Scientific Committee on Consumer Products

SCCP

OPINION ON

HC Violet n° 2

COLIPA n° B98

The SCCP adopted this opinion at its 13th plenary meeting on 2 October 2007

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

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

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

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

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Opinion to be cited as: Opinion of the SCCP on HC Violet n° 2

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1. BACKGROUND

Submission I for HC Violet n° 2 with the chemical name:1-γ-Hydroxypropylamino-2-nitro-4-bis-(ß-hydroxyethylamino)-benzene, also called earlier Imexine FAG, was submitted in September 1994 by COLIPA[1, 2].

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted at its plenary meeting on 23 June 1999 the opinion (SCCNFP/0142/99) stating:

"The SCCNFP is of the opinion that Imexine FAG can be used safely in semi-permanent hair dyes and colour setting lotions at a maximum concentration of 2.0%".

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 14 on the list of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission II for HC Violet n° 2 was submitted by COLIPA in July 2005. According to this submission the substance is used in semi-permanent (non-oxidative) hair dye formulations at a maximum concentration of 2.0%.

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. *Does the Scientific Committee on Consumer Products (SCCP) consider HC Violet n° 2 safe for use as a non-oxidative hair dye with a concentration of maximum 2.0 % taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use of HC Violet n° 2 in any non-oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

[2] According to records of COLIPA

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Violet n° 2 (INCI)

3.1.1.2. Chemical names

1-γ-Hydroxypropylamino-2-nitro-4-bis-(β-hydroxyethylamino)-benzene
3-[4-[bis-(2-hydroxyethyl)-amino]-2-nitro-phenylamino]-propan-1-ol
1-Propanol, 3-[[4-[bis(2-hydroxyethyl)amino]-2-nitrophenyl]amino],

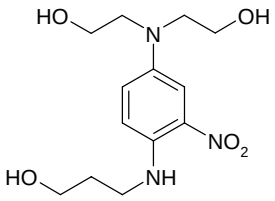
3.1.1.3. Trade names and abbreviations

IMEXINE FAG
COLIPA n° B98

3.1.1.4. CAS / EINECS number

CAS: 104226-19-9
ELINCS: 410-910-3 (Imexine FAG)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{13}H_{21}N_3O_5$

3.1.2. Physical form

Greenish-brown crystalline powder

3.1.3. Molecular weight

Molecular weight: 299

3.1.4. Purity, composition and substance codes

Deduced specifications for the Material used in the market

Purity by potentiometry: between 99.0 g/100g and 100.5 g/100g

Purity (rel.) by HPLC: ~ 99%

Total Impurities content: Not more than 1 %
Loss on drying: < 0.5 g/100g

Heavy Metals
- As, Sb, Hg: < 5 mg/kg each
- Cd: < 10 mg/kg
- Pb: < 20 mg/kg

Batches used
Four batches (see Table below) were used in the toxicological tests. Of them, only batch 0509045 was fully characterized.

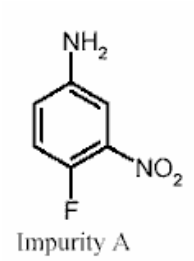
	HC Violet N°2 (B098)			
	0509045	**Op.9**	**Op.15**	**Op.M26**
Appearance	A greenish brown crystalline powder			
Titre by Potentiometry (g/100g)	99.2	100.3	100	99.7
UV_Vis. Spectrum	Comparable			
Water content(g/100g)	0.15			
Melting point(°C)	78.1 (DSC)	72.0 (Capillary tube)	73.4 (Capillary tube)	74.0 (Capillary tube)
H.P.T.L.C. Profile		Conforms to the standard		Conforms to the standard
Impurities(g/100g) H.P.L.C.				
Impurity A	< 0.1 (ND)			
Impurity B	< 0.1 (D)			
Impurity C	~ 0.25			
Residual solvent(g/100g) GC				
Methanol	< 0.1 (ND)			
Ethyl acetate	< 0.05 (D)			
Isopropanol	< 0.1 (ND)			
IR Spectrum	In accordance with the proposed structure			
Mass spectrum	Compatible with the proposed structure			
^{1}H and ^{13}C NMR Spectra	In accordance with the proposed structure			

D: Detected
ND: Not detected

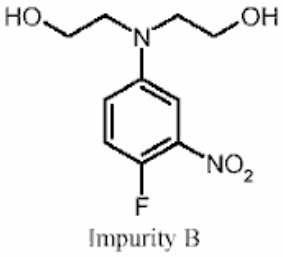
3.1.5. Impurities / accompanying contaminants

Possible impurities which may originate from reagents and intermediate reaction products were checked only in batch 0509045. Two unknown impurities were detected, of which one was tentatively characterized.

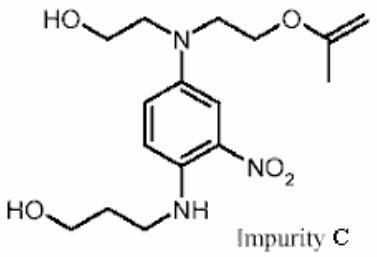
Impurity A: 4-Fluoro-3-nitro-phenylamine (starting material)
Impurity B: 2-[(4-Fluoro-3-nitro-phenyl)-(2-hydroxyethyl)-amino]-ethanol (intermediate reaction product)
Impurity C (t_R = 19.4 min.)**:** Proposed structure: 1-γ-Hydroxypropylamino-2-nitro-4-(β-Hydroxyethyl)-4-(β-acetylhydroxyethyl)-amino-Benzene (detected in batch 0509045 with a relative UV purity above 0.1%)
An other impurity (t_R = 20.1 min.): with a relative UV purity below 0.1%. Not characterized.



Impurity A



Impurity B



Impurity C

- Impurity A: < 0.1 g/100g in batch 0509045
- Impurity B: < 0.1 g/100g in batch 0509045
- Impurity C: ~ 0.25 g/100g in batch 0509045
- Unknown Impurity: < 0.1 g/100g in batch 0509045

3.1.6. Solubility

in water : > 64.8 g/100 ml at 20 °C according to EEC method A6 and Supplier MSDS
in ethanol : 10 ≤ S < 20 g/100 ml at 22 °C after 24 hours
in DMSO : ≥ 20 g/100 ml at 22 °C after 24 hours

3.1.7. Partition coefficient (Log P_{ow})

Log $P_{o/w}$: 0.608 at 23°C (according to EEC method A8)

3.1.8. Additional physical and chemical specifications

Melting point:	70 – 80 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	/
UV_Vis spectrum	λ_{max} : 539 nm ± 5 nm in ethanol solution (ε = 130 - 140)
	λ_{max} : 262 nm and 550 nm

3.1.9. Stability

The stability of the test item in the dosage forms at 0.1 and 200 mg/ml in purified water and at 10 and 500 mg/ml in DMF was satisfactory over a 4-hour period at room temperature, protected from light and under inert gas atmosphere.

General Comments to physico-chemical characterisation

- The test substance is a secondary (and tertiary) amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb. Data on nitrosamine content is not provided.
- Only one batch was fully characterized.
- The stability of the test substance in marketed products is not reported.

3.2. Function and uses

HC Violet N°2 is used in non-oxidative (semi-permanent) hair colouring products at a maximum concentration of 2.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 420 (2001)
Species/strain:	Sprague-Dawley Rj:SD (IOPS Han).
Group size:	1 female sighting test, 4 females main experiment
Test substance:	HC Violet No. 2 (B098)
Batch:	0509045
Purity:	99% (potentiometry)
Dose:	2000 mg/kg bw in water
Route:	oral, gavage
Exposure:	once
GLP:	in compliance

The acute oral toxicity of the HC Violet No. 2 (B098) was evaluated in rats. In a sighting test the test item was administered at the dose of 2000 mg/kg to one female animal. As no mortality was observed at this dose the test item was administered at the dose-level of 2000 mg/kg to a group of four females.

Results
At the 2000 mg/kg dose-level, 1/4 animals died on day 1 within 2 hours following the treatment. Hypoactivity or sedation, piloerection and dyspnea were observed prior to death in this animal as well as in the surviving animals on day 1. Recovery was complete in the surviving animals on day 2. A purple coloration of the fur and tail was observed in all animals from day 1 up to day 15 (end of the observation period). The body weight gain of the surviving animals given 2000 mg/kg was not affected by treatment with the test item. Macroscopic examination of the main organs of the animals revealed no apparent abnormalities.

Conclusion
Under the experimental conditions, the LD50 of the test item HC Violet No. 2 (B098) was higher than 2000 mg/kg.

Ref.: 1

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402 (1981)
Species/strain:	Sprague-Dawley (Charles River)
Group size:	5 per sex
Test substance:	Imexine FAG
Batch:	Op15
Purity:	100% (potentiometry)
Dose:	2000 mg/kg bw, neat
Route:	epidermal, semi-occluded
Exposure:	24 h

GLP: in compliance

5 male and 5 female rats were treated with the test substance on 5 cm x 4 cm clipped skin of the back and flanks. A piece of surgical gauze was placed over the treatment area and semi-occluded with self-adhesive bandage. After 24 h exposure the skin was cleaned.

Results
No deaths were observed. Purple staining of the fur and skin was noted, no signs of systemic toxicity or skin irritation were observed. Body weight changes were as expected and no findings were noted at necropsy.

Conclusion
The acute dermal toxicity of Imexine FAG was higher than 2000 mg/kg bw.

Ref.: 2

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404
Species:	New Zealand White
Group:	3 females
Substance:	IMEXINE FAG
Batch:	OP. 9
Purity:	99%
Dose:	0.5g moistened 2x3 cm patch, 4 hour semi occlusion
Vehicle:	/
GLP:	in compliance

The day before application, the back area of test animals was clipped. On day 1, 0.5g of undiluted, moistened IMEXINE FAG was applied to the intact skin of the shaved area of animals and held in contact with skin for 4 hours by means of a semi-occlusive Metalline dressing. The dressing was then removed, and cutaneous reactions were assessed 1, 24, 48 and 72 hours after dressing removal. The skin area of all animals was shaved again before the observation on day 2, 3 and 4.

Results
There were no cutaneous reactions apart from a purple skin colouration in all animals, which did not interfere with the scoring of irritation.

Conclusion
Undiluted IMEXINE FAG was non-irritant to intact rabbit skin in the conditions of this study.

Ref.: 3

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405
Species:	New Zealand White
Group:	3 females
Substance:	IMEXINE FAG
Batch:	OP. 9
Purity:	99%

Dose:	68 ± 2mg (equivalent to 0.1 ml) into the right conjunctival sac
Vehicle:	/
GLP:	in compliance

On day 1, 68 mg undiluted, moistened IMEXINE FAG was instilled into the right conjunctival sac of test animals. The lids were then gently held together for about one second to prevent loss of the test substance. The eyes were not rinsed following instillation of the test item. The non-treated left eye served as control. The ocular reactions were assessed 1, 24, 48 and 72 hours after instillation.

Results
Ocular reactions were limited to conjunctival reactions. They consisted of a very slight chemosis (grade 1 in 2/3 animals) present only 1 hour after instillation, and a very slight redness (all animals) which did not persist beyond day 2. There were no corneal or iridal reactions.

Conclusion
Undiluted IMEXINE FAG produced transient eye irritation under the conditions of the experiment.

Ref.: 4

3.3.3. Skin sensitisation

Local Lymph Node Assay

Guideline:	OECD 429
Species:	CBA/J female mice
Group:	2 independent experiments, each with 28 animals, (5 groups of 4 treated, negative, positive controls)
Substance:	HC Violet No 2
Batch:	0509045
Purity:	99.2%
Dose:	Experiment 1: 2.5, 5, 10, 25 and 50% Experiment 2: 2.5, 5, 10, 20 and 40%
Vehicle:	dimethylformamide (DMF)
Control:	negative: vehicle; positive α-hexylcinnamaldehyde 25% in DMF
GLP:	in compliance

The test item was then tested in two independent experiments, each consisting of:
- five treated groups receiving the test item HC Violet N°2 at the chosen concentrations,
- one negative control group receiving the vehicle (dimethylformamide = DMF),
- one positive control group receiving the reference item, alpha-hexylcinnamaldehyde (HCA), at the concentration of 25% (v/v) in DMF.

For the first experiment, the highest tested HC Violet N°2 concentration (50% in DMF) was selected based on the solubility and preliminary assays. As positive results were obtained in the first experiment and in order to determine more precisely its EC_3 value, HC Violet N°2 was then tested in a second experiment at 2.5, 5, 10, 20 and 40%.

In each experiment, HC Violet N°2, DMF or HCA was applied over the ears (25 µL per ear) of respective mice for three consecutive days designated as days 1, 2 and 3. After 2 days of resting (on day 6), mice received a single intravenous injection of tritiated methyl thymidine (^{3}H-TdR). Lymph nodes draining the application sites (auricular nodes) were sampled, pooled per group, and the proliferation of lymphocytes was evaluated by measuring the incorporation of ^{3}H-TdR. The values obtained were used to calculate stimulation indices (SI). The irritant potential of the test item was assessed by measuring ear thickness on days 1, 2, 3 and 6.

Results
SI values of 12.8 and 17 were obtained in the HCA positive control group in the first and the second experiments, respectively. There were no irritation reactions attributed to HC Violet n° 2.

In the first experiment, positive lymphoproliferative responses were noted at the concentrations of 10 and 25% (SI values: 3.4 and 4.1, respectively). However, at 50% the SI value decreased markedly (0.9), as well as the amount of lymph node cells (cellularity index: -47%), though the cell viability remained unaffected.

Concentration %	SI
2.5	2.08
5	2.91
10	3.39
25	4.11
50	0.86
HCA 25 (positive control)	12.80

In the absence of local irritation, the positive dose-related lymphoproliferative responses observed at 10 and 25% were attributed to contact sensitisation. The EC_3 value for the test item HC Violet N°2 calculated on the basis of the results obtained in the first experiment was 5.9%.

To investigate the nature of the response observed at high concentrations of HC Violet N°2 in the first assay, a second experiment was conducted using two additional concentrations (20% and 40%).

Concentration %	SI
2.5	4.19
5	5.59
10	2.83
20	2.74
40	2.00
HCA 25 (positive control)	17.00

In this assay, positive, dose-related lymphoproliferative responses were observed at the concentrations of 2.5 and 5% (SI values: 4.2 and 5.6, respectively). At higher concentrations however (10, 20 and 40%), no evidence of a dose-response relationship was noted with the SI values (2.8, 2.7 and 2.0, respectively) or with the concurrent cellularity indexes (1.9, 1.9 and 2.0, respectively). The explanation of this decrease of lymph node cells proliferation observed at high concentrations in the LLNA remained unclear. The results obtained in this second experiment did not allow the calculation of an EC_3 value.

Conclusion
Under the conditions of this study, HC Violet N°2 induced contact sensitisation in mice in the Local Lymph Node Assay and is a moderate sensitiser.

Ref.: 5

3.3.4. Dermal / percutaneous absorption

***In Vitro* Percutaneous Absorption Study through Human Dermatomed Skin**

Guideline: OECD 428
Tissue: human skin; dermatomed thickness 400µm
Group size: total of 11 intact membranes, 7 female donors

Diffusion cells:	flow through
Skin integrity:	tritiated water. K_p < 2.5 x 10^{-3} cm h^{-1} selected
Test substance:	HC Violet n° 2
	[ring-U-^{14}C]-HC Violet n° 2 (labelled)
Batch:	0509045
	CFQ13622 Batch 1 (labelled)
Purity:	99.2% (w/w)
	99.1% (labelled)
Test item:	1.93% HC Violet n° 2 in semi permanent dye formulation
Doses:	20 mg/cm² of hair dye formulation (389µg/cm^2 test substance)
Receptor fluid:	PBS
Solubility receptor fluid:	> 170mg mL^{-1}
Stability:	stable
Method of Analysis:	liquid scintillation counter
GLP:	in compliance

Eleven samples (one to three replicates per donor) were mounted in flow-through diffusion cells with calcium and magnesium free phosphate buffer saline (PBS) as receptor fluid. Their integrity was verified by determination of the permeation coefficient for tritiated water (Kp < 2.5 x 10^{-3} cm/h for all selected membranes).

HC Violet N°2 was tested as a semi-permanent hair dye in a typical formulation containing 1.93% (w/w) of unlabelled and [^{14}C]-labelled dye. 20 mg/cm² of the hair dye formulation (corresponding to a mean application rate of 389 µg/cm² of HC Violet N°2) were applied to the skin surface for 30 minutes. At the end of the exposure period, any of the formulation remaining on the skin surface was removed using a standardized washing procedure. Twenty four hours after application, the percutaneous absorption of HC Violet N°2 was determined by measuring the concentration of the dye by liquid scintillation counting (LSC) for liquid samples and/or combustion/LSC (other samples) in the following compartments: skin wash (dislodgeable dose), stratum corneum, epidermis + dermis, and receptor fluid.

Results
11 samples tested yielded data that could be analysed. Under the experimental conditions, most of the hair dye was washed off after 30 minutes of exposure. The dislodgeable dose represented 98.84% of the applied dose for a mean recovery of 99.41%.

The dermal delivery (sum of the amounts measured in the epidermis + dermis + receptor fluid) was 0.55 ± 0.55 μg_{eq}/cm² (range: 0.06 - 1.44 μg_{eq}/cm²) or (0.14 ± 0.14 %) range: 0.01 – 0.37%)

	μg_{eq}/cm²	**% applied dose**
Dislodgeable dose	381.25 ± 14.03	98.84 ± 3.64
Unabsorbed dose *	382.89 ± 13.99	99.27 ± 3.63
Absorbed dose **	0.08 ± 0.08	0.02 ± 0.02
Dermal delivery ***	0.55 ± 0.55	0.14 ± 0.14
Mass Balance	383.44 ± 13.99	99.41 ± 3.63

* Unabsorbed dose = dislogeable dose + Stratum corneum
** Absorbed dose = receptor fluid + receptor rinse
*** Dermal delivery = skin (except stratum corneum) + absorbed dose

Conclusion
The dermal absorption (sum of the amounts measured in the epidermis + dermis + receptor fluid) of HC Violet N°2 incorporated at 1.93% in a typical semi-permanent hair dye formulation was estimated to be 0.55 ± 0.55 μg_{eq}/cm² (range: 0.06 - 1.44 μg_{eq}/cm²) or (0.14 ± 0.14 %) range: 0.01 – 0.37%) in use conditions. As only 11 test chambers were used, the A_{max} of 1.44 μg_{eq}/cm² may be used for calculating the MOS.

Ref.: 13

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

No data submitted

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

Guideline:	OECD 408
Species/strain:	rats, Crl:CD (SD) BR strain (VAF plus)
Group size:	10 per sex per group
Test substance:	Imexine FAG suspended in 0.5 % aqueous carboxymethylcellulose
Batch:	oP M 26
Purity:	99.7 % (potentiometry)
Dose:	0, 50, 200 and 800 mg/kg bw/d
Route:	oral, gavage
Exposure:	once daily for 13 weeks
GLP:	in compliance

The test substance was applied once daily for 13 weeks at doses of 0, 50, 200 and 800 mg/kg bw/d in 0.5 % aqueous carboxymethylcellulose to the animals by oral gavage. The animals were observed daily for mortality and clinical signs. They were weighed weekly and their feed consumption was recorded. Ophthalmoscopy of all animals was performed before the start of the treatment, controls and high dose groups were also examined during week 13. Urinalysis, haematology and blood chemistry examinations were performed on all animals during week 12 or 13, respectively. At the end of the treatment period all animals were subjected to necroscopy, organs were weighed and several tissues were analysed microscopically.

Results
With the exception of one death due to mis-dosing no mortality and clinical signs of toxicity were found, but fur, extremities and urine were purple-discoloured. Body weight gain, feed consumption and ophthalmoscopy revealed no substance-related changes or abnormalities. Blood biochemistry values were unaffected. In haematology, decreases in red blood cell count (RBC), mean haemoglobin concentration and packed cell volume of females were noted at 800 mg/kg bw/d as well as a slightly prolonged prothrombin time (PT). At 50 and 200 mg/kg bw/d small but statistically significant decreases in RBC and PT were found.
Because of staining of the urine the evaluation of several parameters was inhibited. At 800 mg/kg bw/d increases in liver (absolute and relative) and kidney (relative) weights were found for both sexes, at 200 mg/kg bw/d also liver weight changes in males (absolute and relative) and females (relative) were observed. At this dose only in females the relative kidney weight was affected. But no histopathological changes were seen.

Conclusion
The study authors concluded that the NOEL is 50 mg/kg bw/d.

Ref.: 6

Comment of the SCCP
Since the borderline change of one haematological parameter at all doses was not considered toxicologically significant, the NOAEL of 50 mg/kg bw/d proposed by the study authors was accepted.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline:	OECD 471
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538 and *Escherichia coli* WP2 uvrA$^-$
Replicates:	3 replicates in 2 individual experiments both in the presence and absence of S9-mix.
Test substance:	Imexine FAG
Solvent:	sterile distilled water
Batch:	Op15
Purity:	100% (potentiometry)
Concentrations:	Experiment 1: 8 - 5000 µg/plate without and with S9-mix Experiment 2: 312.5 - 5000 µg/plate without and with S9-mix
Treatment:	direct plate incorporation method with 48 h incubation time without and with S9-mix
GLP:	In compliance

Imexine FAG was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in a preliminary toxicity study with strains TA100 and WP2 uvrA$^-$. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and/or thinning of the bacterial background lawn. Imexine FAG was non toxic in this preliminary toxicity test and thus tested up to the prescribed maximum concentration of 5000 µg/plate. Both experiment 1 and 2 were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Toxicity was only seen in experiment 1 in TA98 without S9-mix at 5000 µg/plate. This was not observed in the second experiment and not seen in any other strain used without or with S9-mix in both experiments.
In both experiments, no significant, biologically relevant increases in the number of revertant colonies of bacteria were recorded for any of the strains used at any dose level either without or with metabolic activation.

Conclusion
Under the experimental conditions used Imexine FAG was not genotoxic (mutagenic) in the gene mutation tests in bacteria.

Ref.: 7

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

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	2 replicates in 3 independent experiments
Test substance:	HC Violet No. 2

Solvent:	purified water
Batch:	0509045
Purity:	99.2% (potentiometry)
Concentrations:	Experiment 1: 0.63 – 10 mM, without and with S9-mix Experiment 2: 6 - 10 mM, without S9-mix 5 - 10 mM with S9-mix Experiment 3: 5 – 10 mM, with S9-mix
Treatment	3 h both without and with S9-mix; expression period of 48, selection growth 11-12 days.
GLP:	In compliance

HC Violet n° 2 was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a preliminary toxicity test measuring adjusted relative suspension growth. In the main test, cells were treated for 3 h followed by an expression period of 48 h to fix the DNA damage into a stable *tk* mutation. Colony sizing was performed to investigate for putative clastogenic effects of HC Violet No. 2. Liver S9 fraction from Arachlor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured as adjusted relative total growth of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
The appropriate level of toxicity (10-20% adjusted relative total growth after the highest dose) was only reached in some experiments at high concentrations (7.7 and 10 mM), while in the other experiments lower and variable levels of toxicity were measured even for the highest concentration of 10 mM.
In both experiments without metabolic activation HC Violet No. 2 did not induce a biologically relevant increase in the mutant frequency. With metabolic activation a 1.9 fold increase was seen in experiment 1 at a dose level of 10 mM and a 2.1 to 2.5 fold increase in experiment 2 at dose levels of 8 and 10 mM were observed. These increases were considered not biologically relevant since they were not in a dose response relationship, they were not reproduced in the third confirmatory experiment and they did not exceed the global evaluation factor of 125 x 10^{-6} for the microwell method (current proposed value). The ratio of small colonies *versus* large colonies was not shifted as compared to the solvent controls in any of the experiments.

Conclusion
Under the experimental conditions used, HC Violet No. 2 was considered not genotoxic (mutagenic and/or clastogenic) in the mouse lymphoma assay at the *tk* locus.

Ref.: 10

In vitro Mammalian Chromosome Aberration Test (1)

Guideline:	OECD 473
Replicates:	duplicate cultures
Cells:	CHO (K_1-BH_4)
Test substance:	Imexine FAG
Solvent:	DMSO
Batch:	Op 15
Purity:	100% (potentiometry)
Concentrations:	experiment 1: 625 – 2500 µg/ml in the absence and presence of S9-mix experiment 2: 625 – 2500 µg/ml in the absence of S9-mix 1250 – 5000 µg/ml in the presence of S9-mix
Treatment:	experiment 1: 20 h without S9-mix; harvest 20 h after start of treatment

		4 h with S9-mix; harvest 16 h after start of treatment
	experiment 2:	44 h without S9-mix; harvest 44 h after start of treatment
		4 h with S9-mix; harvest 40 h after start of treatment
GLP:	In compliance	

Imexine FAG has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. Test concentrations were based on the results of a preliminary cytotoxicity test measuring growth inhibition estimated by counting the number of cells at the end of the culture period and expressing this cell count as a percentage of the concurrent negative control value. In the absence of S9-mix, cells were treated for 20 h (experiment 1) or 44 h (experiment 2) and immediately harvested; in the presence of S9-mix, cells were treated for 4 h and harvested 16 h (experiment 1) or 40 h (experiment 2) after the start of treatment. Two hours before harvest, each culture was treated with colchicine solution (final concentration 0.1 µg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring growth inhibition relative to the concurrent controls. Chromosome (metaphase) preparations were stained with 2% Gurrs Giemsa and examined microscopically for chromosomal aberrations. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Exposure of the cells to Imexine FAG, measured as growth inhibition relative to the concurrent control, was sufficient.
A biologically relevant and more or less dose dependent increase in cells with chromosomal aberrations was seen in both experiments in the absence as well as in the presence of S9-mix.
A dose dependent and biologically relevant increase in polyploid cells was observed in experiment 1 whereas occasional increases were seen in experiment 2. A biologically relevant and dose dependent increase in endoreduplicated cells was also observed after treatment with Imexine FAG in the presence of S9-mix in experiment 1.

Conclusion
Under the experimental conditions used Imexine FAG is genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 8

In vitro Mammalian Chromosome Aberration Test (2)

Guideline:	OECD 473	
Replicates:	duplicate cultures	
Cells:	CHO	
Test substance:	HC Violet No. 2	
Solvent:	purified water	
Batch:	0509045	
Purity:	99.2% (potentiometry)	
Concentrations:	experiment 1:	1250 - 3000 µg/ml without S9-mix
		750 - 3000 µg/ml with S9-mix
	experiment 2:	125 - 1000 µg/ml without S9-mix
		1000 - 3000 µg/ml with S9-mix
	experiment 3:	250 - 1300 µg/ml without S9-mix
		1500 - 3000 µg/ml with S9-mix
	experiment 4:	250 - 1200 µg/ml without S9-mix
		125 - 750 µg/ml without S9-mix
Treatment:	experiment 1:	3 h both without and with S9-mix, harvest time 17 h after start of treatment

	experiment 2:	20 h without S9-mix, harvest time 20 h after start of treatment 44 h without S9-mix, harvest time 44 h after start of treatment 3 h with S9-mix, harvest time 17 h after start of treatment
	experiment 3:	20 h without S9-mix, harvest time 20 h after start of treatment 3 h with S9-mix, harvest time 17 h after start of treatment
	experiment 4:	20 h without S9-mix, harvest time 20 h after start of treatment 44 h without S9-mix, harvest time 44 h after start of treatment
GLP:	In compliance	

HC Violet No. 2 has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. From a range of concentrations up to the top one of 3000 µg/ml (about the prescribed maximum concentration of 10 mM), the dose level causing approximately 50% cell number relative to the concurrent control was used as the highest dose level. In the absence of S9-mix cells were treated for 20 h or 44 h and immediately harvested; in the presence of S9-mix, cells were treated for 3 h and harvested 17 h after the start of treatment. Two hours before harvest, each culture was treated with colchicine solution (final concentration 1.0 µg/ml) to block cells at metaphase of mitosis. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring the decrease in relative cell number. Chromosome (metaphase) preparations were stained with 4% Giemsa and examined microscopically for chromosomal aberrations. Negative and positive controls were in accordance with the OECD draft guideline.

Results
The relative cell number was in most experiments sufficiently reduced to about 50% compared to the negative control both in the absence or presence of S9-mix. Only in experiment 1 (19% reduction) and experiment 4 (20 h treatment, 76 % reduction) deviating values were observed.
In the absence of S9-mix HC Violet No. 2 did not induce a biological relevant and dose related increase in cells with chromosome aberrations. Occasional exceptions fell within the historical negative control ranges.
In the presence of S9-mix, treatment with HC Violet n° 2 resulted in increases of cells with chromosomal aberrations at 3000 µg/ml exceeding the historical negative control ranges in experiment 1 and 3. In experiment 2 at similar toxicity (49% *vs* 52%) an increase was not seen.
There were also small increases in the frequencies of endoreduplicated cells in experiment 1 only: without S9-mix at 2250 µg/ml and with S9-mix at 1500 and 3000 µg/ml.

Conclusion
Under the experimental conditions used HC Violet No. 2 is considered genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 9

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	CD1 mice
Group size:	5 mice/sex/group

Test substance:	Imexine FAG
Batch:	OP 15
Purity:	100% (potentiometry)
Dose level:	5000 mg/kg bw
Route:	gavage, once
Vehicle:	distilled water
Sacrifice times:	24, 48 and 72 h after treatment.
GLP:	In compliance

Imexine FAG has been investigated for the induction of micronuclei in bone marrow cells of mice. The test concentration was based on the result of a range finding toxicity study in which mice were exposed by gavage to 5000 mg/kg. Animals were observed 1 h after dosing and subsequently once daily for 3 days. This dose was selected as the maximum recommended dose as clinical signs were observed without premature deaths. Bone marrow cells were collected 24, 48 and 72 h after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between normochromatic to polychromatic erythrocytes (NCE/PCE ratio). Moreover, all animals were observed daily for signs of overt toxicity and death. Bone marrow preparations were stained and examined microscopically for the NCE/PCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
There were no premature deaths in any of the dose groups. Clinical signs observed after treatment with Imexine FAG were diuresis, blue coloration of the urine, extremities and fur. The red-coloured urine of the treated animals indicated to systemic distribution and thus bioavailability of Imexine FAG. Also the ratio NCE/PCE, which was significantly changed in the 72 h group as compared to the untreated controls indicating to cytotoxic properties in the bone marrow, demonstrated the bioavailability of Imexine FAG
Biological relevant increases in the number of micronucleated PCEs compared to the concurrent vehicle controls were not found following treatment with Imexine FAG.

Conclusion
Under the experimental conditions used, the test substance did not induce micronuclei in bone marrow cells of treated mice and, consequently, Imexine FAG is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 11

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	OECD 414 (1981)
Species/strain:	Sprague-Dawley rat (Charles River)
Group size:	25 mated females
Batch:	Op 9
Purity:	100.3 % (potentiometry)
Test substance :	0, 500 and 2500 mg/kg bw/d Imexine FAG suspended in 0.5 % carboxymethylcellulose and PEG 200 (5/50, w/w)

Route: oral, gavage
Exposure: day 6 to 15 of gestation
GLP: in compliance

The test substance was administered to 25 mated females per dose group from day 6 to 15 of gestation at the doses 0, 500 and 2500 mg/kg bw/d. The animals were observed for mortality and clinical signs. The animals were weighed on days 0, 6, 9, 12, 15 and 20 of gestation and feed consumption was determined for the respective periods. On day 20 of gestation the animals were sacrificed, the foetuses were taken and the common parameters were recorded. Skeletal and soft tissues anomalies were analysed using routine methods.

Results
2 deaths (1 in the control, 1 in the medium dose group) were assigned to misdosing. No clinical signs were observed in the control group while all test substance-treated animals showed purplish coloured urine. No changes in maternal body weight gain and feed consumption were observed. The numbers of resorptions and foetal body weight were unaffected by treatment. One foetus of the medium dose exhibited microphthalmia. No treatment related foetal skeletal and soft tissue anomalies were noted. No treatment-related changes in frequency of variations were found.

Conclusion
Under the experimental conditions HC Violet N°2 showed no maternal and embryotoxicity as well as no teratogenicity at the doses 500 and 2500 mg/kg bw/d. The NOAEL of maternal toxicity, embryo/foetotoxicity and teratogenicity is 2500 mg/kg bw/d.

Ref.: 12

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

(HC Violet n° 2)
(Direct / semi-permanent)

Maximum absorption through the skin	**A (µg/cm²)**	**=**	**1.44 µg/cm²**
Skin Area surface	**SAS (cm²)**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.008 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.017 mg/kg bw**
No observed effect level (90-day, oral, rat)	**NOAEL**	**=**	**50 mg/kg bw**
Margin of Safety	**NOAEL / SED**	**=**	**2941**

3.3.14. Discussion

Physico-chemical properties
HC Violet n° 2 is a secondary (and tertiary) amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb. Data on nitrosamine content is not provided. Only one batch was fully characterized.
The stability of the test substance in marketed products was not reported.

General toxicity
Under the experimental conditions, the LD50 of HC Violet n° 2 was higher than 2000 mg/kg. The acute dermal toxicity was higher than 2000 mg/kg bw.
Since the borderline change of one haematological parameter at all doses was not considered toxicologically significant, the NOAEL of 50 mg/kg bw/d proposed by the study authors was accepted.
The NOAEL of maternal toxicity, embryo/foetotoxicity and teratogenicity was 2500 mg/kg bw/d.

Irritation / sensitisation
Undiluted IMEXINE FAG was non-irritant to intact rabbit skin. It produced transient eye irritation.
HC Violet n° 2 induced contact sensitisation in mice in the Local Lymph Node Assay. It is a moderate sensitiser.

Dermal absorption
The dermal absorption (sum of the amounts measured in the epidermis + dermis + receptor fluid) of HC Violet n° 2 incorporated at 1.93% in a typical semi-permanent hair dye formulation was estimated to be 0.55 ± 0.55 $\mu g_{eq}/cm^2$ (range: 0.06 - 1.44 $\mu g_{eq}/cm^2$) or (0.14 ± 0.14 %) range: 0.01 – 0.37%) in use conditions. As only 11 test chambers were used, the A_{max} of 1.44 $\mu g_{eq}/cm^2$ may be used for calculating the MOS.

Mutagenicity / genotoxicity
The genotoxicity of Imexine FAG/ HC Violet n° 2 was investigated for the three types of mutation: gene mutation, structural chromosome mutation and aneuploidy.
Imexine FAG/ HC Violet No. 2 did not induce gene mutations neither in bacteria nor in mammalian cells at the *tk*-locus of mouse lymphoma cells. Although not observed in the mouse lymphoma assay, Imexine FAG/ HC Violet No. 2 was considered an *in vitro* clastogen inducing an increase in cells with chromosomal aberrations, polyploidy and endoreduplication in two *in vitro* chromosome aberration tests (CHO cells). These positive findings could not be confirmed in an *in vivo* micronucleus test in mice; Imexine FAG/ HC Violet No. 2 did not induce an increase in micronucleated bone marrow cells.

As Imexine FAG/ HC Violet No. 2 did not induce gene mutations and the positive results of the *in vitro* chromosomal aberrations test could not be confirmed in an *in vivo* micronucleus rest, Imexine FAG/ HC Violet No. 2 can be considered to have no relevant genotoxic potential *in vivo*.

Carcinogenicity
No data submitted

4. CONCLUSION

This risk assessment relates to the use of HC Violet n° 2 in non-oxidative hair dye formulations only.

HC Violet n° 2 is a secondary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

Apart from its moderate sensitising potential, the SCCP is of the opinion that the use of HC Violet n° 2 as a non-oxidative hair dye at a maximum concentration of 2.0% on the head does not pose a risk to the health of the consumer.

5. MINORITY OPINION

Not applicable

6. REFERENCES

The references in italics (15-23) were not submitted by the applicant as full reports in the present dossier, since the studies reported therein were not considered to be adequate.

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