EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

Acid Red 33

COLIPA n° C22

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

The SCCP adopted this opinion at its 14th plenary of 18 December 2007

About the Scientific Committees
Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Products (SCCP), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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

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

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1. BACKGROUND

Submission I for Acid Red 33 with the chemical name 5-Amino-4-hydroxy-3-phenylazo-2,7-naphthalenedisulfonic acid (disodium salt) was submitted in September 1984 by COLIPA[1,2].

Submission II for Acid Red 33 was submitted in November 1984 by COLIPA[2].

Acid Red 33 is identical with CI 17200 also used as a colouring agent allowed in all cosmetic products.

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 58 on the List of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission III for Acid Red 33 was submitted by COLIPA in July 2005. According to this submission the substance is used in hair colouring formulations as a non-reactive dye in semipermanent hair dye formulations at a maximum on-head concentration of 0.5%. This non-oxidative dye is used in a formulation where it is common practice to apply 100 ml of the undiluted formulation. The application times covers a period of 30 minutes followed by washing off with water and shampoo. It is assumed that application may be repeated weekly.

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 Products (SCCP) consider Acid Red 33 safe for use as non-oxidative hair dye with an on-head concentration of maximum 0.5% taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use of Acid Red 33 in any non-oxidative hair dye formulations?*

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

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Acid Red 33 (INCI)

The same substance and its permitted lakes and salts is also used as a cosmetic colorant with common name CI 17200, currently regulated by the Cosmetics Directive (76/768/EC), Annex IV, Part 1, column 1, with field of application specified as "Colouring agents allowed in all cosmetic products".

3.1.1.2. Chemical names

Disodium 5-amino-4-hydroxy-3-(phenylazo)-naphthalene-2,7-disulphonate
5-Amino-4-hydroxy-3-phenylazo-2,7-naphthalenedisulfonic acid (disodium salt)
5-amino-4-hydroxy-3-[(*E*)-phenyldiazenyl]naphthalene-2,7-disulfonic acid (disodium salt)

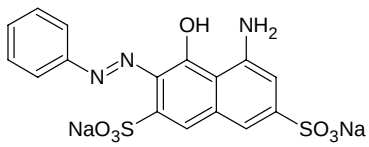
3.1.1.3. Trade names and abbreviations

Red 33	Fuchsia Red
Aka227	D&C Red 33
CI 17200	Covacap Rouge W32103

3.1.1.4. CAS / EINECS number

CAS: 3567-66-6
EINECS: 222-656-9

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{16}H_{11}N_3Na_2O_7S_2$

3.1.2. Physical form

Dark red/brown powder

3.1.3. Molecular weight

Molecular weight: 467

3.1.4. Purity, composition and substance codes

Complete characterization by UV-VIS spectrometry, IR, H^1-NMR, C^{13}-NMR, MS and HPLC is reported for one batch denoted as Batch No. 41/FDA Lot No AJ4513 (apparently corresponding to a lot certified by FDA)

Purity (Area HPLC) greater than 96.7%
Overall Purity (NMR) greater than 82%
Solvent Content less than 6%
Sulphated Ash Content less than 12%

3.1.5. Impurities / accompanying contaminants

4-Amino-5-hydroxy-2,7-naphthalenedisulfonic acid (disodium salt)			< 0.3%
4,5-Dihydroxy-3-(phenylazo)-2,7-naphthalenedisulfonic acid (disodium salt)			< 3%
4-Aminoazobenzene	< 100 ppb	(Annex II, n° 990)	(EU carcinogenic category 2)
4-Aminobiphenyl	< 275 ppb	(Annex II, n° 726)	(EU carcinogenic category 1)
Aniline	< 25 ppm	(Annex II, n° 22)	(EU carc. cat. 3; muta cat 3)
Azobenzene	< 1 ppm	(Annex II, n° 727)	(EU carc. cat. 2; muta cat 3)
Benzidine	< 20 ppb	(Annex II, n° 26)	(EU carcinogenic category 1)
1,3-Diphenyltriazene	< 125 ppb		

Heavy Metal Content

Antimony, Arsenic, Mercury	< 5 ppm
Cadmium	< 10 ppm
Lead	< 20 ppm

3.1.6. Solubility

Water: > 2.5%
DMSO: > 10%
Ethanol: < 1%

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: ≤ 0.5 (extrapolated)

The determination of the partition coefficient (n-octanol/water) of Acid Red 33 was performed according to the EEC Directive 92/69, A.8 'Partition coefficient' (1992) and the OECD Guideline n° 117, ''Partition coefficient (n-octanol/water),High Performance Liquid Chromatography (HPLC) method (1989).

Based on the method calculation during the preliminary test, the Log P_{ow} of Acid Red 33 was estimated to be 0.5. Therefore, the HPLC method was chosen to conduct the main test. Six common standards, which have known Log Pow values in the range of 0.5 to 2.1 were used as reference standards during the main test.

Using the corresponding log P_{ow} values of the reference items, the regression coefficients were calculated and the log P_{ow} of the test item was calculated from the calibration equation. Since from the calibration curve the retention time of the test item was below the retention time of 4-acetylcholine, the log P_{ow} value was not estimated more precisely. In

conclusion, the partition coefficient (n-octanol/water) of Acid Red 33 was determined to be log $P_{ow} \leq 0.5$ using the HPLC method.

Ref.: 6

3.1.8. Additional physical and chemical specifications

Melting point:	/
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	/
UV_Vis spectrum (200-800 nm)	λ_{max} (nm) 235, 311, 531 (neutral), 237, 307, 526 (acidic), 236, 307, 524 (alkaline)

3.1.9. Homogeneity and Stability

Acid Red 33 is stable under normal laboratory conditions. Solutions of this chemical in water or DMSO are stable for at least 48 hours under lab conditions.

General Comments to physico-chemical characterisation

* The overall purity (NMR) of the test item is greater than 82% and the solvent content plus other impurities is less than 10%. Therefore, about 8% of the test item are unknown.
* No data were provided for most physical and chemical constants.
* No data were provided for the stability in marketed products.
* The solvent content of 6% was not characterised.
* Acid Red 33 contains several 'CMR'-impurities.

3.2. Function and uses

Acid Red 33 is used as a non reactive dye up to an on-head concentration of 0.5% in non-oxidative hair dye formulation.
Acid Red 33 (CI 17200) is also allowed for use as a colorant in all cosmetic products (Directive 76/768/EC, Annex IV, Part 1, column 1.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

(Literature data)

LD50 (rat) > 3 160 mg/kg bw [DFG, Kosmetische Färbemittel]
LD50 (dog) > 1 000 mg/kg bw [DFG, Kosmetische Färbemittel]

Ref.: 16

Comment
Even though details on study design and purity of the test material are not available, Acid Red 33 shows no acute lethality in rats even when tested at much higher concentration than requested by current guideline. Repetition of an animal test is not justified due to the low toxicity reported.

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

Literature data

0.5 g/kg bw of a 0, 0.1 and 1% formulation in white petrolatum or a hydrophilic cream was applied on intact skin of rabbits for 65 applications or on damaged skin for 15 applications.
Under the conditions of the study, the test material was considered not be irritant to rabbit skin.

Ref.: 16

3.3.2.2. Mucous membrane irritation

Literature data

0.2 ml of a 10% aqueous solution (20 mg) or suspension of the test material was applied twice daily, five times weekly, for four weeks to the conjunctival sac of one eye of each of a group of six or more albino rabbits (40 applications). One hour after each application, the eyes were examined for evidence of staining and the irritation was scored according to Draize.

Results
The test material caused intense colouring of the iris, lasting for 2 to 7 days.

	Before 1 application on day:				**3 days after last application**	**1st hour after 1st application on day 5**
	2	**5**	**10**	**20**		
Irritation score	0	0	0	0	0	2

Conclusion
The study authors concluded that the test substance was not irritating to the rabbit eye.
Ref.: 11

3.3.3. Skin sensitisation

Contact hypersensitivity, maximisation test

Guideline:	OECD 406 (1992)	
Species/strain:	Ibm: GOHI; SPF-quality Guinea pig	
Group size:	15 female (10 test and 5 control)	
Test substance:	D&C Red 33 (CI 17200)	
Batch:	41 (lot AJ4513)	
Purity:	88%	
Concentrations:	intradermal induction:	5% dilution of test item in 1% CMC and in an emulsion of FCA/physiological saline
	Epidermal induction:	25% dilution in 1% CMC
	Challenge:	25% dilution in 1% CMC
Positive control:	2-mercaptobenzothiazole	
Treatment:		
GLP:	In compliance	

The maximisation test was performed in 15 (10 test and 5 control) female guinea pigs.
The intradermal induction of sensitisation in the test group was performed in the nuchal region with a 5% dilution of the test item in 1% CMC and in an emulsion of Freund's Complete Adjuvant (FCA) / physiological saline. The epidermal induction of sensitisation was conducted for 18 hours under occlusion with the test item at 25% in 1% CMC one week after the intradermal induction and following pre-treatment of the test areas with 10% sodium-laureth-sulfate (SLS), 24 hours prior to application of the test item. The animals of the control group were intradermally induced with 1% CMC and FCA/physiological saline and epidermally induced with 1% CMC under occlusion following pre-treatment with 10% SLS.
Cutaneous reactions were evaluated at 24 and 48 hours after removal of the dressing.

Results
Skin reactions after the challenge procedure

	After 24 hours		After 48 hours	
	Positive / total	**% positive**	**Positive / total**	**% positive**
Control Group				
D&C Red 33, 25% in 1% CMC (left flank)	0 / 5	0	0 / 5	0
1% CMC only (right flank)	0 / 5	0	0 / 5	0
Test Group				
D&C Red 33, 25% in 1% CMC (left flank)	0 / 10	0	0 / 10	0
1% CMC only (right flank)	0 / 10	0	0 / 10	0

Conclusion,
The study authors concluded that the test substance was not a skin sensitiser.
Ref.: 5

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (draft 1996)
Tissue:	porcine ear skin, 600-800 µm thickness (exp. I); 300-500 µm (exp. II)
Number of donors:	/
Diffusion cells:	6 chambers/experiment, exposed membrane area 1.01 cm^2

Skin integrity:	electrical conductivity. Membranes > 2-5 mS excluded
Test substance:	experiment I: 6 mg/ml D&C Red 33 in saline (adjusted to pH 3 with lactic acid) Experiment II: formulation with SC Hair Color Gel C1 L362 2 containing 0.5% D&C Red 33
Batch:	41 (lot AJ4513) (D&C Red 33) C1 L 362 2 (formulation containing D&C Red 33 at 0.5%)
Purity:	88%
Doses:	experiment 1: 1.5 ml of the solution (6 mg/ml) of dye in saline per chamber Experiment 2: 1.0 g of test formulation per chamber
Receptor fluid:	saline, pH 3
Solubility receptor fluid:	/
Stability:	stable in water for > 72 hours (D&C Red 33)
Method of Analysis:	HPLC
Detection limit:	150 ng/ml
GLP:	in compliance

D&C Red 33 was assessed for its potential to permeate porcine skin. The pure dye was tested in the first experiment. In the second experiment, a viscous ready to use mixture including dye and additives (representative hair dye formulation) was directly applied to the skin. Samples of the receptor fluid were taken at 0.5, 1, 2, 4, 6, 8 and 24 hours following application of the test item. The samples were analysed by HPLC.

Results
The mean recovery of the test item was 107.8% in the first and 99.3% in the second experiment.
No measurable permeation through the skin occurred at any time point within the time frame of both experiments.
The calculated flux of the test item across the skin barrier was 6.6 µg/cm² in the first and 5.9 µg/cm² in the second experiment. Together with the skin extracts, the penetration of the test item was 9.3 ± 3.25 µg/cm² in the first and 8.5 ± 3.59 µg/cm² in the second experiment.

	Experiment I		**Experiment II**	
	µg	**%**	**µg**	**%**
Chamber 1	7.5	0.08	10.2	0.20
Chamber 2	9.0	0.10	15.2	0.30
Chamber 3	15.2	0.17	6.8	0.14
Chamber 4	6.0	0.07	6.7	0.13
Chamber 5	10.8	0.12	6.1	0.12
Chamber 6	7.3	0.08	6.3	0.13
Mean	***9.3***	***0.10***	***8.5***	***0.17***

Conclusion
The test authors stated that a worst case consideration results in an upper limit of 9.3 µg/cm² of the test item (0.07% of the applied dose) in the first and 8.5 µg/cm² of the test item (0.12% of the applied dose in the second experiment.

Ref.: 3

Comment
The A_{max} of 15.2 µg/cm² is used for calculating the MOS as too few test chambers were used.

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

No data submitted

3.3.5.3. Chronic (> 12 months) toxicity

A. Multi-generation study in rats (see 3.3.8.1)

In the multi-generation study in rats Charles River COBS CD rats (Sprague Dawley), one control groups and four test groups (100 males and 100 females) were used. The following dose levels were tested: 0.25, 2.5, 7.5 and 25 mg/kg bw/day. The control and treated rats were maintained on their respective diets for the duration of this generation (at least 100 d).

Body weight and parental food consumption were recorded. Parental rats and pups were observed for toxicity signs, changes in behaviour and mortality. Histopathology (14 representative tissues) was performed from control and high dose group from F_1 and F_{3a} generation.

Result
With the exception of discoloration (pink or reddish) of the urine of males and females in the highest dose group (25 mg/kg bw/d) of all generations, no treatment related effects were noted.

Conclusion
The NOAEL of D&C Red 33 in this study is 25 mg/kg bw/d (highest dose used).

Ref.: 10

B. Long term rat feeding study, exposure beginning in utero (see 3.3.7.)

In this long-term feeding study, D&C Red 33 was given in the diet at dosage levels of 0.025, 0.05 or 0.2% (it is stated that this corresponded to 12, 25, and 102 mg/kg bw/d for males and 15, 31, and 129 mg/kg bw/d for females) to rats with exposure beginning *in utero.*

For the F_0 of the study, 60 rats/sex were assigned to each treatment level and also to each of two control groups. After receiving the appropriate diets for 60 days, the rats were mated on a 1:1 ratio for one week. Test diet was administered throughout the mating, gestation and lactation period. A minimum of 35 litters per dosage level was used to select 70 rats/sex/group for the F1 study. Beginning at 21 days of birth, pups of a litter were weaned and continued to receive their respective diets.

Throughout the *in utero* and post weaning segments of the study, rats were observed daily for signs of overt toxicity, morbidity and mortality. Detailed physical examinations were recorded weekly. No effects attributable to compound were seen in survival, body weights, food consumption, ophthalmoscopic examinations, fertility or gestation and lactation indices. Individual body weights and food consumption measurements were recorded weekly throughout the *in utero* segment weekly for the first 14 weeks, biweekly (the second 7 days of every two weeks) the next 12 weeks and once monthly (7 days during the third

week of each month) thereafter for the post-weaning segment of the study. Ophthalmoscopic examinations were performed for all rats during week 16 of the *in utero* segment, during week 1 and months 3, 6, 12, 18 and 24 of the post-weaning segment of the study. For the post-weaning segment, haematological and biochemical evaluations and urinalyses were conducted for 10 rats/sex/group at 3, 6, 12, 18 and 24 months of study. An interim sacrifice and necropsy of 10 rats/sex/group was conducted following 12 months of compound administration.

Results
At various times throughout both segments of the study, there were differences in the colour of urine, faeces, hair or exposed skin areas related to treatment level. No other changes considered to be related to compound were seen.

For the long term F_1 exposure, no compound-related effects were noted in survival, body weights, food consumption and ophthalmoscopic examinations. No definitive compound-related changes were evident in haematological and biochemical studies. Urine analyses at 3, 6 and 12 months of study generally confirmed the orange to red discoloration of the urine of treated rats noted during physical examinations at one or more dosage levels at 18 months, a light-red colour was noted for most rats at the 0.2 % dosage level during the urinalysis. At 24 months, other than colour, no differences were noted between the control and treated rats that could be attributed to the test article. No compound-related changes were noted in macroscopic examinations. No significant changes in the body weights between the different groups were noted. There were no pathological changes in the microscopic pathologic examination.

Conclusion
The NOAEL of D&C Red 33 in this long-term toxicity study is 102 mg/kg bw/d (highest dose used).

Ref.: 9

C. Long term feeding study in mice (see 3.3.7)

Charles River CD-1 mice, two control groups and three test groups (60 males and 60 females) were used for this assay. Groups of 60 male and 60 female mice, each were fed D&C Red 33 in their diet for 24 month. The following dose levels were tested in this study: 0.1, 1.0; and 5.0 %, two control groups were included. The criteria evaluated were haematology (after 3, 6, 12 month), signs of overt toxicity, moribundity, mortality, body weight, food consumption, general behaviour, detailed macroscopic and microscopic examination of organs and tissues.

Results
Compound related reduced survival prompted the termination of high–dose males at week 57 and high dose females at week 74. Changes in body weight and food consumption compared to the control were observed in the high dose group. Changes seen in colour of hair, skin and faeces of the mice were judged to be not toxicologically significant because they are directly linked to the colour of the dye.

The high dose group showed anaemia and elevated reticulocytes in week 74. However, the haemoglobin, haematocrit and erythrocyte values were similar to the control females at 18 month of the study. The mid dose (1 %) showed anaemia and a significant increase in reticulocytes at 18 and 24 month for both sexes. In addition, leucocytes were increased for males and females at this 1% level. For 0.1 % the leucocytes were increased in females at 24 month.

Microscopical examination showed abnormal changes in kidneys at the 5 % dosage level. Pigments were also present in the liver and spleen of this high-dosage group. In addition, splenic weights were increased in high dose group.

Conclusion
The no-effect level was considered to be 0.1% (ca. 150 mg/kg bw/day).

Ref.: 8

Comment of the SCCP
In all dose groups including the lowest dose an increase in the leucocytes/reticulocytes number was observed. This lowest dose of 150 mg/kg bw/d is considered as LOAEL.

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 and *E. coli* WP2 uvrA.
Replicates:	3 replicates in 2 individual experiments both in the presence and absence of S9 mix
Test substance:	D&C Red 33 (C.I. 17200)
Solvent:	deionised water
Batch:	41
Lot:	AJ4513
Purity:	88 % (certified total colour content)
Concentrations:	42 - 5000 µg/plate without and with S9 mix in both experiments
Treatment:	pre-incubation method was used with 30 minutes pre-incubation and at least 48 h incubation time both without and with S9 mix in both experiments.
GLP:	In compliance

D&C Red 33 was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9 fraction from Syrian golden hamsters was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in a pre-experiment with all *Salmonella* and *Escherichia* strains. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and/or a clearing of the bacterial background lawn. Since D&C Red 33 was freely soluble and non toxic in this preliminary toxicity test, it was tested up to the prescribed maximum concentration of 5000 µg/plate. The pre-experiment is reported as main experiment I. Both experiments were performed with the preincubation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Precipitation of D&C Red 33 was not observed. Slight toxicity was exclusively observed in experiment II at the maximum concentration of 5000 µg/plate in TA1535 with S9 mix and TA98 without S9 mix.
No substantial and biological relevant increase in revertant colonies of any of the five tester strains was observed in both experiments following treatment with D&C Red 33 at any dose level neither in the absence nor in the presence of metabolic activation.

Conclusion
Under the experimental conditions used D&C Red 33 was not genotoxic (mutagenic) in the gene mutation tests in bacteria.

Ref.: 1

In Vitro Mammalian Cell Gene Mutation Test (*tk* locus)

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	2 replicates in 2 independent experiments
Test substance:	D&C Red 33
Solvent:	deionised water
Lot:	AJ4513
Batch:	41
Purity:	88 % (certified total colour content)
Concentrations:	Experiment I: 300 - 4800 µg/ml both without and with S9 mix Experiment II: 150 - 4800 µg/ml without S9 mix
Concentrations:	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

D&C Red 33 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test measuring relative suspension growth. Since no toxic effects were observed in the pre-test, D&C Red 33 was tested up to the prescribed maximum concentration of 10 mM (= 4800 µg/ml). In the main test, cells were treated for 4 h (experiment I) or 24 h (experiment II) followed by an expression period of 72 h to fix the DNA damage into a stable *tk* mutation. Liver S9 fraction from Syrian golden hamsters was used as exogenous metabolic activation system. Toxicity was measured as percentage relative survival and total growth of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
Precipitation of D&C Red 33 was not observed. In both experiments in the absence and presence of S9 mix the appropriate level of toxicity (10-20% survival after the highest dose) was not reached. However, D&C Red 33 was tested up to the maximum prescribed concentration of 10 mM (= 4800 µg/ml)
No reproducible or dose dependent increase in mutant colony number was observed in both experiments neither in the absence nor in the presence of S9 mix. The ratio of small colonies *versus* large colonies was not shifted as compared to the solvent controls.

Conclusion
Under the experimental conditions used, D&C Red 33 was not genotoxic (mutagenic and/or clastogenic) in the mouse lymphoma assay at the *tk* locus.

Ref.: 2

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	NMRI mice
Group size:	5 mice/sex/group
Test substance:	D&C Red 33
Batch:	C00042
Purity:	93 % (certified total colour content)
Dose level:	500, 1000 and 2000 mg/kg bw
Route:	orally, once

Vehicle: deionised water
Sacrifice times: 24h and 48h (highest dose only) after the treatment
GLP: In compliance

D&C Red 33 has been investigated for the induction of micronuclei in bone marrow cells of mice. The test concentrations were based on the result of a pre-experiment for toxicity in which 2 mice were orally exposed to 2000 mg/kg bw D&C Red 33. The animals were examined for acute toxic symptoms at intervals of around 1, 2-4, 6, 24, 30, and 48h after administration of D&C Red 33. 2000 mg/kg was selected as the maximum tolerated dose level. In the main experiment bone marrow cells were collected 24h and 48h (highest dose only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between normo-chromatic to polychromatic erythrocytes (PCE/NCE ratio). The animals of the highest dose group were examined for acute toxic symptoms at intervals around 1, 2-4, 6 and 24 h after treatment. Bone marrow preparations were stained and examined microscopically for the NCE/PCE ratio and micronuclei. In order to quantify the concentration of D&C Red 33 in blood serum 2 animals per sex were treated with 2000 mg/kg bw D&C Red 33. 1 and 4 h after treatment the animals were sacrificed, their blood was collected and analysed by HPLC. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-experiment for toxicity, all animals expressed toxic effects like reduction of spontaneous activity, abdominal position, eyelid closure and ruffled fur 1 and 2-4 h after treatment. From 6h after treatment these toxic effects decreased and were lost at 30 h. In the main experiment the similar toxic effects were observed with an almost identical timing.
The ratio PCE/NCE was not substantially changed in the treated animals indicating that D&C Red 33 did not have cytotoxic properties in the bone marrow. However, the urine colour of the treated animals was red which together with the observed toxic effects indicate the systemic distribution and thus bioavailability in the bone marrow of D&C Red 33. This was confirmed by the serum analysis showing substantial amounts of D&C Red 33 in the serum 1 h after treatment; after 4 h the levels dropped below the detection limit.
Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found following treatment with D&C Red 33 at any time point.

Conclusion
Under the experimental conditions used D&C Red 33 did not induce micronuclei in bone marrow cells of treated mice and, consequently, D&C Red 33 was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 4

3.3.7. Carcinogenicity

Oral administration, mice

Guideline: /
Species: Mouse/Charles River CD-1
Group size: 60 animals per sex per dose level
Test substance: D&C Red 33
Vehicle: Diet (Purina Rodent Chow)
Batch: #21 108-8-100
Purity: 88%, impurities not given, a factor of 1.124 was used to compensate for the 88% purity
Dose level: 0.1, 1.0 and 5.0%
Route: Oral, diet
Exposure period: 24 months
GLP: In compliance

In a long-term feeding study in mice, D&C Red 33 was fed in the diet at dosage levels of 0.1, 1.0 and 5.0%. (it is stated that this corresponded to 150, 1502, and 8764 mg/kg bw/d for males and 181, 1809, and 10,362 mg/kg bw/d for females) Sixty male and 60 female mice were used at each dosage level and in each of two control groups. The mice were observed two – three times daily for signs of overt toxicity, morbidity and mortality. Detailed observations were recorded weekly. Individual body weights and food consumption values were recorded weekly during the first 14 weeks of study, biweekly (the second 7 days of every two weeks) during the next 12 weeks and monthly (7 days during the second week of each .month) thereafter. Haematological studies were conducted for 10 mice/sex/group at 3, 6, 12, 18, and 24 months.

For mice at the 0.1% dosage level, the exposed skin areas appeared pink in colour, the faeces appeared brownish red, and the urine of male and female mice appeared orange and pink, respectively. For mice at the 1.0% and 5.0% dosage levels, the hair and exposed skin areas appeared purple in colour and the urine and faeces appeared red. Compound-related reduced survival prompted the termination of high dosed males at week 57 and high-dose females at week 74. Body weight changes, except for high-dose groups and food consumption values were similar for control and treated mice. The survival was similar (about 50%) in all groups except the high dose groups.

Decreases in erythrocyte, haemoglobin and haematocrit values and increases in reticulocytes in most treated groups were considered indicative of a dosage-related anaemia at the 6 and 12 month evaluations.

The terminal values obtained week 74, for the high-dose females showed continued elevated reticulocytes, however, the haemoglobin, haematocrit and erythrocyte values were similar to the values obtained for the control females at 18 months of study. At 18 and 24 months, mean haematological values for the 0.1 and 1% dosage level males and females were similar to the control means with the following exceptions. Toxicologically significant increases in reticulocytes were present for males and females at the 1.0% level at 18 and 24 months. Leucocytes were increased for males and females at the 1.0% level at 18 and 24 months and for the 0.1% females at 24 months. The significance of the leucocyte increase is unknown.

Microscopic examination of the tissues revealed a high incidence of abnormal changes in the kidneys in the 5.0% dosage level group. These changes were characterized by chronic nephritis, hydronephrosis and tubular pigment. Pigment was also present in the liver and spleen at the 5.0% dosage level.

It was concluded that D&C Red 33 was not oncogenic under the conditions of this study.

Ref.: 8

Oral administration, rat

In utero, long term rat feeding study

Guideline:	/
Species:	Rat/Charles River CD, Sprague Dawley
Group size:	F0: 60 animals per sex per dose level, F1: 70 animals per sex per dose level
Test substance:	D&C Red 33
Vehicle:	Diet (Purina Rodent Chow)
Batch:	#21 108-8-100
Purity:	88%, impurities not given, a factor of 1.12 was used to compensate for the 88% purity

Dose level:	0.025, 0.05 and 0.20%
Route:	Oral, diet
Exposure period:	24 months
GLP:	In compliance

In this long-term feeding study, D&C Red 33 was given in the diet at dosage levels of 0.025, 0.05 or 0.2% (it is stated that this corresponded to 12, 25, and 102 mg/kg bw/d for males and 15, 31, and 129 mg/kg bw/d for females) to rats with exposure beginning *in utero.*

For the F0 of the study, 60 rats/sex were assigned to each treatment level and also to each of two control groups. After receiving the appropriate diets for 60 days, the rats were mated on a 1:1 ratio for one week. Test diet was administered throughout the mating, gestation and lactation period. A minimum of 35 litters per dosage level was used to select 70 rats/sex/group for the F1 study. Beginning at 21 days of birth, pups of a litter were weaned and continued to receive their respective diets.

Reproductive parameters were evaluated to determine fertility index, gestation anomalies and effects on parturition and lactation. Indices for live birth and survival to weaning were calculated. Throughout the *in utero* and post weaning segments of the study, rats were observed daily for signs of overt toxicity, morbidity and mortality. Detailed physical examinations were recorded weekly. No effects attributable to compound were seen in survival, body weights, food consumption, ophthalmoscopic examinations, fertility or gestation and lactation indices.

Individual body weights and food consumption measurements were recorded weekly throughout the *in utero* segment weekly for the first 14 weeks, biweekly (the second 7 days of every two weeks) the next 12 weeks and once monthly (7 days during the third week of each month) thereafter for the post-weaning segment of the study. Ophthalmoscopic examinations were performed for all rats during week 16 of the *in utero* segment, during week 1 and months 3, 6, 12, 18 and 24 of the post-weaning segment of the study. For the post-weaning segment, haematological and biochemical evaluations and urinalyses were conducted for 10 rats/sex/group at 3, 6, 12, 18 and 24 months of study. An interim sacrifice and necropsy of 10 rats/sex/group was conducted following 12 months of compound administration.

At various times throughout both segments of the study, there were differences in the colour of urine, faeces, hair or exposed skin areas related to treatment level. No other changes considered to be related to compound were seen.

For the long term F1 exposure, no compound-related effects were noted in survival, body weights, food consumption and ophthalmoscopic examinations. No definitive compound-related changes were evident in haematological and biochemical studies. Urine analyses at 3, 6 and 12 months of study generally confirmed the orange to red discoloration of the urine of treated rats noted during physical examinations at one or more dosage levels at 18 months, a light-red colour was noted for most rats at the 0.2% dosage level during the urinalysis. At 24 months, other than colour, no differences were noted between the control and treated rats that could be attributed to the test article. No compound-related changes were noted in macroscopic examinations. No differences in the survival of the different groups were observed nor were there any significant changes in the body weights between the different groups.

It was concluded that the long-term dietary administration of D&C Red 33 at levels of 0.025, 0.05, and 0.2% of the diet to male and female Charles River CD albino rats did not produce neoplastic, proliferative or degenerative changes compared to male and female rats receiving control laboratory diet.

Ref.: 9

Comment
D&C Red 33 has been studied for carcinogenicity after oral administration to mice for 2 years and by a long term rat feeding study (2 years). It was concluded in both studies that under the conditions of the bioassays D&C Red 33 was not carcinogenic.
In the rat study, higher concentrations of the test substance should have been used.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

Multi-generation study in rats

Guideline:	/
Species:	Charles River COBS CD rats (Sprague Dawley)
Group size:	F_0: 20 animals per sex per dose level F_{1a}: mating on day 100 F_{1b} and F_{1c}: mating at least 10 day later F_{2a}: mating of 20 F_1 animals randomly selected F_3: mating of 20 F_{2b} litters
Test substance:	D&C Red 33
Vehicle:	Diet (Purina Rodent Chow 5002)
Batch:	#21 108-8-100
Purity:	/
Dose level:	0, 0.25, 2.5, 7.5 and 25.0 mg/kg bw/d
Route:	Oral, diet
GLP:	In compliance

Charles River COBS CD rats (Sprague Dawley), one control groups and four test groups (100 males and 100 females) were used for this assay. The following dose levels were tested in this study: 0.25, 2.5; 7.5 and 25 mg/kg bw/day. The control and treated rats were maintained on their respective diets for the duration of this generation. F_0 parental animals were mated twice (each group 20 males and 20 females), to produce two litters and the F_1 parents were mated to produce three litters and the F_2 parents were mated once to produce the F_{3a} litters.
Body weight and parental food consumption were recorded. Parental rats and pups were observed for toxicity signs, changes in behaviour and mortality. Reproductive parameters were evaluated to determine male and female fertility indices, gestation anomalies, viability and survival of the pups. Histopathology (14 representative tissues) was performed from control and high dose group from F_1 and F_{3a} generation.

Results
With the exception of discoloration (pink or reddish) of the urine of males and females in the highest dose group (25 mg/kg bw/d) of all generations, no treatment related effects were noted. One single pup of the F_{2a} generation had exencephaly, spina bifida and great vessel anomalies which was considered an incidental finding.

Conclusion
D&C Red 33 produced no toxic and reprotoxic effects up to 25 mg/kg bw/d.

Ref.: 10

3.3.8.2. Teratogenicity

Long term rat feeding study, exposure beginning in utero (see 3.3.7.)

In this long-term feeding study, D&C Red 33 was given in the diet at dosage levels of 0.025, 0.05 or 0.2% (it is stated that this corresponded to 12, 25, and 102 mg/kg bw/d for males

and 15, 31, and 129 mg/kg bw/d for females) to rats with exposure beginning *in utero.* For the F_0 of the study, 60 rats/sex were assigned to each treatment level and also to each of two control groups. After receiving the appropriate diets for 60 days, the rats were mated on a 1:1 ratio for one week. Test diet was administered throughout the mating, gestation and lactation period. A minimum of 35 litters per dosage level was used to select 70 rats/sex/group for the F1 study. Beginning at 21 days of birth, pups of a litter were weaned and continued to receive their respective diets. Reproductive parameters were evaluated to determine fertility index, gestation anoma1ies and effects on parturition and lactation. Indices for live birth and surviva1 to weaning were ca1cu1ated. Throughout the *in utero* and post weaning segments of the study, rats were observed daily for signs of overt toxicity, morbidity and morta1ity. Detailed physica1 examinations were recorded week1y.

Results
No effects attributable to the test compound were seen in survival, body weights, food consumption, ophthalmoscopic examinations, fertility or gestation and lactation indices.

Conclusion
The NOAEL of parental toxicity was set at 102 mg/kg bw for males and at 129 mg/kg bw for females. The NOAEL for developmental toxicity was set at 129 mg/kg bw/d.

Ref.: 9

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

(4-Hydroxypropylamino-3-nitrophenol)
(Direct / semi-permanent)

Maximum absorption through the skin	**A (µg/cm²) µg/cm²**	**=**	**15.2**
Skin Area surface	**SAS (cm²)**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**10.64 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.18 mg/kg**
No observed adverse effect level (multi-generation, oral, rat)	**NOAEL**	**=**	**25 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**139**

3.3.14. Discussion

Physico-chemical properties
Acid Red 33 is used as a non-reactive dye up to an on-head concentration of 0.5% in non-oxidative hair dye formulation.
The overall purity (NMR) of the test item is greater than 82% and the solvent content plus other impurities is less than 10%. Therefore, about 8% of the test item is unknown.
No data were provided for most physical and chemical constants. No data were provided for the stability in marketed products.
Acid Red 33 contains several 'CMR'-impurities

General toxicity
The acute oral toxicity (LD_{50}) was > 3 160 mg/kg bw in rats and > 1 000 mg/kg bw in dogs.
The NOAEL in a long-term feeding toxicity study in rats was set at 102 mg/kg bw/d. In mice, the LOEL was set at 150 mg/kg bw/day.
In a multi-generation study in rats, the NOAEL was set at 25 mg/kg bw/d.
The NOAEL of parental toxicity was set at 102 mg/kg bw for males and at 129 mg/kg bw for females. The NOAEL for developmental toxicity was set at 129 mg/kg bw/d.

Irritation / sensitisation
Acid Red 33 was considered not to be irritant to rabbit skin and eye. It was found not to be a skin sensitiser in a Guinea pig maximisation test.

Dermal absorption
As too few test chambers were used in the percutaneous absorption study on pig skin *in vitro*, the A_{max} of 15.2 µg/cm² is used for calculating the MOS.

Mutagenicity / genotoxicity
Overall, the genotoxicity program on Acid Red 33 investigated the three types of mutation: gene mutation, structural chromosome mutation and aneuploidy.
Acid Red 33 did not induce gene mutations in the gene mutation assay neither in bacteria nor in mammalian cells at the *tk* locus of mouse lymphoma cells. An *in vitro* cytogenetic

assay was not performed. Instead the structural chromosome aberration and aneuploidy endpoint was covered with an *in vivo* micronucleus assay. In the latter test Acid Red 33 did not induce an increase in micronucleated erythrocytes in mice.
As it did not induce gene mutations *in vitro* nor chromosome aberrations or aneuploidy *in vivo*, Acid Red 33 can be considered to have no relevant mutagenic potential *in vivo*. Additional tests are not necessary.

Carcinogenicity
Acid Red 33 has been studied for carcinogenicity after oral administration to mice for 2 years and by an *in utero* long term rat feeding study (2 years). It was concluded in both studies that under the conditions of the bioassays Acid Red 33 was not carcinogenic.

4. CONCLUSION

The SCCP is of the opinion that the use of Acid Red 33, at a maximum concentration of 0.5% in non-oxidative hair dye formulations, does not pose a risk to the health of the consumer.

Acid Red 33 contains several 'CMR'-impurities. 8% of the content are unknown.

The Margin of Safety relates to the use of Acid Red 33 in hair dye formulations only. Acid Red 33 may also be used as a colorant in other types of cosmetic products. These other types of exposure have not been considered.

5. MINORITY OPINION

Not applicable

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