyes were rinsed with distilled water and evaluated and scored 1 hour as well as 1, 2 and 3 days after application.

Conjunctival redness and lid swelling were observed in all animals after 1 hour, discharge in three of the four animals. All eyes were clear after 3 days in all animals. No corneal opacity or iris irritation was observed.

Conclusion
HC Orange n° 1 is considered to have the potential to produce transient mild eye irritation.

Ref.: 3

3.3.3. Skin sensitisation

Guinea Pig Maximisation Test

Guideline:	/	
Species:	Hartley albino guinea pig	
Group:	10 females	
Substance:	HC Orange n° 1	
Batch:	731078	
Purity:	/	
Concentration:	intradermal induction:	0.1% (w/v) in propylene glycol
	topical induction:	25% (w/v) in propylene glycol
	topical challenge:	25% (w/v) in propylene glycol
GLP:	not in compliance	
Study period:	December 1979	

The aim of the study was to evaluate the skin sensitising properties of seven hair dyes, one of which was HC Orange n° 1. The method used was the GPMT as described by Magnusson and Kligman.
10 female guinea pigs were used for each test substance.

Induction: 6 injections (3 each side) of 0.05 ml were made intradermally as follows:

- site 1: 2 x 0.05 ml of the adjuvant alone
- site 2: 2 x 0.05 ml of the test material
- site 3: 2 x 0.05 ml of test substance in complete adjuvant (1:1)

A closed patch exposure was performed over the injection site (4x6 cm, shoulder) one week later.

Challenge: after 2 weeks of rest, the animals were challenged topically. The sites were evaluated 24, 48 and 72 hours after patch removal.

Results
No positive results were recorded.

Conclusion
No evidence of sensitisation was observed.

Ref.: 5

Local Lymph Node Assay (LLNA)

Guideline: predates OECD guideline

Species: CBA/CaJ female mice
Group: 20 females, 5 per group
Substance: TM#2043
Batch: /
Purity: /
Dose: 0.25, 0.50, 1.0 and 2.0%
Vehicle: dimethylsulfoxide (DMSO)
Control: p-phenylenediamine (PPD)
GLP: not in compliance
Study period: 22 – 27 March 1999

CBA/CaJ female mice were treated on the dorsal surface of both ears once per day for 3 days with 0.25, 0.50, 1.0 or 2.0% (w/v) of TM#2043, the positive control (PPD) or the vehicle (DMSO). On Day 5, the mice were injected with 20 µCi of ^{3}H-thymidine. Five hours later, the mice were euthanized and the draining auricular lymph nodes were removed. The lymph node cells were precipitated with 5% trichloro-acetic acid (TCA) and the pellets counted in a β-scintillation counter to determine incorporation of the ^{3}H-thymidine.

Results
The positive control (PPD at 2.0%) resulted in test/control ratios greater than 3 (7.10) indicating a positive response. This response was also statistically significant compared to the vehicle control group. The test article was negative in the assay at any of the concentrations tested.

Treatment	**Dose**	**DPM** (mean ± sem)	**Test/control Ratio**
Vehicle	-	1380 ± 328	-
TM#2043	0.25%	1196 ± 526	0.87
TM#2043	0.50%	892 ± 256	0.65
TM#2043	1.0%	1352 ± 631	0.98
TM#2043	2.0%	1205 ± 241	0.87
PPD	0.25%	806 ± 303	0.58
PPD	0.50%	2294 ± 995	1.66
PPD	1.0%	3055 ± 831	2.21
PPD	2.0%	9803 ± 1093	7.10

Conclusion
The study authors concluded that the results of this assay indicate that TM#2043 did not induce a hypersensitivity response.

Ref.: 6

Comment
The highest concentration tested was too low. No conclusion regarding the sensitising potential of HC Orange n° 1 can be drawn from the data in this study.

Human Repeated Insult Patch Test, study 1

Guideline: /
Species: Human volunteers (males and females, age 18 – 78)
Group: 98 individuals completed the study
Substance: HC Orange n° 1
Batch: 2421186

Purity: /
Dose: 3% HC Orange n° 1 in a base formulation (12% isopropanol, 2% Tween, 2% natrosol, 0.05% sodium sulfite, water q.s. 100%)
GLP: /
Study period: December 1984

The purpose of this study was to determine whether the test material is capable of sensitising the skin of humans under controlled patch test conditions. The patches were applied under occlusion to the infra-scapular area of the back, either to the right or left of the midline.

The induction phase consisted of nine consecutive applications of the test material and subsequent evaluations of the test sites. The subjects were required to remove the patches approximately 24 hours after application. They returned to the facility at 48-hour intervals to have the sites evaluated, and identical patches reapplied. Following the ninth evaluation, the subjects were dismissed for a fourteen days rest period.

The challenge phase was initiated during the sixth week of the study, with identical patches applied to sites previously unexposed to the test material. These patches were removed after 24 hours. The sites were graded 24 and 48 hours after removal, i.e. 48 and 72 hours after application.

Results
Two subjects developed reactions on challenge which required further testing to rule out sensitization. The results were indicative of irritation rather than sensitization.

Conclusion
The study authors concluded that there was no evidence of sensitisation to the test substance under the conditions employed in this study.

Ref.: 7

Comment
The SCCP considers HRIPT-studies as unethical.

Human Repeated Insult Patch Test, study 2

Guideline: /
Species: Human volunteers (males and females, age 18 – 79)
Group: 101 completed the study
Substance: HC Orange n° 1
Batch: 2421183
Purity: /
Dose: 3% HC Orange n° 1 in a base formulation (12% isopropanol, 2% Tween, 2% natrosol, 0.05% sodium sulfite, water q.s. 100%)
GLP: /
Study period: 21 January – 8 March 1985

The purpose of this study was to determine whether the test material is capable of sensitising the skin of humans under controlled patch test conditions. The patches were applied under occlusion to the infra-scapular area of the back, either to the right or left of the midline.

The induction phase consisted of nine consecutive applications of the test material and subsequent evaluations of the test sites. The subjects were required to remove the patches approximately 24 hours after application. They returned to the facility at 48-hour intervals to have the sites evaluated, and identical patches reapplied. Following the ninth evaluation, the subjects were dismissed for a 14-day rest period.

The challenge phase was initiated during the sixth week of the study, with identical patches applied to sites previously unexposed to the test material. These patches were removed after 24 hours. The sites were graded 24 and 48 hours after removal, i.e. 48 and 72 hours after application.

Results
A large number of reactions scored "?" (? = doubtful response, barely perceptible erythema, only slightly different from surrounding skin) were recorded throughout the induction period. Because HC Orange n° 1 was a brown liquid, it is likely that a portion of the "?" reactions resulted from epidermal staining rather than irritation.

Conclusion
The study authors concluded that there was no evidence of sensitisation to the test substance under the conditions employed in this study.

Ref.: 8

Comment
The SCCP considers HRIPT-studies as unethical.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD draft 428 (2004)
Tissue:	Human (*post mortem*) dermatomed skin, 400 µm thickness
Group size:	12 membranes from 5 different donors
Diffusion cells:	glass diffusion cells, 2.54 cm² membrane area
Skin integrity:	trans-dermal electrical resistance; at least 10 kΩ
Test substance:	HC orange n° 1 (technical grade) [^{14}C]-HC Orange n° 1; 2.886 GBq/mmol or 78 mCi/mmol
Batch:	I01916, lot 32 507-045-078 (radio-labelled)
Purity:	100.3% 99.4% (radio-chemical)
Test item:	hair dye cream formulation containing 1% HC Orange n° 1
Doses:	20 mg/cm²
Receptor fluid:	4% polyoxyethylene-20-oleyl ether solution in phosphate buffered saline (PBS/A)
Solubility in receptor fluid:	0.72 mg/ml
Stability:	/
Method of Analysis:	liquid scintillation counting
GLP:	in compliance
Study period:	15 November 2004 – 2 January 2005

The penetration and distribution of HC Orange 1 from a nominal 1% w/w formulation was measured *in vitro* through human skin, following the incorporation of [^{14}C]-HC Orange 1.

The formulation was applied to 12 human dermatomed skin membranes (nominally 400 µm thick), mounted in glass diffusion cells, at a nominal rate of 20 mg/cm². After 30 minutes, the dose was washed from the surface of the skin using natural sponges soaked in 3% Teepol. Samples of the receptor fluid were taken at recorded intervals over a 48 hour period, during which time the applications remained unoccluded. At the end of the experiment, the surface of the skin was washed again and layers of the *stratum corneum* removed using a tape stripping technique. The receptor fluid samples, sponges, tape strips, residual skin and donor chambers were analysed for radioactivity, which was representative of the HC Orange 1 content.

Results

The results for the penetration of HC Orange 1 from the formulation are summarised in the following tables.

	Amount recovered (µg/cm²)													
Cell number	**1**	**2**	**6**	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**17**	**18**	**mean**	**SD**
Flange	0.01	0.02	0.05	0.04	0.06	0.03	0.14	0.04	0.03	0.03	0.03	0.02	**0.04**	**0.03**
Donor chamber	0.08	0.09	0.04	0.05	0.06	0.09	0.18	0.19	0.30	0.27	0.12	0.17	**0.14**	**0.09**
Skin wash, 0.5h	194	199	177	187	184	189	201	211	208	191	181	183	**192**	**10.1**
Skin wash, 48h	0.26	0.17	0.32	0.22	0.19	0.31	0.32	0.27	0.27	0.28	0.32	0.26	**0.27**	**0.05**
Stratum corneum	0.08	0.06	0.23	0.08	0.14	0.17	0.11	0.08	0.20	0.12	0.04	0.12	**0.12**	**0.06**
Remaining	0.03	0.03	0.05	0.04	0.03	0.03	0.06	0.05	0.06	0.06	0.04	0.03	**0.4**	**0.01**
dermis/epidermis	0.71	0.53	1.24	2.02	1.07	0.79	1.00	1.07	0.51	0.46	0.51	0.29	**0.85**	**0.47**
Systemically available*	**0.74**	**0.56**	**1.29**	**2.06**	**1.10**	**0.82**	**1.06**	**1.12**	**0.57**	**0.52**	**0.55**	**0.32**	**0.89**	**0.47**
Total	196	200	179	190	186	190	203	213	209	192	182	184	**194**	**10.8**

	Amount recovered (%)													
Cell number	**1**	**2**	**6**	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**17**	**18**	**mean**	**SD**
Flange	0.01	0.01	0.02	0.02	0.03	0.01	0.07	0.02	0.02	0.02	0.01	0.01	**0.02**	**0.02**
Donor chamber	0.04	0.04	0.02	0.02	0.03	0.05	0.09	0.09	0.15	0.13	0.06	0.08	**0.07**	**0.04**
Skin wash, 0.5h	97.1	100	88.3	93.6	92.1	94.5	101	105	104	95.2	90.4	91.4	**96.0**	**5.40**
Skin wash, 48h	0.13	0.08	0.16	0.11	0.10	0.16	0.16	0.14	0.13	0.14	0.16	0.13	**0.13**	**0.03**
Stratum corneum	0.04	0.03	0.12	0.04	0.07	0.08	0.05	0.04	0.10	0.06	0.02	0.06	**0.06**	**0.03**
Remaining	0.01	0.2	0.02	0.02	0.01	0.02	0.03	0.02	0.03	0.03	0.02	0.01	**0.02**	**0.01**
dermis/epidermis	0.36	0.26	0.62	1.01	0.54	0.39	0.50	0.54	0.26	0.23	0.26	0.15	**0.43**	**0.23**
Systemically available*	**0.37**	**0.28**	**0.64**	**1.03**	**0.55**	**0.41**	**0.53**	**0.56**	**0.29**	**0.26**	**0.28**	**0.16**	**0.45**	**0.24**
Total	**97.7**	**100**	**89.2**	**94.8**	**92.9**	**95.2**	**102**	**106**	**105**	**95.8**	**90.9**	**91.9**	**96.7**	**5.41**

* Systemic available is the sum of the remaining epidermis and the receptor fluid data.

Conclusion
The study authors concluded that 0.89 µg/cm² (0.45%) of the applied HC Orange 1 was regarded as being systemically available.

Ref.: 9

Comment
As too few chambers were used, the Amax of 2.06 µg/cm² (1.03% of the applied dose) could be used for the calculation of the Margin of Safety.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

14 day oral range finding study in rats

Guideline:	OECD 407
Species/strain:	Rat, Sprague Dawley Crl:CD IGS BR
Group size:	5 per dose/sex; 50 in total
Test substance:	GTS03977
Batch:	# 32
Purity:	99.9%
Dose:	0, 50, 150, 300, or 750 mg/kg bw/day
Route:	oral gavage
Volume/Vehicle:	5 mL/kg in polyethylene glycol 400
Exposure:	14 days
GLP:	in compliance
Date:	02.02.2005

Dose preparations were prepared twice in this study and stored in a refrigerator, set to maintain 2 – 8 °C. For each dose formulation, the required amount of GTS03977 was added to a volume of vehicle and mixed using a laboratory homogenizer until the test substance appeared uniformly dispersed.
The animals were observed twice daily for abnormalities, and signs of pain or distress or death. Detailed clinical observations were performed and body weights recorded once prior to treatment and on Days 1, 4, 8, 11, and 15. Food consumption data were measured from Days 1 to 4, 4 to 8, 8 to 11, and 11 to 15. Blood and urine samples for haematology, clinical

chemistry, and urinalysis were collected at the scheduled sacrifice. On Day 17, animals were anesthetized, weighed, and killed. Macroscopic observations were recorded, selected organs weighed, and selected tissues collected and preserved.
All animals survived. Clinical observations noted were orange urine and orange fur, mainly around the mouth, at all doses and skin of the tail and paws stained orange in animals at the mid-low, mid-high and high doses.
At Day 15, mean body weights were not affected (low, mid-low, mid-high and high doses were males: 99.4, 99.1, 96.2, and 95.9%; females: 96.8, 98.4, 98.4, and 91.3% of controls, respectively). There was a significant decrease in mean body weight gain for Days 11 to 15 for high dose females. It was not associated with any adverse health effects, but was attributed to the test substance. Overall body weight gains (Days 1 to 15) for high dose females were slightly decreased, but not statistically significant.
Mean food consumption was decreased for mid-high dose males and high dose males and females. These were statistically significant in high dose females for Days 1 to 4 and 8 to 11. The reduced mean food consumption was attributed to the test substance and correlated with decreases in mean body weight gain for females for Days 11 to 15.
The haematological parameters were not affected. Statistically significantly raised cholesterol levels were seen in mid-high dose males and high dose males and females. This was associated with increased total bilirubin in mid-high and high dose males. Discoloured urine was seen at all dose levels and ranged in colour from orange at the low dose to red or black in the high dose animals. This hampered reading reagent test strips but there were no abnormalities in the urinary sediment.
Absolute and relative liver and kidney weights were increased in high dose animals. The increases in liver- and kidney-to-body weight percentages were statistically significant. These increases were considered dose related and possibly adverse.
There were no dose-related macroscopic observations.

The toxicological significance of clinical pathological changes at mid-high and high doses could not be determined since though the tissues were preserved, no histopathology was performed. Based on relative changes in liver and kidney weights and related clinical pathology findings at 750 mg/kg/day, the NOAEL following oral gavage of GTS03977 was considered to be 300 mg/kg/day.

Ref.: 10

Comment
This was a range-finding study. The NOAEL of 300 mg/kg/day was derived without assessing the histopathology.

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

Guideline:	OECD 408 (1998)
Species/strain:	Rat, Sprague Dawley Crl:CD IGS BR
Group size:	5 per dose/sex; 50 in total
Test substance:	GTS03977
Batch:	# 32
Purity:	100.3%
Dose:	0, 2.5, 10, 25 mg/kg bw/day
Route:	oral gavage
Volume/Vehicle:	5 mL/kg in polyethylene glycol 400
Exposure:	91 days
Recovery period:	4 weeks
GLP:	in compliance
Date:	13.09.2005

The animals were observed twice daily for abnormalities, and signs of pain or distress or death. Detailed clinical observations were performed and body weights recorded once prior to treatment, on Day 1, weekly thereafter, and on the day of each scheduled sacrifice.

Neurobehavioral clinical observations were performed weekly; hand-held and open-field expanded clinical observations were done pre-study and during Weeks 4, 8, and 13; elicited behaviour observations were done pre-study and during Week 13; and motor activity data were collected pre-study and during Week 13. Ophthalmic examinations were done prior to treatment and during Week 13. Body weights were collected twice prior to treatment, on Day 1, and weekly thereafter. Food consumption was measured weekly. From Week 10, vaginal cytology data were collected daily for 21 consecutive days. Blood and urine samples for haematology, clinical chemistry, urinalysis, and urine chemistry were collected prior to scheduled sacrifice. On Day 93 (all males and one female) or 94 (remaining females), up to 15 animals/sex/group were anesthetized, weighed, and killed.
On Day 121, all surviving recovery group animals were anesthetized, weighed, and killed. Macroscopic observations were recorded; selected organs weighed, and selected tissues collected and preserved for peer reviewed histopathology. Male reproductive potential (sperm motility, morphology and counts) was assessed at each scheduled sacrifice.
All animals survived to their respective culling with the exception of two control (Group 1) females that died on Day 25. No abnormal clinical observations were noted for these animals prior to death. Organ weights were not recorded for these two animals. Deaths were attributed to a gavage error or aspiration as the lungs were diffusely red with signs of pulmonary haemorrhage and/or congestion.
No substance-related effects on mean body weights, body weight changes, or food consumption were observed. There was a dose-related increase in the incidence of orange staining of fur and skin in animals dosed at 10 or 25 mg/kg/day. Urine was darker or discoloured for males at all dose levels and females given 10 or 25 mg/kg/day. No other clear effects on clinical pathology test were recorded.

Conclusion
There were no other statistically significant treatment-related effects in any dose group at the end of the treatment and recovery phases. The NOAEL for GTS03977was determined to be 25 mg/kg bw/d in this study.

Ref.: 11

Comment
According the OECD guidelines, the highest dose should induce toxicity.

3.3.5.3. Chronic (> 12 months) toxicity

See 3.3.7. Carcinogenicity

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2*uvr*A (pKM101)
Replicates:	duplicates or triplicates in 2 individual experiments both in the presence and absence of metabolic activation
Test substance:	GTS03977
Solvent:	DMSO
Batch:	32
Purity:	99.8 %
Concentrations:	Experiment 1: 2.5, 5, 20, 50, 200, 500, 2000 and 5000 µg/plate without and with S9-mix

	Experiment 2: without	10, 25, 50, 100, 250, 500, 1000 and 3000 µg/plate and with S9-mix
Treatment:	Experiment 1: and	pre-incubation method with 60 minutes pre-incubation 48 - 72 h incubation.
	Experiment 2: without	direct plate incorporation with 60 ± 12 h incubation S9-mix
GLP:	in compliance	
Date:	27 July – 27 September 2004	

HC Orange n° 1 was investigated for the induction of gene mutations in both *Salmonella typhimurium* and *Escherichia coli* (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 in a preliminary toxicity and mutagenicity test with all strains both without and with S9-mix. Toxicity was evaluated up to the prescribed maximum concentration of 5000 µg/plate on the basis of a thinning of the bacterial background lawn and a reduction in the number of revertant colonies. The results of the initial assay were used to select the doses for the confirmatory assay (experiment 2). Experiment 1 was performed with the pre-incubation method; experiment 2 with the direct plate incorporation method. Negative and positive controls were in accordance with the guideline.

Results
In experiment 1, toxic effects in the form of a thinning of the background bacterial lawn were found in all strains tested both in the presence and absence of metabolic activation from 200 µg/plate; in experiment 2 from 200 µg/plate in the absence of metabolic activation and from 500 µg/plate in the presence of metabolic activation. In experiment 1 precipitation of HC Orange #1 was observed in all strains from 2000 µg/plate both in the presence and absence of metabolic activation. In experiment 2, precipitation was found from 500 µg/plate in the presence of metabolic activation and from 250 µg/plate in the absence of metabolic activation.
In both experiments, no biological relevant, dose related and reproducible increases in revertants were observed in any of the strains tested in the absence or presence of metabolic activation.

Conclusion
Under the experimental conditions used HC Orange #1 was not mutagenic in this gene mutation tests in bacteria.

Ref.: 12

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476
Cells:	L5178Y $tk^{+/-}$ mouse lymphoma cells
Replicates:	single cultures per concentration in one experiment
Test substance:	GTS03977
Solvent:	DMSO
Batch:	32
Purity:	99.8 %
Concentrations:	15, 20, 25, 30, 35, 37.5, 40 and 42.5 µg/ml (without S9-mix) 10, 15, 20, 25, 30, 35, 37.5 and 40 µg/ml (with S9-mix)
Treatment	4 h both without and with S9-mix; expression period 2 days and a selection period of 12 days
GLP:	in compliance
Date:	27 July – 18 August 2004

HC Orange n° 1 was assayed for mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. Test concentrations were based on the

results of a toxicity test measuring reduction in cell growth relative to the concurrent vehicle control cell cultures. In the main test, cells were treated for 4 h followed by an expression period of 2 days to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from Arochlor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as percentage relative total growth (relative suspension growth of the cells over the 2-day expression period multiplied by the relative cloning efficiency at the time of selection). To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Negative and positive controls were in accordance with the OECD guideline.

Results
Both in the absence and presence of S9-mix the appropriate level of toxicity (10-20% survival after the highest dose) was reached. Both in the absence and presence of S9-mix, a biological relevant and dose dependent increase in mutant colonies was observed. This increase appeared to be the result of an increase in the number of small colonies.

Conclusion
Under the experimental conditions used, the positive results obtained with HC Orange #1 in this mouse lymphoma assay at the *tk* locus indicate a mutagenic potential. Because an increase in the frequency of small colonies was observed, the result indicates a clastogenic mode of action.

Ref.: 14

In vitro Mammalian Chromosome Aberration Test

Guideline:	OECD 473
Cells:	CHO-WBL cells
Replicates:	duplicates in 2 independent experiments
Test substance:	GTS03977
Solvent:	DMSO
Batch:	32
Purity:	99.8 %
Concentrations:	4 h treatment without S9-mix: 10, 40 and 60 µg/mL 4 h treatment with S9-mix: 10, 20 and 30 µg/mL 20 h treatment without S9-mix: 5, 7.5 and 10 µg/mL
Treatment:	4 h treatment and harvest time ~20 h after start of treatment both in the absence and presence of S9-mix; ~20 h treatment and harvest immediately after the end of treatment in the absence of S9-mix only
GLP:	in compliance
Date:	13 July – 7 December 2004

HC Orange n° 1 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 an initial toxicity assay on cell count, cell growth and cell growth inhibition a.o. after 4 h and 24 h treatment. Cells were treated for 4 h and harvested ~20 h after the start of treatment or for 20 h and harvested immediately after the end of treatment. Approximately 2 h before harvest, each culture was treated with colcemid (final concentration 0.1 µg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from Arochlor 1254-induced rats was used as exogenous metabolic activation system. Chromosome (metaphase) preparations were stained with 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 initial toxicity assay, HC Orange n° 1 precipitated at doses > 80 µg/ml; in the main tests precipitation was not observed at the doses tested. Biologically relevant increases in

polyploid metaphases were not found. In the cultures after 4 h treatment with S9-mix an increase in cells with endoreduplication was observed. Cytotoxicity was demonstrated by an increase of the cell growth inhibition.
After 4 h treatment in the presence of S9-mix and after 20 h treatment in the absence of S9-mix, biologically relevant increases in cells with chromosomal aberrations were found with the highest increase at the mid dose. After 4 h treatment in the absence of S9-mix a dose dependent and biologically relevant increase in cells with chromosomal aberrations was observed.

Conclusion
Under the experimental conditions used the increase in cells with structural chromosomal aberrations indicates that HC Orange n° 1 was clastogenic *in vitro*.

Ref.: 13

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	CD-1®(ICR)BR mice
Group size:	5 male mice/dose group
Test substance:	GTS03977
Batch no:	32
Purity:	99.8 %
Dose level:	500, 1000 and 2000 mg/kg bw
Route:	oral gavage
Vehicle:	PEG 400
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the control and highest dose group only.
GLP:	in compliance
Date:	26 August – 27 September 2005

HC Orange n° 1 has been investigated for the induction of micronuclei in bone marrow cells of mice. Test concentrations were based on the results of a dose range-finding study in male and female mice on toxic signs and mortality at 1, 4, 24 and 48 h after start of treatment. Since no relevant differences in toxicity between sexes were observed, only male mice were used in the main experiment. In the main experiment mice were exposed by gavage to single doses of 0, 500, 1000 and 2000 mg/kg bw. Mice were examined immediately after dosing, at 1 and 4 h after dosing and at least daily for the duration of the experiment for signs of clinical toxicity and mortality. 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 normochromatic erythrocytes (PCE/NCE). An additional satellite group of 15 mice, treated with 2000 mg/kg bw, was included for determination of plasma concentrations of the test article. Blood was collected at 1, 2, 4, 6, 8, 24 and 48 h after dosing (3 mice per time-point). Bone marrow preparations were stained with May-Grünwald/Giemsa and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD guideline.

Results
Both in the preliminary study and the main experiment, all mice remained healthy until the end of the observation period. In the main experiment all animals from the 1000 and 2000 mg/kg bw groups showed pail ears and tails 1 h after dosing and orange urine 1 day after treatment with HC Orange n° 1. Treatment with HC Orange n° 1 did not result in decreased PCE/NCE ratios compared to the untreated controls. However, toxicokinetic results confirmed biological evidence of bone marrow exposure. Elevated plasma concentrations were detected up to 24 h after administration with a peak mean plasma concentration

approximately after 1 h. Moreover, clinical signs indicated to systemic availability of HC Orange n° 1.
Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found at any dose tested, neither 24 nor 48 h after treatment.

Conclusion
Under the experimental conditions used HC Orange #1 did not induce an increase in the number of micronucleated PCEs in bone marrow cells of treated mice and, consequently, HC Orange n° 1 is not clastogenic and/or aneugenic in bone marrow cells of mice.

Ref.: 15

Unscheduled DNA synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline:	OECD 486
Species/strain:	male Sprague Dawley rats
Group size:	3 rats per dose
Test substance:	GTS03977
Batch:	32
Purity:	100.3%
Dose level:	1000 and 2000 mg/kg bw
Route:	oral gavage
Vehicle:	PEG 400
Sacrifice times:	2-4 h and 12-16 h after dosing
GLP:	in compliance
Date:	27 June – 22 July 2005

HC Orange n° 1 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test concentrations were selected on information supplied by the sponsor. Hepatocytes for UDS analysis were collected 2-4 h and 12-16 h after administration of HC Orange n° 1. 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 8 days. UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. Also the percentage of cells in repair (defined as cells with a net grain count of ≥ 5) was calculated for each animal. Negative and positive controls were in accordance with the OECD guideline.

Results
Mortality was not observed. Most animals both of the 2-4 h and the 12-16 h treatment time appeared normal immediately following dosing and prior to harvest. Only the control animals of the 12-16 h group suffered from diarrhoea prior to harvest. Prior to scoring, slides were examined for toxic effects of treatment, such as irregularly shaped or very darkly stained nuclei, of which none was detected. 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- 4h and the 12-16 h treatment time.

Conclusion
Under the experimental conditions used HC Orange n° 1 did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 16

3.3.7. Carcinogenicity

Topical application, mice

Guideline:	/
Species/strain:	Swiss Webster mice
Group size:	50 animals per sex and dose
Test substance:	Hair dye formulation (P-24; non-oxidative formulation) containing 0.15% HC Orange n° 1 (unknown purity and specifications). The hair dye formulation is not given in ref. 20, but is specified in Appendix II to the COLIPA submission. P-24 contained 13 dye ingredients.
Batch:	/
Purity:	/
Dose:	0.05 ml
Route:	Topical, 1 application weekly.
Exposure:	23 months.
GLP:	not in compliance
Date:	1980 (publication)

The experiment involved 12 treatment groups (9 oxidative and 3 non-oxidative hair dye formulations) and 3 negative control groups.

The hair dye formulations were applied topically to a 1 cm² area on a clipped (24 hours prior to application) site in the interscapular region. The mice received a dose of 0.05 ml topically without occlusion once weekly from 8 – 10 weeks of age for 23 months. The animals were observed daily for mortality and signs of toxicity, and were weighed monthly. A continuous weekly record was maintained for any skin lesions noted. After 9 months of treatment, 10 males and 10 females per group were necropsied and the study was terminated after 23 months. Skin and internal organs were evaluated histologically.

5 males and 9 females survived to 23 months in the group receiving HC Orange n° 1 (P-24) while 3 males and 8 females survived in the concurrent negative control group. There were no significant differences in absolute or relative liver or kidney weights in the groups of 10 male and 10 female mice necropsied after 9 months.

No unusual tumour types were observed in either the test or control groups. There were no statistically significant differences in the distribution of tumours between the test group and the control groups.

Ref.: 20

Comment
2,4-Diaminoanisole (EU, carcinogenic category 2) was tested in the same experiment and no response was found. It should also be noted that the concentration of HC Orange n° 1 was only 0.15%. No conclusion with regard to carcinogenicity can be made from the study.

Topical application, rats

Guideline:	/
Species/strain:	Charles River rats
Group size:	60 animals per sex and dose (obtained from the first mating of a multi-generation reproduction study where parents had received topical application of the hair dye formulation from the time of their weaning to the weaning of the offspring)
Test substance:	Hair dye formulation (P-24; non-oxidative formulation) containing 0.15% HC Orange n° 1 (unknown purity and specifications). The hair dye formulation is not given in ref. 21, but is specified in Appendix II to the COLIPA submission. P-24 contained 13 dye ingredients.
Batch:	/
Purity:	/

Dose:	Initially 0.2 ml increasing by increments of 0.1 ml per application weekly until reaching 0.5 ml per application.
Route:	Topical, 2 applications per week.
Exposure:	23 months.
GLP:	not in compliance
Date:	1979 (study report)

The experiment involved 12 treatment groups (9 oxidative and 3 non-oxidative hair dye formulations) and 3 negative control groups. In the submission (ref 21), the results from with 4 hair dye formulations (1 oxidative and 3 non-oxidative dye formulations) are presented.

60 male and 60 female weanling rats obtained from the first mating (F_{1a}) of a multi-generation reproduction study in rats were used in the study. The F_0 parents had received topical application of the hair dye formulation from the time of their weaning to the weaning of their offspring. The non-oxidative dye formulation containing 0.15% HC Orange N° 1 (P-24) was administered topically to the shaved (24 hours prior to application) neck and back area twice weekly. An initial dosage level of 0.2 ml/rat was increased incrementally by 0.1 ml per week until 0.5 ml was achieved. There were three independent control groups each containing 60 males and 60 females, which received no treatment.

The rats were observed daily for overt signs of toxicity and for mortality. Detailed observations were recorded weekly. Individual body weights were recorded weekly for the first 14 weeks and monthly thereafter. Group food consumption was recorded weekly. Haematological, biochemical and urinalysis studies were done on 5 males and 5 females per group at 3, 12, 18, and 24 months of study. After 12 months of treatment, 5 males and 5 females from each group were sacrificed and necropsied and all rats of a sex group were sacrificed and necropsied when survival reached 20%. Histopathological evaluations were performed on 18 tissues (plus tumour masses) including treated skin.

Results
Survival just prior to terminal sacrifice (at week 117, 118 or 119) was 11 males and 15 females for the exposed group. Survival was 15 males and 14 – 18 females for the control groups. After 114 weeks, group mean body weights in the treated group were 740 g in males and 496 g in females. Control group values ranged from 682 to 759 g in males and 477 to 513 g in females.

Haematology: Isolated variations in haematological values included decrease in total erythrocytes, haemoglobin and haematocrit in one treated female at 12 months and in one treated male at 24 months.
Biochemistry: Increases in SGOT, glucose, urea nitrogen and alkaline phosphatase were observed in one female rat at 12 months.
Urine: Rats in the treatment group had dark straw coloured urine at 3, 12 and 24 months.
Autopsy: Gross observations considered to possibly be test material related were skin lesions including ulceration, scabbing, abscesses and thickening, colouring of the fur and skin at the application site and increased incidences of enlarged and/or firm livers.
Histology: The incidence of parathyroid hyperplasia was higher in treated male and female rats than in control groups. The incidence of hyperkeratosis and dermatitis was considered higher in treated animals than in controls. The incidence of haematopoiesis in the livers of treated rats was greater than that observed in control rats however the significance of this was not determined. Wide variations in haematopoiesis were noted between groups and between organs.

The most common tumour observed was pituitary adenoma. The incidence of this tumour type was statistically significantly greater in treated female rats (88%; 45/51) compared with the control groups (68%; 34/50, 71%; 36/51, 72%; 36/50). No pituitary adenocarcinomas were, however, found among the treated females, while 2 – 3

adenocarcinomas occurred in each of the control groups. If pituitary adenomas and adenocarcinomas are counted together, the frequencies in the treated females are statistically significantly higher than in control group 1, but not when compared to control group 2 or 3. The number of pituitary adenomas was statistically significantly higher in treated male rats (67%; 31/46) compared with control group 1 (29%; 14/49). However, the number of pituitary adenomas among males was statistically significantly lower in control group 1 (29%; 14/49) compared with control group 2 (64%; 30/47) and control group 3 (53%; 25/47). The incidences of this tumour in rats of both sexes was comparable to that routinely observed in aging rats of this strain at the testing facility and the present findings were not considered to be of biological significance.

The incidence of mammary adenocarcinoma/carcinoma was statistically significantly increased among the treated females (31%; 16/51) compared to control group 1 females (12%; 6/50), but not compared with control group 2 (20%; 10/51) and control group 3 females (26%; 13/50) or with all control females combined (19%; 29/151). Therefore this finding was not considered to be of biological significance.

Ref.: 21

Comment
2,4-Diaminoanisole (EU, carcinogenic category 2) was tested in the same experiment and no response was found. It should also be noted that the concentration of HC Orange n° 1 was only 0.15%. Moreover, several pages were missing in the submission.
No conclusion with regard to carcinogenicity can be made from the study.

3.3.8. Reproductive toxicity

3.3.8.1. Multi-generation reproduction toxicity

HC Orange n° 1 in Formulation

Guideline:	/
Species/strain:	Charles River rats
Group size:	F_0: 80 per group (40 males and 40 females) F_{1b}: 40 per group (20 males and 20 females) F_{2b}: 40 per group (20 males and 20 females)
Test substance:	Hair dye formulation (P-24; non-oxidative formulation) containing 0.15% HC Orange #1 (unknown purity and specifications).
Batch:	/
Purity:	/
Dose:	Initially 0.2 ml increasing by increments of 0.1 ml per application weekly until reaching 0.5 ml per application.
Route:	Topical, 2 applications per week dorso-scapular area
Exposure:	23 months, continuous through growth, mating, gestation, lactation and weaning of litters,
Gestational Exposure:	GD 1, 4, 7, 10, 13, 16 and 19
Negative control:	3 independent groups not treated with hair dye formulations
Route:	Topical, dorso-scapular area
GLP:	not in compliance
Study period:	17 October 1974 – 24 September 1976

Four different formulations were applied separately in this multi-generation study. Treatment was continuous through growth, mating, gestation, lactation and weaning of F_{1b}, F_{2b}, F_{3b} litters of respective generations.
Topical application twice a week was on the neck and back where the hair had been clipped at least 24h previously. The application was as even as possible to ensure skin contact and avoid run off. No standard area of coverage was mentioned in the report. Skin irritation was minimised by applying the formulation on adjacent sites over on successive days.

The test formulation was applied to the parental generation (F_0) until they reached 100 days of age after which they were mated. The F_0 parents were reduced to 20 males and 20 females and rebred to produce F_{1b} litters. Twenty males and twenty females from the F_{1b} litter were then administered test material until they reached 100 days of age and were mated twice to produce the F_{2a} and F_{2b} litters, avoiding brother-sister pairings.
Twenty males and twenty females from the F_{2b} litter were then administered test material until they reached 100 days of age and mated to produce the F_{3a}, F_{3b} and F_{3c} litters.

Results
Parental generations
There was no significant difference in body weight, food consumption or survival between test and control groups in the F_0 and F_1 generations. Other occasional effects were soft faeces, respiratory congestion and nasal discharge. It is stated that some skin effects; scabbing, fissuring, atonia and leathery texture, were seen in the treated animals but this was a generic statement for all the formulations tested. No further details were provided.
Reproductive performance of F_0, F_1 and F_2 parental rats was similar with no differences between test and control groups in fertility, gestation and live birth indices. The F_2 parents had markedly reduced fertility indices for three separate matings to produce the F_{3a-c} litters of both the control groups and the test groups. There were no significant differences between the control and dosed groups with regard to fertility. Thus, it was considered to be due to a spontaneous reduction in sperm motility and not treatment related. A possible cause was that both treated and control groups developed sialadenitis followed by respiratory congestion.
Therefore it was concluded that this test formulation did not cause reduction in fertility.

Offspring
There were no significant differences between body weights, litter size and survival rates between the test and control groups.

Ref.: 22

3.3.8.2. Teratogenicity

Range finding study

Guideline:	OECD 414
Species/strain:	Rat, Crl:CD (SD)IGS BR VAF/Plus
Group size:	8
Test substance:	GTS03977 (H.C. Orange 1)
Batch:	Lot 32
Purity:	99.9%
Dose:	0, 25, 75, 200 and 500 mg/kg/d in Polyethylene Glycol 400
Route:	Gavage, 5 ml/kg
Exposure:	Gestation Day (GD) 6-20
GLP:	in compliance
Date:	8 August 2005

Female rats with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed in situ were considered to be at DG 0 and assigned to individual housing. Animals were also examined for clinical observations, general appearance, abortions, premature deliveries and death daily. Food consumption and body weight were recorded on GD 0, 6, 9, 12, 15, 18, 20 and 21. On GD 21, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section.

Results
In the 200 mg/kg/day group, two deaths and one premature delivery and subsequent culling of the dam occurred. These were not considered related to the test substance but no cause for the deaths could be determined. Body weight and food consumption were similar

to the others at this dose. All tissues appeared normal at *post-mortem* between 13-15 foetuses in utero. This was comparable to the controls. All other rats survived to GD21.
Clinical observations considered to be test-substance related included discoloured urine (orange or red) and fur (red and/or orange) observed in all groups, discoloured tail (red and/or orange) and rales observed in the 75, 200 and 500 mg/kg/day groups and red perivaginal substance and audible breathing observed in the 500 mg/kg/day group. Soft or liquid faeces were observed in at least one rat in each of the groups, including the control group, most occurred in the 500 mg/kg/day group. Orange perioral substance was observed in rats in the 25, 75 and 200 mg/kg/day dosage groups. All other clinical observations were unrelated to the test substance because: 1) these clinical signs occurred in only one to two rats in any dosage group; and/or 2) the number of affected rats in each dosage group did not occur in a dosage-dependent manner. These observations included chromorhinorrhea, dehydration, red substance in cage pan, excess salivation, localized alopecia (back or limbs) and urine-stained abdominal fur.
At *post-mortem*, some lymph nodes and abdominal adipose tissue appeared yellow or orange in the 200 and 500 mg/kg/day groups. At the highest dose, kidneys, stomach, small intestine, mesentery and mesenteric lymph nodes were yellow or orange. This was considered to be related to the colour of the test substance. All other tissues appeared normal.
Compared with control group, mean body weights and body weight gains were decreased in the 500 mg/kg/d dose group throughout the study. Mean gravid uterine weights and corrected maternal body weights on GD 21 were decreased. Corrected maternal body weight gains revealed a body weight loss for the dosage period, and reduced body weight gain during the gestation period in the 500 mg/kg/day dosage group. Mean body weight gains were decreased in the 75 and 200 mg/kg/day dosage groups on GD 6 to 9 compared with the controls.
Mean absolute and relative food consumption values were decreased in the throughout the study period in 500 mg/kg/day group compared with the controls. Mean absolute food consumption values were decreased in the 200 mg/kg/day group throughout the study period.
At all dose levels, the number of corpora lutea and implantations were similar to the controls.

Foetal parameters:
Mean foetal body weights (total, male and female) were decreased in the 500 mg/kg/day group, in compared with the control group. The mean litter sizes and the number of live foetuses were similar to the controls. One dam in the 500 mg/kg/d dose group had whole litter loss consisting of 15 resorbed conceptuses. There were no significant differences in foetal abnormalities between the treated and control litters
Based on these data, dosages of 0 (Vehicle), 25, 75 and 250 mg/kg/day of GTS03977 were selected for the developmental toxicity study in rats.

Ref.: 17

Main study

Guideline:	OECD 414
Species/strain:	Rat, Crl:CD (SD)IGS BR VAF/Plus
Group size:	25
Test substance:	GTS03977 (H.C. Orange 1)
Batch:	Lot 32
Purity:	99.9%
Dose:	0, 25, 75 and 250 mg/kg/d in Polyethylene Glycol 400
Route:	Gavage, 5 ml/kg
Exposure:	Gestation Day (GD) 6-20
GLP:	in compliance
Date:	9 August 2005

Female rats with spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed in situ were considered to be at DG 0 and assigned to individual housing. Animals were also examined for clinical observations, general appearance, abortions, premature deliveries and death daily. Food consumption and body weight were recorded on GD 0, 6, 9, 12, 15, 18, 20 and 21. On GD 21, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section.

Results
Maternal parameters
No deaths occurred. From GD14 to 21, mean body weights were significantly reduced ($p \leq 0.05$ or $p \leq 0.01$) in the 250 mg/kg/day dosage group, in comparison with the controls. Gravid uterine weights were comparable among the four dosage groups. Once corrected for the weight of the gravid uterus, significantly reduced ($p \leq 0.01$) mean body weights on GD 21 and mean body weight gain during the GD 6-21 were seen the 250 mg/kg/day group. At the lower doses the body weights and body weight gains were unaffected.
Absolute and relative food consumption values were decreased the throughout the study period in 250 mg/kg/day group compared with the controls. There was a significantly reduction ($p \leq 0.05$) over GD 15-21. At the lower doses, the absolute and relative food consumption were similar to the controls.
At all dose levels, the litter averages for corpora lutea, implantations, litter sizes, live foetuses, early and late resorptions, foetal body weights, percent resorbed conceptuses, and percent live male foetuses were similar to the controls. No dams had litters of only resorbed conceptuses, and there were no dead foetuses. All placentae appeared normal.
The numbers of rats with light orange and/or orange skin; orange perioral substance; and dark orange, light orange and/or orange urine were significantly increased ($p \leq 0.05$ or $p \leq 0.01$) in all dosage groups compared with the controls. A significant increase ($p \leq 0.01$) in yellow, light orange and/or orange fur occurred in rats in the 75 and 250 mg/kg/day dosage groups compared with the controls. In the 25 mg/kg/day group, there was also an increase, but not statistically significant compared with the controls. These were considered related to the colour of the test substance and not adverse clinical observations. Rales occurred in rats in all treated groups. This was significantly increased ($p \leq 0.01$) in the 250 mg/kg/day group.
All other clinical observations were considered unrelated to the test substance because: 1) the sign was observed in a similar number of rats in the control group; 2) the number of affected rats in each dosage group did not occur in a dosage-dependent manner; and/or 3) the sign was considered to be a common finding in this species and strain in the laboratory environment.
These observations included soft or liquid faeces, urine-stained abdominal fur, ungroomed coat, sparse hair, chromorhinorrhea, localized alopecia (limbs and/or neck), and missing/broken incisors.

Foetal parameters
No gross external, soft tissue or skeletal alterations (malformations or variations) were caused by doses as high as 250 mg/kg/day. There were no dosage-dependent, significant differences in the litter or foetal incidences of any gross external, soft tissue or skeletal alterations.
Ossification site averages were comparable among the groups and no biologically important differences occurred.

Conclusions
On the basis of these data, the maternal no-observable-adverse-effect-level (NOAEL) for HC Orange n° 1 (GTS03977) is 75 mg/kg/day (the 250 mg/kg/day dosage produced clinical signs of rales and decreases in body weight, body weight gain and absolute feed consumption). The developmental NOAEL of GTS03977 is 250 mg/kg/day (no effects were observed at the highest dose tested).

Ref.: 18

HC Orange n° 1 in formulation

Guideline:	/
Species/strain:	Charles River rats
Group size:	20 rats female; 6 rabbits male and female.
Test substance:	Hair dye formulation (P-24; non-oxidative formulation) containing 0.15% HC Orange #1 (unknown purity and specifications).
Batch:	/
Purity:	/
Dose:	2ml/kg.
Positive control:	acetylsalicylic acid – 250 mg/kg (GD 6-16)
Negative control:	3 independent groups not treated with hair dye formulations
Route:	Topical, dorsoscapular area
Exposure:	GD 1, 4, 7, 10, 13, 16 and 19
GLP:	not in compliance
Date:	1976 (publication)

A number of different hair dyes were included in the formulation P-24.

Results
Maternal parameter:
The skin and fur at the site of application was coloured. No signs of irritation were noted. No signs of toxicity were seen throughout the study. There were no significant differences in body weight gain or food consumption in treated animals versus the negative control groups The number of corpora lutea, implantations, resorptions and abortions were similar to the negative controls.

Foetal parameters:
The sex ratio and number of live foetuses, dead or resorbed foetuses resorptions and foetal abnormalities were similar to the negative controls.
A maternal and foetal NOAEL in this hair dye formulation was derived of 0.15% HC Orange #1.

Ref.: 19

Comment
This study indicates that HC Orange N°1 in this hair dye formulation is not teratogenic.
Included in this publication was a 13 week topical application study on New Zealand white rabbit that suggested that the use of this hair dye formulation twice a week, at the same concentrations as in the above rat study, had no toxic effect.

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

No data submitted

3.3.11. Human data

See point 3.3.3 - Sensitisation (HRIPT)

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 Orange n° 1)
(Non-oxidative / semi-permanent)

Maximum absorption through the skin	**A (µg/cm²)**	**=**	**2.06 µg/cm²**
Skin Area surface	**SAS (cm²)**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.442 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.024 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**25 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**1042**

3.3.14. Discussion

Physico-chemical properties
HC Orange n° 1 is used as an ingredient in non-oxidative hair dye formulations at levels up to 1%.
The stability of HC Orange n° 1 in marketed products is not described. Only one batch of HC Orange n° 1 has been analyzed and characterized. Calculated values of Log P_{ow} cannot be accepted as an estimate of the true physical constant without justification. The absolute content of the dye in any of the batches used is not reported.

General toxicity
The no-observable-adverse-effect-level (NOAEL) for HC Orange n° 1 was determined to be 25 mg/kg bw/d in the 90 study. In the reproductive studies, the NOAEL for maternal toxicity was 75 mg/kg bw/day and 250 mg/kg bw/day for developmental toxicity.

Irritation / sensitisation
Under the test conditions, HC Orange n° 1 was not a skin irritant. HC Orange n° 1 is considered to have the potential to produce transient mild eye irritation.
No evidence of sensitisation was observed in a Guinea Pig Maximisation Test. It did not induce a hypersensitivity response in an LLNA. However, as the tested concentrations were too low, a skin sensitising potential cannot be excluded.

Dermal absorption
Too few chambers were used. Therefore, the Amax of 2.06 µg/cm² (1.03% of the applied dose) should be used for the calculation of the Margin of Safety.

Mutagenicity / genotoxicity
Overall, the genotoxicity of HC Orange n° 1 is sufficiently investigated in valid genotoxicity tests for the 3 types of genotoxic endpoints: gene mutations, structural and numerical chromosome aberration. HC Orange n° 1 did not induce gene mutations in bacteria. In the gene mutation test in mammalian cells HC Orange n° 1 induced an increase in mutant frequency which appeared due to an increase in small colonies which in turn is an indication

for a clastogenic effect. The latter was confirmed in the in vitro chromosome aberration test which showed an increase in cell with chromosomal aberrations.
The putative clastogenic potency of HC Orange n° 1 could not be confirmed in a mouse bone marrow micronucleus tests. Moreover, an *in vivo* UDS test was negative as well.
As the clastogenic effects found *in vitro* were not confirmed in *in vivo* tests, HC Orange n° 1 itself can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
A non-oxidative hair dye formulation containing 0.15% HC Orange n° 1 together with 12 other dye ingredients was tested for carcinogenicity in mice and rats by topical application. No increase in tumour frequency was found. No conclusion with regard to carcinogenicity can, however, be made from the studies due to the low concentration of HC Orange n° 1 used and the fact that the experimental procedure used did not give any response when 2,4-diaminoanisole (EU, carcinogenic category 2) was tested.

4. CONCLUSION

The SCCP is of the opinion that the use of HC Orange n° 1 as an ingredient in non-oxidative hair dye formulations with an on-head concentration of 1% (assuming 100% absolute dye content, does not pose any risk to the health of the consumer.

5. MINORITY OPINION

Not applicable

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