

EUROPEAN COMMISSION  
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL  
Directorate C - Public Health and Risk Assessment  
**C7 - Risk assessment**

## **SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS**

### **SCCP**

#### **Opinion on**

#### **Acid Violet 43**

COLIPA N° C63

Adopted by the SCCP  
during the 7<sup>th</sup> plenary meeting of 28 March 2006

**TABLE OF CONTENTS**

1.	BACKGROUND	.....	3
2.	TERMS OF REFERENCE	.....	3
3.	OPINION	.....	3
4.	CONCLUSION	.....	23
5.	MINORITY OPINION	.....	23
6.	REFERENCES	.....	24
7.	ACKNOWLEDGEMENTS	.....	25

## 1. BACKGROUND

Submission I on Benzenesulfonic acid, 2-[(9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthracenyl)amino]-5-methyl-, monosodium salt was submitted by COLIPA (European Cosmetics Toiletry and Perfumery Association) in March 1984.

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (<http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf>) within the framework of the Cosmetics Directive 76/768/EEC.

## 2. TERMS OF REFERENCE

1. *Is Benzenesulfonic acid, 2-[(9,10-dihydro-4-hydroxy-9,10-dioxo-1-anthracenyl)amino]-5-methyl-, monosodium salt safe for use in hair dye formulations taken into account the data provided?*
2. *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?*

## 3. OPINION

### 3.1. Chemical and Physical Specifications

3.1.1. Chemical identity
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3.1.1.1. Primary name and/or INCI name
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Acid Violet 43 (INCI name)

3.1.1.2. Chemical names
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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
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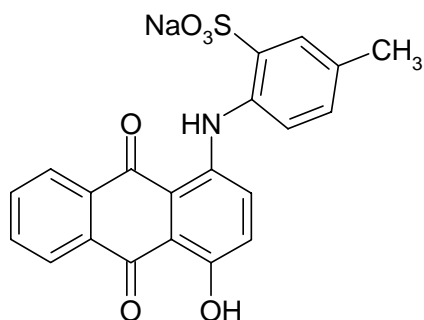
Trade names: Jarocol Violet 43  
Ext. D&C Violet No. 2  
Colour Index: C.I. 60730  
COLIPA: C063

## Opinion on Acid Violet 43

## 3.1.1.4. CAS / EINECS number

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

## 3.1.1.5. Structural formula



## 3.1.1.6. Empirical formula

Formula: C<sub>21</sub>H<sub>14</sub>NO<sub>6</sub>S, 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 No. 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 No. 2 (Batch 0609RA).

## Opinion on Acid Violet 43

Description		Jarocol Violet 43			Ext. D&C Violet No. 2*
		Batch No.			Batch No.
		2060208	10130	437/3	0609RA
Titre <sup>1</sup>	[%]	54.4	60.5	59.3	93.8
Impurities (HPLC)					
Acid Green 25		d	d	-	-
Isomer of Acid Green 25		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
p-Toluidine sulfonic acids, sodium salt	[%]	-	-	-	< 0.2
Subsidiary colors	[%]	-	-	-	< 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/100g	25.2	25.9	-	-
Water content (K.F.Method)	g/100g	9.3	8.26	-	-
Starch content <sup>2</sup> (HPIC)	g/100g	-	26	-	-
Sulphate ions <sup>3</sup> (HPIC)	g/100g	0.43	6.5	-	-
Chloride ions <sup>4</sup> (potentiometry)	g/100g	4.2	4.42	-	-
Sum of volatile matter	[%]	-	-	-	< 18
Water-insoluble matter	[%]	-	-	-	< 0.4
Residual solvents (GC)	-	n.d.	n.d.	-	-

- \* Data according to colour additive certificate and analytical specification required by FDA
- 1 spectrophotometry, Jarocol Violet 43: Purity by HPLC, UV-Vis spectra (Area % without response factor, UV detection at 570 nm). Infrared and <sup>1</sup>H-NMR spectra in accordance with proposed structure.
- 2 expressed as glucose
- 3 expressed as Na<sub>2</sub>SO<sub>4</sub>
- 4 expressed as NaCl
- d detected but not quantified due to lack of reference standard
- n.d. not detected
- no data

## 3.1.5. Impurities / accompanying contaminants

See 3.1.4.

## Opinion on Acid Violet 43

## 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

3.1.7. Partition coefficient (Log P<sub>ow</sub>)

n-octanol/water partition coefficient calculated:

Log P<sub>ow</sub>:

acid	:	3.1	(Jarocol Violet 43, batch 10130)
salt	:	1	(Jarocol Violet 43, batch 10130)

## 3.1.8. Additional physical and chemical specifications

melting point:	/
flash point:	/
vapour pressure:	/
boiling point:	/
density at 20 °C:	/
viscosity:	/
pKa:	/
UV absorption spectrum:	/
Refractive index at 20 °C:	/
Storage:	at room temperature, protected from light and under inert gas atmosphere

## 3.1.9. 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 2060208. Content of heavy metals were only submitted for Jarocol Violet 43, Batch No 10130. For External D&C Violet No. 2 only the analytical specification from FDA was submitted, and additionally the colour additive certificate for batch 0609RA;
- \* A difference in sulphate content was noted between batch 2060208 and 10130;
- \* Log P<sub>ow</sub> (calculated) is not acceptable;

- \* the stability in marketed formulations was not reported;
- \* no data were given on the solubility in the receptor fluid (dermal absorption study).

### 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 No. 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 No. 2. Toxicological evaluation was related to the amount of active dye in the test substance used for the investigations.

3.3.1. Acute toxicity
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3.3.1.1. Acute oral toxicity
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Guideline:	/
Species/strain:	Sprague-Dawley albino rats
Group size:	5 males per group
Test substance:	External D&C Violet No. 2 in a 0.5 % aqueous methyl cellulose solution
Batch:	/
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.

## Opinion on Acid Violet 43

## Results

No mortality occurred. The LD<sub>50</sub> 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

## 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. Eye irritation

Guideline:	OECD 405 (2002)
Species/strain:	New Zealand White rabbits
Group size:	3 males
Test substance:	Jarocol Violet 43
Batch:	10130
Purity:	60.5 %
Dose:	1 % active dye
GLP:	in compliance

A single dose of 0.1 ml Jarocol Violet 43 at a concentration of 1% active dye (w/v) 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 very slight chemosis was observed in 1/3 animals on days 1 and 2, and very slight or 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. Discharge was not scored on day 1 because of residual test item. All ocular reactions had disappeared by day 4.

Ref.: 3



## Opinion on Acid Violet 43

## 3.3.3. Skin sensitisation

Guideline:	OECD 429 (2002)
Species/strain:	CBA/J mice
Group size:	4 females / group
Test substance:	Jarocol 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)
GLP:	in compliance

Animals were separated in 7 groups (4 mice/group) consisting of 5 treated groups receiving Jarocol Violet 43, a negative control group receiving the vehicle (AOO) alone and a positive control group receiving alpha-hexylcinnamaldehyde (HCA), at 25% (v/v) in AOO. The vehicle was selected in a previous solubility study showing that 15% (w/v) active Jarocol 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 discrete erythema. No lymphoproliferation was observed at any tested concentration (SI ranging from 0.9 for 0.6% to 1.6 for 15% active dye).

Ref.: 4

## 3.3.4. Dermal / percutaneous absorption

## Study 1

Guideline:	/
Species/strain:	Porcine ear skin
Test substance:	Ext. D&C Violet No. 2
Batch:	0609RA
Purity:	95 %
Dose:	Experiment 1: 2 mg/ml; solution in 20 % (v/v) ethanol in water Experiment 2: 0.6 % in semi-permanent hair dye formulation six replicates in both experiments
Exposure time:	30 minutes
GLP:	in compliance

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 the first and 800-900 µm in the second experiment) 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 first experiment, the donor chamber of the diffusion cell was filled with 1 ml of the test solution, covered and left for 30 minutes. In the second experiment, 1.25 ml of the hair dye formulation was applied in the same conditions (1000 mg/cm<sup>2</sup> corresponding to 5 mg/cm<sup>2</sup> 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  $88.8 \pm 4.08$  % in the first and  $90.6 \pm 4.67$  % in the second experiment 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<sup>2</sup> in experiment 1 and 19.0 or 24.8 µg/cm<sup>2</sup> in experiment 2) and values for skin extracts ( $1.85 \pm 0.01$  µg or  $1.83 \pm 0.01$  µg/cm<sup>2</sup> in experiment 1 based on the detection limit of 500 ng/ml and  $5.82 \pm 2.67$  µg or  $5.75 \pm 2.6$  µg/cm<sup>2</sup> in experiment 2) the following rates for skin absorption might be estimated as worst case: 20.93 µg/cm<sup>2</sup> (approximately 1 %) in experiment 1 and 30.55 µg/cm<sup>2</sup> (approximately 0.6 %) in experiment 2.

Ref.: 17

## Study 2

Guideline:	/
Species/strain:	human (dermatomed skin)
Test substance:	Jarocol Violet 43
Batch:	437/3
Purity:	59.3 %
Dose:	0.12% in semi-permanent hair dye formulation (appr. 20 mg/cm <sup>2</sup> , corresponding to 25.4 µg active dye/cm <sup>2</sup> ) eight replicates
Exposure time:	30 minutes
GLP:	not in compliance

## Opinion on Acid Violet 43

Human skin samples were obtained from four female donors subjected to plastic surgery. Skin samples ( $380 \pm 25 \mu\text{m}$  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 Jarocol 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

Most of the test substance applied on the skin surface was removed with the washing procedure (about 104 % of the applied dose, in mean), and the total recovery rate was about 105 %. No Jarocol Violet 43 was measured in the receptor fluid. The mean absorbed amounts of Jarocol 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):  $0.11 \pm 0.06 \mu\text{g active dye}/\text{cm}^2$  ( $0.53 \pm 0.33$  % of the applied dose).

Ref.: 18

## Comment

Differences in assessed percutaneous absorption are related (a) to amounts in the stratum corneum, which was not separated from the epidermis and dermis in study No. 1 and (b) the calculation of a flux rate, which is based on a higher detection limit in study No. 1. Additionally, in study No. 1 the amount of formulation applied to the skin sample is higher than normally used for testing of skin penetration of hair dyes ( $1000 \text{ mg formulation}/\text{cm}^2$  used vs.  $20 \text{ mg formulation}/\text{cm}^2$  recommended by the SCCNFP). Study 1 therefore is considered to be inadequate.

Deficiencies were also noted in study 2: the batch was not adequately characterized, no data on solubility in the receptor fluid were provided and a high variability of the data was observed.

## 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

**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, 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, 27, 109 or 435 mg active dye/kg/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 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 macroscopic examination, selected organs were weighed, and organs/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 died during the study. Purplish colour and/or purplish and blue contents were observed in the lungs, in buccal and thoracic cavities and in the trachea of these females. Their death, therefore, was attributed to a gavage error and not to the test substance. No adverse clinical signs, no ocular findings or changes in body weight and food intake were reported. There were no intergroup differences in organ weights. Coloured urine, faeces, fur and extremities were observed at all dose levels and were related to the staining properties of Jarocol Violet 43. Findings at necropsy were dose-related discolorations of the digestive tract. Increased salivation was observed at all dose levels with a dose-dependent incidence. Loud breathing was noted in 1/10 females given 27 mg active dye/kg bw, 1/10 females given 109 mg active dye/kg bw, 2/10 females and 1/10 males given 435 mg active dye/kg bw. Regurgitation occurred in all treatment groups (10 – 30 %). No relevant histopathological changes were noted at examination of tissues and organs of treated animals.

Moderate but statistically significant changes in clinical laboratory parameters were: a decrease in inorganic phosphorus content for males in all dose groups; in the highest dose group decreases in values for leukocytes, lymphocytes, urea, alkaline phosphatase and alanine aminotransferase for males and decrease in glucose content for females. Furthermore in this group increases in mean clotting time values (males and females), activated partial thromboplastin time and mean cell haemoglobin concentration (males) were noted as well as increases in fibrinogen content and pH of urine in females.

The No Observed Adverse Effect Level was 109 mg active dye/kg bw.

Ref.: 5

## Oral, study 2

Guideline:	OECD 408
Species/strain:	Wistar rats
Group size:	10 animals per sex and group
Test substance:	External D&C Violet No. 2
Batch:	0609RA
Purity:	95%
Dose:	0, 95, 285 or 950 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), 95, 285 or 950 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 cageside 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 or changes in organ weights were reported. Dark blue feces were observed at all dose levels and were related to the staining properties of the test substance. Statistically significant decreases in motor activity were observed for females given 285 or 950 mg active dye/kg bw/day as well as increases in motor activity in males given 950 mg active dye/kg bw/day. In females effects were evident after 45 minutes and persisted in animals treated with 950 mg/kg bw/day until the end of the observation period. In males effects were observed after 15 minutes only. The dose-dependency of effects in females might give evidence, that effects could be related to the test substance. In the absence of other behavioural changes indicative of decreased activity or excitability of the central nervous system, however, effects were assessed to be not adverse.

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.

Moderate but statistically significant changes in several clinical laboratory parameters including increases in mean clotting time values and in activated partial thromboplastin time in males were reported for the highest dose group. The only findings at necropsy were a blue discolouration of the digestive tract observed for a few animals given 950 mg active dye/kg/day. No relevant histopathological changes were noted at examination of tissues and organs of treated animals. The No Observed Adverse Effect Level (NOAEL) was reported to be 950 mg active dye/kg bw. The SCCP, however, considers the effects on mean clotting time to be adverse and sets the NOAEL to 285 mg active dye/kg bw.

Ref.: 6

### 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 No. 2
Batch:	/
Purity:	> 80 %

## Opinion on Acid Violet 43

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

#### 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

#### 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 No. 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 No. 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 No. 2 did not increase the number of revertants in any strain in the presence or absence of S9.

Ref.: 8

***In Vitro* Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1997)
Species/strain:	Mouse lymphoma cell line L5178Y/TK <sup>+/-</sup>
Replicates:	Duplicates, two independent tests
Test substance:	Ext. D&C Violet No. 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 No. 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  $\beta$ -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 (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 No. 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 were 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 ( $\pm$ S9, 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, experiment 2).

Ref.: 9

***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



## Opinion on Acid Violet 43

Treatment time: 68, 272 and 408 µg dye/ml in experiment 2 with metabolic activation  
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 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 (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

## 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. 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, 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

### 3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

#### 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

### 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 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 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

### 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 No. 2  
 Batch: lot W-4566  
 Purity: 84 %  
 Dose: aqueous solution (first dose), 2 % dispersion in propylene glycol (2<sup>nd</sup> to 6<sup>th</sup> dose), 1 % dispersion in propylene glycol (7<sup>th</sup> to final dose), average estimated: 23 mg active dye/kg per application  
 Treatment: 103 weekly dermal applications over 107 weeks  
 GLP: not in compliance

## Opinion on Acid Violet 43

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. 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 No. 2-treated mice were not different from controls.

Ref.: 14

### 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
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3.3.8.1. Two generation reproduction toxicity
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No data submitted

3.3.8.2. Teratogenicity
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### Study 1

Guideline:	/
Species/strain:	Sprague Dawley rats
Group size:	25 females
Test substance:	Jarocol Violet 43
Batch:	2060208
Purity:	54.4 %
Dose:	0, 27.2, 108.8, and 435.2 mg active dye/kg/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 b.w. 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, 27, 109 and 435 mg active dye/kg/day groups, respectively. No deaths were reported. Clinical signs were increased salivation at 435 mg active dye/kg/day and discoloured faeces at 109 mg active dye/kg/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 could be attributed to treatment with the test substance. The No Observed Adverse Effect Level (NOAEL) is 435 mg active dye/kg/day for teratogenicity and for maternal toxicity.

Ref.: 15

### Study 2

Guideline:	OECD 414
Species/strain:	Wistar rats
Group size:	22 females
Test substance:	Ext. D&C Violet No. 2
Batch:	0609RA
Purity:	95 %
Dose:	0, 95, 285, and 950 mg active dye/kg/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 95 mg active dye/kg/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 to be incidental as a dose relation was missing. No deaths were reported, and clinical signs were limited to discoloured faeces at 950 mg active dye/kg/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. The No Observed Adverse Effect Level (NOAEL) for teratogenicity and maternal toxicity is 950 mg active dye/kg/day.

Ref.: 16

## Opinion on Acid Violet 43

3.3.9. Toxicokinetics
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No data submitted

3.3.10. Photo-induced toxicity
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3.3.10.1. Phototoxicity / photoirritation and photosensitisation
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No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity
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No data submitted

3.3.11. Human data
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No data submitted

3.3.12. Special investigations
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No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)
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#### CALCULATION OF THE MARGIN OF SAFETY

Not applicable

3.3.14. Discussion
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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 No. 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 No. 2.

#### *Physico-chemical specifications*

Physico-chemical characterisation of test substances was incomplete. 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.

#### *General toxicity*

In an acute oral toxicity study in rats, the maximal non-lethal dose Ext. D&C Violet No. 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. No Observed Adverse Effect Level (NOAEL) derived were 109 mg active dye/kg/day for Jarocol Violet 43 and 285 mg active dye/kg/day for Ext. D&C Violet No. 2.

Both test substances were not embryotoxic or teratogenic up to 435 mg active dye/kg/day for Jarocol Violet 43 and 950 mg active dye/kg/day for Ext. D&C Violet No. 2.

#### *Irritation / sensitisation*

Irritation studies showed that at 1% active dye is not irritant to rabbit skin (Ext. D&C Violet No. 2) and rabbit eye (Jarocol Violet 43). Jarocol Violet 43 was considered to be non-sensitising when tested in a murine Local Lymph Node Assay.

#### *Dermal absorption*

The studies were not performed according to the SCCP Notes of Guidance: the test substance was not properly characterised and the solubility in the receptor fluid was not given.

#### *Carcinogenicity*

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

#### *Mutagenicity*

The test substances were not mutagenic in two separate bacterial reverse mutation tests performed either with Jarocol Violet 43 or with Ext. D&C Violet No. 2. Ext. D&C Violet No. 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.

## **4. CONCLUSION**

The SCCP is of the opinion 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.

## **5. MINORITY OPINION**

Not applicable

## 6. REFERENCES

References in italics are not submitted as full reports in the present dossier. They consist of reports for studies which were considered to be inadequate for submission (19-25), reports for range finding toxicity studies (26-27, 30-31) or publications (28-29); they can be provided upon request.

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