EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

Basic Yellow 57

COLIPA n° C10

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

The SCCS adopted this opinion at its 6th plenary meeting
of 23 March 2010

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

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (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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ISSN 1831-4767
doi:10.2772/21779
ISBN 978-92-79-11110-5
ND-AQ-09-002-EN-N

http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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, C10, Basic Yellow 57, CAS 68391-31-1, EC 269-943-5, directive 76/768/EEC

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Basic Yellow 57, 23 March 2010

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1. BACKGROUND

Submission I and II for Basic Yellow no 57, with the chemical name [5-Hydroxy-3-methyl-1-phenyl-4-(3'-trimethylammoniophenylazo)-pyrazol, were submitted by COLIPA[1] in October 1993 and in November 2002 respectively.

The Scientific Committee on Consumer Products and Non Food Products intended for Consumers (SCCNFP) adopted its opinion SCCNFP/0679/03 at the 24th plenary meeting of 24-25 June 2003 with the conclusion, that *"The SCCNFP is of the opinion that the information submitted is inadequate to assess the safe use of the substance. Before any further consideration, a complete new re-submission is required on the substance(s) to which the consumer is presently exposed."*

According to current submission III, submitted by COLIPA in April 2006, Basic Yellow no 57 is used as an ingredient in direct hair colour products. The final concentration on the scalp is proposed up to 2.0%.

Submission III 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 Basic Yellow no 57 safe for the consumers, when used as an ingredient in non-oxidative hair dye formulations with a concentration on the scalp of maximum 2.0% taking into account the scientific data provided?*

2. *Does the SCCS recommend any restrictions with regard to the use of Basic Yellow no 57 in hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Basic Yellow 57 (INCI name)

3.1.1.2. Chemical names

Benzenaminium, 3-[(4,5-dihydro-3-methyl-5-oxo-1-phenyl-1H-pyrazol-4-yl)azo]-N,N,N-trimethyl-, chloride (9CI, CAS name)
N,N,N-trimethyl-3-[(3-methyl-5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-4-yl)diazenyl]-benzenaminium chloride

3.1.1.3. Trade names and abbreviations

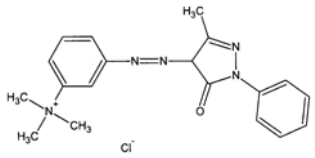
Arianor Straw Yellow
Arianor Straw Yellow 306005
Basic Yellow 57
C.I. 12719
C.I. Basic Yellow 57
Jarocol Straw Yellow

COLIPA C10

3.1.1.4. CAS / EC number

CAS: 68391-31-1
EC: 269-943-5

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{19}H_{22}N_5O^+ Cl^-$

3.1.2. Physical form

Orange-yellow fine power

3.1.3. Molecular weight

Molecular weight: 371.87 g/mol

3.1.4. Purity, composition and substance codes

Analytical description of batches used in toxicity studies

Chemical characterisation was performed by NMR-spectroscopy IR-spectroscopy, UV/Vis-spectrometry and HPLC.

	00656555101 (SAT 050016)*	**Batch 15 (SAT 040269)****	**RS68216101**
Purity by NMR assay	78.7% (w/w)	61% (w/w)	65% (w/w)
Purity by HPLC assay	99.9% (area)	98.7% (area)	99.0% (area)
Solvent content (water)	9.2% (w/w)	5.5% (w/w)	5.9% (w/w)
Chloride	13.1% (w/w)	13.1% (w/w)	7.7% (w/w)
Methylsulphate	/	10.6% (w/w)	/
Sodium	3.7% (w/w)	6% (w/w)	1.05% (w/w)
Chloromethane	1.6% (w/w)	1.0% (w/w)	/
Sulfate	0.7% (w/w)	0.8% (w/w)	0.3% (w/w)
Sulphated ash	12.1% (w/w)	24.9% (w/w)	3.6% (w/w)
Calcium	/	/	/
Magnesium	/	0.3% (w/w)	/
5-Methyl-2-phenyl-2,4-dihydro-3H-pyrazol-3-one	< 10 ppm (detection limit)	0.11% (w/w)	200 ppm
3-Amino-N,N,N-trimethyl-benzenaminium chloride	< 0.2% (w/w) (detection limit)	/	Not determined
Saccharose	/		24% (w/w)

Declarations in the dossier

* This batch was not standardised with respect to colour strength by addition of saccharose or sodium chloride.

** The high values of chloride, sodium and sulphated ash are due to the fact that this batch represents an actual market material containing the extender sodium chloride which has been used to adjust the colour strength to a certain predefined value.

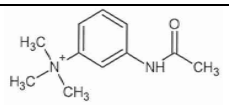
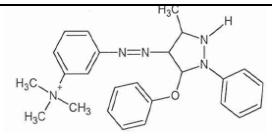
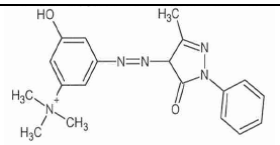
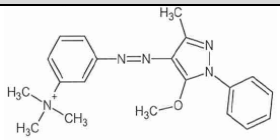
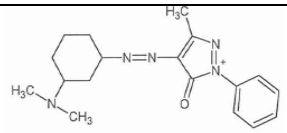
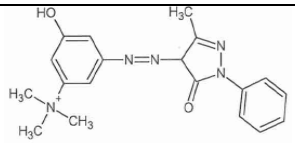
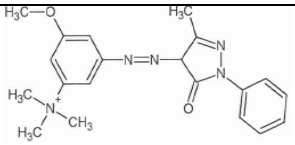
In relation to the Methylsulphate content of 10.6% in batch 15, the applicant declared:

Actually, for the synthesis of C 010 3-Amino-N,N,N-trimethylbenzenaminium chloride and not the corresponding methylsulfate salt is used as starting material. This is diazotized and coupled with 3-Methyl-1-phenyl-5-pyrazolone. Therefore, no dimethylsulfate or monomethylsulfate is used or produced in the actual technical process. Accordingly, this parameter was not relevant to mention in the raw material description. The former marketed batch 15 is representing a specification which is not any longer used in cosmetic products.

In relation to the Chloromethane content initially measured by GC/MS, the applicant declared that this is assumed to have been caused by heating of the substance during analysis. Upon re-analysis of batches 00656555101 and 15, using an alternative method without heating, no chloromethane was detected.

Ref. 18, 19

Additional data on impurities in the three batches of Basic Yellow 57by based on HPLC-MS-MS

Peak No.	Retention Time (min)	#0065655101	#RS68216101	#15	m/z	Possible chemical structures of impurities based on HPLC-MS-MS analysis
		% Peak area	% Peak area	% Peak area		
1	2.1		1.2		365.1	Saccharose
2	3.3			0.1	193.1	
3	5.6			0.3	163.1	No structure could be assigned
4	17.0	0.1	0.2		416.2	
5	17.4			0.1	324.1	No structure could be assigned
6	18.2		0.1	0.1	352.2	
7	21.1	96.9	96.8	99.5	336.2	Basic Yellow 57
8	23.5	0.2			350.2	
9	27.1		1.3		519.3	No structure could be assigned
10	27.2	1.9			513.3	No structure could be assigned
11	35.4	0.2	0.2		359.1	No structure could be assigned
12	36.1	0.2		0.0	326.4	
13	39.5	0.4	0.2		352.1	
14	43.1	0.1			366.2	

RT: HPLC Retention time

Ref.: 17

Comments
- The NMR purity of Basic Yellow 57 of Batch 15 (61%) and of batch RS68216101 (65%) is much lower than the declared purity (75%) of Basic Yellow 57 in the marketed material.

- Batch 15 contains 10.6% methyl sulphate. Monomethyl sulphate is used as an anion to the dye. No toxicity data for monomethyl sulphate was submitted. However, the applicant declared that no monomethyl sulphate is present in the product as currently manufactured.

Declaration of the applicant

Batch KS 3131, used in the acute oral toxicity test, is not fully analytically described. However, information is available from the laboratories that have synthesized this batch concerning the identity and purity of the material produced at that time. From this information it can be concluded that the former not fully described batch is representative and the specification of the dye is quite similar to the fully characterized batch 00656555101.

Purity and composition of marketed material

Purity by NMR assay:	> 75% (w/w)
Purity by HPLC assay:	> 98% (area)
Solvent content (water):	< 12% (w/w)
Chloride:	< 17% (w/w)
Chloromethane:	< 2% (w/w)
Sulfate:	< 1% (w/w)
Sulphated ash	< 15% (w/w)
5-Methyl-2-phenyl-2,4-dihydro-3H-pyrazol-3-one	< 1000 ppm
Pb	< 20 ppm
Sb, Ni	< 10 ppm
As, Cd	< 5 ppm
Hg	< 1 ppm

Before marketing, sodium chloride or saccharose is usually added to the neat dye in order to adjust the colour strength to a certain predefined value.

3.1.5. Impurities / accompanying contaminants

See above

3.1.6. Solubility

Water:	> 100 g/l	at room temperature
Ethanol:	3 – 30 g/l	at room temperature
DMSO:	10 – 100 g/l	at room temperature

Comment
Water solubility has not been determined by EEC method.

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 0.84 (calculated)

Log P_{ow}: 0.0632 (measured by EU A.8 method)

Ref.:16

3.1.8. Additional physical and chemical specifications

Melting point:	163-169 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	λmax: 384 nm, 248 nm

3.1.9. Homogeneity and Stability

A HPLC method was developed and validated for the analysis of Basic Yellow 57 in aqueous solutions. Basic Yellow 57 was shown to be stable when an aqueous solution containing approximately 1 mg/ml Basic Yellow 57 was stored for 21.7 hours at room temperature. This method was used for the determination of stability of Basic Yellow 57 in the test formulations used for 90 day subchronic toxicity:
The nominal concentrations of Basic Yellow 57 in the test formulations varied from 92 -95% of the target concentrations of 10 mg/g, 30 mg/g and 100 mg/g.
Test formulations containing 10 mg/g and 100 mg/g Basic Yellow 57 were shown to be homogenous, % CV 1.8% and 4% respectively.
Test formulations containing 10 mg/g and 100 mg/g Basic Yellow 57 were shown to be stable for 8 days in a refrigerator; maximum variation up to 10%.

General Comments to physico-chemical characterisation

- The NMR purity of Basic Yellow 57 of Batch 15 (61%) and of batch RS68216101 (65%) is much lower than the declared purity (75%) of Basic Yellow 57 in the marketed material.
- Batch 15 contains 10.6% methyl sulphate. Monomethyl sulphate is used as an anion to the dye. No toxicity data for monomethyl sulphate was submitted. However, the applicant declared that no monomethyl sulphate is present in the product as currently manufactured.
- Water solubility of Basic Yellow 57 has not been determined by the EEC method.
- Stability of Basic Yellow 57 in typical hair dye formulations has not been reported.

3.2. Function and uses

Basic Yellow 57 is used as a direct dye for hair colouring products. It is used without mixing with an oxidising agent (e.g. hydrogen peroxide). The final concentration of Basic Yellow 57 on head can be up to 2.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401
Species/strain:	Sprague-Dawley CD rat
Group size:	10 (five males and five females)
Test substance:	Arianor Straw Yellow
Batch:	KS 3131
Purity:	/
Dose:	2.0 g/kg bw
Vehicle:	distilled water
Route:	oral, gavage
GLP:	not in compliance
Study period:	27 December 1985 – 10 January 1986

Animals were observed soon after dosing and frequent intervals on Day 1. On subsequent 14 days the animals were observed at least twice per day. Clinical signs were recorded at each observation and bodyweights were recorded on Days 1, 8 and 15.

Results
There were no mortalities. Signs of reaction to treatment observed shortly after dosing in all rats were increased salivation, pilo-erection, hunched posture and abnormal gait (waddling). Recovery was complete by Day 3.

Conclusion
The acute lethal oral dose to rats of Arianor Straw Yellow was greater than 2.0 g/kg bw.
Ref.: 5

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:	OECD 404 (2002)
Species/strain:	New Zealand white rabbit, SPF
Group size:	3 (1 male and 2 females)
Test substance:	C10 (SAT 040269)
Batch:	15
Purity:	99.3% (HPLC)
Dose level:	0.5 g, moistened with 0.1 ml purified water
Observ. Period:	10 days
GLP:	in compliance
Study period:	11 – 27 May 2004

Approximately 24 hours prior to the treatment, the dorsal fur was shaved, to expose an area of about 100 cm². 0.5 g of the moistened test substance was applied under a 4 cm x 4 cm semi-occlusive patch to the intact shaved back skin of each animal. The patch was removed 4 hours after contact.
Animals were examined for signs of erythema, eschar and oedema formation. The skin reactions were assessed approx. 1 hour, 24, 48 and 72 hours, 7 and 10 days after exposure.

Results
Slight yellow staining of the treated skin was observed in two animals at the 1- and 24-hour reading and persisted in one animal up to the 7-day examination. There was no sign of irritation at any observation point.

Conclusion
Under the conditions of the study, C10 was not irritating to rabbit skin.

Ref.: 6

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405 (2002)
Species/strain:	New Zealand white rabbit, SPF
Group size:	3 (1 male and 2 females)
Test substance:	C10 (SAT 040269)
Batch:	15
Purity:	99.3% (HPLC)
Dose level:	0.1 g neat substance
Observ. Period:	10 days
GLP:	in compliance
Study period:	3 – 18 June 2004

0.1 g of C10 was placed into the conjunctival sac of one eye of the test animals. The substance remained in permanent contact with the eyes until rinsing with warm tap water, 24 hours after instillation. The other eyes served as controls. The eye irritation reactions were scored approx. 1 hour, 24, 48 and 72 hours, 7 and 10 days after instillation of the test solution.

Results
C10 caused mild to moderate, early-onset and transient ocular changes, such as reddening of the conjunctivae and sclerae, and chemosis. These effects were reversible and had resolved at 10 days in the three animals. No abnormal findings were observed in the cornea or iris of any animal at any of the examinations.
No staining of the treated eyes by the test item was observed.

Conclusion
Under the conditions of the study, the undiluted test substance was irritating to the rabbit eye, however, did not fulfil the EU criteria for classification as eye irritant (R36).

Ref.: 7

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species:	Mice, CBA/CaOlaHsd (nulliparous and non-pregnant)
Group:	16 (3 test and 1 control group)
Substance:	C10 (SAT 040269)
Batch:	15

Purity: 99.3%
Dose: 2.5%, 5% and 10% (w/v) solutions
Vehicle: ethanol:water, 7:3 (v/v)
Positive control: α-hexylcinnamaldehyde at 5, 10 and 25% in acetone:olive oil (4:1 v/v)
GLP: in compliance
Study period: 12 – 26 May 2004

Three dose groups and a control group (receiving the vehicle only) of four female mice each, were chosen. The test item was topically applied to the dorsal surface of the ears to analyse the sensitization activity by measuring the proliferative response of lymph node cells.

Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with the different test item concentrations. The application volume, 25 µl, was spread over the entire dorsal surface of each ear lobe once daily for 3 consecutive days. The control group was treated with the vehicle. Five days after the first topical application, all mice were administered with radio-labelled thymidine (^{3}HTdR) by intravenous injection via the tail vein.

Approximately 5 hours after ^{3}HTdR application all mice were killed. The draining lymph nodes were excised and pooled for each experimental group. After preparation of the lymph nodes, disaggregation and overnight precipitation of macromolecules, these precipitations were re-suspended and transferred to scintillation vials.

The level of ^{3}HTdR incorporation was then measured by scintillation counting. The proliferative response of lymph node cells is expressed as the ratio of ^{3}HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index).

An appropriate reference (α-hexylcinnamaldehyde) was used as positive control, to show distinct increases in the stimulation index.

The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of ^{3}H-methyl thymidine.

Results
The Stimulation Index (S.I.) was below 3 in all dose groups. No dose response relation was noted.

Concentration	**Stimulation Index**
Test item	
2.5%	1.2
5%	1.5
10%	1.5
α-Hexylcinnamaldehyde	
5%	1.5
10%	2.3
25%	8.4

Calculation of the EC 3 value was not performed as the S.I. value did not reach or exceed 3 for any test concentration.
The positive control used affected an increase in stimulation index with an EC3 of 11.7%.

Conclusion
Based on the criteria of the test system, C10 was not a non-sensitizer when tested up to 10% in ethanol:water (7:3 v/v) in mice.

Ref.: 8

Comment
In a pre-study evaluation on 2 mice, 1%, 2.5%, 5% and 10% solutions were evaluated. The highest dose (10%) in the main study was considered by the study authors to be "*the highest technically applicable concentration whilst avoiding systemic toxicity and excessive local irritation*". It cannot be excluded that C10 is a skin sensitiser as the maximum test concentration (10%) is too low.

3.3.4. Dermal / percutaneous absorption

Guideline:	Draft OECD. European Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP, 2000)
Tissue:	Dermatomed pig skin, 400 µm of thickness
Group size:	Six membranes (from 3 pigs) per application
Diffusion cells:	Glass diffusion cell with an exposed membrane area of 2.54 cm²
Skin integrity:	Measured by trans-dermal electrical resistance ($\geq$3kΩ)
Test substance:	Basic Yellow 57
Batch:	RS68216101
Purity:	> 99%
Test item:	Direct dye formulation containing 2% (w/w) Basic Yellow 57; Aqueous solution containing 2% (w/w) Basic Yellow 57
Doses:	10 mg/cm² formulation (= 180 µg Basic Yellow 57 per cm²) 10 µl/cm² aqueous solution (= 197 µg Basic Yellow 57 per cm²)
Receptor fluid:	25% ethanol in physiological saline
Solubility receptor fluid:	/
Stability:	/
Method of Analysis:	HPLC
GLP:	in compliance
Study period:	4 February – 8 March 2002

The formulation of the direct dye used was:

Ingredient	Concentration
Basic Yellow 57	2.00%
Cetearyl alcohol	8.50%
Sodium laureth sulphate	5.40%
Cocoamidopropylbetaine	3.75%
Coconut alcohol	2.00%
Phenoxyethanol	0.80%
Ceteareth-12	0.75%
Aminomethylpropanol	0.70%
Methylparaben	0.30%
Propylparaben	0.20%
Water	ad 100%

The content of Basic Yellow 57 in the final application formulations (direct dye cream and aqueous solution) was 2%. About 10 mg formulation per cm² pig skin was applied and therefore the dose of the test substance was approximately 0.2 mg/cm² skin. Skin discs of 2.54 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with physiological saline.
The formulation and the aqueous solution were analysed in two experiments with six replicates per experiment for adsorbed, absorbed and penetrated amount of the test substance.
The receptor fluid used was 25% ethanol in physiological saline. Although C10 is soluble in physiological saline alone, the ethanolic receptor fluid was chosen to reduce interference during analysis. This choice of receptor fluid is unlikely to significantly influence the integrity of the membrane or the rate of penetration.
In the static system, samples of the receptor fluid were drawn before the application of the

test substance formulation and 0.5, 2, 6, 12, 24, 30 and 48 hours after application. The removed volume was replaced by fresh receptor fluid.

Aqueous solution

	Dose recovered (%)							
Cell number	**31**	**32**	**39**	**40**	**45**	**46**	**mean**	**SD**
Donor chambers	0.915	1.59	0.362	0.218	0.716	1.40	**0.867**	**0.550**
Skin wash at 0.5h	94.6	87.8	79.7	80.8	72.1	88.3	**83.9**	**7.97**
Skin wash at 48h	6.84	6.54	9.82	11.4	10.3	7.60	**8.75**	**2.02**
Stratum corneum	2.21	1.87	4.67	5.46	6.15	1.90	**3.71**	**1.94**
Remaining epidermis/dermis	2.44	6.29	1.52	1.20	4.12	2.39	**2.99**	**1.91**
Receptor fluid	0.745	0.212	2.36	1.20	1.91	0.749	**1.20**	**0.805**
Bioavailable (%)	3.185	6.502	3.88	2.40	6.03	3.139	**4.19**	**1.68**

Formulation

	Dose recovered (%)							
Cell number	**33**	**37**	**41**	**42**	**47**	**48**	**mean**	**SD**
"Spreaders"	11.4	5.00	10.1	15.4	10.8	8.74	**10.2**	**3.41**
Donor chambers	1.55	0.799	1.00	4.59	1.75	0.605	**1.72**	**1.48**
Skin wash at 0.5h	68.7	80.0	84.1	73.0	76.9	77.5	**76.7**	**5.36**
Skin wash at 48h	4.27	2.77	1.12	1.99	2.65	2.25	**2.51**	**1.04**
Stratum corneum	0.243	0.984	0.458	1.125	1.279	1.650	**0.957**	**0.524**
Remaining epidermis/dermis	3.33	3.19	1.25	0.930	1.69	1.40	**1.96**	**1.03**
Receptor fluid	0.233	0.498	0.441	0.802	0.728	0.160	**0.477**	**0.257**
Bioavailable (%)	3.563	3.688	1.691	1.732	2.418	1.560	**2.442**	**0.965**

The quantities that had penetrated during the 30 minute exposure to an aqueous solution and to the hair dye formulation containing Basic Yellow 57 within 48 hours after application are shown in the following table. Both the amounts absorbed and penetrated were taken as systemically available.

ANALYSED SAMPLE	Direct dye formulation without H_2O_2		Aqueous solution	
	[% of dose]	[µg/cm²]	[% of dose]	[µg/cm²]
Skin rinsings	91.13	164.034	93.517	184.229
Adsorption (stratum corneum)	0.957	1.723	3.71	7.309
Not Bioavailable	92.087	165.757	97.227	191.538
Absorption (epidermis/dermis)	1.96	3.528	2.99	5.89
Penetration (receptor fluid)	0.477	0.859	1.2	2.364
Bioavailable	2.442	4.387	4.19	8.254
Total recovery / mass balance	94.524	170.144	101.417	199.792

In this *in vitro* dermal penetration study the amount of Basic Yellow 57 systemically available from a standard cream formulation containing 2% (w/w) Basic Yellow 57 was found to be 4.387 ± 1.737µg/cm² (range 2.808 to 6.638 µg/cm²) or 2.442 ± 0.965% (range 1.56 to 3.688%).

Ref.: 15

Comment
The batch of the test material was not characterised. The number of chambers used (6) are considered too few. An ethanolic receptor fluid was used. However, the experiment was otherwise well conducted and the value of 7.87 (mean + 2SD; 4.39 + 2 x 1.74) µg/cm² may be used for calculating the MOS.

3.3.5. Repeated dose toxicity

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

No data submitted

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

Guideline:	OECD 408 (1998)
Species/strain:	Wistar rat Crl:(WI) BR (outbred, SPF-Quality)
Group size:	12 per sex/dose; Recovery group: 5 per sex/dose control and high dose group
Test substance:	C10
Batch:	00656555101
Purity:	99.9 area% (HPLC), 78.7% (NMR)
Vehicle:	water (Milli-U)
Dose levels:	0, 100, 300 and 1000 mg/kg bw
Dose volume:	10 ml/kg bw
Route:	oral, gavage
Exposure:	once daily for 90 days, 28 days recovery
GLP:	in compliance
Study period	26 May – 23 September 2005

The animals were dosed by gavage for at least 90 consecutive days at 0, 100, 300 and 1000 mg/kg bw based on pre-test results for the prenatal developmental toxicity study (200, 400, 800 mg/kg bw/day). Gavage with a stainless steel stomach tube caused 4 deaths (three males, 2 mid dose, 1 high dose and one female, high dose) on day 2. From day 3 a rubber catheter was used. These animals were replaced with reserve animals of approximately the same weight. These animals were dosed for 88 days but this was not considered a problem.

The following parameters were evaluated: clinical signs daily; functional observation tests in week 12/13; body weight and food consumption weekly; ophthalmoscopy at pre-test and in week 13; clinical pathology and macroscopy at termination; organ weights and histopathology on a selection of tissues. The recovery groups were monitored for a 4-week treatment-free period and then killed for routine pathology.

Results
There were deaths at the high dose [2 males: day 26 and 82; 4 females; days 29, 56, 56, and 79]. The authors considered two deaths to be due to gavage errors as the thoracic cavity contained yellow fluid. However, since all 6 showed enlarged spleen and extramedullary haemopoiesis these were treatment related. A further three high dose females died at blood sampling at the end of treatment. Based on the mortality incidence at 1000 mg/kg bw/day, the cause of death for these females was considered to be (in part) related to treatment with the test substance.

Body weight and food consumption were similar to the controls.

Haematology showed a dose related effect on red blood cell turnover. Increased extramedullary haemopoiesis in the spleen was seen in the low dose animals. This was more severe at the mid and high dose. Combined with higher and increased severity of extramedullary haemopoiesis in the liver at high dose, there would seem to be a regenerative response to the lower red blood cell counts. Generalized bone marrow stimulation was indicated by increased reticulocyte counts at all doses, and increased number of platelets and sternal myelopoiesis at the high dose. There appeared to be a direct effect on haemoglobin with increased Heinz bodies at the high dose and increased methaemoglobin formation at all doses. The authors considered slightly higher percentage of methaemoglobin at the low dose was not an adverse effect. The increase plasma bilirubin and potassium levels in the mid and high dose suggest that haemolysis occurred.

The splenic terminal congestion at all doses may reflect increased extravascular sequestration of red blood cells by macrophages. These morphological alterations in the spleen were supported by necropsy observations of enlargement and irregular surface of the spleen with higher spleen weights.

At the end of the recovery period red blood cell counts were normal, but other haematological changes were seen (increased red cell distribution width, haemoglobin, haematocrit and mean corpuscular haemoglobin level). Increased spleen weights were noted in females but without morphological correlates.

Periacinar hepatocytic hypertrophy at 1000 mg/kg bw/day correlated with increased liver weight and liver enlargement. The higher alanine aminotransferase activity values at the high dose in males were considered to be due to the enlarged liver. In females, the decreased total protein level at mid and high dose, and increased prothrombin time at the high dose suggest that liver function effects since the liver is the major source of protein synthesis including the blood clotting pathways.

Red/orange/yellow staining of urine and various body parts, red contents of the urinary bladder, tinctorial change in the keratin of the stomach and tongue, and yellowish discolouration of various organs (including tongue, caecum, stomach mucosa and mesenteric adipose tissue) at high dose bw/day were considered to be related to staining properties of the test substance formulation (orange/red).

At the mid and high doses, there was clear evidence of haematotoxic effects, seen by the significant reduction of red blood cell counts and a regenerative haematopoietic response. The presence of Heinz bodies and increased levels of methaemoglobin at these dose levels suggest a direct adverse effect of the test substance on haemoglobin. Also, deaths at 1000 mg/kg bw/day were considered to be test substance related. At the low dose, there were morphological effects (see above) in the spleen and marginal haematological effects in females (reduced erythrocyte counts and haemoglobin levels) that suggested this dose could be the threshold of an adverse effect on red blood cells.

Conclusion
Based on haematotoxic effects and spleen congestion, a Lowest Observed Adverse Effect Level (LOAEL) for Basic Yellow 57 of 100 mg/kg bw/day was established. According to the dye content of 79% (NMR) this value should be corrected to 79 mg/kg bw/day.

Ref.: 12, 13

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 Test

Guideline:	OECD 471 (1997)
Species/Strain:	Salmonella typhimurium TA98, TA100, TA102, TA1535, TA 1537
Test item:	C10
Batch:	00656555101
Purity:	99.9% (HPLC)
Replicate:	Two experiments with triplicate plates

Concentrations:	experiment I and II 3.16, 10, 31.6, 100, 316 and 1000 µg/plate with and without S9-mix
Treatment:	plate incorporation assay and pre-incubation assay
Positive controls:	sodium azide, 2-nitrofluorene, 2-aminofluorene, 9-aminoacridine, mitomycin C, 1,8-dihydroxyanthraquinone, 2-aminoanthracene
Negative control:	water (second test only)
Solvent control:	DMSO
GLP:	in compliance
Study period:	14 March - 11 April 2005

C10 was tested in the *Salmonella*/microsome assay in five tester strains according to OECD guideline. In a preliminary toxicity test (plate incorporation test) the test substance was tested in TA 98 and TA 100 at six concentrations up to 2000µg/plate. The dose selections in the main test were based on cytotoxicity and precipitation at the highest concentrations in this pre-test. DMSO was used as solvent. Appropriate negative and positive controls were included.

Results
Precipitation was observed at the two highest concentrations with and without metabolic activation in both tests. In the first experiment cytotoxicity was observed at the highest concentration without S9 mix in all strains and in some strains also slight toxicity at the second highest concentration. No toxicity was observed with S9-mix. In the second experiment strong toxicity was observed at the two highest concentrations without S9-mix and the highest concentration with S9 mix.

In both experiments of the main test there was no significant increase in the number of revertant colonies in any of the tester strains neither with nor without metabolic activation and no concentration related increase was observed either.

Conclusion
The test item did not induce gene mutations in bacteria under the experimental conditions reported

Ref.: 9

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)
Species/strain:	mouse lymphoma cell line L5178Y
Test item:	C10
Batch:	00656555101
Purity:	99.9% (HPLC)
Concentrations:	Experiment I without S9-mix: 118.8, 237.5, 475.0, 712.5, 950.0 µg/ml with S9-mix: 118.8, 237.5, 475.0, 712.5, 950.0 µg/ml Experiment II without S9-mix: 59.4, 118.8, 237.5, 475.0, 950.0 µg/ml Experiment IIA: without S9-mix: 400, 500, 600, 700 µg/ml
Replicates:	3 independent experiments, using 2 parallel cultures each
Treatment:	Experiment I: 4 h treatment with and without S9-mix Experiment II and IIA: 24 h treatment without S9-mix
Positive controls:	Methyl methane sulfonate (- S9-mix) Cyclophosphamide (+ S9-mix)
GLP:	in compliance
Study period:	11 July – 4 October 2005

The mouse lymphoma cell line L5178Y was used to examine the potential of C10 to induce

mutations at the thymidine kinase locus. The assay was performed in the presence and absence of phenobarbital and β-naphthoflavone stimulated rat liver S9-mix.
The assay was performed in three independent experiments, using two parallel cultures each. Experiment I was performed with and without S9-mix and a treatment period of 4 hours. Experiment II was performed solely without metabolic activation and a treatment period of 24 hours. An additional experiment IIA without metabolic activation was performed (24 hour treatment) to verify a dose related increase of the mutant frequency observed in the second experiment. The test substance was dissolved in deionised water and appropriate negative and positive controls were included.

Results
No precipitation was observed by the naked eye in any of the experiments performed.
In the first two main experiments the test item induced strong toxic effects in both parallel cultures at 950 µg/ml. In the first experiment the relative total growth (RTG) was below 10% except for culture one without S9-mix, at the second highest concentration (712.5 µg/ml) RTG was between 30 and 60%. In the second experiment RTG was 8.8% and 6.5% at the highest concentration and 43% and 40% at the second highest concentration. In the third experiment the RTG, at the highest tested concentration, was 11.5% and 13.6% in the two cultures respectively.
No relevant and reproducible increase of the mutant frequency was observed in the first experiment with and without metabolic activation. In the second experiment, performed solely without metabolic activation, the number of mutant colonies/10^6 cells exceeded the range of the historical control data at 237.5 µg/ml (culture II), and at 475 µg/ml (both cultures) and there was a concentration related increase except at the highest concentration, where the decline in mutant frequency could be due to strong toxicity. In culture two of the second experiment the mutant frequency was increased 2.2 times compared to the corresponding solvent control and the induced mutant frequency was 163 x 10^{-6} at 475 µg/ml, which is above the recommended increase for a positive result. In culture I there was a minor increase (1.5 times compared to the negative control and induction of 75 mutants) and no concentration response relationship.
To verify this minor increase a repeat experiment was performed using a rather narrow concentration range. The toxic range of 10 – 20% of survival was covered but no relevant increase of the mutant frequency was observed in any of both cultures. Therefore, the minor effects noted in experiment II were judged as irreproducible fluctuations with no biological relevance.

Conclusion
In the study described and under the experimental conditions reported, C10 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation. Therefore C10 is considered to be not mutagenic in this mouse lymphoma assay.

Ref.: 10

Comment
The required toxic range of approximately 10 to 20% in the relative total growth was not covered in the first two experiments, due to a steep gradient of toxicity between the two highest concentrations (712.5 and 950 µg/ml) the 10% limit for RTG was not quite reached. In the third experiment the RTG, at the highest tested concentration, was 11.5% and 13.6% in the two cultures respectively. However, under the experimental conditions used, toxicity values slightly below 10% can be accepted.

In vitro Micronucleus Test

Guideline: OECD 487 (2004)
Species/strain: V79 Chinese hamster cells
Test item: C10

Batch:	00656555101
Purity:	99.9% (HPLC)
Concentrations:	Experiment I
	without S9-mix: 1000, 1500, 2000 µg/ml
	with S9-mix: 1000, 1200, 1400 µg/ml
	Experiment II
	without S9-mix: 1000, 1500, 2000 µg/ml
Treatment:	Experiment I: 4h treatment + 18h incubation
	Experiment II: 24h treatment
Positive controls:	Mitomycin C (- S9-mix)
	Cyclophosphamide monohydrate (+ S9-mix)
GLP:	in compliance
Study period:	11 April – 26 August 2005

Chinese Hamster V79 cells were used to examine the potential of C10 to induce micronuclei in the presence and absence of phenobarbital and β-naphthoflavone stimulated rat liver S9-mix.
In each experimental group two parallel cultures were set up. Per culture 1000 cells were scored for micronuclei. The test article was dissolved in Dulbecco's Modified Eagle Medium (DMEM).
A pre-test on cytotoxicity (MTT assay) was performed in order to determine the toxicity of the test item, the solubility during exposure and changes in osmolarity and pH value at experimental conditions. The concentrations used in the main study were based on this pre-test.

Results
No signs of precipitation were observed at the end of incubation. No decrease in the number of attached cells was observed at the end of incubation of both tests. Determination of the cytokinesis-block proliferation index (CBPI) showed a substantial cytotoxic effect at and above 2000 µg/ml in the first test (with and without metabolic activation) and 1400 µg/ml (without metabolic activation) in the second test.
C10 did not induce an increase in the number of cells with micronuclei either with or without metabolic activation (4h incubation) in the first test or in the second test after a continuous treatment time of 24 h without S9-mix.
The micronucleus frequency of negative controls was partly below the range of the historical data. These slightly low values did not impair the outcome of the study as the functionality of the test system was demonstrated by the corresponding positive controls. These induced the expected increase in micronucleus frequency, with and without metabolic activation, respectively, thus demonstrating the sensitivity of the test system used for the endpoints investigated in this study.

Conclusion
In conclusion, it can be stated that in the study described and under the experimental conditions reported, C10 did not induce micronuclei in Chinese hamster V79 cells in the absence and in the presence of metabolic activation, and is considered to be not clastogenic and/or aneugenic *in vitro*.

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

No data submitted

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Prenatal development toxicity study

Guideline:	OECD 414 (2001)
Species/strain:	female Wistar rat Crl:(WI) BR (outbred, SPF-Quality)
Group size:	24 females/dose (96 in total), nulliparous and non-pregnant
Test substance:	C10
Batch:	00656555101
Purity:	99.9 area% (HPLC), 78.7% (NMR)
Vehicle:	water (Milli-U)
Dose levels:	0, 100, 300 and 1000 mg/kg bw
Dose volume:	10 ml/kg bw
Route:	oral, gavage
Exposure:	once daily from day 6 to day 20 *post-coitum*
GLP:	in compliance
Study period	8 May – 16 November 2005

The females were paired with male rats of the same strain one to one with an accurate day of mating (GD0), fixed by the presence of vaginal plugs or sperm in the vaginal smear. Dosages were based on the results of the previous dose range-finding study (200, 400, 800 mg/kg/day).

The animals were checked daily for the clinical signs. Body weights and food consumption was determined at periodically during pregnancy. On GD 21, all females were subjected to an examination *post-mortem* and external, thoracic and abdominal macroscopic findings were recorded. The ovaries and uterine horns were dissected and examined for the number of *corpora lutea*, the weight of the gravid uterus, the number of implantation site scars, the number and distribution of live/dead foetuses and embryo-foetal deaths, the weight of each live foetus and corresponding placenta, foetal sex and externally visible foetal macroscopic abnormalities. Alternate foetuses of each litter were preserved in 96% aqueous ethanol or Bouin's fluid, and subjected to skeletal or visceral examinations respectively.

Results
Maternal effects
There was one death in the high dose group on GD 13, but there were no relevant toxicologically clinical or pathological signs. Since the cause of death is unclear, a treatment-related effect can not be excluded.
Body weight and body weight gain were comparable to the control in the low and mid dose group, but there was a statistically significant decrease in the high dose group. This decrease was mirrored in the food consumption in the high dose group, In the mid dose group, food consumption decreased from GD12.
Orange to red urine was noted in all animals in the mid and high dose groups and in some of the low dose group.
Macroscopic observation of enlarged spleen in one mid dose and 5 high dose animals was observed. In addition, 2 high dose animals had crateriform retractions in the stomach. Abdominal fat and the forestomach appeared stained yellow in some of the high dose group. These were considered to be treatment related.

Reproductive effects
Eight animals were not pregnant (3 control; 2 low dose, 2 mid dose and 1 high dose) and one mid dose animal only showed implantation sites. Post-implantation losses increased at

the high dose resulting in decreased foetal numbers and increased foetal deaths. Embryonic resorption was not affected. No treatment related effects were seen at the other doses.

Foetal findings
The foetal sex ratio was not affected by treatment. In the low and mid dose groups, no treatment related effects were seen. Maternal treatment with C10 at dose levels up to 300 mg/kg bw/day did not elicit any teratogenic effects.
At the high dose, there was a high incidence of foetuses with major visceral abnormalities, i.e. severe umbilical hernia and associated visceral changes, including displacement of organs and absence of diaphragm. A number of the abnormal foetuses also exhibited mal-rotated hind limbs, atypical skeletal ossification. Some ossification parameters showed slight retardation that could be explained by the reduction in mean foetal weight recorded, whilst other parameters were slightly better ossified. The toxicological significance of the latter finding is unclear.

Conclusion
Based on the results in this prenatal developmental toxicity study, the maternal No Observed Effect Level (NOEL) was considered to be 100 mg/kg bw/day (79 mg/kg bw/day corrected for dye content) and the developmental No Observed Adverse Effect Level (NOAEL) 300 mg/kg bw/day (237 mg/kg bw/day corrected for dye content).

Ref.: 14

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

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

Basic Yellow 57
(non-oxidative conditions)

Absorption through the skin (mean + 2SD)	**A**	**=**	**7.87 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**4.56 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.076 mg/kg bw**
Lowest observed adverse effect level (90-day, oral, rat)	**LOAEL**	**=**	**100 mg/kg bw**
LOAEL corrected for 79 % dye content		**=**	**79 mg/kg bw**
Adjusted LOAEL (/3)		**=**	**26 mg/kg bw**
Margin of Safety	**adj LOAEL / SED**	**=**	**342**

3.3.14. Discussion

Physico-chemical properties
Basic Yellow 57 is used as a direct dye in hair colouring products in a non-oxidative environment. The final concentration of Basic Yellow 57 on head can be up to 2.0%.
The NMR-purity of Basic Yellow 57 in one of the three batches reported is much lower than that described in the composition of the marketed material. Batch 15, used in hair dye formulations, contains 10.6% methyl sulphate. Monomethyl sulphate is used as an anion to the dye. No toxicity data for monomethyl sulphate was submitted. However, the applicant declared that no dimethylsulfate or monomethylsulfate is used or produced in the actual technical process.
Water solubility of Basic Yellow 57 has not been determined by the EEC method. Stability of Basic Yellow 57 in typical hair dye formulations has not been reported.

Toxicity
In the sub-chronic oral toxicity study in rats, the LOAEL was determined to be 100 mg/kg bw/day. For the calculation of the Margin of Safety, a conservative ten-fold factor could be used for the adjustment of this LOAEL as proposed by the applicant, resulting in a value of 10 mg/kg bw/day.
Based on the results of a prenatal developmental toxicity study in rats, the maternal No Observed Effect Level (NOEL) was considered to be 100 mg/kg bw/day and the developmental No Observed Adverse Effect Level (NOAEL) 300 mg/kg bw/day.
No two generation reproduction study was submitted.

Skin/eye irritation and sensitisation
Under the conditions of the study, Basic Yellow 57 was not irritating to rabbit skin. The undiluted test substance was irritating to the rabbit eye.
In an LLNA study, the highest test concentration (10%) was considered by the study authors to be "*the highest technically applicable concentration whilst avoiding systemic toxicity and excessive local irritation*". It cannot be excluded that Basic Yellow 57 is a skin sensitiser as the maximum test concentration (10%) is too low.

Percutaneous absorption
The number of chambers used (6) are considered too few. An ethanolic receptor fluid was used. However, the experiment was otherwise well conducted and the value of 7.87 µg/cm² (mean + 2 SD) may be used for calculating the MOS.

Mutagenicity/genotoxicity
Basic Yellow 57 was tested for the three genotoxicity endpoints: gene mutation, structural and numerical chromosomal aberrations. It did not induce gene mutations in bacteria and mammalian cells *in vitro* and did not have clastogenic and/aneugenic potential in an *in vitro* micronucleus test. Therefore it can be concluded that Basic Yellow 57 does not have genotoxic potential *in vitro*, and no further genotoxicity tests are required on the substance.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCS is of the opinion that the use of Basic Yellow 57 as an ingredient in non-oxidative hair dye formulations at a maximum on-head concentration 2.0% does not pose a risk to the consumer.

This opinion refers to Basic Yellow 57 not containing methyl sulphate.

A sensitising potential of Basic Yellow 57 cannot be excluded.

5. MINORITY OPINION

Not applicable

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