Scientific Committee on Consumer Safety

SCCS

OPINION ON

Basic Red 76

COLIPA n° C8

The SCCS adopted this opinion at its 10th plenary meeting

of 22 March 2011

About the Scientific Committees

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

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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ISSN 1831-4767 ISBN 978-92-79-30713-3
Doi:10.2772/97445 ND-AQ-11-006-EN-N

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

ACKNOWLEDGMENTS

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Keywords: SCCS, scientific opinion, hair dye, Basic Red 76, C8, CAS 68391-30-0, EC 269-941-4, Directive 76/768/EEC

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on Basic Red 76, 22 March 2011

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

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1. BACKGROUND

Submission I for Basic Red 76 with the chemical name [7-Hydroxy-8-[(2-methoxyphenyl)azo]-2-naphthyl]-trimethylammonium chloride was submitted in August 1992 by COLIPA [1].

Submission II for Basic Red 76 was submitted in July 2001 by COLIPA.

The Scientific Committee on Consumer Products and Non Food Products intended for Consumers (SCCNFP) adopted its opinion SCCNFP/0661/03 at the 23rd plenary meeting of 18th March 2003 with the conclusion, that:

"Commercial grade dye of different batches has been used for various tests, but purity (>98%) of the dye has been described only in one case. The impurities in the dye should be described. The test substance is an azo-dye, therefore, free aromatic amine (2-methoxy aniline) content in the dye (in all batches) is required for the evaluation of carcinogenic potential of the dye. The dye formulation contains 16% sugar and 14.5% inorganic salts. A complete description of the sugar and salts is required. Following physical properties are also required: density and Log Pow.
The sensitisation data in the dossier used a concentration too low for intradermal induction. The studies on percutaneous absorption are inadequate. The test concentrations were well below the expected use concentration.
Basic Red 76 has been tested for the induction of gene mutation in bacterial cells (positive results) and in the mammalian cells, in vitro (negative results), and for chromosome aberration in mammalian cells in vitro (negative results). The in vitro UDS study was inadequate. The in vivo micronucleus test gave negative results; no firm evidence that the bone marrow was reached by the test agent was noted. No conclusion can be drawn".

According to current submission III Basic Red 76 is used as a direct dye for hair colouring products. The final concentration on the scalp is proposed up to 2.0%.

2. TERMS OF REFERENCE

1. *Does SCCS consider Basic Red 76 safe for use as an ingredient in non-oxidative hair dye formulations with a concentration on the scalp of maximum 2.0% taken into account the scientific data provided?*

2. *And/or does the SCCS have any other concerns with regard to the use of Basic Red 76 in non-oxidative hair dye formulations?*

[1] 1 COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

The identity of the test substance in batch 0050644101 (SAT 040267), 0057891101 (SAT 050017) and 12/13B (SAT 050529) was confirmed by ^{1}H-, ^{13}C-, DEPT and 2D-NMR-, IR- and UV-spectrometry. All spectra were in good accordance with the expected results.

3.1.1.1. Primary name and/or INCI name

Basic Red 76

3.1.1.2. Chemical names

2-Naphthalenaminium, 7-hydroxy-8-[(2-methoxyphenyl)azo]-N,N,N-trimethyl-, chloride (9CI)
[7-Hydroxy-8-[(2-methoxyphenyl)azo]-2-naphthyl]-trimethylammonium chloride,
7-Hydroxy-8-[(2-methoxyphenyl)diazenyl]-N,N,N-trimethylnaphthalen-2-aminium chloride
8-(2'-Methoxyphenylazo)-7-hydroxy-2-naphthyl-trimethylammonium chloride,

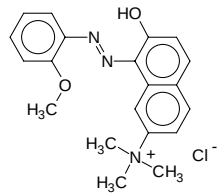
3.1.1.3 Trade names and abbreviations

Arianor Madder Red
Basic Red 76
CI 12245
CI Basic Red 76
COLIPA C8

3.1.1.4 CAS /EC number

CAS: 68391-30-0
EC: 269-941-4

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_{20}H_{22}N_3O_2^+ Cl^-$

3.1.2 Physical form

Red fine powder

3.1.3 Molecular weight

Molecular weight: 371.86 g/mol

3.1.4 Purity, composition and substance codes

Purity of the test substance was quantified in D_2O against Na-acetate as standard based on ^{1}H-NMR. ^{1}H-, ^{13}C-, DEPT and 2D-NMR- were performed in D_2O and DMSO for further confirmation of identity.
UV-Vis-spectra showed maxima at the wavelength 235 nm, 332 nm and 503 nm.

Purity and impurities	**Batch 0050644101 (SAT 040267)**	**Batch 0057891101 (SAT 050017)**	**Batch 12/13B (SAT 050529)**	**material used in the market**
reference	2	3	4	1
Dye content by NMR	62%(w/w)	80.5% (w/w)	89.1% (w/w)	> 77%(w/w)
Purity by HPLC	98.1%(area)	98.6% (area)	98.5% (area)	> 97%(area)

3.1.5 Impurities / accompanying contaminants

Purity of batch 0050644101 (SAT 040267), 0057891101 (SAT 050017) and 12/13B (SAT 050529) was determined by HPLC-UV. The purity was calculated via area percentage. Impurities of o-anisidine were determined by HPLC-UV against o-anisidine standard solutions.

Water content was determined by Karl-Fischer-Titration. Methyl sulphate and chloride were determined by ion-chromatography. Sodium and calcium were determined by ICP-metal ion screening.

Chloromethane and methyl formate were determined by gas chromatography.
Sulphate ash was determined by sulphate ash assay.
Saccharose was determined by HPLC with refractive index detection.

	Batch 0050644101 (SAT 040267)	**Batch 0057891101 (SAT 050017)**	**Batch 12/13B (SAT 050529)**	**material used in the market**
reference	2	3	4	1
water content	5,1%(w/w)	4.1% (w/w)	3.1% (w/w)	<6%(w/w)
Monomethyl sulphate	11.8%(w/w)	15.9% (w/w)	11.4% (w/w)	<18%(w/w)
o-anisidine	5 ppm	19 ppm	11 ppm	<10 ppm
Chloromethane		0.3% (w/w)	0.1% (w/w)	
Methyl acetate		0.1% (w/w)		
Methyl formate		0.4% (w/w)		
7-Hydroxy-N,N,N-trimethyl-naphthalen-2-aminium chloride		< 500 ppm (detection limit)	< 500 ppm (detection limit)	
methanol			0.7 % (w/w)	
Sulphated ash	0.4% (w/w)	0.3% (w/w)	0.1% (w/w)	< 0.5% (w/w)
Chloride	1.6%(w/w)	2.7% (w/w)	4.4% (w/w)	< 5%(w/w)

	Batch 0050644101 (SAT 040267)	Batch 0057891101 (SAT 050017)	Batch 12/13B (SAT 050529)	material used in the market
Sodium	630 ppm	190 ppm	240 ppm	
Calcium	590 ppm			
Saccharose*	25.8%(w/w)			

* Note: As this batch represents an actual market material it contains the extender saccharose which has been used to adjust the colour strength to a certain predefined value

All batches analysed contain (mono)methyl sulphate 11.4 to 18%. SCCS considers that it is used as an anion to the dye. No toxicity data for monomethyl sulphate were provided.

3.1.6 Solubility

Water: 10 – 100 g/l room temperature
Ethanol: 0.3 – 3 g/l room temperature
DMSO: 1 – 10 g/l room temperature

3.1.7 Partition coefficient (Log P_{ow})

Log P_{OW}: - 1.7834 +/- 0.1131

Ref.: 17

Comment
For the determination of Log Pow a shake flask method had been used, which according to the applicants "complies with EU test method L383A/63-73(EU A.8)".

3.1.8 Additional physicochemical specifications

Melting point: > 200 °C (decomposition)
Boiling point: /
Flash point: /
Vapour pressure: /
Density: /
Viscosity: /
pKa: /
Refractive index: /

3.1.9. Stability

The neat test substance is reported to be stable in the refrigerator in the dark (ref 5).
Accuracy, stability and homogeneity of the test substance formulations have been tested for the subchronic and developmental toxicity investigations.
The formulations used for subchronic toxicity tests proofed to be accurately prepared (86 to 106%) and homogeneous (0.18-to 3.6%) (with an outlier of 11%). In the refrigerator the aqueous formulations were stable for 8 days (0.036 to 3.6%)
The preparations used for developmental toxicity tests were accurately prepared (98 to 105%) and were homogeneous (0.8 to 1.4%). Stability has not been tested.
Recent experiments of the applicants show that the formulations used for the toxicity tests are stable for at least 24h.
No data have been supplied on the stability of the test substance in typical hair dye formulations.

General Comments on Physico-chemical characterisation

- All batches are characterised for identity, purity and impurities.
  Next to NMR, HPLC has been used for purity testing of the test substance. Using HPLC no standard reference material has been used for calibration. The HPLC results are therefore semi-quantitative. Major components like methyl sulphate (11.4-18.0%), water (3.1-5.1%) and chloride (1.6-5.0%) have been quantified by other analytical methods than HPLC.
- The purity and identity of the raw material of C8, which is used in marketed hair dyes is specified in ref. 1. Original analytical results like chromatograms etc. are not supplied.
- Recent experiments of the applicants show that the formulations used for the toxicity tests are stable for at least24h.
- All batches analysed contain (mono)methyl sulphate(11.4 to <18%), which is an anion to the dye. No specific toxicity data for monomethyl sulphate were provided.

3.2. Function and uses

Basic Red 76 is used as a direct dye for hair colouring products. The final concentration on head of Basic Red 76 can be up to 2.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 423 (2001)
Species/strain:	Wistar rats
Group size:	three female and three male rats
Test substance:	C 008 in propylene glycol
Batch:	00506441 01
Purity:	reference in table 3.1.5
Dose:	2000 mg/kg bw
Observation period:	15 d
GLP:	in compliance
Study date:	August 2004

C 008 was administered by oral gavage to a group of three female Wistar rats and subsequently to a group of three male Wistar rats at 2000 mg/kg bw. Animals were subjected to daily observations and weekly determination of body weight. Macroscopic examination was performed after terminal sacrifice (day 15).

Results
No mortality occurred. Hunched posture was noted in one female and 3 males on day 1. Red staining of the back and/or snout and/or head was noted in 1 female and 2 males between days 1 and 11. Red and/or yellow faeces and/or yellow urine were seen among the animals on days 2 and/or 3. The mean body weight gain over the study period was considered to be normal. No abnormalities were found at macroscopic post mortem examination of the animals. The oral LD_{50}-value of C 008 in Wistar rats was established to exceed 2000 mg/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:	adult male albino rabbits; New Zealand White strain
Group size	3
Test substance:	C008 (SAT 040267)
Batch:	0050644101
Purity:	98.1%
Dose:	500 mg
Observation period:	1, 24, 48, 72 hours
GLP:	in compliance
Study date:	May 2004

Approximately 24 hours prior to the treatment, the dorsal fur was shaved to expose an area of about 150 cm².

The intact shaved back skin of each animal was exposed to an aliquot of 0.5 g of the moistened test substance. The patch was removed 4 hours after semi-occlusive contact.
Animals were examined for signs of erythema, eschar and oedema formation. The skin reactions were assessed approximately 1, 24, 48 and 72 hours after termination of the exposure.

Results
There were no visible signs of irritation. However, slight red staining was observed in all animals from the 1-hour reading up to 72 hours after treatment. Staining persisted in one animal up to a 14-day examination, the end of the observation period for all animals.
Under the conditions of the study, the undiluted test substance was not irritating to rabbit skin.

Ref.: 6

Comment
The red staining of the skin may have masked signs of erythema caused by semi-occlusive application of neat C008 on the skin of the rabbit.

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405 (2002)
Species/strain:	adult albino rabbits; New Zealand strain (one male, two females).
Group size:	3
Test substance:	C008 (SAT 040267)
Batch:	0050644101
Purity:	98.1%
Dose:	100mg
Observation period:	1 hour, 24, 48 and 72 hours and 7 days
GLP:	in compliance
Study date:	June 2004

100 mg of C008 was instilled into the conjunctival sac of the left 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 right eyes served as controls.
The eye irritation reactions were scored approximately 1, 24, 48 and 72 hours and 7 days after instillation of the test solution.

Results
The instillation of the undiluted C008 into the eyes resulted in mild, early-onset and transient ocular changes, such as reddening of the conjunctivae and sclerae, discharge and chemosis.
These effects were reversible and were no longer evident 72 hours after treatment. No abnormal findings were observed in the cornea or iris of any animal at any of the examinations. No corrosion was observed at any of the measuring intervals. Slight red staining of the treated eyes by the test item was observed in all animals at the 1- and 24-hour reading and persisted in two animals up to 48 hours and in one animal up to 72 hours after treatment. The study was terminated seven days after instillation.

Conclusion
Under the conditions of the study, the undiluted test substance was slightly irritating to the rabbit eye.

Ref.: 7

Comment
Undiluted C008 was irritating to rabbit eyes.

3.3.3. Skin sensitisation

Guideline:	OECD 429 (2002)
Species/strain:	CBA/CaOlaHsd mice
Group size:	4 female mice per groups
Test substance:	C008 (SAT 040267)
Batch:	0050644101
Purity:	98.1%
Dose levels:	2.5%, 5% and 10% in ethanol:water (7:3 v/v)
Route:	topical
GLP:	in compliance
Study date:	May 2004

A dilution of the test item in a mixture of ethanol:water (7:3 v/v) was made shortly before each dosing. Although acetone:olive oil (4:1 v/v) is recommended, ethanol:water (7:3 v/v) was selected as the vehicle due to pre-tests on the solubility of the test item. The highest non-irritating, "technically applicable" test item concentration was determined in a pre-test with 4 mice. Based on these test results 2.5%, 5% and 10% solutions were chosen for the main study.

Each test group of mice was treated by topical 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 exclusively. Five days after the first topical application, all mice were administered with radio-labelled thymidine (^{3}H-TdR) by intravenous injection via the tail vein.

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

The level of ^{3}H-TdR incorporation was then measured by scintillation counting. The proliferative response of lymph node cells is expressed as the ratio of ^{3}H-TdR 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 demonstrate the sensitivity of the test system. 5%, 10% and 25% dilutions in acetone:olive oil were used.

The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of ^{3}H-methylthymidine. A test item is regarded as a sensitizer if the exposure to at least one concentration resulted in an at least 3-fold increase in incorporation of ^{3}H-TdR compared with concurrent controls, as indicated by the stimulation index (S.I.) together with consideration of dose-response.

Results
The Stimulation Index (S.I.) was below 3 in all dose groups.

Test tem concentration	S.I.
2.5% (w/v)	0.9
5% (w/v)	1.1
10% (w/v)	1.3

Calculation of the EC 3 value was not performed as no test concentration produced a stimulation index of 3 or above.
The positive control induced a distinct increase of the stimulation index and gave an EC3 of 11.7% w/v.

Conclusion
Based on the criteria of the test system, C008 was found to be a non-sensitizer when tested up to a concentration of 10% (w/v) in ethanol:water (7:3 v/v) in mice.

Ref.: 8

Comment
The vehicle used to test C008 was non-standard and different to that used for the peri-contemporaneous control. The highest dilution tested was 10% in ethanol:water. Therefore, a sensitising potential cannot be excluded. Curiously, concentrations above 10% C008 in ethanol:water were found to be irritating whereas moistened C008 was non irritating to the skin.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (2004)
Tissue:	pig skin; dermatomed 0.75mm
Group size:	one male and one female donor 4 replicates from each animal per experiment
Skin integrity:	trans-dermal electrical resistance (at least 7 kΩ)
Diffusion cell:	Static 1.0 cm^2
Test substance:	C008 (SAT 050017)
Batch:	0057891101
Purity:	98.6%
Dose volume:	20 mg formulation/cm^2 (0.4mg/ cm^2) Experiment A: 2% in direct dye Experiment B: 2% in water
Receptor fluid:	Dulbecco´s phosphate buffered saline
Solubility receptor fluid:	/
Stability receptor fluid:	1-2% reduction after 24 hours in saline
Method of Analysis:	HPLC; quantification limit 2.32 ng/ml
GLP:	in compliance
Study date:	Sept-Dec 2005

The direct dye cream used consisted of:

Ingredient	Concentration
C008	2.0%
Hydrenol D	6.0%
Lorol techn.	6.0%
Eumulgin B1	3.0%
Eumulgin B2	3.0%
PHB-methyl ester	0.3%
PHB-propyl ester	0.2%
Phenoxyethanol	1.0%
Polyglycol 400	5.0%
Eumulgin RO 40	1.0%
Natrosol 250 HR	1.0%
NaOH	for pH adjustment

Tartaric Acid	for pH adjustment
Water	ad 100%
	pH 8.97

The content of C008 in the final application formulations (direct dye cream and aqueous solution) was 2%.
The dermal absorption/percutaneous penetration of the test substance was investigated for the open application of about 20 mg formulation/cm² pig skin. Therefore the resulted dose of the test substance was approximately 0.4 mg/cm² skin. Skin discs of 1.0 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with a 0.01% Tween 80 solution and water.
The direct dye cream and the aqueous solution were evaluated in two experiments with four replicates from each animal per experiment for adsorbed, absorbed and penetrated amount of the test substance. In the static system samples of the receptor fluid were drawn before the application of the test substance formulation and 0.5, 1, 2, 4, 6, 24, 28 and 48 hours after application. The removed volume was replaced by fresh receptor fluid.

Results
The quantities that had penetrated during the 30 minutes exposure to C008 containing formulations and within the 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 cream		Aqueous solution	
	[% of dose]	[µg/cm²]	[% of dose]	[µg/cm²]
Skin rinsings	81.2	337	65.6	239
Adsorption (stratum corneum)	1.44	5.88	3.87	14.10
Absorption (epidermis/dermis)	0.46	1.94	1.77	6.48
Penetration (receptor fluid)	0.0049	0.020	0.012	0.042
Bioavailable	0.47 ± 0.17	1.96 ± 0.83	1.78 ± 0.96	6.52 ± 3.58
Total recovery / mass balance*	83.4	-	73.6	-

* Slight differences to the sum of the results may occur due to 1) rounding and 2) residual masses in the flange region of the penetration cell.

The low mass balance was most likely caused by the fact that the cellulose pads and the cellulose filters used in the rinsing procedure were stained red by the test substance. This colour could not be removed from the cellulose by extraction processes and was therefore not included in the mass balance. An additional experiment on extraction of pads and filters was performed and demonstrated the impact of such persistent staining on the mass balance.

In this *in vitro* dermal penetration study the amount of C008 systemically available from a direct dye cream formulation containing 2% C008 was found to be 1.96 ± 0.83 µg/cm² (0.47±0.17%).

Ref.: 16

Comment
The recovery/mass balances were outside the 100 ± 15% required by the study protocol. A total of eight replicates were used from only 2 donors. Therefore, the mean + 2SD (3.62 µg/cm²) may be used for estimating the MOS for direct hair dye formulations containing 2% C008.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 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 rats
Group size:	12/sex/dose group; 5/sex/dose group for recovery (4 weeks)
Test substance:	C 008 in distilled water
Batch:	SAT 050017
Purity:	98.6 area% (HPLC)
Dose levels:	0, 60, 250 and 1000 mg/kg bw/day
Exposure period:	91 days
GLP:	in compliance
Study date:	May to Dec 2005

The test substance was administered as an aqueous solution in a single dose by gavage for 91 consecutive days in daily doses of 60, 250 and 1000 mg/kg bw/day based on the results of the dose range finding study of the prenatal developmental toxicity study (0, 200, 600, 1000 mg/kg bw/day). The control animals received the vehicle alone (distilled water).

During the study the mortality and signs of intoxication (daily), the body weight and the food consumption (weekly) were recorded. Functional observation tests were performed in week 12, and ophthalmoscopy before the start of treatment and in week 13.

The animals of the recovery groups were additionally examined during the 4-week treatment-free period. At the end of the study, the animals were sacrificed and subjected to pathological investigations.

Results
No mortality occurred that was considered to be due to toxicity of the test substance. One female of the 60 mg/kg bw/day dose group was found dead on day 60. Based on microscopic and macroscopic findings the cause of death for this animal was considered to be misgavage. All other rats survived the scheduled duration of the study.

Red staining of various body parts and red discolouration of faeces and urine observed among the dose groups were considered to be related to the staining properties of the test substance (a red-brown powder) and/or a metabolite, and considered to be of no toxicological significance.

Clonic spasms observed in some animals of all dose groups after dosing, including the control group in the last few weeks of treatment were considered not to be significant in toxicological terms. This clinical sign was of an intermittent and infrequent nature and was not supported by abnormalities during functional observation tests.

No toxicologically relevant changes in body weight and food intake levels were noted, and no abnormalities were noted during ophthalmoscopic examination.

At 250 and 1000 mg/kg bw/day effects on haematology parameters were indicative of destruction of red blood cells, and included increased tissue iron stores in the spleen (hemosiderosis) and liver (brown pigment in the Kupffer cells) and increased serum bilirubin levels. Elevated numbers of reticulocytes, increased severity of extramedullary haemopoiesis in the spleen and erythropoiesis in the sternal bone marrow indicate a regenerative response to the lower red blood cell counts. Splenic congestion (considered reflecting increased extravascular sequestration of red blood cells by macrophages) and increased splenic weights (with enlargement at necropsy) were related to these processes.

Increased methaemoglobin formation at 250 and 1000 mg/kg bw/day indicates that the test substance may convert haem to the ferric state. The increased incidence/severity of hemosiderin pigment in the spleen at 1000 mg/kg bw/day at the end of the recovery period may indicate a residual presence of tissue iron stores. Also, based on normal red blood cell counts and absence of extramedullary haematopoiesis it was considered that haematological effects had widely resolved during the 28-day recovery period.

Also at 60 mg/kg bw/day, haematological changes were observed. The haematological changes at 60 mg/kg bw/day were considered an early indicator for adverse effect on red blood cell turn over.

Thyroid follicular cell hypertrophy in combination with hypertrophy of the adenohypophyseal cells of the pituitary, as seen at 1000 mg/kg bw/day, probably reflect a perturbation of thyroid – pituitary hormone homeostasis.

At 250 and 1000 mg/kg bw/day there was clear evidence of an adverse effect of the test substance on red blood cell life span as indicated by evidence for haemolysis and increased methaemoglobin levels at these dose levels, which indicate a direct adverse effect of the test substance on haemoglobin. At 60 mg/kg bw/day, evidence for an effect on red blood cell turn over was marginal in nature and occurred in the absence of any evidence of red blood cell destruction or increased extramedullary haematopoiesis, and was considered not to reflect an adverse effect on red blood cell turn over. Therefore, from the results presented in this report a definitive No Observed Adverse Effect Level (NOAEL) for Basic Red 76 of 60 mg/kg bw/day was established.

Ref.: 13

Comment
Also at 60 mg/kg bw/day, the lowest dose investigated, an early indication for an effect on red blood cell turn over was observed (decreased red blood cells counts in males, decreased haemoglobin levels in both sexes, decreased haematocrit levels in males, decreased mean corpuscular haemoglobin concentration in females). Therefore, the SCCS is of the opinion that from the results presented a No Observed Adverse Effect Level (NOAEL) for Basic Red 76 cannot be established and the dose of 60 mg/kg bw/day is considered a LOAEL.

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

Guideline:	OECD 471 (1997)	
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	
Replicates:	triplicates in 2 individual experiments	
Test substance:	C 008	
Solvent:	DMSO	
Batch nr:	0057891101	
Purity:	98.6 area%	
Concentration:	experiment I:	10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
	experiment II:	33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
Treatment:	experiment I:	direct plate incorporation with 48 h incubation without and with S9-mix

	experiment II:	pre-incubation method with 60 minutes pre-incubation and at least 48 h incubation without and with S9-mix
GLP:	in compliance	
Study date:	17 February – 15 April 2005	

C 008 was investigated for the induction of gene mutations in strains of *Salmonella typhimurium* (Ames test). Liver S9-fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-experiment with strains TA98 and TA100 for toxicity and mutation induction both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of revertant colonies and/or clearing of the bacterial background lawn. Because relevant toxic effects were not observed in any of the strains at the maximal concentration, 5000 µg/plate was used as the top concentration. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in the strains used, the pre-experiment is reported in experiment I. Experiment I was performed according to the direct plate-incorporation test, experiment II with the pre-incubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
In the main tests toxic effects evident as clearing of the bacterial background lawn were observed in experiment I in TA98 and TA100 and in experiment II in all strains predominantly at higher concentrations. Toxic effects evident as a reduction in the number of revertants were observed at higher concentrations without and with metabolic activation in nearly all strains tested.
A biologically relevant increase in revertant colonies was not observed in any of the strains tested at any dose level in the absence or presence of S9-mix in both experiments.

Conclusion
Under the experimental conditions used, C 008 was not mutagenic in this gene mutation tests in bacteria both in the absence and the presence of S9 metabolic activation.

Ref.: 9

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)	
Species/strain:	mouse lymphoma cell line L5178Y	
Replicates:	duplicate cultures in 2 independent experiments	
Test substance:	C 008	
Batch:	0057891101	
Purity:	98.6 area%	
Vehicle:	deionised water	
Concentrations:	experiment I:	26.6, 53.1, 106.3, 212.5 and 318.8 µg/ml without and with S9-mix
	experiment II:	53.1, 106.3, 212.5, 318.8 and 425.0 µg/ml without S9-mix
Treatment:	experiment I:	4 h both without and with S9-mix; expression period 72 h, selection growth 10-15 days
	experiment II:	24 h without S9-mix; expression period 48 h, selection growth 10-15 days.
GLP:	in compliance	
Study period:	2 February – 3 June 2005	

C 008 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9-fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. C 008

test concentrations were based on the results of a pre-test with treatments up to 1700 µg/ml both in the presence and absence of S9-mix measuring suspension growth relative to the concurrent vehicle control cell cultures. In the main test, cells were treated for 4 h (experiment I: both without and with S9-mix) or 24 h (experiment II: without S9-mix only), followed by an expression period of 72 h (experiment I) or 48 h (experiment II) to fix the DNA damage into a stable tk mutation and a selection growth 10-15 days. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. If the increase in mutant frequency is accompanied by a reproducible and dose dependent shift in the ratio of small versus large colonies clastogenic effects are indicated. Toxicity was measured in the main experiments as percentage relative total growth and relative suspension growth of the treated cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test after 4 h treatment, precipitation was observed at 425 µg/ml both in the absence and presence of S9-mix and at 212.5 µg/ml after 24 h treatment in the absence of S9-mix. No relevant increase of the osmolarity and pH-value was observed at the maximum concentration. In the main experiments, precipitation was observed at 318.8 µg/ml (experiment I without S9-mix) and 212.5 µg/ml (experiment I with S9-mix) and in experiment II).
In both experiments in the absence and presence of S9-mix the appropriate level of toxicity (about 10-20% survival after the highest dose) was reached; only in experiment II in one culture treated with the highest concentration of C 008 the appropriate level of toxicity was not reached. Both in the absence and presence of metabolic activation, a biologically relevant increase in the mutant frequency due to exposure to C 008 was not found.

Conclusion
Under the experimental conditions used, C 008 was not mutagenic in this mouse lymphoma assay using the *tk* locus as reporter gene.

Ref.: 10

In vitro Micronucleus Test

Guideline:	draft OECD 487 (1st draft) and OECD 473 (1997)	
Cells:	V79 cells	
Replicates:	duplicates in 2 independent experiments	
Test substance:	C 008	
Batch:	0057891101	
Purity:	98.6 area%	
Vehicle:	deionised water	
Concentrations:	experiment IA:	53.1, 106.3 and 212.5 µg/ml without S9-mix. 53.1, 106.3, 212.5 and 425.0 µg/ml with S9-mix
	experiment IB:	150.0, 200.0 and 300.0 µg/plate with S9-mix
	experiment II:	106.3, 212.5, 425, 850 and 1700 µg/ml without S9-mix. 106.3, 212.5 and 425 µg/ml with S9-mix
Treatment	experiment I:	4 h treatment without and with S9-mix, harvest time 24 hours after the beginning of treatment
	experiment II:	20 h treatment without S9-mix, harvest time 24 hours after the beginning of treatment 4 h treatment with S9-mix, harvest time 48 hours after the beginning of treatment
GLP:	in compliance	
Date:	19 April – 8 September 2005	

C 008 has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in V79 cells. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. A pre-test on cell growth inhibition (XTT assay) with 4 and 24 h treatment and concentrations up to 1700 µg/ml was performed in order to determine the toxicity of C 008, the solubility during exposure and thus the test concentrations for the main micronucleus test. The highest concentration should produce clear toxicity with reduced cell growth.
The treatment period in experiment I was 4 h without and with S9-mix and a harvest time of 24 hours after the start of treatment. In experiment II cells were treated for 20 h without S9-mix and a harvest time of 24 h or for 4 h with S9-mix and a harvest time of 48 h after the start of treatment. For assessment of cytotoxicity the relative cell count and the XTT activity of the test groups were determined as reduction of cells (in %) as compared to the respective solvent/negative control. Negative and positive controls were in accordance with the draft OECD guideline.

Results
In the pre-test in the presence of S9-mix precipitation of C 008 occurred at 1700 µg/ml. In the pre-test in the absence of S9-mix and in the main test in the presence and in the absence of S9-mix precipitation was observed at concentrations of 200 µg/ml and above. Clear toxic effects indicated by reduced cell numbers and/or XTT activities below 40% of control were not observed after treatment with C 008 except for experiment II in the absence of S9-mix. In this experimental part the cell numbers were reduced after treatment with 1700 µg/ml.
In experiment I in the absence of S9-mix a biologically relevant increase in cells with micronuclei was not observed. With S9-mix a first experiment (IA) did show a biologically relevant and dose dependent increase in cells with micronuclei but a second confirmatory experiment (IB) did not. In experiment II in the absence and presence of S9-mix biologically relevant increases in cells with micronuclei were observed; in the presence of S9-mix the increase in cells with micronuclei was dose dependent.

Conclusion
Under the experimental conditions used C 008 induced an increase in cells with micronuclei and, consequently, is genotoxic (clastogenic and/or aneugenic) in V79 cells.

Ref: 11

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

In vivo Mammalian Erythrocytes Micronucleus Test

Guideline:	OECD 474 (1997)
Species:	NMRI mice
Group sizes:	5 mice/sex/group
Test substance:	C 008
Batch:	0057891101
Vehicle:	deionized water
Purity:	98.6 area% (HPLC)
Dose levels:	0, 25, 50 and 100 mg/kg bw
Route:	*intraperitoneal* injection
Sacrifice times:	24 h after treatment for all concentrations, 48 h for the high dose only.
GLP:	In compliance
Date:	14 September – 6 December 2005

C 008 has been investigated for induction of micronuclei in the bone marrow cells of male and female mice. Test doses were based on the results of a pre-experiment on acute toxicity. Mice were treated *intraperitoneally* with C 008 up to 200 mg/kg bw and examined for acute toxic symptoms at 1, 2-4, 6, 24, 30 and 48 h after treatment. In the main experiment mice were exposed orally to 0, 25, 50 and 100 mg/kg bw. The animals of the

highest dose groups were examined for acute toxic symptoms at 1, 2-4, 6 and 24 h after treatment. Bone marrow cells were collected 24 h or 48 h (high dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Negative and positive controls were in accordance with the OECD guideline.

Results
In the a pre-experiment on acute toxicity 3 of the 4 animals of the 200 mg/kg bw group, 3 of the 4 of the 150 mg/kg bw group and one animal of the 125 mg/kg bw group died within 24 h. Until death these animals as well as the surviving animals showed reduction of spontaneous activity, abdominal position, eyelid closure, ruffled fur and apathy. These clinical signs were also observed in the 100 mg/kg bw group. Since no animals from this group died 100 mg/kg bw was chosen as the highest dose. In the main experiment the animals treated with 100 mg/kg bw showed reduction of spontaneous activity, abdominal position, eyelid closure, ruffled fur and apathy. These clinical signs gradually declined at lower doses; at 25 mg/kg bw exclusively reduction of spontaneous activity remained. All treated animals, even those from the lowest dose (25 mg/kg bw), showed red to pink coloured urine.
A substantial decrease in the PCE/TE ratio was not observed indicating that C 008 is not cytotoxic for bone marrow cells. However, the urine of the treated animals had taken the colour of C 008 indicating its systemic distribution and thus its bioavailability.
At both sacrifice times (24 and 48 h) a biologically relevant increase in the number of polychromatic erythrocytes with micronuclei over the concurrent vehicle control values was not observed for any dose level.

Conclusions
Under the experimental conditions used C 008 did not induce an increase in the number of bone marrow cells with micronuclei and, consequently, C 008 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 12

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 developmental study

Guideline:	OECD 414
Species/strain:	Wistar (Crl(WI)BR) rats
Group size:	24 per dose group
Test substance:	C 008
Batch:	12/13B
Purity:	98.5 area% (HPLC)
Vehicle:	water
Dose levels:	0, 60, 250, 1000 mg/kg bw/d
Dose volume:	10 ml/kg bw
Route:	oral gavage
Administration:	day 6 to 20 *post coitum*

GLP statement: in compliance
Study date: 2005 - 2006

The test substance at dose levels of 0, 60, 250 and 1000 mg/kg bw/day, was administered daily (day 6-20 of gestation) by gavage. Milli-U water was used as vehicle. Dosages were based on the results of a dose-range-finding study. The mortality and the body weight gain were observed daily. Females were checked daily for the presence of clinical signs. Body weights and food consumption of females were determined at periodic intervals during pregnancy. On day 21 *post-coitum*, 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 sites, 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
At the low dose group (60 mg/kg bw/day), no maternal, reproductive or developmental toxicity was observed. At the intermediate dose group (250 mg/kg bw/day), maternal toxicity consisted of decreased body weights, body weight gain, for uterus corrected body weight gain, and decreased (absolute and relative) food consumption. Also at 250 mg/kg bw/day, developmental toxicity consisted of decreased foetal body weights and decreased placental weights. There were slight increases in thinning of the central tendon region of the diaphragm and left-sided umbilical artery. In general, skeletal ossification, particularly of the cranial bones and the proximal long bones, was better than that recorded for the concurrent and historical control data. One exception, however, was ossification of the nasal bones, which was considerably retarded compared with both the concurrent and historical control data. The toxicological significance of this atypical pattern of foetal ossification is unclear.

At the high dose group (1000 mg/kg bw/day), maternal toxicity consisted of decreased body weights, body weight gain, for uterus corrected body weight gain, and decreased (absolute and relative) food consumption. Also at 1000 mg/kg bw/day there were slight increases in thinning of the central tendon region of the diaphragm and left-sided umbilical artery. The incidences of two other visceral observations also showed minimal changes namely an increase in the incidence of foetuses with extension of one or both lobes of the thymus gland into the neck region and absence of any foetus with distension of the urinary bladder. A similar, but more pronounced, atypical ossification pattern (see 250 mg/kg bw/day) was seen. Generally minor changes in thoracic centra slightly above the historical control range are considered to be of no toxicological significance.

Based on available data in this study, no explanation can be given for the observation that some toxicological findings (maternal body weights and food consumption, foetal body weights, placental weights) were more pronounced at the mid dose level (250 mg/kg bw/day) when compared to the high dose level (1000 mg/kg bw/day).

In conclusion, based on the results in this prenatal developmental toxicity study, the maternal No-Observed-Adverse-Effect-Level (NOAEL) was established as being 60 mg/kg bw/day. The developmental No-Observed-Effect-Level NOEL was considered to be 60 mg/kg bw/day.

Ref.: 15

Comment
The NOAEL of developmental toxicity in this study for Basic Red 76 is considered 60 mg/kg bw/day due to reduction in foetal body weight at 250 mg/kg bw/day.

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Basic Red 76

Absorption through the skin	**A (µg/cm^2)**	**=**	**3.62 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.035 mg/kg bw**
LOAEL		**=**	**60 mg/kg bw/d**
(subchronic toxicity study, oral gavage, rats)			
Adjusted by factor 3		**=**	**20 mg/kg bw/d**
MOS		**=**	**571**

3.3.14. Discussion

Physico-chemical specification
Basic Red 76 is used as a direct dye for hair colouring products.
The batches of the test material used for toxicological testing are well characterized with respect to identity and purity. Major components are methyl sulphate (11.4-18.0%), water (3.1-5.1%) and chloride (1.6-5.0%).
No data have been supplied on the stability of the test substance in typical formulations of hair colouring products.
All batches analysed contain ((mono)methyl sulphate 11.4 to 18%), which is used as an anion to the dye. No specific toxicity data for monomethyl sulphate were provided. However, the toxicity testing was performed in the presence of this anion.

General toxicity
The oral LD_{50}-value of Basic Red 76 in Wistar rats was established to exceed 2000 mg/kg bw. In a subchronic toxicity study, treatment by oral gavage, in rats a LOAEL of 60 mg/kg bw/day was determined due to haemato-toxicity. In a prenatal developmental toxicity study in rats, the maternal and the developmental NOAEL were both established as being 60 mg/kg bw/day. No data on reproductive toxicity were submitted.

Irritation, sensitisation
Under the conditions of the study, the undiluted test substance was not irritating to rabbit skin. The red staining of the skin may have masked signs of erythema caused by semi-occlusive application of neat Basic Red 76 on the skin of the rabbit.
Undiluted Basic Red 76 was irritating to rabbit eyes.
The sensitising potential of Basic Red 76 has not been excluded, as only concentrations up to 10% were used in the LLNA. Curiously, concentrations above 10% Basic Red 76 in ethanol:water were found to be irritating whereas moistened Basic Red 76 was non irritating to the skin.

Dermal absorption
The mean + 2SD (3.62 µg/cm²) determined in an *in vitro* dermal absorption assay with pig skin, may be used for estimating the MOS for direct hair dye formulations containing 2% C008.

Mutagenicity
Overall, the genotoxicity of Basic Red 76 is sufficiently investigated for the three endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Basic Red 76 did not induce gene mutations neither in bacteria nor in mammalian cells. However, in an *in vitro* micronucleus test Basic Red 76 treatment did result in an increase in V79 cells with micronuclei. The positive finding found in this *in vitro* micronucleus test was not confirmed in an adequately performed bone marrow micronucleus test in mice.
Consequently, Basic Red 76 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data submitted

4. CONCLUSION

Based on the data provided, Basic Red 76 containing up to 18% methyl sulphate does not pose a risk to the health of the consumer when used as a non-oxidative hair dye with a maximum on-head concentration of 2.0%.

A sensitising potential of Basic Red 76 cannot be excluded.

5. MINORITY OPINION

Not applicable

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