Scientific Committee on Consumer Safety

SCCS

OPINION ON

Acid Violet 43

COLIPA n° C63

The SCCS adopted this opinion at its 18th plenary meeting

of 26 February 2013

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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1. BACKGROUND

Submission I on Acid Violet 43 with the chemical name Benzenesulfonic acid, 2-[(9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthracenyl)amino]-5-methyl-, monosodium salt was submitted by COLIPA[1] in March 1984.

Submission II contains full and updated scientific data on the above substance which is in line with the second step of the strategy on the evaluation of hair dyes.

The SCCP adopted during the 7th plenary meeting of 18 March 2006 the opinion (SCCP/0964/05) on Acid violet 43 with the conclusion that "*the information submitted is inadequate to assess the safe use of the substance as a hair dye.*

Before any further consideration, the following information is required:

- *Complete physico-chemical characterisation of the test substances;*
- *Further data to exclude the clastogenic potential".*

The current submission provides the data requested by the Committee.

2. TERMS OF REFERENCE

1. *Is benzenesulfonic acid, 2-[(9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthracenyl)amino]-5-methyl-, monosodium salt safe for use in semi-permanent hair dye formulations at a maximum concentration of 0.5% of active dye, taken into account the data provided ?*

2. *And/or does the SCCP recommend any restrictions with regard to the use of benzenesulfonic acid, 2-[(9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthracenyl)amino]-5-methyl-, monosodium salt in hair dye formulations ?*

[1] The European Cosmetic Toiletry and Perfumery Association

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Acid Violet 43 (INCI name)

3.1.1.2. Chemical names

Benzenesulfonic Acid, 2-[(9,10-Dihydro-4-Hydroxy-9,10-Dioxo-1-Anthracenyl)Amino]-5-Methyl-, Monosodium Salt
Sodium, 4-[(9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthryl)amino]toluene-3-sulphonate

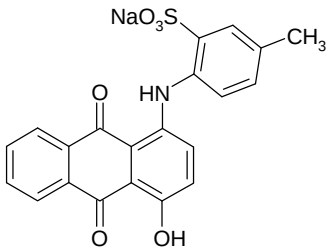
3.1.1.3. Trade names and abbreviations

Jarocol Violet 43
Ext. D&C Violet n° 2
C.I. 60730
COLIPA n° C063

3.1.1.4. CAS / EC number

CAS: 4430-18-6
EC: 224-618-7

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{21}H_{14}NO_6S$, Na

3.1.2. Physical form

Dark violet crystalline powder

3.1.3. Molecular weight

Molecular weight: 431.4

3.1.4. Purity, composition and substance codes

Acid Violet 43 is commercially available in different forms with different analytical specifications and impurity profiles. When Acid Violet 43 meets the standards and specifications of the United States Food and Drug Administration (FDA) indicated in Title 21 — Food and Drugs, Code of Federal Regulations (CFR), Part 74, "Listing of Colour Additives Subject to Certification" as well as the impurity specifications set out by the FDA, it is referred to as External D & C Violet n° 2.

For the toxicity studies performed, two commercial forms were used: Jarocol Violet 43 (Batch 2060208, Batch 10130, Batch 437/3) and External D&C Violet n° 2 (Batch 0609RA, K7116 and Batch 21CFR 9.411).
Chemical characterisation of Acid Violet 43 in the batches 2060208, 10130 and 0609RA was performed by NMR, IR and MS. UV-absorption spectra and HPLC chromatograms of these batches were also submitted.

Description		**Jarocol Violet 43**			**Ext. D&C Violet n° 2 Batch 0609RA**		
		2060208	**10130**	**437/3**	**0609RA**	**K7116**	**21CFR 9.411***
Titre [1]	[%]	54.4	60.5	59.3	93.8	84%	84%
Impurities (HPLC)							
Acid Green 25		d	d	-	-	-	-
Isomer of Acid Green		d	d	-	-	-	-
Isomer of Acid Violet 43		d	d	-	-	-	-
1,4-dihydroxyanthraquinone	[%]	< 0.25	< 0.25	-	< 0.2	-	-
1-hydroxy-9,10-anthracenedione	[%]	-	-		< 0.2	-	-
p-toluidine	[%]	-	< 0.1	-	< 0.1	-	0.2%
p-toluidine sulfonic acids, sodium salts	[%]	-	-	-	< 0.2	-	-
Subsidiary colours	[%]	-	-	-	< 1	-	-
Heavy metals (ICP-OES)							
Fe	mg/kg	-	270	-	-	-	-
Al	mg/kg	-	61	-	-	-	-
Sn	mg/kg	-	10	-	-	-	-
Cu	mg/kg	-	4	-	-	-	-
Mn	mg/kg	-	3	-	-	-	-
Ag, As, Ba, Bi, Cd, Co, Cr, Mo, Ni, Pb, Pd, Pt, Sb, Se, Ti, V, Zn	mg/kg	-	< 1	-	As < 3 Pb < 20	-	-
Hg	mg/kg	-	< 0.1	-	< 1	-	-
Ash content	g/100 g	25.2	25.9	-	-	-	-
Water content (K.F. method)	g/100g	9.3	8.26	-	-	-	-
Starch content [2] (HPIC)	g/100g	-	26	-	-	-	-
Sulphate ions [3] (HPIC)	g/100g	0.43	6.5	-	-	-	*
Chloride ions [4] (potentiometry)	g/100g	4.2	4.42	-	-	-	*
Sum of volatile matter	[%]	-	-	-	< 18	-	10
Water-insoluble matter	[%]	-	-	-	< 0.4	-	1
Residual solvents (GC)	-	n.d.	n.d.	-	-	-	
Mixed oxides	[%]	-	-	-	-	-	1

* Sodium chloride + Sodium sulphate 8%
1 spectrophotometric determination, at 570 nm. For the batch 2060208, HPLC peak area by detection at 570 nm was described as 98-100%. .
2 expressed as glucose
3 expressed as Na_2SO_4
4 expressed as NaCl
d detected but not quantified due to lack of reference standard
n.d. not detected
- no data

Comments
For the characterisation of Acid Violet 43 in the batches 437/3, K7116 and 21CFR 9.411, no documentation was provided. For the batch 0609RA FDA, only a certificate was submitted, and for the batch 437/3, a certificate of analysis of the applicants laboratory was submitted.

The documentation for the quantitative data (in the table above) was provided only for the batches 2060208 and 10130.

3.1.5. Impurities / accompanying contaminants

See 3.1.4.

3.1.6. Solubility

Jarocol Violet 43, 10130, (at 22 °C after 24 h)
Water: < 1g/100 ml
Ethanol: < 1g/100 ml
DMSO: < 1g/100 ml

Jarocol Violet 43, 2060208
Water: < 0.1g/100 ml
Ethanol: < 0.1g/100 ml
Water/Ethanol: ≥ 0.1g/100 ml

Comment:
Water solubility was not determined by EU Method A.6

3.1.7. Partition coefficient (Log Pow)

n-Octanol/water partition coefficient determined by EU Method A.8
Log P_{ow}: 3.1

3.1.8. Additional physical and chemical specifications

Melting point:	/
Flash point:	/
Vapour pressure:	/
Boiling point:	/
Density at 20 °C:	/
Viscosity:	/
pKa:	/
UV-Vis absorption spectrum:	λmax 253 nm, 570 nm
Refractive index at 20 °C:	/
Storage:	at room temperature, protected from light and under inert gas atmosphere

3.1.9. Homogeneity and Stability

Stable for 4 hours in acetone/olive oil (6.05 and 151 mg active dye/ml) and purified water (3.03 and 121 mg active dye/ml) at room temperature, protected from light and under inert gas atmosphere. Deviation from initial value was between – 4% and 6% after 4 hours (only tested for Jarocol Violet 43, Batch No. 10130)

General Comments on physico-chemical characterisation

* Analytical data are incomplete for Jarocol Violet 43, Batch No. 437/3 and for External D&C Violet n° 2 batches K7176 and 21CFR 9.411.
* The content of Acid Violet 43 in various batches, assayed by spectrophotometric method, can only be considered as semi-quantitative determination.
* Content of heavy metals was only submitted for Jarocol Violet 43, Batch No 10130.
* A difference in sulphate content was noted between batch 2060208 and 10130;
* Stability of Acid Violet 43 in typical hair dye formulations was not reported.

3.2 Function and uses

Acid Violet 43 is an anthraquinone-colour used in semi-permanent hair dye formulations at a maximum concentration of 0.5% active dye.

According to the EU Cosmetics Directive 76/768/EEC, Annex IV Part I List of Colouring Agents, Acid Violet 43 is allowed as CI 60730 in cosmetic products except those intended to come into contact with mucous membranes.

3.3 Toxicological Evaluation

Two commercial forms were used for the toxicity studies submitted, Jarocol Violet 43 and External D&C Violet n° 2. Several toxicity endpoints (subchronic toxicity, induction of gene mutations or chromosome aberrations, embryo-foetal development toxicity and percutaneous penetration) were covered by both test materials though different test systems were often used. Acute eye irritation and skin sensitisation studies were conducted only on Jarocol Violet 43, skin irritation and acute oral toxicity studies were only available for External D&C Violet n° 2. Toxicological evaluation was related to the amount of active dye in the test substance used for the investigations.

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Guideline:	/
Species/strain:	Sprague-Dawley albino rats
Group size:	5 males per group
Test substance:	External D&C Violet n° 2 in a 0.5% aqueous methyl cellulose solution
Batch:	K7116
Purity:	84%
Dose:	100, 215, 464, 1000, 2150, 4640 mg active dye/kg bw
Observation:	seven days
GLP:	not in compliance

5 male Sprague-Dawley albino rats (body weight 190-236 g) were treated with single doses of the test substance by gavage. Animals were observed for mortality and toxic effects immediately, one, four, and 24 h after administration and once daily thereafter for a total of seven days. At the end of the observation period animals were weighed, sacrificed and autopsied.

Results
No mortality occurred. The LD_{50} of the test substance administered to rats by the oral route was > 4640 mg active dye/kg bw. One animal of the highest dose group, three animals of the group which received 2150 mg/kg bw and all animals of the group which received 464 mg/kg bw showed granular appearing spleens. For three animals of the highest dose group congestion of the kidneys was observed. Coloration of faeces for the two higher dose groups at 24 hours and coloration of urine for the highest dose group at four-hour observation were reported.

Comment
The study was not performed according to modern standards, but gives an indication of the acute toxicity.

Ref.: 1 (subm II)

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

See 3.3.5.2 subchronic dermal toxicity and 3.3.7 lifetime skin painting.

3.3.2.2 Mucous membrane irritation

Guideline:	OECD 405 (2002)
Species/strain:	New Zealand White rabbits
Group size:	3 males
Test substance:	54115 (Acid Violet 43)
Batch:	10130
Purity:	60.5%
Dose:	1% active dye (%w/w of 'pure' dye taking into account the titre of 60.5%)
GLP:	in compliance
Date:	2004

A single dose of 0.1 ml 54115 (Acid Violet 43) at a concentration of 1% active dye (w/w) in water was instilled into the conjunctival sac of the left eye of test animals (day 1). The ocular reactions were assessed 1, 24, 48 and 72 hours after instillation.

Results
A slight chemosis was observed in 1/3 animals on days 1 and 2, and slight redness of the conjunctiva was observed in all animals from day 1 up to days 2 or 3. A clear discharge was also noted in 1/3 animals on day 2. The discharge was not scored on day 1 because of residual test item. All ocular reactions had disappeared by day 4.

Ref.: 3 (subm II)

Comment
Under the conditions of this experiment 54115 (Acid Violet 43) at a concentration of 1% w/w active dye caused some eye irritation. It is unknown whether the 'impurities' in the tested product had an effect on this.

3.3.3 Skin sensitisation

Guideline:	OECD 429 (2002)
Species/strain:	CBA/J mice
Group size:	4 females / group
Test substance:	54115 (Acid Violet 43)
Batch:	10130
Purity:	60.5%
Concentration:	0.6, 1.5, 3, 6 and 15% active dye (w/v) in acetone/olive oil (AOO) mixture (4/1, v/v) (%w/w of 'pure' dye taking into account the titre of 60.5%)
Controls:	vehicle; 25% v/v α-hexylcinnamaldehyde

GLP: in compliance
Date: 2004

Animals were separated in 7 groups (4 mice/group) consisting of 5 treated groups receiving 54115 (Acid Violet 43), a negative control group receiving the vehicle (AOO) alone and a positive control group receiving α-hexylcinnamaldehyde (HCA), at 25% (v/v) in AOO. The vehicle was selected in a previous solubility study showing that 15% (w/v) active 54115 (Acid Violet 43) was the "maximal practicable concentration, and that this concentration was non-irritant" in a preliminary irritation test.
During induction period test substances were applied over the ears (25 µL per ear) for three consecutive days (designated as days 1, 2 and 3). After 2 days of resting, the proliferation of lymphocytes in the lymph nodes draining the application sites was measured by incorporation of tritiated methyl thymidine (3H-TdR, day 6). The values obtained were used to calculate stimulation indices (SI). The irritant potential of the test item was assessed in parallel by measurement of ear thickness on days 1, 2, 3 and 6.

Results
No cutaneous reactions and no increases in ear thickness were observed in animals treated with the test substance. A black coloration of the skin of the ears was noted in all treated animals on days 2 and or from day 2 up to day 6. This coloration could have masked a possible erythema. No lymphoproliferation was observed at any tested concentration.

Test substance	**SI**
54115 (Acid Violet 43) 0.6% w/v	0.86
54115 (Acid Violet 43) 1.5% w/v	1.21
54115 (Acid Violet 43) 3.0% w/v	0.85
54115 (Acid Violet 43) 6.0% w/v	0.96
54115 (Acid Violet 43) 15.0% w/v	1.55
α-hexylcinnamaldehyde 25% v/v	6.55

Ref.: 4 (subm II)

Comment
The maximum test concentration of 54115 (Acid Violet 43) was too low to exclude a sensitising potential.

3.3.4 Dermal / percutaneous absorption

Study 1

Guideline: /
Species/strain: Porcine ear skin (number of donors not stated)
Chambers: Glass flow through; 6 per experiment
Membrane integrity: conductivity
Test substance: Ext. D&C Violet n° 2
Experiment 1:
Batch: 0609RA
Purity: 95% (certificate)
Dose: Experiment 1: 2 mg/ml; solution in 20% (v/v) ethanol in water; 1000mg/cm^2
Experiment 2:
Batch: /
Purity: /
Dose: 0.5% in semi-permanent hair dye formulation; 1000mg/cm^2
Exposure time: 30 minutes
Receptor: 20% ethanol in water

Solubility in receptor: /
Stability in receptor: /
Detection: HPLC
GLP: in compliance
Date: 1999

Porcine ears were obtained from a slaughterhouse. Skin from the outer ear region was removed by dissection and skin samples (thickness 100-450 µm in Experiment 1 and 800-900 µm in the Experiment 2) were mounted in glass flow-through (1-2 ml/h) diffusion chambers (diameter: 1.135 cm), using a 20% (v/v) solution of ethanol in water as a receptor fluid. Skin integrity was checked over the entire duration of the study by measuring conductivity across the skin (maintained at 32°C) at each sampling time.

In the Experiment 1, the donor chamber of the diffusion cell was filled with 1 ml of the test solution, covered and left for 30 minutes. In the Experiment 2, 1.25 ml of the hair dye formulation was applied in the same conditions (1000 mg/cm^2 corresponding to 5 mg/cm^2 test substance). After this time period, the remaining formulation was removed using a standardized washing procedure with a shampoo solution. Following exposure period in both experiments, the donor chambers were filled with 1 ml of receptor fluid (20% ethanol in water). The receptor fluid in collecting vials was collected after 0, 0.5, 1, 2, 4, 6, 8 and 24 hours and analysed by HPLC. The amounts of test material in receptor solution plus that in skin extracts were considered to be absorbed, since the stratum corneum was not separated from the epidermal and dermal compartments.

Results
The mean total recovery rate of test material was 88.8 ± 4.08% in Experiment 1 and 90.6 ± 4.67% in the Experiment 2 when excluding from this evaluation two or one of the six diffusion cells, respectively, for which solubility problems were encountered. Test substance could not be measured in the receptor fluid at any time point and in skin extracts from Experiment 1 (detection limit 500 ng/ml). Assuming concentrations of test material at the detection limit in the total volume of receptor fluid collected at all time-points maximal flux rates were calculated. To quantify skin absorption amounts measured for skin extracts were added to the calculated fluxes. Within the report, the values for fluxes and skin absorption differ slightly. Based on the highest values calculated for the flux (19.3 µg or 19.1 µg/cm^2 in Experiment 1 and 19.0 or 24.8 µg/cm^2 in Experiment 2) and values for skin extracts (1.85 ± 0.01 µg or 1.83 ± 0.01 µg/cm^2 in Experiment 1 based on the detection limit of 500 ng/ml and 5.82 ± 2.67 µg or 5.75 ± 2.6 µg/cm^2 in experiment 2) the following rates for skin absorption might be estimated as worst case: 20.93 µg/cm^2 (approximately 1%) in Experiment 1 and 30.55 µg/cm^2 (approximately 0.6%) in Experiment 2.

Ref.: 17 (subm II)

Comment
It is not stated how many donor were used. Too few chambers were available. The vehicle was not appropriate. The dosing was too high. The stratum corneum was not separated from the epidermis and dermis. These were not guideline studies.
This study is considered inadequate and cannot be used.

Study 2

Guideline: /
Species/strain: human (dermatomed skin)
Donors: 4 donors; 2 chambers each
Chambers: Static diffusion cells
Membrane integrity: Trans epidermal water loss (TEWL)
Test substance: Acid Violet 43
Batch: 437/3

Purity:	59.3%
Dose:	0.12% (as active dye) in semi-permanent hair dye formulation (20 mg/cm^2, corresponding to 25.4 µg active dye/cm²)
Exposure time:	30 minutes
Receptor:	0.9% saline
Solubility in receptor:	11.43mg/L
Stability in receptor:	/
Detection:	LC/MS/MS
GLP:	in compliance
Date:	2001

Human skin samples were obtained from four female donors subjected to plastic surgery. Skin samples (380 ± 25 µm in thickness) were dermatomed and mounted in diffusion cells, using 0.9% NaCl in water as a receptor fluid. Skin integrity was checked before application of the formulation by measuring Trans Epidermal Water Loss. After exposure period, the remaining formulation on the skin surface was removed using a standardized washing procedure. Twenty-four hours after application, the percutaneous absorption of Acid Violet 43 was estimated by measuring its concentration by LC/MS/MS in the following compartments: skin excess, stratum corneum (isolated by tape strippings), epidermis + dermis and receptor fluid.

Results

	Formulation 473219 (n = 8)
Skin excess	
µg/cm²	26.43 ± 2.50
% of the applied dose	104.27 ± 3.19
Stratum corneum (SC)	
µg/cm²	0.08 ± 0.05
% of the applied dose	0.33 ± 0.20
Epidermis + dermis	
µg/cm²	0.07 ± 0.06
% of the applied dose	0.28 ± 0.28
Receptor fluid (RF)	
µg/cm²	**0.04 ± 0.00**
% of the applied dose	**0.25 ± 0.07**
Total recovery	
% of the applied dose	**105.12** ± 3.07

	473219 (n = 8)
Total skin + receptor fluid[1]	
µg/cm²	0.20 ± 0.11
% of the applied dose	0.86 ± 0.51
Absorbed amount[2]	
µg/cm²	0.11 ± 0.06
% of the applied dose	0.53 ± 0.33

[1] Total skin + receptor fluid = SC + epidermis + dermis + receptor fluid
[2] Absorbed amount = Epidermis + dermis + receptor fluid

Most of the test substance applied on the skin surface was removed with the washing procedure (about 104% of the applied dose), and the total recovery rate was about 105%. No Acid Violet 43 was measured in the receptor fluid (limit of detection used as default). The mean absorbed amounts of Acid Violet 43 were estimated as follows (sum of amounts measured in epidermis, dermis and receptor fluid when assuming concentrations at the detection limit in the receptor fluid of 40 ng): 0.11 ± 0.06 µg active dye/cm^2 (0.53 ± 0.33% of the applied dose).

Ref.: 18 (subm II)

Comment
This was a non-guideline study. The amount of Acid Violet 43 considered available from a semi-permanent (non-oxidative) hair dye formulation containing 0.12% (as active dye) is mean +2SD. This is 0.23 µg active dye/cm^2 or 1.19% of the applied dose.
The applied concentration was too low. An absorption of 4 x 0.23 (0.92) may be used for the calculation of the MoS.

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (30 days) oral toxicity

No data submitted

3.3.5.2 Sub-chronic (90 days) toxicity (oral, dermal)

Oral, study 1

Guideline:	/
Species/strain:	Sprague-Dawley rats
Group size:	10 animals per sex and group
Test substance:	Jarocol Violet 43
Batch:	2060208
Purity:	54.4%
Dose:	0, 50, 200 or 800 mg/kg bw/day (0, 27, 109 or 435 mg active dye/kg bw/day) in water (5 ml/kg) by gavage
Exposure:	13 weeks
GLP:	in compliance

Daily oral gavage of 0, 50, 200 or 800 mg/kg bw/day (0, 27, 109 or 435 mg active dye/kg bw/day) in 5 ml/kg water was performed for 13 weeks. These doses were selected on the basis of a previous 14-day oral toxicity study in rats. Evaluations and measurements included mortality, daily clinical observations, weekly body weight and food intake, ophthalmoscopy (in acclimation once before the beginning of the treatment period and in week 13 on control and high dose animals), haematology, blood clinical chemistry and urinalysis (week 13). At the end of the dosing period, surviving animals were killed and subjected to a complete macroscopic examination, principal organs (adrenals, heart, kidneys, liver, ovaries, spleen, testes, thymus) were weighed, and a full spectrum of tissues were preserved. Microscopic examination was performed for specified tissues/organs from all decedent rats, control and high dose rats killed at the end of the study, as well as for gross anomalies, lungs, liver and kidneys from all animals.

Results
There were no treatment-related deaths. Two treated females (one given 200 mg/kg bw/day and one given 800 mg/kg bw/day) died during the study. Purplish colour and/or purplish and blue contents were observed in the lungs, in buccal and thoracal cavities and in the trachea of these females. No remarkable clinical signs were observed just before death; at the microscopic examination, alveolar oedema together with pigmented granular material was noted. Their death, therefore, was attributed by the study authors to a gavage error and not to the administration of the test substance. Increased salivation was observed at all dose levels with a dose-dependent incidence. Loud breathing was noted in 1/10 females given 50 mg/kg bw/day, 1/10 females given 200 mg/kg bw/day, 2/10 females and 1/10 males given 800 mg /kg bw/day. Regurgitation occurred in all treatment groups (10 – 30%). Coloured urine, faeces, fur and extremities were observed at all dose levels and were related to the staining properties of Jarocol Violet 43. No other clinical signs, no ocular findings or changes in body weight and food intake were reported. There were no significant differences in organ weights between treated and control groups. Findings at necropsy were dose-related greenish contents or greenish colorations of the mucosa of the digestive tract. The histopathological changes noted at the microscopic examination of tissues and organs of treated animals were similar in incidence, severity and morphological characteristics to those observed in the control group and therefore, not considered by the study authors to be treatment-related.

Statistically significant changes in blood clinical chemistry parameters included slight decreases in inorganic phosphorus (males in all dose groups), glucose (mid-dose males and high-dose females), urea (high-dose males), alkaline phosphatase (high-dose males) and

alanine aminotransferase (high-dose males). These changes were considered to be of no toxicological importance.

Statistically significant changes in haematological parameters included decreased values for white blood cells (high-dose males), leukocytes, (high-dose males), prothrombin time (high-dose females) and fibrinogen (high-dose females); and increased values for packed cell volume (high-dose females), mean cell volume (mid- and high-dose females), mean cell haemoglobin (mid-dose females), mean cell haemoglobin concentration (high-dose males), prothrombin time (high-dose males) and activated partial thromboplastin time (high-dose males). The only finding in the urinalysis was an increased pH of urine in females (high-dose). The only finding considered by the study authors to be treatment-related was the increased activated partial thromboplastin time in high-dose males (+30% in mean).

Conclusion
The No Observed Adverse Effect Level was 200 mg/kg bw/day (109 mg active dye/kg bw/day) based on the increased activated partial thromboplastin time in high-dose males.
Ref.: 5 (subm II)

Oral, study 2

Guideline:	OECD 408
Species/strain:	Wistar rats
Group size:	10 animals per sex and group
Test substance:	External D&C Violet n° 2
Batch:	0609RA
Purity:	93.8%
Dose:	0, 100, 300 and 1000 mg/kg bw/day (0, 94, 282 or 940 mg active dye/kg bw/day) in 1% aqueous solution of carboxymethylcellulose (10 ml/kg) by gavage
Exposure period:	13 weeks
GLP:	in compliance

Daily oral gavage at 0 (vehicle, 1% aqueous solution of carboxymethylcellulose), 100, 300 and 1000 mg/kg bw/day (0, 94, 282 or 940 mg active dye/kg/day) at a dosing volume of 10 ml/kg was performed for 13 weeks. These dose levels were selected on the basis of a previous 14-day oral toxicity study in rats. Evaluations and measurements included mortality, daily cage-side observations, weekly body weight, food intake and detailed clinical observations, ophthalmoscopy (on all animals in acclimation period and on control and high dose animals in week 13), as well as functional parameters, haematology, blood clinical chemistry and urinalysis (week 13). At the end of the dosing period, animals were killed and subjected to macroscopic examination, selected organs were weighed, and organs/tissues were preserved. Microscopic examination was performed for specified tissues/organs from control and high dose rats, and for gross anomalies from all animals.

Results
There were no deaths, no adverse clinical signs or changes in body weights and food intake. No substance related ocular findings were reported. Dark blue faeces were observed at all dose levels and were related to the staining properties of the test substance. In males given 1000 mg/kg bw/day, slightly increased locomotor activity was observed after 15 minutes; in absence of a clear dose-relationship this finding was considered by the study authors to be fortuitous. Statistically significant decreased locomotor activity was observed after 45 minuts in females given 300 or 1000 mg/kg bw/day and persisted until the end of the measurement period in females given 1000 mg/kg bw/day. The toxicological significance was unclear according to the study authors, but in absence of this finding in males and in absence of similar parameters (general and detailed clinical observations) a test article relation was considered by the study authors to be unlikely.

Changes in grip strength were reported in treated males and females. However, data did not show dose-relationship and no correlation between fore- and hind-limb strength. Effects therefore might not be related to the test substance.

Statistically significant changes in haematological parameters at low dose included increased prothrombin time (low- and high-dose males) and activated partial thromboplastin time (high-dose males).
The changes observed in blood clinical chemistry laboratory parameters and in urinalysis were considered by the study authors to be of no toxicological significance.
Statistically significant changes in relative organ weights included increased kidney weight in low- and mid-dose males, and decreased heart weight in mid-dose females. In absence of any dose relationship these findings were considered by the study authors to be fortuitous.

The only findings at necropsy were a blue discolouration of the mucosal surface of the stomach and/or intestines observed for a few animals given 1000 mg/kg bw/day. The histopathological changes noted at the microscopic examination of tissues and organs of treated animals were similar in incidence, severity and morphological characteristics to those observed in the control group and therefore, not considered by the study authors to be treatment-related.

Conclusion
The No Observed Adverse Effect Level (NOAEL) was reported to be 1000 mg/kg bw/day (940 mg active dye/kg bw/day).

Ref.: 6 (subm II)

Comment
The SCCS, however, considers the increase (approximately 10 %) in APTT in high dose males to be adverse and sets the NOAEL to 300 mg/kg bw/day (282 mg active dye/kg bw/day).

Dermal

Guideline:	/
Species/strain:	male albino rabbits
Group size:	5 animals per treatment group, 10 animals in control group
Test substance:	External D&C Violet n° 2
Batch:	21CFR 9.411
Purity:	84%
Dose:	0, 0.1% and 1% active dye in base ointment
Exposure:	91 days
GLP:	not in compliance

The day before the first dosing, the application site of test animals (back area) was clipped free of hair. Clipping was repeated as necessary during the study. 5 days a week a 0.5 g sample of a base ointment (USP hydrophilic ointment) containing 0% (control group, 10 rabbits) 0.1% or 1% of the dye (5 rabbits/group) was spread over the back of the animals and left uncovered. Treatment was performed for a total of 65 applications over a 3-month interval. Skin was evaluated for signs of irritation and clinical signs were recorded prior to the daily application. Animals were weighed at weekly intervals. At the end of the dosing period, animals were killed and subjected to gross examination; kidney and liver weights were recorded. Additionally, portions of liver, kidneys and skin from treated area were preserved for subsequent microscopic examination. These tissues were examined from 5 control animals and those receiving the 1% ointment.

Results

None of the animals died during the study. The skin at the test site appeared slightly pink in animals of the control group and was discoloured violet in animals of the test groups. Erythema and oedema were not observed. Thickening of the skin, nasal or eye discharge occurred occasionally and were equally distributed among control and test animals. Significant differences in mean body weights or mean relative kidney weights between test and control animals were not observed. Mean relative liver weights were statistically decreased for animals of the test group as compared to controls, but a significant difference in absolute liver weights was not detected. These effects seem to be related to the high standard deviation for liver weight in the control group. Gross or macroscopic lesions due to the test substance were not reported.

Ref.: 2 (subm II)

3.3.5.3 Chronic (> 12 months) toxicity

No data submitted

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Study 1

Guidelines:	Directive No. 92/69/EEC
Species/strain:	*Salmonella typhimurium* TA 1535, TA 1537, TA 98, TA 100 *Escherichia coli* WP2uvrA
Replicates:	Triplicates, two independent tests
Test substance:	Jarocol Violet 43
Batch:	2060208
Purity:	54.4%
Concentrations:	170, 340, 680, 1360, 2720 µg active dye/plate, with and without metabolic activation
GLP:	in compliance

Jarocol Violet 43 was evaluated in two independent experiments in the absence and presence of metabolic activation (S9-mix prepared from the livers of rats given Aroclor 1254). The highest dose was the limit of solubility of the test compound in the test conditions used. Known mutagens were used as positive controls, and cultures treated with distilled water (solvent) were used as negative controls. Three plates per treatment condition were used. The tests were conducted according to the direct plating incorporation method, except the second test with S9-mix which was performed according to the pre-incubation method.

Results
The test substance was toxic at doses higher than 680 µg/plate in the TA 100 strain without metabolic activation. In both experiments, Jarocol Violet 43 did not increase the number of revertants in any strain in the presence and absence of metabolic activation.

Ref.: 7 (subm II)

Study 2

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA 1535, TA 1537, TA 98, TA 100 *Escherichia coli* WP2uvrA

Replicates:	Triplicates, two independent tests
Test substance:	Ext. D&C Violet n° 2
Batch:	0609RA
Purity:	95%
Concentrations:	31.35, 95, 316.35, 950, 2375, 4750 µg active dye/plate, with and without metabolic activation
GLP:	in compliance

Ext. D&C Violet n° 2 was evaluated in two independent experiments in the absence and presence of metabolic activation (S9-mix prepared from the livers of β-naphthoflavone and sodium phenobarbitone-treated rats). Known mutagens were used as positive controls, and concurrent untreated and solvent (DMSO) controls were performed. Three plates per treatment condition were used. The first experiment was conducted according to the direct plating incorporation method, and the second experiment was performed according to the pre-incubation method.

Results
No cytotoxicity was observed in any of the test conditions used. Ext. D&C Violet n° 2 did not increase the number of revertants in any strain in the presence or absence of S9-mix.

Ref.: 8 (subm II)

In Vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)
Species/strain:	Mouse lymphoma cell line L5178Y/TK$^{+/-}$
Replicates:	Duplicates, two independent tests
Test substance:	Ext. D&C Violet n° 2
Batch:	0609RA
Purity:	95%
Concentrations:	5.3, 10.7, 21.4, 42.8, 85.5 and 171 µg active dye/ml; with and without metabolic activation (experiment 1) 21.4, 42.8, 85.5, 171 and 342 µg active dye/ml; without metabolic activation (experiment 2)
Treatment time:	4 h (experiment 1) 24 h (experiment 2)
GLP:	in compliance

Ext. D&C Violet n° 2 was evaluated in two independent experiments using duplicate cultures each. The first experiment used a pulse (4-hour) treatment procedure and was conducted in the absence and presence of metabolic activation (S9-mix prepared from the livers of β-naphthoflavone and sodium phenobarbitone-treated rats). The second experiment was performed only in the absence of metabolic activation (24-hour treatment). Known mutagens in the presence (3-methylcholanthrene) or absence of S9-mix (methyl methane sulfonate) were used as positive controls. Negative controls consisted of untreated cultures and cultures treated with the solvent alone (DMSO).

Results
Ext. D&C Violet n° 2 was not mutagenic in the mouse lymphoma assay (TK locus) in the presence and absence of metabolic activation as no increased number of mutant colonies was observed up to the maximal concentrations of the substance. Precipitation was observed at 342 µg active dye/ml in the absence and presence of S9-mix. Strong cytotoxicity was observed at 171 µg active dye/ml after 4-hour treatment (+ or - S9-mix, experiment 1), and relevant toxic effects were observed at the highest concentration of 342 µg active dye/ml under precipitation after 24-hour treatment (- S9-mix, experiment 2).

Ref.: 9 (subm II)

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

Study 1

Guideline:	OECD 473
Species/strain:	Human lymphocytes
Replicates:	Duplicates, two independent tests
Test substance:	Jarocol Violet 43
Batch:	2060208
Purity:	54.4%
Concentrations:	68, 136 and 272 µg dye/ml in experiment 1 without metabolic activation (active dye) 85, 170 and 340 µg dye/ml in experiment 1 with metabolic activation 68, 204 and 272 µg dye/ml in experiment 2 without metabolic activation 68, 272 and 408 µg dye/ml in experiment 2 with metabolic activation
Treatment time:	2 h, 24 h (experiments 1, 2) and 48 h (experiment 1)
GLP:	in compliance

Jarocol Violet 43 was investigated in two independent experiments in the absence and presence of metabolic activation (S9-mix mix prepared from the livers of Aroclor 1254-treated rats). Duplicate cultures were treated with each concentration of Acid Violet 43 or with known clastogens in the presence (cyclophosphamide) or absence of S9-mix (mitomycin C), and untreated cultures were used as negative controls. The highest concentration selected for each of these tests was the lowest concentration achieving a reduction of the mitotic index in the range 50-75%. In both experiments, continuous treatment (until harvesting) was performed in the absence of S9-mix, whereas pulse (2-hour) treatment was performed in the presence of S9-mix. Cells were harvested 24 hours after the beginning of treatment in both experiments and additionally at 48 hours in the second experiment (except for positive controls). Two hours prior to harvest, cell cultures were treated with a colcemid solution to block them in metaphase. Chromosome preparations were stained and examined microscopically for mitotic index and for aberrations when selected.

Results
For the 24-h harvest time the test substance did not induce any significant increase in the aberrant cells frequency, with and without metabolic activation in both experiments. For the 48-h harvest time performed during the repeat test, a significant increase in the aberrant cells frequency was recorded in the 2-h treatment group at the highest concentration (408 µg active dye/ml) with metabolic activation as well as in the 48-h treatment group at the highest concentration (272 µg active dye/ml) without metabolic activation. Aberrations consisted almost of chromatid deletions, and the proportion of cells bearing numerical aberrations was also slightly increased. These changes were observed together with a reduction in mitotic index to 46 and 52% of controls.

Conclusion
Jarocol Violet 43 has the potential to induce chromosome aberrations in cultured mammalian cells.

Ref.: 10 (subm II)

Study 2

Guideline:	OECD 473
Species/strain:	Chinese Hamster Ovary (CHO Cells)
Replicates:	Duplicates, two independent tests
Test substance:	Jarocol Violet 43

Batch:	2060208
Purity:	54.4%
Concentrations:	272, 544 and 2720 µg active dye/ml in experiment 1 with and without metabolic activation 102, 204 and 408 µg active dye/ml in experiment 2 for the first harvesting time (20h) without metabolic activation 51, 102 and 204 µg active dye/ml in experiment 2 for the second harvest time (44h) without metabolic activation 680, 1360 and 2720 µg active dye/ml in experiment 2 with metabolic activation for both harvest times
GLP:	in compliance

Jarocol Violet 43 was evaluated in two independent experiments in the absence and presence of metabolic activation (S9-mix prepared from the livers of Aroclor 1254-treated rats). The highest concentration for each experimental condition was selected on the basis of solubility or cytotoxicity criteria. Duplicate cultures were treated with each concentration of Jarocol Violet 43 selected or with known clastogens in the presence (cyclophosphamide, CPA) or absence of S9-mix (methyl methane sulfonate, MMS). Vehicle (distilled water) treated cultures were used as negative controls. In the first experiment, a pulse (3- or 4-hour) treatment procedure was used. In the second experiment, the same pulse-treatment procedure was used in the presence of S9-mix whereas cultures were treated continuously until harvesting in the absence of S9-mix. Cells were harvested 20 hours after the beginning of treatment in both experiments and additionally at 44 hours in the second experiment.

Results
Treatment of cultures with Jarocol Violet 43 resulted at concentrations from 102 µg active dye/ml in the presence and absence of S9-mix, in both experiments and at both harvest times, in statistically significantly increased numbers of cells bearing structural aberrations, chromatid and/or chromosome deletions and exchanges). Some of these increases were observed in the absence of overt cytotoxicity.

Ref.: 11 (subm II)

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

New study, submission III, 2007

Bone Marrow Micronucleus Test

Guideline:	OECD 474 (1997)
Species:	mouse, Swiss Ico: OF1 (IOPS Caw).
Group sizes:	2 groups of 5 males and 5 females per dose 1 group of 5 males and 5 females for positive control
Test substance:	Acid Violet 43
Batch:	10130
Purity:	59.8%
Vehicle:	purified water
Dose:	0, 2000 mg active substance/kg bw 50 mg/kg bw (positive control)
Dose volume:	20 mL/kg bw (test substance and vehicle) 10 mL/kg bw (positive control)
Positive control:	cyclophosphamide
Administration:	once by oral route
GLP:	in compliance
Study period:	12 September – 7 November 2006

Groups of mice (5/sex/dose level/sampling time + 3 spare animals/sex) received a single oral (gavage) dose of Jarocol Violet 43, at 0 or 2000 mg active dye/kg bw (approximately

3348 mg Jarocol Violet 43/kg bw) in water at 20 mL/kg. This dose level is the test limit dose recommended by appropriate guidelines and did not produce mortality or clinical signs in a preliminary experiment.
Animals were killed either 24 or 48 hours after treatment. A positive control group of 5 mice/sex was given a single oral dose of cyclophosphamide (CPA) at 50 mg/kg and was killed 24 hours after dosing.
For each animal, smears were prepared from femoral bone marrow, stained with Giemsa and scored blind for the incidence of micronucleated polychromatic erythrocytes (MN-PCE, 2000 PCE counted) and for the polychromatic/normochromatic erythrocyte ratio (PCE/NCE ratio, 1000 erythrocytes counted). In the same study a toxicokinetic study was performed with 3 mice/sex/sampling time-point. Blood samples were taken 15, 30 or 60 minutes after dosing (single sample per animal,).

Results
Treatment with Jarocol Violet 43 produced no clinical signs or mortality. Mean MN-PCE frequency was similar for Jarocol Violet 43-treated and control animals. There was no indication of bone marrow toxicity since PCE/NCE ratios were similar for Jarocol Violet 43 and control groups. However, the oral bioavailability of the test material was demonstrated in the toxicokinetic part of the study, with high blood levels of Acid Violet 43 observed in all animals (range 5.7-34.6 µg/mL).
When compared to controls, the incidence of MN-PCE was statistically significantly increased (approximately 15-fold) in animals given the positive control CPA, showing the adequate sensitivity of the test system and procedure used.

Conclusion
Under the conditions of the study, Jarocol Violet 43 did not produce cytogenetic damage leading to micronucleus formation in the bone marrow of mice treated orally up to the limit dose level of 2000 mg active dye/kg (3344 mg Jarocol Violet 43).

Ref.: 8 (subm III)

Bone Marrow Micronucleus Test

Guideline:	OECD 474
Species:	Swiss mice
Group sizes:	Group 1: 5 animals/sex/dose/killing time Group 2: 3 animals/sex/dose/killing time
Test substance:	Jarocol Violet 43
Batch:	2060208
Purity:	54.4%
Dose:	0 and 1088 mg active dye/kg bw in water, 20 ml/kg (gavage)
GLP:	in compliance

Swiss mice received a single oral dose of 2000 mg/kg bw Jarocol Violet 43. This dose level was not associated with any signs of toxicity. An additional positive control group of 5 mice/sex was given a single oral dose of cyclophosphamide (CPA) at 50 mg/kg. Animals from Jarocol Violet 43 or vehicle control groups were killed either 24 or 48 hours after dosing, whereas CPA-treated animals were killed 24 hours after dosing. For each animal, smears were prepared from femur bone marrow and were scored blindly for the incidence of micronucleated polychromatic erythrocytes and for the polychromatic/normochromatic erythrocyte (PCE/NCE) ratio.

Results
In all groups treated with Jarocol Violet 43, the mean values of micronucleated polychromatic erythrocytes were similar to those of their respective vehicle group at each sampling time and no statistically significant differences were observed. At the 48-h sampling time, the PCE/NCE ratio was lower than in controls but this difference was mainly

due to a high control value and does not clearly indicate bone marrow toxicity of the test substance.

Ref.: 12 (subm II)

Unscheduled DNA synthesis

Guideline:	OECD 482
Species:	Wistar rats
Group sizes:	3 males + 1 spare male/dose level/killing time
Test substance:	Jarocol Violet 43
Batch:	2060208
Purity:	54.4%
Dose levels:	0, 82 and 816 mg active dye/kg bw in water, 10 ml/kg (gavage)
GLP:	in compliance

Wistar rats received a single oral dose of Jarocol Violet 43. Dose levels were selected on the basis of a sighting test in which 816 mg active dye/kg was the Maximal Tolerated Dose, associated with piloerection and blue-coloured urine. A higher dose (1088 mg active dye/kg) was lethal for one animal. An additional group of 4 male rats was given a single oral dose of 2-Acetylaminofluorene (2-AAF, 100 mg/kg) and acted as a positive control group. Animals given 816 mg active dye/kg were killed either 2 or 16 hours after dosing, whereas rats given the vehicle alone, 82 mg active dye/kg or 2-AAF, respectively, were killed 16 hours after dosing. For each animal, hepatocytes were isolated from the liver and at least three primary cultures were established. Autoradiographic slides from 2 cultures/animal were prepared and the unscheduled synthesis of DNA was evaluated by the incorporation of tritiated methyl thymidine in 50 cells/slide.

Results
No changes from controls in the number of nuclear and net grain counts were observed in Jarocol Violet 43-treated rats at both dose levels and both killing times.

Ref.: 13 (subm II)

3.3.7 Carcinogenicity

Guideline:	/
Species/strain:	Swiss female mice
Group size:	six consecutive groups with 100 females treated and 200 control animals in total
Test substance:	Ext. D&C Violet n° 2
Batch:	21CFR 9.411
Purity:	84%
Dose:	aqueous solution (first dose), 2% dispersion in propylene glycol (2nd to 6th dose), 1% dispersion in propylene glycol (7th to final dose), average estimated: 23 mg active dye/kg per application
Treatment:	103 weekly dermal applications over 107 weeks
GLP:	not in compliance

The application site was gently clipped free of hair prior to the application of each dose. The average of the mean of the 103 doses was estimated to be 23 mg active dye/kg bw/day. The test dispersions were applied to the back of the animals using a hairbrush and then left uncovered. An additional group of 200 mice received propylene glycol alone under the same conditions and acted as a control group. Body weight of mice was taken at 6-month intervals. Surviving mice were killed and necropsied from week 102 to week 107, when surviving treated animals was close to 30%. Organs and tissues were preserved. Neoplasms and gross lesions sampled were examined microscopically for all animals, and 10 mice per group were selected for histological examination. Necropsy and histological examination of

lesions/tumours from decedent animals was undertaken on those showing only moderate autolysis.

Results
Throughout the study, the proportion of surviving mice was similar in treated and control groups. The tumour and leukaemia incidences in Ext. D&C Violet n° 2-treated mice were not different from controls.

Ref.: 14 (subm II)

Comment
No information has been provided demonstrating the ability of the testing procedure to detect carcinogens. Thus, no conclusion with regard to carcinogenicity can be made from the experiment.

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

No data submitted

3.3.8.2 Teratogenicity

Study 1

Guideline:	/
Species/strain:	Sprague Dawley rats
Group size:	25 females
Test substance:	Jarocol Violet 43
Batch:	2060208
Purity:	54.4%
Dose:	0, 50, 200 or 800 mg/kg bw/day (0, 27, 109 or 435 mg active dye/kg bw/day)
Treatment	day 6 - 15 of gestation
GLP:	in compliance

The test substance was given in water daily at dose volumes of 5 ml/kg bw by oral gavage. The doses were selected on the basis of the results of a preliminary study in rats. Maternal evaluations and measurements included daily clinical signs and body weight/food intake recorded at designated intervals. The females were killed on gestation day 20, subjected to macroscopic examination, and foetuses were removed by Caesarean section. Common litter parameters were recorded and foetuses were sexed, weighed and submitted to external examination. About one half of the foetuses were examined for soft tissue anomalies whereas remaining foetuses were examined for skeletal anomalies following alizarin red staining.

Results
There were 18, 23, 21 and 21 pregnant females in the 0, 50, 200 or 800 mg/kg bw/day groups, respectively. No deaths were reported. Clinical signs were increased salivation at 800 mg/kg bw/day and discoloured faeces at 200 mg/kg bw/day and higher. Discoloration of placenta was also noted in the highest dose group. No effects on litter parameters or foetal weight were observed. There were no external soft tissue or skeletal anomalies that, according to the study authors, could be attributed to treatment with the test substance.

Conclusion
The No Observed Adverse Effect Level (NOAEL) is 800 mg/kg bw/day (435 mg active dye/kg bw/day) for teratogenicity and for maternal toxicity.

Ref.: 15 (subm II)

Study 2

Guideline:	OECD 414
Species/strain:	Wistar rats
Group size:	22 females
Test substance:	Ext. D&C Violet n° 2
Batch:	0609RA
Purity:	93.8%
Dose:	0, 100, 300 and 1000 mg/kg bw/day (0, 94, 282 or 940 mg active dye/kg bw/day)
Treatment:	day 6 - 17 of gestation
GLP:	in compliance

The test substance (in 1% carboxymethylcellulose in water) was given daily at dose volumes of 10 ml/kg bw by oral gavage. The doses were selected on the basis of the results of a preliminary study in rats. Maternal evaluations and measurements included daily clinical signs and body weight/food intake recorded at designated intervals. The females were killed on gestation day 21, subjected to macroscopic examination, and foetuses were removed by Caesarean section. Common litter parameters were recorded and foetuses were sexed, weighed and submitted to external examination. About one half of the foetuses were also examined for soft tissue anomalies whereas remaining foetuses were examined for skeletal anomalies following alizarin red staining.

Results
There were 20 to 22 pregnant females per group. In the group who received 100 mg /kg bw/day one female had only embryonic resorptions and in the highest dose group two females were not pregnant, one female had only empty implantation sites and a further one only embryonic resorptions at Caesarean section. These findings were considered by the study authors to be incidental as a dose relation was missing. No deaths were reported, and clinical signs were limited to discoloured faeces at 1000 mg/kg bw/day. No effects on litter parameters or foetal weight were observed. Foetal and litter incidences of external, soft tissue and skeletal anomalies were similar for control and treated groups.

Conclusion
The No Observed Adverse Effect Level (NOAEL) for teratogenicity and maternal toxicity is 1000 mg/kg bw/day (940 mg active dye/kg bw/day).

Ref.: 16 (subm II)

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

(Acid Violet 43)

(non-oxidative conditions)

Absorption through the skin	**A**	**=**	**0.92 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.534 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.009 mg/kg bw/d**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**282 mg/kg bw/d**
50% bioavailability *		**=**	**141 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**15667**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14 Discussion

Different commercial forms of Acid Violet 43 with different analytical specifications and impurity profiles are available. Two commercial forms were used for the toxicity studies submitted, Jarocol Violet 43 (minimum titer 50%) and External D&C Violet n° 2 (minimum titer 80%).

Several toxicity endpoints (subchronic toxicity, induction of gene mutations or chromosome aberrations, embryo-foetal development toxicity and percutaneous penetration) were covered by both test materials though different test systems were often used. Acute eye irritation and skin sensitisation studies were conducted only on Jarocol Violet 43, skin irritation and acute oral toxicity studies were only available for External D&C Violet n° 2.

Physico-chemical specifications
Physico-chemical characterisation of test substances was incomplete. Analytical data are incomplete for Jarocol Violet 43, Batch No. 437/3 and for External D&C Violet n° 2 batches K7176 and 21CFR 9.411. The content of Acid Violet 43 in various batches, assayed by spectrophotometric method, can only be considered as semi-quantitative determination. Quantitative determination of Acid violet 43 in various batches using a reference standard is required. Content of heavy metals were only submitted for Jarocol Violet 43, Batch No 10130. A difference in sulphate content was noted between batch 2060208 and 10130. Stability of Acid Violet 43 in typical hair dye formulations was not reported.

Two forms were used for the toxicity studies submitted with different specifications and impurities and further forms with unknown specifications may be on the market.
Complete qualitative and quantitative chemical characterisation of all batches and quantitative determination of Acid violet 43 in all batches using a reference standard are required

According to the EU Cosmetics Directive 76/768/EEC, Annex IV Part I List of Colouring Agents, Acid Violet 43 is allowed as CI 60730 in cosmetic products except those intended to come into contact with mucous membranes.

General toxicity
In an acute oral toxicity study in rats, the maximal non-lethal dose Ext. D&C Violet n° 2 was higher than 4640 mg active dye/kg. In the in vivo/in vitro UDS assay with Jarocol Violet 43, however, the Maximal Tolerated Dose was 816 mg active dye/kg bw in rats.
In two separate oral toxicity studies in rats subchronic effects were evaluated. The No Observed Adverse Effect Level (NOAEL) was 109 mg active dye/kg bw/day for Jarocol Violet 43 (purity: 54.4%) and 282 mg active dye/kg bw/day for Ext. D&C Violet n° 2 (purity: 94%). The NOAEL from the study performed with Ext. D&C Violet n° 2 is used for the MOS calculation as this study has been performed with the highest purity of the test substance and according to OECD TG 408 and therefore, is considered as more reliable than the study performed with Jarocol Violet 43.
Both test substances were not embryotoxic or teratogenic up to 435 mg active dye/kg bw/day for Jarocol Violet 43 (purity: 54.4%) and 940 mg active dye/kg bw/day for Ext. D&C Violet n° 2 (purity: 94%).
No additional study on reproductive toxicity was submitted.

Irritation / sensitisation
Irritation studies showed that at 1% active dye is not irritant to rabbit skin (Ext. D&C Violet n° 2).
Under the conditions of this experiment 54115 (Acid Violet 43) at a concentration of 1% w/w active dye caused some eye irritation. It is unknown whether the 'impurities' in the tested product had an effect on this.
The maximum test concentration of 54115 (Acid Violet 43) was too low to exclude a sensitising potential.

Dermal absorption
This was a non-guideline study. The amount of Acid Violet 43 considered available from a semi-permanent (non-oxidative) hair dye formulation containing 0.12% (as active dye) is mean + 2SD. This is 0.23 µg active dye/cm² or 1.19% of the applied dose.
Since the test concentration was too low, it was corrected to 4 x 0.23 or 0.92 µg active dye/cm².

Carcinogenicity
The test procedure used was not adequate. Thus, no conclusion regarding carcinogenicity can be made

Mutagenicity
The test substance was not mutagenic in two separate bacterial reverse mutation tests performed either with Jarocol Violet 43 or with Ext. D&C Violet n° 2. Ext. D&C Violet n° 2 was negative in a Mouse Lymphoma Assay. A clastogenic potential *in vitro* was shown for Jarocol Violet 43, which was positive in human lymphocytes at high concentrations associated with overt cytotoxicity and clearly clastogenic in CHO cells. However, Jarocol

Violet 43 was not genotoxic when tested *in vivo* in an oral mouse bone marrow micronucleus study at 2000 mg/kg bw (1088 mg active dye) and also not genotoxic in an oral rat UDS test performed at the Maximal Tolerated Dose. No evidence of target organ exposure was demonstrated. In an additional *in vivo* micronucleus assay, where target organ exposure was demonstrated, no genotoxic effect was observed when tested at 2000 mg/kg bw (3344 mg Jarocol Violet 43). It is therefore concluded that Jaracol Violet 43 has no genotoxic potential *in vivo*.

4. CONCLUSION

The safety assessment of Acid Violet 43 relates to batch Ext D&C Violet n° 2 0609RA (purity of 94%).

The SCCS is of the opinion that the use of Acid Violet 43 as a non-oxidative hair dye with a maximum on head concentration of 0.5% active dye does not pose a risk to the health of the consumer.

A sensitising potential cannot be excluded.

Acid Violet 43 is also used as a colorant but this use has not been assessed in this opinion.

5. MINORITY OPINION

Not applicable

6. REFERENCES

The references in italics were not used.

Submission III, 2007

1. COLIPA. Response to the Comments of SCCP opinion on Submission II (SCCP/0964/05) on Acid Violet 43 (C063), September 14, 2006
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