EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Yellow n° 4

COLIPA n° B38

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

The SCCS adopted this opinion at its 5th plenary meeting
of 8 December 2009

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 (EMEA), 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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ACKNOWLEDGMENTS

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

Keywords: SCCS, scientific opinion, hair dye, B38, HC Yellow n° 4, directive 76/768/ECC, CAS 59820-43-8, EINECS 258-002-4

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on HC Yellow n° 4, 8 December 2009

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1. BACKGROUND

Submission I for HC Yellow No. 4 with the chemical name 1-Nitro-3-(2-hydroxyethyl)-oxy-4-(2-hydroxyethyl)-aminobenzene was submitted in September 2003 by COLIPA [1, 2].

Submission II with additional data for this substance was submitted in July 2005 by COLIPA. According to this submission HC Yellow No. 4 is used in semi-permanent hair colouring products at a maximum concentration of 1.5%.

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 Safety (SCCS) consider HC Yellow No. 4 safe for use as a non-oxidative hair dye with a concentration of maximum 1.5 % taken into account the scientific data provided?*

2. *Does the SCCS recommend any further restrictions with regard to the use of HC Yellow No. 4 in non- oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to 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 Yellow n° 4 (INCI name)

3.1.1.2. Chemical names

2-[2-(2-hydroxyethylamino)-5-nitrophenoxy]ethanol (IUPAC)
2-[[2-(2-hydroxyethoxy)-4-nitrophenyl]amino]-ethanol (CA Name)
N,O-di(2-hydroxyethyl)-2-amino-5-nitrophenol
Ethanol, 2-[[2-(2-hydroxyethoxy)-4-nitrophenyl]amino]-
2-[[2-(2-hydroxyethoxy)-4-nitrophenyl]amino]ethanol
2-(Bis(2-hydroxyethyl)amino)-5-nitrophenol
2-(3-nitro-6-(β-hydroxyethylamino)-phenoxy)ethanol
1-nitro-3-(2-hydroxyethyl)-oxy-4-(2-hydroxyethyl)-aminobenzene

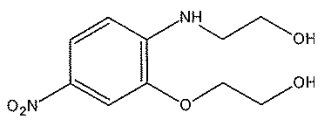
3.1.1.3. Trade names and abbreviations

Colorex HCY4
Rodol 2G Y4
COLIPA B038

3.1.1.4. CAS / EC number

CAS: 59820-43-8
EC: 258-002-4

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{10}H_{14}N_2O_5$

3.1.2. Physical form

Yellow to yellow/green crystalline powder

3.1.3. Molecular weight

Molecular weight: 242.23 g/mol

3.1.4. Purity, composition and substance codes

HC Yellow n° 4 was characterized by FTIR, 1H-NMR, 13C-NMR, HPLC-UV and for NDELA-content. (ref 1a).

Purity: > 95% by HPLC vs external standard

Impurities	
2-(2-amino-5-nitrophenoxy)ethanol	< 0.7% (HPLC area)
N,N,O-tris-(2-hydroxyethyl)-2-amino-5-nitrophenol	< 0.3% (HPLC area)
N-2-[(2-hydroxyethoxy)ethyl]-O-(2-hydroxyethyl)-2-amino-5-nitrophenol	< 0.5% (HPLC area)
N-(2-hydroxyethyl)-O-2-[(2-hydroxyethoxy)]ethyl]-2-amino-5-nitrophenol	< 0.18% (HPLC area)
Nitrosodiethanolamine (NDELA)	< 50 ppb

Comments
There is no information on the analytical method used for determination of NDELA

Heavy metals	
As, Hg, Sb	< 5 ppm
Cd	< 10 ppm
Pb	< 20 ppm

Loss on drying:	< 1%
Residue on ignition:	< 1%

Characterisation of GTS03976 (batch 17)

Batch 17 of GTS03976 was selected as a typical commercial lot of HC Yellow n° 4, used for toxicological experiments 2004/2005.
This batch was used for stability tests of the formulations (3.1.9).

Purity: 99.2% (HPLC at 395nm)

Impurities	
2-(2-amino-5-nitrophenoxy)ethanol	0.312 % (HPLC area)
N-(2-hydroxyethyl)-O-2-[(2-hydroxyethoxy)]ethyl]-2-amino-5-nitrophenol	0.100% (HPLC area)
N,N,O-tris-(2-hydroxyethyl)-2-amino-5-nitrophenol	0.194% (HPLC area)
N-2-[(2-hydroxyethoxy)ethyl]-O-(2-hydroxyethyl)-2-amino-5-nitrophenol	0.190% (HPLC area)
N-Nitroso-N-phenyl-N-hydroxyethylnitrosamine	< 20 ppb
Loss on Drying:	0.097%
Residue on Ignition:	0.26%

The mean purity of HC Yellow n° 4 was determined by fourfold determination as peak area of analysed vs. total peak area after injection of sample by HPLC-UV at 395 nm. Identification of the impurities was performed due to retention times and a reference chromatogram. No standards were used for identification or quantification (ref 1b).

3.1.5. Impurities / accompanying contaminants

See point 3.1.4. Purity, composition and substance codes

3.1.6. Solubility

Water:	1.39 - 2.09	mg/ml
Ethanol:	26.1 - 39.2	mg/ml
DMSO:	252 - 378	mg/ml

Comment
The water solubility is not determined by the EC method A6.

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 0.81 (calculated)

3.1.8. Additional physical and chemical specifications

Melting point:	142 – 147 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	/

3.1.9. Homogeneity and Stability

Stability tests were conducted using GTS03976 (batch 17). Analysis was performed by HPLC-UV at 395 nm. The analytical method is described in ref 1b.
The purity of the same batch (GTS03976, batch 17) was analyzed in 2004 and 2005 to demonstrate the stability of the solid HC Yellow n° 4. The mean purity of four replicate tests was 99.2 and 99.3 % respectively (ref 1a).
The formulations used for the repeated toxicity studies had shown to be stable for at least 14 days. These formulations were used within this time.

Determination of HC Yellow n° 4 in toxicological solutions was performed by HPLC-UV at 395 nm against a standard solution of HC Yellow n° 4 (ref 1a).
PEG 400 solutions were stored at room temperature. Preparations of two concentrations, 1 mg/ml and 200 mg/ml were used for stability tests, four sets of duplicate samples for each dose level.
Three sets of samples were refrigerated and analyzed in duplicate on day 5, 8 and 16. One set was kept at room temperature and analyzed in duplicate after 24 hours.
PEG 400 solutions of HC Yellow n° 4 (1 mg/ml and 200 mg/ml) were shown to be stable (within ± 5% of the initial value) for 24 hours when stored at room temperature and 16 days when stored at 5 ± 3°C (ref 1b).

The stability of HC Yellow n° 4 in DMSO formulations was determined at two concentrations, 0.05 mg/ml and 300 mg/ml. One sample of each concentration was analyzed on the day of preparation and after storage in a freezer (-20°C) for 7 days. DMSO solutions of HC Yellow n° 4 (0.05 mg/ml and 20 mg/ml) were shown to be stable (within ± 3% of the initial value) for 7 days when stored at -20 ± 10°C (ref 1b).

General Comments to physico-chemical characterisation

- Only for one batch, data on identity and purity was provided. For other batches used, the stated purity was between >93 to 99.9%. For many batches used no information on purity was available
- The impurities were not quantified by appropriate standards but relative to absolute resulting HPLC area.
- Only UV-active impurities were identified, because of the lack of other examinations of purity.
- HC Yellow n° 4 is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb. In the absence of a study report, the NDELA content could not be evaluated.
- The stability in typical hair dye formulation is not reported.
- The Log P_{ow} strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log P_{ow}, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.

3.2. Function and uses

HC Yellow n° 4 is used in non-oxidative hair dye formulations with a maximum on-head concentration of 1.5%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401 (1981)
Species/strain:	rat, BOR:WISW, SPF TNO
Group size:	10 (5 males and 5 females)
Test substance:	HC Yellow n° 4
Batch:	/
Purity:	/
Vehicle:	distilled water
Dose:	5000 mg/kg bw
Route:	oral gavage
GLP statement:	/
Study period:	22 January – 12 February 1985

Male and females Wistar Rats of the BOR: WISW strain, in the weight range 193 to 209g (male) and 155-193 g (female) were starved 16h before treatment. HC Yellow n°4 was prepared as a 50 % suspension in distilled water and administered by oral intubation at the doses of 1, 2.5 and 5 g/kg bw in range finding test (2 males per dose). As no mortalities occurred at these doses, 5 g/kg bw was chosen for the primary test (5 males and 5 females). During the observation period of 14 days, a record was kept for mortalities and signs of toxicity. Body weights were recorded on day 0 and day 14 for the surviving animals.

All rats that died were investigated macroscopically to identify organ changes in the skull, thorax and abdomen and surviving animals were similarly examined at the end of the 14-day post-observation.

Results
No mortalities occurred at the dose of 5 g/kg bw. Yellow colorations of the skin and mucosae were observed until the fourth day after administration of the tested dose. No other signs of reaction to treatment were observed.

The results of the test indicated that the lethal oral dose (LD_{50}), was above 5 g/kg bw.

Ref.: 2

Guideline:	/
Species/strain:	rat, Sprague-Dawley
Group size:	25 (2 groups of 5 males and 3 groups of 5 females)
Test substance:	HC Yellow n° 4
Batch:	/
Purity:	/
Vehicle:	3% Acacia in water
Dose:	males: 1250 and 5000 mg/kg bw females: 1250, 2500 and 5000 mg/kg bw
Route:	oral gavage
GLP statement:	/
Study period:	14 January – 5 February 1987

Male and females Sprague-Dawley Rats, in the weight range 190 to 240 g were treated via oral gavage with HC Yellow n°4 in a 10 % suspension in 3% acacia in water at the doses of 1250 and 5000 mg/kg bw for male rats and 1250, 2500 and 5000 mg/kg for female rats (5 rats per dose). Animals were observed during 14 days after treatment.

Results

The results of this study indicated that the median lethal oral dose (LD_{50}), was in the region of 1250 to 5000 mg/kg bw in the male rats and in the region of 1250 and 2500 mg/kg bw in females. Dosing was then extended to larger groups of rats (five males and five females) in order to set the median lethal dose more precisely. Signs of reaction to treatment were not recorded.

Ref.: 3

Comment
The experiment did not conform to a guideline and the experimental description is incomplete.

Guideline:	Acute oral toxicity test (fixed dose procedure) EC Directive 84/449/EEC Annex V, test B1
Species/strain:	rat, Sprague-Dawley
Group size:	20 (2 groups of 5 males and 5 females)
Test substance:	HC Yellow n° 4
Batch:	9307000805
Purity:	/
Vehicle:	0.5% aqueous carboxymethylcellulose
Dose:	2000 and 500 mg/kg bw
Route:	oral gavage
GLP statement:	in compliance
Study period:	29 September – 3 November 1994

Rats of the Sprague-Dawley strain, in the weight range 127 to 186 g were starved overnight before treatment. HC Yellow n°4 was prepared as a suspension in 0.5 % aqueous carboxymethylcellulose and administered by gavage at a constant dose volume of 10 ml/kg. 5 male and 5 female rats were treated at the dose of 2000 mg/kg bw. As this dose produced mortality, a further group of 5 male and 5 female rats were treated at the dose of 500 mg/kg bw. Rats were weighed prior to dosing (day 0), day 7 and day 14. During the observation period of 14 days, a record was kept of mortalities and signs of toxicity. All rats that died were examined macroscopically to identify organs changes and surviving animals were similarly examined.

Results
The dose of 2000 mg/kg bw produced mortality in 4 male and 4 female rats. Signs of piloerection, soiled coat, reduced activity, ataxia, subdued behaviour, prostration, hunched appearance and laboured breathing were observed for all animals in day of dosing. Most of these signs were also observed in the 2 surviving rats at day 5 after dosing. Yellow colorations of urines and extremities were observed in all animals after dosing. These colorations were also observed in surviving rats after the end of the 14 days observation period. Yellow colorations of stomach, intestines, skin, coat and tail were observed.

The dose of 500 mg/kg bw produced no mortality in male and female rats. Signs of piloerection, reduced activity and hunched appearance were observed for all animals from the day of dosing until 5 days after dosing. Yellow colorations of urines and extremities were observed in all animals after dosing. These colorations were also observed after the end of the 14 days observation period. No other effects were observed at this dose.

Conclusion
Under the conditions of the study, HC Yellow n°4 is considered as having acute oral toxicity above 500 mg/kg/bw.

Ref.: 4

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:	rabbit
Group size:	6 (4 males and 2 females)
Test substance:	HC Yellow n° 4
Batch:	/
Purity:	/
Vehicle:	water
Dose volume:	500 mg test substance moistened with water (slurry)
GLP:	/
Study period:	12 – 15 January 1987

500 mg of an aqueous slurry of the test substance was applied to intact skin. Site and conditions were not stated.

Results
Readings 1 day and 3 days after applications showed no redness or oedema.

Conclusion
Under the conditions of the test, HC Yellow n° 4 was not irritant to rabbit skin.

Ref.: 7

Comment
The experiment did not conform to a guideline and the experimental description is incomplete.

3.3.2.2. Mucous membrane irritation

Guideline:	/
Species/strain:	rabbit, New Zealand White
Group size:	4 (1 male and 3 females)
Test substance:	HC Yellow n° 4
Batch:	/
Purity:	/
Vehicle:	/
Dose level:	neat substance
Dosing volume:	100 mg
GLP:	/
Study period:	13 – 16 January 1987

100 mg test substance was instilled into left conjunctival sac of each animal; the right eyes served as the controls. After 20 seconds, the eyes of 2 animals were rinsed with 20 ml water. Reading times: 1 hour, 1, 2 and 3 days after instillation.

Results
Moderate conjunctival redness, mild lid swelling and mild to moderate discharge in treated eyes of all animals at 1 hour post dosage. At day 1, clearing was noted in one out of four animals. All treated eyes were clear at day 2. Rinsed eyes showed similar pattern to non-rinsed.

Conclusion
HC Yellow n° 4 was considered to be an eye irritant in rabbits.

Ref.: 6

Comment
The experiment did not conform to a guideline.

3.3.3. Skin sensitisation

Buehler test

Guideline:	"conforms to OECD"; study began as a GPMT but was changed	
Species/strain:	Guinea pig, Dunkin-Hartley	
Group size:	20 females, 10 controls	
Test substance:	HC Yellow n° 4	
Batch:	9307000805	
Purity:	/	
Vehicle:	corn oil	
Concentration:	induction:	0.5ml 75% in corn oil, 1 application per week for 3 weeks
	challenge:	0.5ml 75% in corn oil, 1 application 2 weeks after third application

Adjuvant	none
Control	0.5% aqueous carboxymethylcellulose
GLP:	in compliance
Study period:	15 September – 14 November 1994

Test animals received 0.5 ml 75% HC Yellow n° 4 in maize oil under occlusion with foiled back patches (2.5 cm x 2.5 cm patch) on shaved flanks for 6 hours, on 3 occasions one week apart; control group was similarly treated, but with 0.5% aqueous carboxymethylcellulose.

2 weeks after induction, challenge was with 0.5ml 75% HC Yellow n° 4 in maize oil under occlusion with foiled back patches (2.5 cm x 2.5 cm patch) on shaved flanks for 6 hours. Reading times were 24 and 48 hours after patch removal.

Results
No abnormal changes were observed.

Conclusion
Under the conditions of the study, HC Yellow n°4 is non-sensitising in guinea pigs.

Ref.: 8

Magnusson & Kligman Guinea pig maximisation test

Guideline:	/	
Species/strain:	Guinea pig, Pirbright, Hoe:DHPK (SPF-LAC.) /Boe	
Group size:	20 test and 10 control (positive and negative) of both sexes	
Test substance:	HC Yellow n° 4	
Batch:	/	
Purity:	/	
Vehicle:	distilled water	
Positive control:	1-chloro-2,5-dinitrobenzene (DNCB)	
Negative control:	distilled water	
Adjuvant	Freund's (FCA)	
Concentration:	Intradermal:	FCA, 1:1 in distilled water
		HC Yellow n° 4, 1% in distilled water
		DNCB, 0.005% in distilled water
	Dermal:	HC Yellow n° 4, 1.0% in petrolatum
		DNCB, 0.025% in white petrolatum
		Distilled water, 1% in white petrolatum
	Intradermal:	HC Yellow n° 4, 1% in FCA, diluted 1:1 in arachis oil
		DNCB, 0.005% in FCA, diluted 1:1 in arachis oil
		Distilled water, 1% in FCA, diluted 1:1 in arachis oil
	Challenge:	HC Yellow n° 4, 0.1, 0.5 and 1% in distilled water
		DNCB, 0.001, 0.01 and 0.2%
		Distilled water
Dosing volume:	Intradermal:	0.05 ml
	Dermal and challenge:	0.5 ml
GLP:	not in compliance	
Study period:	15 January – 2 February 1985	

After the first two intradermal injections of 0.05 ml of 1% HC Yellow n° 4 in distilled water, the left half of the shaved shoulder region was pretreated with 10% sodium lauryl sulphate in petrolatum to induce slight irritation. 6-8 hours later, 0.5 ml of the test material was mixed with petrolatum and applied to the skin. The treated area was covered with a piece of gauze and animals were wrapped in a strip of adhesive tape.

The following day (2 days after the two intradermal treatments), the bandage was removed and the third intradermal treatment of 0.05 ml of 1% HC Yellow n° 4 in Freund's complete adjuvant was administered.

14 days after induction, challenge was performed as a "closed patch test", using three different concentrations per animal. 0.5 ml each of 1%, 0.5% and 0.1% HC Yellow n° 4 in distilled water was topically administered on the shaved flank in Hilltop Chambers. Shaved region was covered with a gauze swab and adhesive tape. One day later the bandage was removed.

Reading times were 24 and 48 hours after application of challenge.

Results
No reactions were observed in the test animals.

Conclusion
Under the conditions of the study, 1% HC Yellow n° 4 was non-sensitising in this animal model.

Ref.: 9

Comments
The test concentration is lower than the intended use concentration of 1.5%.
In this experiment, the dose of the test substance was too low for hazard assessment.

Local Lymph Node Assay (LLNA)

Guideline:	/
Species/strain:	CBA/CaJ female mice
Group size:	5 per group (45 in total)
Test substance:	1-nitro-3-(2-hydroxyethyl)-oxy-4-(2-hydroxyethyl)-aminobenzene TM#2040
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	0.25, 0.5, 1 and 2% (w/v)
Positive control:	p-phenylenediamine (0.25, 0.5, 1.0 and 2.0%)
GLP:	in compliance
Study period:	15 - 20 March 1999

25 µl/ear of 0.25, 0.5, 1.0 and 2.0% of TM#2040 in DMSO were applied to the dorsal surface of both ears, once per day for 3 days.
On day 5, animals received an intravenous injection of 20 µCi of 3H-thymidine in 250 µl. Five hours later the mice were killed and the draining auricular lymph nodes removed. Cells were washed twice with phosphate buffered saline and precipitated with 1 ml of 5% trichloro-acetic acid overnight. The pellets were recovered by centrifugation and re-suspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid to be analysed.

Concentration	Stimulation Index
TM#2040	
0.25%	1.09
0.50%	2.19
1.0%	1.28
2.0%	0.96
p-phenylenediamine	
0.25%	1.38
0.50%	1.89
1.0%	5.06
2.0%	13.79

Conclusion
TM#2040 was not a contact sensitiser in this study.

Ref.: 10

Comment
The highest concentration tested of 2% was considered too low for hazard assessment.

Human Repeated Insult Patch Test, study 1

Guideline:	/
Species/strain:	Human volunteers (male and female)
Group size:	103 (6 male and 97 female)
Test substance:	C-6838-10; sample "A" (HC Yellow n° 4), described as a "mustard gel"
Batch:	/
Purity:	/
Amount applied:	0.2 ml on the infra-scapular area
Application:	Induction: 9 consecutive applications under occlusion (Webril patch) for 24h, 3 times per week, followed by a 2 week rest period Challenge: identical patches as during induction on unexposed sites
GCP:	/
Study period:	9 March – 16 April 1987

Sites graded 48 and 72 hours after challenge patch application.

Results
4 Subjects showed a reaction (just perceptible erythema) by 72h after challenge.

Conclusion
Under the conditions of this study, C-6838-10; sample "A" was considered non-sensitising.
No information on the concentration of B38 used was given.

Ref.: 11

Comment
The SCCS considers such studies to be unethical.

Human Repeated Insult Patch Test, study 2

Guideline:	/
Species/strain:	Human volunteers (male and female)
Group size:	104 (5 male and 99 female)
Test substance:	C7119-37 "B" HC Yellow n° 4 '3%'
Batch:	/
Purity:	/
Amount applied:	0.2 ml on the infrascapular area
Application:	Induction: 9 consecutive applications under occlusion (Webril patch), 3 times per week, followed by a 2 week rest period Challenge: identical patches as during induction on unexposed sites
GCP:	/
Study period:	21 November 1988 – 10 February 1989

0.2 ml applied to infrascapular area of the back under occlusive patch, either to the right or left of the midline, three times a week, for a total of 9 applications.
Identical patches to those used in induction phase were applied to sites previously unexposed to the test material.

Sites graded 48 and 72 hours after challenge patch application.

Results
No reaction was observed on challenge.

Conclusion
Under the condition of the experiment, C7119-37 "B" HC Yellow n° 4 '3%' was non-sensitising.

Ref.: 12

Comment
The SCCS considers such studies to be unethical. No concentration mentioned for reference 11.

General comment
None of the submitted studies were conformed to guidelines. Although there is no evidence from the available data that HC Yellow n° 4 is a sensitiser, this cannot be excluded.

3.3.4. Dermal / percutaneous absorption

***In vitro* Human Skin penetration, submission I**

Guideline:	/
Tissue:	human female skin, split skin
Group size:	4 donors, total of 12 membranes for commercial and 11 membranes for prototype formulations
Skin integrity:	$^{3}H_2O$ flux < 1.5 mg/cm² per hour
Diffusion cell:	static horizontal diffusion cell, 1.04 - 154 cm²
Test substance:	HC Yellow n° 4 [^{14}C]-HC Yellow n° 4, 34 µCi/mg
Batch:	CN22900186 (non-labelled) CFQ7536 (labelled)
Purity:	/ 99% (radio-labelled)
Test item:	1.0% HC Yellow n° 4 in commercial and in a prototype hair dye base
Dose volume:	10 mg/cm²
Receptor fluid:	Dulbecco's phosphate buffered saline
Solubility receptor fluid:	/
Stability receptor fluid:	/
Method of Analysis:	liquid scintillation counter
GLP:	/
Study period:	January – February 1995

The amount of the test substance in the skin compartments was not determined. At the end of the experiments the skin was "solubilised" to determine the amount of the test substance in the whole skin.

48 hour receptor fluid value was 0.15 ± 0.05 µg/cm² for the commercial dye base, and 0.12 ± 0.04 µg/cm² for the prototype dye base.
Amount remaining in "solubilised" skin after 48 hours = 2.10 ± 0.63% (calculated as being equivalent to 2.1 µg/cm²) for the commercial dye base, and 2.23 ± 0.83% for the prototype dye base.
Total recovery was over 95.18 ± 1.56% for the commercial dye base and 93.29 ± 1.53% for the prototype dye base.

Ref.: 13, submission I

Comment
The experiment did not conform to a guideline and was not in compliance with GLP. The amount of HC Yellow n° 4 which may be considered to be systemically available cannot be calculated from the data.

In vitro Human Skin penetration, submission II

Guideline:	OECD 428 (2004a, 2004b)
Tissue:	dermatomed human skin, 400 µm thickness
Group size:	12 membranes from 3 donors
Skin integrity:	electrical resistance > 10 kΩ
Diffusion cell:	glass diffusion cell, 2.54 cm² membrane area
Test substance:	HC Yellow n° 4 (technical grade) [^{14}C]-HC Yellow n° 4, 50 mCi/mmol; 1.85 GBq/mmol; 7.64 MBq/mg
Batch:	L01956 lot #17
Purity:	99.9% 98.6% (HPLC) (radio-labelled)
Test item:	hair dye cream formulation containing 1.5% HC Yellow n° 4
Dose volume:	20 mg/cm²
Receptor fluid:	4% polyoxyethylene-20-oleyl ether in phosphate buffered saline
Solubility receptor fluid:	< 1.03 mg/ml
Stability receptor fluid:	/
Method of Analysis:	liquid scintillation counter
GLP:	in compliance
Study period:	13 – 20 December 2004

The 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. Samples of the receptor fluid (4% polyoxyethylene 20 oleyl ether solution in phosphate buffered saline) were taken at recorded intervals over a 48h 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 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 Yellow n°4 content. Penetration rates and distribution of HC Yellow n°4 in the test system were calculated.

Two cells were excluded because they were damaged during the first wash. Therefore, results from 10 chambers are used:

	Amount recovered (µg/cm²)											
Cell	**2**	**3**	**6**	**7**	**8**	**9**	**14**	**15**	**17**	**18**	**Mean**	**SD**
Flange	0.124	0.072	0.054	0.057	0.027	0.030	0.036	0.060	0.035	0.031	*0.053*	*0.030*
Donor chamber	0.039	0.127	0.164	0.140	0.038	0.035	0.059	0.504	0.125	0.246	*0.148*	*0.142*
Skin wash @ 0.5h	338	322	307	302	290	301	301	295	317	276	*305*	*17.3*
Skin wash @ 48h	0.317	0.839	0.668	0.763	0.736	0.580	0.937	0.497	1.04	1.538	*0.792*	*0.337*
Stratum corneum	0.091	0.084	0.210	0.164	0.085	0.082	0.208	0.188	0.297	0.315	*0.172*	*0.088*
Epidermis/dermis	0.049	0.044	0.072	0.080	0.062	0.086	0.160	0.073	0.073	0.162	*0.086*	*0.042*
Receptor fluid	0.009	0.011	0.009	0.015	0.009	0.007	0.024	0.028	0.022	0.027	*0.016*	*0.008*
Bioavailable	**0.058**	**0.055**	**0.081**	**0.095**	**0.071**	**0.093**	**0.184**	**0.101**	**0.095**	**0.189**	***0.102***	*0.050*
Bioavailable (%)	0.019	0.019	0.027	0.032	0.024	0.031	0.062	0.034	0.032	0.064	*0.034*	*0.017*

Total (%)	114	109	104	102	97.9	102	102	99.8	107	93.7	*103*	*5.45*

Conclusion
Under the conditions of this experiment in which 20 mg/cm² of a hair dye cream formulation containing 1.5% HC Yellow n° 4 were applied, the amount considered to be systemically available was 0.102 ± 0.050 µg/cm² or 0.034 ± 0.017% of the applied dose.
Ref.: 13, submission II

Comment
The mean value + 1SD (0.102 + 0.050 µg/cm²) should be used in the calculation of MOS.

3.3.5. Repeated dose toxicity

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

Guideline: /
Species/strain: F344/N rat, B6C3F$_1$ mice
Group size: 5 male and 5 female rats, and 5 male and 5 female mice per dose
Test substance: HC Yellow n° 4
Batch: Lot 0-218
Purity: > 93%
Dose levels: 0, 5000, 10000, 20000, 40000 and 80000 ppm (rat)
0, 1250, 2500, 5000, 10000 and 20000 ppm (mice)
Route: oral
Administration: in diet for 14 days
GLP statement: /
Study period: 1992 (date report)

In this 14-day Oral Toxicity Study in F344/N rats and B6C3F$_1$ mice, HC Yellow n°4 was administered daily in diet in both sexes at dose levels of 0, 5000, 10000, 20000, 40000 and 80000 ppm in male and female rats (5 animals per dose), and 0, 1250, 2500, 5000, 10000 and 20000 ppm in male and female mice (5 animals per dose). Animals were observed twice daily for signs of toxicity. Clinical observations were recorded on the day of necropsy. Animals were weighed at the start of the study, on days 7 and 14, and at necropsy. Feed consumption per cage was determined weekly. Complete necropsies were performed on all animals. The brain, heart, right kidney, liver, lung, right testis, and thymus of survivors were weighed at necropsy. Histopathology was performed on selected tissues from all rats in the 0, 20000, 40000 and 80000 ppm dose groups and mice in the 20000 ppm dose groups.

Results

Rats:

All animals survived to the end of the studies.

The final mean body weights and mean body weight changes of males that received doses of 20000 ppm and above and females that received doses of 10000 ppm and above were significantly lower than those of controls.

Feed consumption by males that received doses of 20000 ppm or greater and females that received doses of 10000 ppm or greater was lower than that of the controls during the first week. During the second week, feed consumption by males in the 40000 ppm dose group was lower than controls; feed consumption by other male and female dose groups was similar to or higher than that of the control. Because rats that received 40000 ppm did not gain weight, and the final mean body weights of rats that received 80000 ppm were

decreased approximately 30 %, it was concluded that the feed consumption values were high and may have included feed scattered by animals searching for non-dosed food.

No clinical findings were attributed to HC Yellow n°4 administration. Significant changes in absolute and relative organ weights were observed but were considered to be secondary to decreases in body weights.

Mice:

All mice survived to the end of the studies.

The final mean body weights and mean body weight changes of females and the mean body weight changes of males that received doses of 20000 ppm were significantly lower than those of controls. Final mean body weights and mean body weight changes of other dose groups were similar to those of the controls.

Feed consumption by dosed groups was generally similar to that of the controls during the first week. During the second week, feed consumption by males and females in the 10000 and 20000 ppm dose groups was higher than controls.
No clinical findings in mice were related to HC Yellow n° 4 administration. No biologically significant changes in absolute and relative organ weights were observed. No gross or microscopic lesions were related to HC Yellow n° 4 administration.

Conclusion
In the 14-day rat feed studies, doses of 20000 ppm (males) or 10000 ppm (females) and above caused decreases in body weight and feed intake. In the 14-day mouse feed studies, doses of 1250 to 20000 ppm (equivalent to 150 to 3200 mg/kg) caused no toxic effects.
Ref.: 14

4-Week feeding study

Guideline:	/
Species/strain:	Sprague-Dawley rats
Group size:	100 (50 males and 50 females, 5 groups of 10 each)
Test substance:	HC Yellow n° 4
Batch:	CN22900186
Purity:	/
Dose levels:	0, 0.5, 1.0, 1.25 and 1.5% HC Yellow n° 4 in diet (Purina certified Chow 5002)
Route:	oral
GLP statement:	/
Study period:	1987

In this 4-week oral range-finding toxicity study in the Sprague Dawley Rat, HC Yellow n° 4 was fed to male and female rats at dose levels of 0, 0.5, 1.0, 1.25 and 1.5% in diet. A total of 100 rats were used in this study. The groups comprised 10 animals per sex which were sacrificed after 4 weeks of treatment. Pharmacologic and/or toxicological effects, food consumption and body weights were recorded periodically during acclimatization and the treatment period. At the end of the treatment period, all animals were killed, necropsied and examined post mortem. Macroscopical examinations were performed on organs and tissues and kidneys and liver were weighed.

Results
No mortalities occurred during the study. No pharmacologic and/or toxicological effects were reported except discoloured fur and urine.

The mean body weights were significantly decreased in males treated at the doses of 0.5% at the week 1, 1.0% during weeks 1-3, and 1.25% and 1.50% during weeks 1-4. The mean body weights were significantly decreased in females treated at all doses at week 4. Mean terminal body weight were significantly decreased in the males treated at the doses of 1.0%, 1.25% and 1.50% and in the females treated at the doses of 1.25% and 1.50%.

No noteworthy findings were observed at the terminal necropsy.

No significant difference was observed in the absolute liver weight of the male rats but a significant increase in the relative liver weight in all tested groups for the male rats was reported. There was a significant decrease in both absolute and relative liver weights for the female rats treated at the dose of 0.5% and 1.0%. A significant increase in the relative liver weight in the female rats treated at the dose of 1.5% was observed.

A significant decrease in the absolute kidney weight in the male rats treated at the dose of 1.25% and 1.50% and in all treated female rats was observed. No significant differences were observed in the relative kidney weights.

Conclusion
Based on the results of this study, dose levels of 1%-1.25% of HC Yellow n°4 were proposed for the combined subchronic feeding study.

Ref.: 15, submission I

Comments
The experiment did not conform to a guideline

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

Guideline:	/
Species/strain:	F344/N rat, B6C3F$_1$ mice
Group size:	10 male and 10 female rats, and 10 male and 10 female mice per dose
Test substance:	HC Yellow n° 4
Batch:	0-218
Purity:	> 93%
Dose levels:	0, 2500, 5000, 10000, 20000 and 40000 ppm (rat) 0, 5000, 10000, 20000, 40000 and 80000 ppm (mice)
Route:	oral
Administration:	in diet for 13 weeks
GLP statement:	/
Study period:	1992 (date report)

In this 13-week oral Toxicity Study in F344/N rats and B6C3F$_1$ mice, HC Yellow n° 4 was fed in diet at dose levels of 0, 2500, 5000, 10000, 20000 and 40000 ppm to male and female rats (10 animals per dose), and 0, 5000, 10000, 20000, 40000 and 80000 ppm to male and female mice (10 animals per dose). Animals were observed twice daily for signs of toxicity. Clinical observations were recorded daily. Animals were weighed at the start of the study and weekly thereafter. Feed consumption per cage was determined weekly. Complete necropsies were performed on all animals. The brain, heart, right kidney, liver, lung, right testis, and thymus of survivors were weighed at necropsy. Histopathology was performed on all animals that died or killed moribund prior to the end of the studies, on all control animals, on all rats that received 40000 ppm and on all mice that received 80000 ppm. Tissues examined for rats in the 2500, 5000, 10000 and 20000 ppm dose groups were the kidney, thyroid gland and uterus. The thyroid gland was examined for mice in the 5000, 100000, 20000 and 40000 ppm dose groups.

Results

Rats

All animals survived to the end of the studies.

The final mean body weights of male that received doses of 10000 ppm and above and females that received doses of 20000 ppm or 40000 ppm were significantly lower than those of controls.

Feed consumption by males in all dose groups was generally higher than that of the controls throughout the study. Feed consumption by females that received 40000 ppm was generally higher than that of the controls. Feed consumption by females in other dose groups was lower than that of the controls. The values for feed consumption by rats receiving 40000 ppm were nearly twice that of other groups and are probably due to spillage of unpalatable diet.

No clinical findings were attributed to HC Yellow n°4 administration. Significant changes in absolute and relative organ weights were observed but were considered to be secondary to decreases in body weights.

Lesions related to chemical administration were seen in the thyroid gland of males and females, the kidney in males and the uterus in females. A mineralization of the renal papilla occurred in all male rats in the 40000 ppm dose. Thyroid pigmentation occurred in male and female rats receiving 40000 ppm. Uterine atrophy occurred in female rats in the 20000 and 40000 ppm groups. The severity of all lesions ranges from minimal to mild except for uterine atrophy in the 40000 ppm female group which ranged from mild to moderate.

Mice:

8 males and 7 females in the 80000 ppm dose groups died; 9 of these deaths occurred during week 1, 5 occurred during week 2 and one occurred during week 11. One male that received 40000 ppm died during week 7.

The final mean body weights and mean body weight changes of males and females that received doses of 10000 ppm were significantly lower than those of controls.

High feed consumption by dosed mice was observed, particularly by those received the three highest dose levels, maybe due to spillage of unpalatable feed and therefore might not reflect the actual amount of feed consumed.

No biologically significant clinical findings were observed that were related to HC Yellow n° 4 administration. Statistically significant changes in absolute and relative organ weights were considered to reflect decreases in body weight.

Thyroid pigmentation occurred in mice at all dose levels. Minimal to mild uterine atrophy occurred in female mice in the 40000 and 80000 ppm groups. Lymphoid depletion and subsequent atrophy of the spleen and thymus occurred in male and female mice that received 80000 ppm. These findings were considered to be secondary to the decreased body weights in these groups.

Conclusion
Rats:
The decreases in mean body weights of male rats in the 10000, 20000 and 40000 ppm dose groups were considered quite dramatic (8%, 19% and 29%) and suggested that for male rats, 10000 ppm may exceed an exposure compatible with long-term survival in the 2-year study, thus doses of 2500 and 5000 ppm were selected for the males in the 2 year study. In the females, the weight decreases were much less dramatic (20000 ppm 5%, 40000 ppm

11%) but all females receiving 20000 ppm or more had uterine atrophy. Thus, doses of 5000 and 10000 ppm were selected for the female rats in the 2-year study.
The NOAEL was determined as 5000 ppm for male rats (equivalent to 250 mg/kg/d) and 10000 ppm for female rats (equivalent to 500 mg/kg/d).

Mice:
A combination of deaths and decreased mean body weights relative to controls precluded the selection of doses above 10000 ppm. Therefore, doses of 5000 and 10000 ppm were selected for mice in the 2-year studies.
The NOAEL was determined as 5000 ppm for mice (equivalent to 750 mg/kg/d body weight).

Ref.: 14

Comments
Rats: A NOAEL of 5000 ppm (equivalent to 250 mg/kg/d body weight) for male rats and of 10000 ppm (equivalent to 500 mg/kg/d body weight) for female may be derived from this study.

Mice: A NOAEL of 5000 ppm (equivalent to 750 mg/kg/d body weight) for male and female mice may be derived from this study.

13 Week dermal toxicity

Guideline:	/
Species/strain:	New Zealand White rabbit
Group size:	12 (6 males and 6 females)
Test substance:	HC Yellow n° 4, 0.4% in hair dye formulation (unknown purity and specifications)
Batch:	/
Purity:	/
Vehicle:	/
Dose levels:	/
Dose volume:	1 ml/kg bw
Administration:	topical application, twice weekly for 13 weeks
GLP:	/
Study period:	1976

Hair dye formulation containing 0.4% HC Yellow n° 4 was applied at the dose level of 1 ml/kg bw twice weekly to alternative sides of the thoracic-lumbar area on three animals per sex per group on the first treatment day of each week (6 males and 6 females). 3 independent groups were treated without any dyes. One hour after application test material was removed by shampooing and subsequent rinsing and drying.
The animals were weight weekly during the study. Hematologic and clinical chemistry determinations and examination of urine was performed on all animals at 0, 3, 7 and 13 week. All survivors were sacrified after 13 week and examined for gross lesions. Organ-body weight ratios were determined for liver, kidneys, adrenals, heart, thyroid, spleen and brain. Main organs and tissues were microscopically examined.

Results
No evidence of compound-induced toxicity was seen. Body weight gain of rabbits was at least equal to that of controls. A statistically significant increase in white blood cells in males and in blood urea nitrogen in males and females was reported. Microscopic examinations of 25 tissues from each animal gave no indication of histomorphologic evidence of toxicity. No dye discoloration of the urine was seen during the test.

Conclusions

There was no evidence of HC Yellow n° 4-induced systemic effects.

Ref.: 21

Comment
The experiment did not conform to a guideline and was not performed according to GLP. The study is reported only as an article in J. Toxicol. Env. Health but not provided as a full report. HC Yellow n° 4 was applied twice weekly for 13 weeks. The test concentration of HC Yellow n°4 was lower than the in use concentration in non-oxidative hair dye (1.5%).

90-day feeding study (Combined Sub-chronic feeding, teratology and Dominant Lethal study)

Guideline:	/
Species/strain:	rat, Sprague-Dawley (TAC:N(SD)fBR)
Group size:	360 (40 males (all doses) and 45 (0, 0.1%) or 55 (0.3, 1.0%) females)
Test substance:	HC Yellow n° 4
Batch:	S022900186
Purity:	99.65% Dose levels: 0, 0.1, 0.3 and 1.0% HC Yellow n° 4 in diet
GLP:	/
Study period:	1987

After 13 weeks, 10 rats per sex per group were killed, blood samples were taken for clinical chemistry and haematology studies and liver, kidneys, gonads, thyroids, brain, pituitary, adrenals, and heart were weighed and microscopically examined.

Following the 13 weeks, 25 of the remaining females in each group were mated to untreated males in a teratology study (results described in 3.3.8.2). They were killed on day twenty of gestation for foetal examination. 10 of the remaining females in each group were maintained on their respective diets for a total of 26 weeks. The 10 remaining females in groups C and D were kept on their respective diets for a total of 21 weeks and were then placed on the control diet for 5 weeks.

20 of the males per group remaining after the 13 week necropsy were kept on their respective diets for an additional 7 weeks at which time the test diet were replaced by the control diet and the males were mated to 2 virgin untreated females each week for 2 weeks. These females were then evaluated for a dominant lethal mutagenicity effect due to treatment of the males with HC Yellow n°4 (results described in 3.3.6.2).

The 10 remaining males per group were kept on their respective diets for a total of 26 weeks at which time they, along with all remaining animals, were killed and selected organs were weighed (including testis) and a complete set of tissues were taken for possible microscopic examination. The results are described in the following section.

Results

During the first week of the study, one female from the 1% dose group was found dead and was therefore replaced. During the rest of the 13-week study, one female from the control group and one female from the 0.1% dose group died. These deaths were not considered related to the test material.

During the first 13 weeks of the study, sporadic increases and decreases in weight gain and food consumption occurred in both the males and females. The high level males gained significantly less weight than the controls at the early onset of the study, with the low and mid level males sporadically gaining significantly more weight than the control during the

remaining 13 weeks. The high level females gained significantly less weight than the controls during the entire study and the mid level females sporadically less.

Food consumption by male rats in the high, mid and low dose groups was significantly increased from the control at different time periods during the early weeks of the study. Food consumption by females from the high and mid dose groups was sporadically increased or decreased from the control during the study. In the low dose group, female food consumption significantly decreased from the control.

Only minor differences were seen in the haematology and clinical chemistry values at 13 weeks and no consistent dose related changes were seen in liver and kidney weights. Testis weights were unaffected.

Microscopic examinations were performed on selected tissues and no dose-related histomorphologic alterations were noted in any of the tissues examined.

Ref.: 17, submission I

Comment
The experiment did not conform to a guideline and was not performed according to GLP.

6-months feeding study (Combined Sub-chronic feeding, teratology and Dominant Lethal study)

Guideline:	/
Species/strain:	rat, Sprague-Dawley (TAC:N(SD)fBR)
Group size:	10 males and 10 females per group
Test substance:	HC Yellow n° 4
Batch:	22900186
Purity:	98.9% (approximately)
Dose levels:	0, 0.1, 0.3 and 1.0% HC Yellow n° 4 in diet
GLP:	/
Study period:	1987

Results

During the 6 month phase of this study, a decrease in the weight of rats from the high dose group when compared to controls was observed. This decrease was only significant in females during week 25. The low and mid dose level females also weighed significantly less than the controls during week 25. A significant decrease in food consumption was observed in females from the 0.1% dose group. Significant changes in food consumption were not observed in males.

In the recovery groups, males from the 0.1% and 0.3% dose groups exhibited a significant increase in body weights during week 14-27 except week 23 where it was only significantly increased in the rats treated at the dose of 0.3%. No significant changes in body weight were observed in females.

A significant increase in the relative weight of the liver was observed in rats from all treated groups. A significant decrease in the in the absolute and relative testes weight was observed in the high dose treated rats.
Conclusion:
HC Yellow n°4 when fed to male and female rats at a dose level up to 1% in the diet is toxic, producing a significant decrease in body weights in the female rat and an increase in relative liver weight at all doses.

Ref.: 17, submission I

Comment
The experiment did not conform to a guideline and was not performed according to GLP.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Assay, study 1

Guideline:	OECD 471
Species/Strain:	*Salmonella typhimurium* TA 98, TA100, TA 1535, TA 1537
Replicates:	triplicate, two independent experiments
Method:	plate incorporation
Test substance:	1708
Batch:	/
Purity:	/
Vehicle:	DMSO
Concentration:	8, 40, 200, 1000 and 5000 µg/plate, without and with S9-mix
Control:	without S9-mix: sodium azide, 9-aminoacridine, 2-nitrofluorene With S9-mix: 2-aminoanthracene
GLP:	in compliance
Study period:	April – May 1987

Results
HC yellow n° 4 induced concentration related significant increases in revertant numbers in all strains tested without S9-mix. No toxicity was observed up to 5000mcg/plate (50mg/ml). Increased numbers of revertants were observed for all tester strains without metabolic activation and with all strains, except for TA 1535, with metabolic activation.

Conclusion
The compound is a directly acting frameshift / base substitution mutagen

Ref.: 18, submission I

Comment
No data on batch number and purity were provided.

Bacterial Reverse Mutation Assay, study 2

Guideline:	/
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537
Replicates:	triplicate
Method:	pre-incubation assay
Test substance:	HC Yellow n° 4
Batch:	/
Purity:	93.4%
Vehicle:	DMSO
Concentration:	0, 3, 10, 33, 100, 333, 1000, 3333 and 10000 µg/plate, without and with S9-mix
Control:	without S9-mix: sodium azide, 4-nitro-o-phenylenediamine, 9-aminoacridine With S9-mix: 2-aminoanthracene

GLP: /
Study period: 1986 (date publication)

Results
HC Yellow n° 4 induced concentration dependent increases in numbers of revertants with and without S-9-mix from rats and hamsters.

Conclusion
The compound is a bacterial mutagen inducing frameshift/base substitution mutations
Ref.: 19, submission I

***In vitro* Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1997)
Species/strain:	mouse lymphoma cell line L5178Y $tk^{+/-}$
Replicates:	single cultures, two independent experiments
Test substance:	HC Yellow n° 4 (GTS 03976)
Batch:	17
Purity:	95.0%
Vehicle:	DMSO
Concentrations:	initial assay without S9-mix: 200, 400, 500, 600, 750, 800, 900 and 1000 µg/ml with S9-mix: 400, 500, 600, 750, 800, 900, 1000 and 1100 µg/ml confirmatory assay without S9-mix: 50, 75, 100, 150, 200, 250, 300 and 400 µg/ml
Treatment	Initial assay 4 h treatment without and with S9; expression period 48 h and selection period of 12 days confirmatory assay Experiment II: 24 h treatment without S9; expression period 48 h and selection period of 12 days
Control:	without S9-mix: methyl methane sulfonate With S9-mix: methylcholanthrene
GLP:	in compliance
Study period:	21 September – 22 December 2004

Results
HC Yellow n° 4 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. HC Yellow n° 4 was tested up to the required level of toxicity (10-20 % survival compared to the concurrent negative controls) both in the initial as well as in the confirmatory assay. In the initial assay (4 hours treatment without S-9) at concentrations from 200 to 1000 µg/ml no positive response was observed. A confirmatory assay was performed (- S9-mix, approximately 24 hours) at concentrations of 50.0 to 400 µg/ml. Again none of the treatments induced a mutant frequency that met criteria for a positive response. In the activation mutation assay with a 4-hour treatment period, eight concentrations ranging from 400 to 1100 µg/ml were tested for mutant induction. No increase in the mutant frequency was observed.

Conclusion
HC Yellow n° 4 **(**GTS03976) did not induce forward mutations with and without metabolic activation at the *tk*-locus in L5178Y mouse lymphoma cells.
Ref.: 16

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

Guideline:	OECD 473 (1997)
Species/strain:	Chinese hamster ovary cell (CHO-WBL)
Replicates:	duplicate cultures (initial toxicity assay: single cultures)
Test substance:	HC Yellow n° 4
Batch:	17
Purity:	95.0%
Vehicle:	DMSO
Concentrations:	250, 500, and 1200 µg/ml, without S9 mix, 4 h treatment 250, 375 and 500 µg/ml, without S9 mix, 20 h treatment 250, 500 and 1000 µg/ml, with S9 mix, 4 h treatment
Treatment	4 h treatment, harvest time 20 h after beginning of treatment, without and with S9 mix 20 h treatment, harvest time 20 h after beginning of treatment, without S9 mix
Control:	without S9-mix: mitomycin C with S9-mix: cyclophosphamide
GLP:	in compliance
Study period:	8 September – 30 June 2005

Results
A small but statistically significant increase in structural and numerical (polyploidy and endoreduplication) chromosomal aberrations was observed in the cultures treated with 1200 µg/ml at 4-hour without S- 9 activation (47% cell growth inhibition). With 20 h treatment, a small but statistically significant increase in cells with structural chromosomal aberrations was observed at 375 µg/ml (16% cell growth inhibition), but no statistically significant increase in numerical (polyploidy and endoreduplication) chromosomal aberrations. At 4 hr treatment with S-9 activation no significant increase in cells with structural chromosomal aberrations was observed but a small, statistically significant increase in numerical (polyploidy and endoreduplication) chromosomal aberrations was observed at 1000 µg/ml (59% CGI). The small increase in numerical aberrations was within the historical range for polyploidy and endoreduplicated cells and therefore was not considered to be biologically significant.

Conclusion

HC Yellow n° 4 was exclusively positive for inducing structural and numerical chromosomal aberrations in Chinese hamster ovary (CHO) cells without metabolic activation. Under the experimental conditions used HC Yellow n° 4 was positive in this in vitro chromosomal aberration assay and, consequently, is genotoxic (clastogenic and possibly aneugenic) in CHO cells.

Ref.: 15

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

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

Guideline:	/
Species/strain:	mouse, CFLP
Group size:	70 (35 males and 35 females0
Test substance:	HC Yellow 4
Batch:	/
Purity:	/
Vehicle:	DMSO, 10% in deionised water
Dose level:	300 mg/kg bw

Dosing volume:	60 ml/kg bw
Route:	intraperitoneal injection
Sampling:	24, 48 and 72 hours after treatment; positive control: 24 hours after treatment
Control:	cyclophosphamide
GLP:	in compliance
Study period:	January – March 1985

HC Yellow n° 4 was tested at the single dose level of 300 mg/kg (approximate MTD, because at 500 mg/kg 1/5 male and female, respectively died within 1 hour). 30 mice (15 male, 15 female) were treated with vehicle or HC Yellow n° 4 and were killed 24, 48 and 72 hours later. The positive control, (5 males, 5 females) received CPA (40 mg/kg) and was killed 24 hours later. Bone marrow was obtained from both femurs and 1000 polychromatic erythrocytes (PCE) were counted from each animal (where possible), from coded slides.

Results
No toxic effects of HC Yellow n° 4 on erythropoiesis indicating exposure were observed. There were more cells with micronuclei in treated versus control animals but these increases were not statistically significant at all time points tested. The positive control showed a marked increase in micronucleated PCE versus controls.

Conclusion
Under the experimental conditions used HC Yellow n° 4 was not clastogenic and/or aneugenic in this *in-vivo* micronucleus test in mice.

Ref.: 17

Comment
Toxic effects on erythropoiesis indicating exposure were not observed. However, systemic availability is expected following intraperitoneal application. Batch number and purity were not given.

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo*

Guideline:	/
Species/strain:	male Fisher-344 rat
Group size:	18 (3 males per dose point)
Test substance:	C7634/43 (HC Yellow n° 4)
Batch:	/
Purity:	/
Vehicle:	carboxymethylcellulose, 1% w/v
Dose level:	100, 250, 500 and 1000 mg/kg bw
Dosing volume:	10 ml/kg bw
Route:	oral gavage
Control:	dimethylnitrosamine
GLP:	in compliance
Study period:	1 September – 1 December 1992

HC Yellow n° 4 (dose range 100-1000 mg/kg), vehicle control and positive control (DMN, 10 mg/kg) were administered by gavage to 3 rats/dose point and DNA repair (tritiated thymidine incorporation into nuclear DNA of hepatocytes not in S-phase) was assessed approximately 16 hours later. Hepatocytes isolated from treated animals were incubated in WME medium containing 10% calf serum. Six cultures per dose were seeded. UDS was quantified by counting net nuclear silver grain increase from 50 hepatocytes/ coverslip, 3 coverslips / rat and 3 rats /dose point.

Result

HC Yellow n° 4 did not induce a mean net nuclear grain count ≥ 5 and thus no DNA repair synthesis at any of the dose levels scored.
Vehicle and positive control induced NNG counts within the criteria of a valid test.

Conclusion
HC Yellow n° 4 does not induce unscheduled DNA repair in rat hepatocytes under the conditions tested.

Ref.: 18

Comment
Batch number and purity were not given.

Dominant Lethal study (Combined Sub-chronic feeding, teratology and dominant Lethal study)

Guideline:	/
Species/strain:	rat, Sprague-Dawley (TAC:N(SD)fBR)
Group size:	20 males per group
Test substance:	HC Yellow n° 4
Batch:	22900186
Purity:	98.9% (approximately)
Dose levels:	0, 0.1, 0.3 and 1.0% HC Yellow n° 4 in diet prior to mating basal laboratory chow through mating until end of study
GLP:	/
Study period:	1987

Results
There was a significant increase in body weights over the controls at the doses of 0.1% and 0.3% during weeks 14-27 with the exception of week 23 where the rats treated at the dose of 0.3% only exhibited a significant increase in body weight over the controls.

Several significant differences in the reproductive parameters were observed. HC Yellow n°4 when fed to male Sprague Dawley rats at dose levels up to 1.0% in the diet for 21 weeks does not have a dominant lethal effect. It does however cause a significant decrease in the fertility of the male rats. A significant decrease was indeed noted in the average number of corpora lutea per dam, the average number of implant sites per dam, and the average number of live foetuses per dam at the higher dose. A significant decrease was also noted in the number of animals pregnant in all treated groups.

5 rats (one at the dose of 0.3% and 4 at the dose of 1%) which had failed to sire a litter with any of 8 females after being on test diet for 21 weeks were killed and testes were macroscopically and microscopically examined. In all testes, there was a severe diffuse atrophy affecting the whole of both testes leaving only Sertoli cells in the tubules. Aspermatogenesis was present with total loss of spermatogonia, spermatids, and spermatocytes. The interstitial cells appeared normal. In two rats from the high dose group (1%), mineralization within the lumen of some of the seminiferous tubules, as well as clumping of eosinophilic proteinaceous material, and degeneration and loss of Sertoli cells were present.

Conclusion
Males fed with HC Yellow n°4 for 21 weeks prior to mating showed a significant decrease in fertility. HC Yellow n°4 appeared to have caused a severe diffuse atrophy of the testes in some animals at 0.3% and 1% during the dominant lethal study resulting in permanent sterility. The mechanism by which this occurred is yet unknown.

Ref.: 17, submission I

Comment

The experiment did not conform to a guideline and was not performed according to GLP. The low dose level of 0.1% of HC Yellow n°4 in the diet was not an unequivocal NOAEL in the dominant lethal phase.

Dominant Lethal study (combined teratology and dominant lethal study)

Guideline:	/
Species/strain:	rat, Sprague-Dawley (TAC:N(SD)fBR)
Group size:	60 (20 males per group)
Test substance:	HC Yellow n° 4
Batch:	22900186
Purity:	/
Dose levels:	0, 0.03 and 0.1% HC Yellow n° 4 in diet (for 10 weeks until mating) basal laboratory chow during mating
GLP:	/
Study period:	1988 - 1989

The 60 male Sprague-Dawley rats received their appropriate test diets for a period of ten weeks prior to the initiation of mating. At the initiation of mating, all males were placed on basal laboratory chow and untreated females were placed in the male cages at a ration of 2 females per male.
Viabilities, clinical observations, body weights and feed consumption values were recorded. All pregnant female rats were sacrificed on day 17 of gestation. The gravid uterus was weighed and examined for gross external alterations and sex. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results
No evidence of a reduction in body weight gain was seen. A significant decrease in body weight gain for day 17 was noted for rats treated at the dose of 0.03% and 0.1% during the second mating.

The number of pregnancies, pre-implantation and post-implantation loss and the number of live pups was comparable among all groups.

Conclusion
HC Yellow n°4 did not produce a dominant lethal effect or cause infertility of male Sprague-Dawley rats when fed in the diet for ten weeks prior to mating at dose levels of 0.03% and 0.1%.

Ref.: 22, submission I

Comment
As in the previous study (ref 17) an unequivocal NOEL was not determined in the teratology and dominant lethal phases, this study was done to serve as a repeat of the reproductive phases using a control level and two dose levels.
An examination of testis should have been done as an atrophy was observed at the dose of 0.3% in the combined sub-chronic feeding, teratology and dominant lethal study (ref 17).

3.3.7. Carcinogenicity

Oral administration

Rats

Guideline:	/
Species/strain:	F344/N rats
Group size:	70 animals per sex and dose

Test substance:	HC Yellow n° 4
Batch:	Southland Corporation (lots 0-218 and 3-074) and Prochimie International (lot 8103).
Purity:	Lot 0-218 > 93% pure, lot 3-074 > 97% pure, and lot 8103 > 98% pure. The most important impurity was identified as N-(2-hydroxyethyl)-2-hydroxy-4-nitroaniline.
Dose level:	Diet containing: Males; 0, 2500, or 5000 ppm HC Yellow n° 4 Females; 0, 5000 or 10000 ppm HC Yellow n° 4
Route:	Oral.
Exposure period:	104 weeks
GLP:	In compliance
Study period:	16 March 1983 – 24 April 1985.

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

F344/N rats, groups of 70 males and 70 females (6 weeks old), were exposed to HC Yellow n°4 in the diet for 104 weeks. The male rats received 0 (control), 2500 (low dose), and 5000 ppm (high dose), corresponding to an average intake of HC Yellow n°4 of 123 and 245 mg/kg bw/day during week 14 – 52 and 82 and 164 mg/kg bw/day during week 52 – 104 for low and high dose animals respectively. The females received 0 (control), 5000 (low dose), and 10000 ppm (high dose), corresponding to an average intake of HC Yellow n°4 of 294 and 592 mg/kg bw/day during week 14 – 52 and 189 and 388 mg/kg bw/day during week 52 – 104 for low and high dose animals, respectively. The animals were observed twice daily. Interim evaluations were performed on 10 rats from each dose group at 6 and 15 months. No biologically significant changes in absolute or relative organ weight or haematology or clinical values were found. No compound-related lesions were seen in exposed rats. The mean body weight of female rats that received 10000 ppm was significantly lower than that of the controls. The survivals of exposed rats were similar to that of the controls.

Pituitary gland pars distalis adenomas were marginally increased in exposed male rats (0 ppm, 17/45; 2500 ppm 20/49; 5000 ppm 28/49), and there was a concomitant dose-related increase in the incidence of hyperplasia (8/45, 13/49, 18/49). There was no increase in the incidence of pituitary gland adenomas or carcinomas in female rats (34/49, 35/48, 30/49).

The authors concluded that under the conditions of the 2-year feed study, there was equivocal evidence of carcinogenic activity of HC Yellow n°4 in male F344 rats based on the increased in pituitary gland adenomas and hyperplasia. The male rats may have been able to tolerate a slightly higher dose of the chemical. There was no evidence of carcinogenic activity in female F344 rats.

Ref.: 14

<u>Mice</u>

Guideline:	/
Species/strain:	B6C3F1mice
Group size:	70 animals per sex and dose
Test substance:	HC Yellow n°4
Batch:	Southland Corporation (lots 0-218 and 3-074) and Prochimie International (lot 8103).
Purity:	Lot 0-218 > 93% pure, lot 3-074 > 97% pure, and lot 8103 > 98% pure. The most important impurity was identified as N-(2-hydroxyethyl)-2-hydroxy-4-nitroaniline.
Dose level:	Diet containing: 0, 5000, or 10000 ppm HC Yellow n°4.
Route:	Oral.
Exposure period:	104 weeks

GLP: In compliance
Study period: 16 March 1983 – 24 April 1985.

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

B6C3F1 mice, groups of 70 males and 70 females (6 weeks old), were exposed to HC Yellow n°4 in the diet for 104 weeks. The mice received 0 (control), 5000 (low dose), and 10000 ppm (high dose), corresponding to an average intake of HC Yellow n°4 for the male mice of 777 and 2283 mg/kg bw/day during week 14 – 52 and 932 and 2673 mg/kg bw/day during week 52 – 104 for low and high dose animals, respectively. The average intake of the females of HC Yellow n°4 of 1075 and 2676 mg/kg bw/day during week 14 – 52 and 984 and 2736 mg/kg bw/day during week 52 – 104 for low and high dose animals, respectively. The animals were observed twice daily. Interim evaluations were performed on 10 mice from each dose group at 6 and 15 months. No biologically significant changes in absolute or relative organ weight or haematology or clinical values were found. Pigmentation of the thyroid gland was observed at the 6-month interim evaluations; pigmentation and hyperplasia of the thyroid gland were seen at the 15-month interim evaluations. The mean body weights of mice receiving 10000 ppm were 20% to 30% lower than those of the controls during the second year of the studies. The survivals of exposed mice were similar to that of the controls.

No neoplasms were considered related to chemical administration of the mice. However, a dose-related increased incidence of thyroid gland pigmentation and follicular cell hyperplasia occurred in both sexes of mice.

The authors concluded: Under the conditions of the 2-year feed study, there was no evidence of carcinogenic activity in male and female B6C3F1mice.

Ref.: 14

Comment
HC Yellow n° 4 did not induce tumours in mice or rats after oral administration under the conditions of an US National Toxicology Program study.

<u>Dogs</u>

Guideline: /
Species/strain: Beagles
Group size: 6 Animals per sex and dose
Test substance: Hair dye formulation containing 0.31% HC Yellow n° 4
Batch: /
Purity: /
Dose: 0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation (0, 60 and 300 µg/kg bw/day of HC Yellow n° 4)
Route: Oral - diet
Exposure period: 24 months
GLP: not in compliance
Study period: Before 1975

Diets were prepared daily with the incorporation of the hair dye formulation 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 0.31% HC Yellow n° 4 in formulations up to 97.5 mg/kg bw/day did not result in any signs of toxicity.

Ref.: 5

Comment
No conclusions concerning potential carcinogenic effects can be made from the study with dogs due to the low concentration of HC Yellow n° 4. 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

Rats

Guideline:	/
Species/strain:	Male and female weanling Sprague Dawley rats, 60 per sex per group
Group size:	60 animals per sex and dose
Test substance:	One semipermanent hair dye formulation (P24) containing 0.4% HC Yellow n° 4
Batch:	/
Purity:	/
Dose level:	0.5 ml of a solution containing 0.4% HC Yellow n° 4
Route:	Topical. 1 application twice weekly
Exposure period:	114 weeks
GLP:	not in compliance
Study period:	May 1975 to May 1977

The experiment involved altogether 12 different dye formulations and 3 control groups.

Groups of 60 male and 60 female were obtained from the first mating (F_{1a}) of a multi-generation reproduction study in rats treated with one semipermanent hair dye formulation containing 0.4% HC Yellow n° 4. 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 dye formulations were 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. No positive control group was used.

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. Histopathological evaluations were performed on 18 tissues (plus tumour masses) including treated skin.

Changes in body weight and food consumption have been similar for control and treated rats. Survival just prior to terminal sacrifice (at week 117 119) the survival 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, the mean body weight in the treated group was 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.

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. 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. The incidence of parathyroid hyperplasia was higher in treated male and female rats than in control groups, which was considered possibly compound related. The incidence of hyperkeratosis and dermatitis was considered higher in treated animals than in controls and was considered possibly compound related.

The most common tumour observed was pituitary adenoma. The statistically significant variations in incidence of pituitary adenoma between rats of different control groups as well as their statistically significant decrease in an experimental group when compared with two control groups tends to discount any biological significance of these findings. In addition, 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. The incidence of mammary lobular hyperplasia was statistically significantly greater in control group 3 females than in control group 1 females. When treated females were compared to control group 1 females the incidence of mammary adenocarcinoma/carcinoma was statistically significantly increased. However when treated females were compared with control group 2 and 3 females or with all control females combined, there was no significant increase in this tumour type. Therefore this finding is not considered to be of biological significance.

The authors concluded that no evidence of carcinogenic activity was seen.

Ref.: 23

Mice

Guideline: /

Species/strain:	Swiss-Webster mice
Group size:	50 animals per sex
Test substance:	One semipermanent hair dye formulation (P24) containing 0.4 % HC Yellow No 4
Batch:	/
Purity:	/
Dose level:	0.05 ml of a solution containing 0.4% HC Yellow n° 4
Route:	Topical, 1 application weekly
Exposure period:	23 months
GLP:	not in compliance
Study period:	Before 1980

The experiment involved 12 treatment groups and 3 negative control groups.

The dye was applied topically to a 1 cm^2 area on a clipped (24 hours prior to application) site in the interscapular region. 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.

There were no overt sign of systemic toxicity in any of the dye-treated groups. Five male and 9 female survived 23 months compared to 3 males and 8 females in the control group. There were no significant differences in absolute or relative liver or kidney weights in groups of 10 male and 10 female mice necropsied after 9 months. Average body weights were comparable in all groups throughout the study. There were no statistically significant differences in the distribution of tumours among treated and control groups.

The authors concluded that no evidence of carcinogenic activity was seen.

Ref.: 22

Comments
One study with HC Yellow n° 4 in a semipermanent hair dye formulation (P24) involving topical application of mice and one involving topical application on rats have been identified. The concentration of HC Yellow n° 4 was in both studies 0.4% (the maximum concentration on the human scalp is 1.5%). A number of different hair dye formulations were tested in the same studies. Although some of the formulations contained 2,4-diaminoanisol (classified as carcinogen category 2 in EU), none of the formulations induced tumours in the mice or rats. Thus, no conclusion with regard to carcinogenicity can be made from the studies.

Conclusion on carcinogenicity
HC Yellow n° 4 did not induce tumours in mice or rats after oral administration under the conditions of an US National Toxicology Program long-term study. No conclusions concerning potential carcinogenic effects can be made from an oral study with dogs and skin painting studies with mice and rats. In these three latter studies HC Yellow n° 4 was present in low concentrations (0.4% or less) in semipermanent hair dye formulations. Moreover, although substances classified as carcinogens were present in the formulations studied, no carcinogenic effects were found in any of the studies indicating low sensitivity.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Multi-generation reproduction study

Guideline: /

Species/strain:	Charles River CD rats
Group size:	320 (40 males and 40 females per group; 3 neg. control, 1 test group)
Test substance:	HC Yellow n° 4, 0.4% in hair dye formulation
Batch:	/
Purity:	/
Dose volume:	initially 0.2 ml, increased weekly by 0.1 ml to 0.5 ml
Route:	dermal
Administration:	twice weekly
GLP statement:	/
Study period:	17 October 1974 – 24 September 1976

Test material was applied to the parental generation (F0) until they reached 100 days of aged after which they were mated. The F0 parents were reduced to 20 males and 20 females and rebred to produce F1b litters.

20 males and females from the F1b litter were then administered test material until they reached 100 days of age and were mated twice to produce the F2a and F2b litters. 20 males and females from the F2b litters were then administered test material until they reached 100 days of age and mated to produce the F3a, F3b and F3c litters.

Results
Parental generations:

No significant difference in body weight between test and control groups was observed.

No significant difference in food consumption between test and control groups was observed.

No significant difference in survival between test and control groups was observed.

No treatment related gross or microscopic pathologic lesions were seen in any F1b parental rats or F3b weanling rats which were sacrificed and necropsied. No treatment related gross pathologic lesions were seen at necropsy in any rats which died during the course of study.

Skin reactions consisting of mild scabbing, fissuring, atonia and leathery texture were observed intermittently throughout the treatment period in rats of the tested group.

Reproductive performance:

Performance of F0, F1 and F2 parental rats showed no differences between test and control groups in fertility, gestation and live indices. The F2 parents had markedly reduced fertility indices for three separate matings to produce the F3 litters.

There were no significant differences between control groups and the test group with regard to fertility. Therefore it was concluded that the tested formulation did not cause the reduction in fertility.

Offspring:

No significant differences between body weights, litter size and survival rats between the test and control groups were observed.

Conclusion
A NOAEL of 0.4% HC Yellow n°4 in the hair dye formulation was determined.

Ref.: 24

Comments

The experiment did not conform to a guideline and was not performed according to GLP. The purity and specifications of test article is not known and assumed to be commercial raw materials. Different hair dye formulations were tested in this study. The dose applied to the F0 rats was increasing weekly during the study form 0.2 ml to 0.5 ml. No NOAEL can be derived from this study.

3.3.8.2. Teratogenicity

Prenatal developmental study, range finding study

Guideline:	OECD 414 (2001)
Species/strain:	rat, Crl:CD® (SD)IGS BR VAF/Plus®
Group size:	40 (8 presumed-pregnant females per group)
Test substance:	HC Yellow n° 4 (GTS 03976)- purity 99.2 %
Batch:	17
Purity:	99.2%
Vehicle:	100% polyethylene glycol 400
Dose levels:	0, 50, 200, 500 and 1000 mg/kg bw
Dose volume:	5 ml/kg bw
Route:	oral, gavage
Administration:	once daily on Day 6 through Day 20
GLP statement:	in compliance
Study period:	23 August – 9 September 2004

In this oral dosage range finding developmental toxicity study, HC Yellow n° 4 was administered orally once daily to 40 presumed-pregnant Crl:CD® (SD)IGS BR VAF/Plus® from day 6 to day 20 of gestation at the doses of 0, 50, 200, 500 and 1000 mg/kg bw/d in 100% polyethylene glycol 400 (8 rats per dose). The dosage volume was 5 ml/kg bw.

Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on day 21. The gravid uterus was weighed and examined for gross external alterations and sex. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results

Maternal parameters:

One female rat treated at the dose of 500 mg/kg bw/d delivered and was sacrificed on day 21 and one female rat treated at the dose of 1000 mg/kg bw/d was found dead on the day 21.

Yellow or orange urine discoloration, black faeces and yellow or red fur were observed in all of the groups treated with HC Yellow n° 4. Yellow skin and yellow perioral coloration were observed in the rats treated at the dose of 200, 500 and 1000 mg/kg bw/d. Other clinical observations included red perivaginal substance and red perioral substance were observed in 2 rats treated at the dose of 500 mg/kg bw/d and in one rat treated at the dose of 1000 mg/kg bw/d. Brown stained or urine-stained abdominal fur was observed in 3 rats treated at the dose of 1000 mg/kg/d. Red substance in the cage pan, dehydratation and brown skin were also recorded in rats treated at the dose of 1000 mg/kg bw/d. These effects were considered related to the colour of the HC Yellow n°4 but were not considered as adverse effects.

Mean body weights were reduced in the rats treated at the dose of 1000 mg/kg bw/d throughout gestation. On gestation day 21, gravid uterine weight and/or corrected maternal body weights were reduced in the rats treated at the dose of 500 and 1000 mg/kg bw/d. A mean body weight loss occurred in the rats treated at the dose of 1000 mg/kg bw/d and

body weigh gains were reduced in the other rats on gestation days 6-9 correlating with the beginning of dosing. Mean body weight gains were reduced in the rats treated at the dose of 200, 500 and 1000 mg/kg bw/d in a dosage dependant manner for the entire dosage and gestation periods. Reductions or losses were also observed once corrected for gravid uterine weights.

Mean absolute and relative feed consumptions were reduced in the rats treated at the dose of 500 and 1000 mg/kg bw/d for the entire dosage and gestation periods.

No significant differences were observed in the number of corpora lutea in all treated rats when comparing with the control rats. No significant differences in the number of implantations were observed in the treated rats when comparing with the control rats.

Resorptions and percent of resorbed conceptuses per litter increased in the rats treated at the dose of 1000 mg/kg bw/d with one dam having a litter consisting only of resorbed conceptuses. Late resorptions increased in the rats treated at the dose of 500 mg/kg bw/d.

Foetal parameters:

Body weight were reduced in the foetus of rats treated at the dose of 1000 mg/kg bw/d. Thirteen foetuses from five litters in the 1000 mg/kg bw/d dosage group were observed with whole body oedema (anasarca). Mean litter sizes and number of live foetuses were decreased in 1000 mg/kg dose group.

Conclusion:
Based on the results of this study, the doses of 0, 50, 150 and 300 mg/kg bw/d were selected for the developmental toxicity study in rats.

Ref.: 19

Comments:
A maternal NOAEL of 50 mg/kg/d and a developmental NOAEL of 500 mg/kg/d may be derived from this study.

Prenatal developmental, main study

Guideline:	OECD 414 (2001)
Species/strain:	rat, Crl:CD® (SD)IGS BR VAF/Plus®
Group size:	100 (25 presumed-pregnant females per group)
Test substance:	HC Yellow n° 4 (GTS 03976)
Batch:	17
Purity:	99.2%
Vehicle:	100% polyethylene glycol 400
Dose levels:	0, 50, 150 and 300 mg/kg bw
Dose volume:	5 ml/kg bw
Route:	oral, gavage
Administration:	once daily on Day 6 through Day 20
GLP statement:	in compliance
Study period:	17 October – 4 November 2004

In this oral prenatal developmental toxicity study, HC Yellow n°4 was administered orally once daily to 100 presumed-pregnant Crl:CD® (SD)IGS BR VAF/Plus® from day 6 to day 20 of gestation at the doses of 0, 50, 150, 300 mg/kg bw/d in 100% polyethylene glycol 400 (25 rats per dose). The dosage volume was 5 ml/kg bw.

Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrified on day 21. The gravid uterus were 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 alterations, sex and either soft tissue or skeletal alterations. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results:

Maternal parameters:

One rat treated at the dose of 300 mg/kg bw/d was found dead on day 19. There was no indication of cause of death from the necropsy observations. All other rats survived to day 21.

Significant increases in yellow or orange urine, yellow or orange fur, yellow and/or orange skin and yellow or red perivaginal substance occurred in the treated rats, and yellow perioral substance was observed in four, five and six rats treated at the dose of 50, 150 and 300 mg/kg bw/d, respectively. Urine-stained abdominal fur was observed in two rats treated at the dose of 300 mg/kg bw/d, one of which was found dead on day 19.

Body weight gains were significantly reduced in the rats treated at the dose of 300 mg/kg bw/d on days 15 to 18. Corrected maternal body weights were significantly reduced in the rats treated at the dose of 300 mg/kg/day. Corrected maternal body weight gains were significantly reduced in the rats treated at the dose of 300 mg/kg bw/d for the entire dosage period and in the rats treated at the dose of 150 mg/kg bw/d dosage for the entire gestation period.

Absolute feed consumption values were significantly reduced in the rats treated at the dose of 150 and 300 mg/kg bw/d on days 15 to 18.

No significant differences were observed in the number of corpora lutea, implantations or resorptions in all treated rats when comparing with the control rats.

Foetal parameters:

No significant differences were observed in the number of either live foetuses, dead or resorbed foetuses or foetal abnormalities in all treated rats when compared with the control rats. A significant increase in the foetal body weight was observed the high dose group.

Conclusion
A maternal NOAEL of 50 mg/kg bw/d and a developmental NOAEL of 300 mg/kg bw/d were determined from this study since the increase in foetal body weight was not considered as an adverse effect.

Ref.: 20

Teratogenicity study, topical application

Guideline:	/
Species/strain:	Charles River CD rats
Group size:	100, 20 mated females per group (3 neg. control, 1 pos. control, 1 test)
Test substance:	HC Yellow n° 4, 0.4% in hair dye formulation
Batch:	/
Purity:	/ unknown purity and specifications assume commercial raw material
Vehicle:	/
Dose levels:	/
Dose volume:	2 ml/kg bw
Route:	topical application

Administration: Day 1, 4, 7, 10, 13, 16 and 19 of gestation
Control: acetylsalicylic acid, 250 mg/kg bw by gavage on Day 6 through 16
GLP statement: /
Study period: 1976

In this study the test formulation was applied to the skin of rats every three days during days 1-19 of gestation at a dose of 2 ml/kg.

Results:

Maternal parameters:

No significant difference in body weight between test and control groups was observed.

No significant difference in food consumption between test and control groups was observed.

No significant difference in number of corpora lutea, implantations, resorptions, abortions and sex ratio between test and control groups was observed.

Foetal parameters:
No differences in the number of live, dead or resorbed foetuses and number of foetal abnormalities were observed.

Conclusion
The administration of formulation P-24 containing 0.4% of HC Yellow n°4 every third day of the gestation period produces no embryotoxic or teratogenic effects. A NOAEL of 0.4% HC Yellow n°4 in the hair dye formulation was determined.

Ref.: 21

Comment
The experiment did not conform to a guideline and was not performed according to GLP. The study is reported only as an article in J. Toxicol. Env. Health but not provided as a full report. HC Yellow n° 4 was applied every three days. The purity and specifications of test article is not known and assumed to be commercial raw materials. The test concentration of HC Yellow n°4 was lower than the use concentration in non-oxidative hair dye (1.5%). No NOAEL can be derived from this study.

Teratology study (Combined Sub-chronic feeding, teratology and Dominant Lethal study)

Guideline: /
Species/strain: rat, Sprague-Dawley (TAC:N(SD)fBR)
Group size: 100 (25 females per group)
Test substance: HC Yellow n° 4
Batch: 22900186
Purity: 98.9% (approximately)
Dose levels: 0, 0.1, 0.3 and 1.0% HC Yellow n° 4 in diet (until mating (2 weeks)) basal laboratory chow during mating
0, 0.1, 0.3 and 1.0% HC Yellow n° 4 in diet (from Day 0 through Day 20)
GLP: /
Study period: 1987

Results

There was a significant increase in 0.3% dose group mean change in maternal body weight during days 0-15 of gestation when compared to the control.

No significant changes were noted in food consumption between the test groups.

There were significant increases in the number of total resorptions and in the number of dead foetuses at the dose of 1.0% when compared to the control. The type and incidence of malformations observed in this study did not suggest a teratogenic effect produced by the test compound. The incidence of developmental variations, including unossified sternebrae 5 and 6, dilated cerebral ventricle and distended ureters, was higher in the mid and high dose groups, suggesting a retardation in foetal development.

Conclusions
Administration of HC Yellow n°4 in the diet at a level of 1% affected intrauterine survival severely. A dose level of 1% was severely foetotoxic. A possible indication of foetal toxicity was noted in the 0.3% group but at a dose level of 0.1%; HC Yellow n°4 did not produce any observable foetal toxicity. HC Yellow n°4 was however not teratogenic at any of the dose levels in this study.

Ref.: 17, submission I

Comment
The experiment did not conform to a guideline and was not performed according to GLP. No NOAEL can be derived from this study.

Teratology study (Combined teratology and dominant lethal study)

Guideline:	/
Species/strain:	rat, Sprague-Dawley (TAC:N(SD)fBR)
Group size:	75 (25 females per group)
Test substance:	HC Yellow n° 4
Batch:	22900186
Purity:	/
Dose levels:	0, 0.03 and 0.1% HC Yellow n° 4 in diet (for 6 weeks until mating) basal laboratory chow during mating 0, 0.03 and 0.1% HC Yellow n° 4 in diet (from Day 0 through Day 20)
GLP:	/
Study period:	1988 - 1989

75 females received their appropriate test diets for a period of 6 weeks prior to the initiation of mating. During mating all females were maintained on basal laboratory chow. On day 0 of gestation each female was returned to its appropriate test diet until it was sacrificed on day 20.

Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on day 20. The gravid uterus was weighed and examined for gross external alterations. Caesarean-sectioning and subsequent foetal observations were conducted.

Results
No evidence in a reduction in body weight gain was observed.

The number of pregnancies, pre-implantation and post-implantation losses and the number of live pups was comparable among all groups. No gross malformations were noted in any foetuses.

Conclusion

HC Yellow n°4 does not appear to produce a teratogenic effect or any observable evidence of foetal toxicity when fed in the diet for six weeks prior to mating and during gestation to female Sprague-Dawley rats at dose levels of 0.03% and 0.1%.

Ref.: 22, submission I

Comment
No NOAEL can be derived from this study.
This study was done to serve as a repeat of the reproductive phases using a control level and two dose levels.

Teratology study

Guideline: /
Species/strain: rat, Sprague-Dawley (TAC:N(SD)fBR)
Group size: 40 (20 females per group)
Test substance: HC Yellow n° 4
Batch: 22900186
Purity: 99.65%
Dose levels: 0 and 1.0% HC Yellow n° 4 in diet
GLP: /
Study period: 1988

Evidence of foetotoxic effect of HC Yellow n°4 was found in this study. 47 foetuses were dead in the treated group (0 in the group control and 1 in a group treated with another dye).

Ref.: 23, submission I

Comment
The experiment did not conform to a guideline and was not performed according to GLP. The study is badly reported but it confirmed the foetotoxicity observed in the ref 17, submission I.

Summary table on toxicity

Study	Species	Sex	Route	Effects	Critical dose	Ref
Acute	Rat, Wistar	m and f	oral	non specific	> 5g/kg	2
Acute	Rat, Sprague-Dawley	m and f	oral		1250 mg/kg< <5000 (m) 1250 mg/kg< <2500 (f)	3
Acute	Rat, Sprague-Dawley	m and f	oral		500< <2000	4
14-day	Rat, F344 mice, B6C3F1	m and f	oral	**0.5% (m and f) and above**: ↓ bw and changes in liver and kidney weight	ND	14
4-week	Rat, Sprague-Dawley	m and f	oral	**0.5% (m and f) and above**: ↓ bw and changes in liver weight **1.25% (m and f)** and above changes kidney weight		15 (I)

Study	Species	Sex	Route	Effects	Critical dose	Ref
13-week	Rat, F344 mice, B6C3F1	m and f	oral	**Rat** **10000 ppm (m) and 20000 ppm (f) and above**: ↓ **bw**; 2**0000 ppm (f)** uterine atrophy; **40000 ppm (m)**: renal mineralization and thyroid pigmentation **Mice** **80000 ppm**: 15 † (m and f) **40000 ppm**: 1† (m) **10000 ppm:** ↓ bw	**Rat** m: 5000 ppm (250 mg/kg bw/d) f: 10000 ppm (500 mg/kg bw/d) **Mice** m and f: 5000 ppm (750 mg/kg bw/d)	14
13-week	New Zealand White rabbit	m and f	dermal		formulation applied no NOAEL can be derived	21
90-day	Rat, Sprague-Dawley	m and f	oral	0, 0.1%, 0.3% and 1%: no specific related effect		17 (I)
6 months	Rat, Sprague-Dawley	m and f	oral	**0.1% and above**: ↑ in liver weight **1%**: ↓ in body weights (m and f) and in testes weight		17 (I)
dominant lethal study	Rat, Sprague-Dawley	m	oral	No dominant lethal effect but at **0.1% and above**: ↓ in fertility (↓ in nb of pregnant females)		17 (I)
dominant lethal study	Rat, Sprague-Dawley	m	oral	**0.03 and 0.1%:** no effect		22 (I)
teratology	Rat, Sprague-Dawley	m and f	oral	**0.3%**: retardation in foetal development **1%**: severely foetotoxic		17 (I)
teratology	Rat, Sprague-Dawley	f	oral	**0.03 and 0.1%:** no effect		22 (I)
teratology	rat crl-CD	f (G6-G20)	oral	**200 mg/kg bw/d and above:** coloration and ↓ in mean body weight gains **500 mg/kg bw/d and above**: ↓ in feed consumption and ↑ in late resorption **1000 mg/kg bw/d**: ↓ in mean body weight and ↑ resorptions; ↓ in foetal body weight; oedema in foetuses and ↓ in mean litter sizes and number of live foetuses	NOAEL maternal: 50 mg/kg bw/d developm: 500 mg/kg bw/d	19
teratology	Rat, crl-CD	f (G6-G20)	oral	**50 mg/kg bw/d and above:** coloration **150 mg/kg bw/d and above**: ↓ in body weight and food consumption **300 mg/kg bw/d**: ↑ in foetal body weight	NOAEL maternal: 50 mg/kg bw/d developm: 300 mg/kg bw/d	20
teratology	Rat, crl-CD	f (G1-G19) every 3 days	topical		formulation applied no NOAEL can be derived	21

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

Photo-sensitisation in Guinea Pig

Guideline:	/
Species/strain:	Hartley albino guinea pig
Group size:	8 males and 8 females; 4 males and 4 females for positive control
Test substance:	HC Yellow n° 4
Batch:	/
Purity:	/
Vehicle:	80% DAE (40% dimethylacetamide, 30% acetone, 30% ethanol) and 20% saline
Concentration:	induction: 10% HC Yellow n° 4 (and Freund's adjuvant) challenge: 5% HC Yellow n° 4
Dosing volume:	0.1 ml/2.54 cm²
Light Source:	150W Xenon arc lamp with WG-354 glass filter when required to remove UVB.
Control:	musk ambrette 5% for induction and challenge
GLP:	/
Study period:	1986

0.1 ml of 10% HC Yellow n°4 was topically administered for four consecutive days during the first, second and third week of the study. During the first week of the study, one hour following application of the test material, animals were irradiated with 0.5 MED of UVA. During the second and third week of the study, one hour following application of the test material, animals were irradiated with 1 MED of UVB. Results indicate that 24 hours after the initiation of the challenge exposure there was no evidence of irritation on the test sites and no evidence of either a photoallergic or contact allergic reaction in the guinea pig.

Ref.: 12, submission I

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° 4)

(non-oxidative)

Absorption through the skin	**A**	**=**	**0.152 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.088 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.001 mg/kg bw**
No observed adverse effect level (maternal, oral, rat)	**NOAEL**	**=**	**50 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**50000**

3.3.14. Discussion

Physico-chemical properties
HC Yellow n° 4 is used in non-oxidative hair dye formulations with a maximum on-head concentration of 1.5%.
Only for one batch, data on identity and purity was provided. For other batches used, the stated purity was between >93 to 99.9%. For many batches used no information on purity was available.
Impurities were not quantified by appropriate standards but relative to absolute resulting HPLC area.
Only UV-active impurities were identified, because of the lack of other examinations of purity.
HC Yellow n° 4 is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb. In the absence of a study report, the NDELA content could not be evaluated.
The stability in typical hair dye formulation is not reported.
The Log P_{ow} strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log P_{ow}, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.

Toxicity
Based on 3 acute oral toxicity studies in rats, HC Yellow n° 4 is considered as having no significant acute oral toxicity.
After 90 days of exposure by oral route, the thyroid gland in male and female rats and mice, the kidney in male rats and the uterus in female rats and mice are the main target organs of systemic toxicity of HC Yellow n° 4. Thyroid pigmentation occurred in rats receiving 40 000 ppm and in mice at 5000 ppm and above. A mineralization of renal papilla occurred in male rats at 40 000 ppm. Uterine atrophy occurred in female rats and mice at 20 000 ppm and 40 000 ppm. Moreover, lymphoid depletion and subsequent atrophy of the spleen and the thymus occurred in male and female mice at 80 000 ppm and were considered secondary to the decreased body weights. NOAELs of 250 mg/kg/day in rats and 750 mg/kg/days in mice was derived from the 90 days oral toxicity study.

In the 6 month oral toxicity study, a significant decrease in the absolute and relative testes weight was observed in rats at the dose of 1% in food. This decrease is probably related to the severe diffuse testes atrophy seen at high dose in the dominant lethal study.
No appropriate two generation reproduction toxicity study was submitted. However, because of the low exposure and the magnitude of MOS no additional reproductive toxicity study seems to be necessary.

In the teratogenicity studies performed in rats by oral route, foetotoxicity was observed at high doses. A maternal NOAEL of 50 mg/kg/d and a developmental NOAEL of 300 mg/kg/d were determined. The maternal NOAEL of 50 mg/kg/d was used to calculate the margin of safety.

Skin/eye irritation and sensitisation
HC Yellow n° 4 was not irritant to rabbit skin. HC Yellow n° 4 was considered to be an eye irritant in rabbits. HC Yellow n° 4 was not a contact allergen in a LLNA. It was not a photo-allergen.
None of the submitted studies on sensitisation were conform to guidelines. Although there is no evidence from the available data that HC Yellow n° 4 is a sensitiser, this cannot be excluded.

Percutaneous absorption
Under the conditions of an experiment in which 20 mg/cm² of a hair dye cream formulation containing 1.5% HC Yellow n° 4 were applied, the amount considered to be systemically available was 0.102 µg/cm² (SD ± 0.050) or 0.034% (SD ± 0.017) of the applied dose.
The mean value + 1SD (0.152 µg/cm²) was used in the calculation of MOS.

Mutagenicity/genotoxicity
Overall, the genotoxicity of HC Yellow n° 4 is investigated for the three endpoints. HC Yellow n° 4 treatment resulted in the induction of gene mutations in bacteria but not in mammalian cells at the *tk* locus of mouse lymphoma cells. In an *in vitro* chromosome aberration test HC Yellow n° 4 induced an increase in the number of cells with chromosome aberrations. Moreover, there are indications for an aneugenic potential of HC Yellow n° 4.
The positive effects found in the *in vitro* experiments could not be neutralized with *in vivo* tests. An *in vivo* bone marrow micronucleus tests in mice did not induce an increase in the number of micronucleated erythrocytes but there were no indications (toxicity) that HC Yellow n° 4 reached the target cells. However, systemic availability is expected following intraperitoneal application. A UDS test in rats was negative, overruling the positive findings in the gene mutation assay in bacteria.
Two dominant lethal tests gave contradictory results using the same strain of rats and identical doses of HC Yellow n° 4.

Carcinogenicity
HC Yellow n° 4 did not induce tumours in mice or rats after oral administration under the conditions of an US National Toxicology Program long-term study. No conclusions concerning potential carcinogenic effects can be made from an oral study with dogs and skin painting studies with mice and rats. In these three latter studies HC Yellow n° 4 was present in low concentrations (0.4% 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° 4 as a non-oxidative hair dye with a maximum concentration on the head of 1.5 % does not pose a risk to the health of the consumer.

A sensitising potential of HC Yellow n° 4 cannot be excluded.

HC Yellow n° 4 is a secondary amine, and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

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