EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

4-Hydroxypropylamino-3-nitrophenol

COLIPA n° B100

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

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

About the Scientific Committees

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

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

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

SCCP

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

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http://ec.europa.eu/health/ph_risk/risk_en.htm

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Opinion to be cited as: SCCP (Scientific Committee on Consumer Products), Opinion on 4-hydroxypropylamino-3-nitrophenol, 18 December 2007

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1. BACKGROUND

Submission I of 4-hydroxypropylamino-3-nitrophenol (B100) was submitted by COLIPA[1] in September 1994 according to COLIPA. The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) expressed its opinion (SCCNFP/0186/99) in the meeting on 23 June 1999 with the conclusion, that:

the substance can be used safely as an oxidative hair dye at a maximum concentration of 5.2% and in semi permanent hair dying products with a concentration of 2.6%. Both product types should bear a label with the warning of risk of sensitisation.
The substance had a margin of safety at 89 for use as an oxidative hair dye based on a NOAEL of 10 mg/kg bw from a subchronic oral rat study.

The substance and its salts are currently regulated in annex III/2, 7.

Submission II was submitted by COLIPA in June 2005. 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 4-hydroxypropylamino-3-nitrophenol and its salts safe for use in non-oxidative or oxidative hair dye formulations within the specified concentrations taken into account the data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of 4-hydroxypropylamino-3-nitrophenol in non-oxidative or oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics 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

4-Hydroxypropylamino-3-nitrophenol (INCI)

3.1.1.2. Chemical names

Phenol, 4-[(3-hydroxypropyl)amino]-3-nitro- (CA INDEX NAME, 9CI)
4-[(3-Hydroxypropyl)amino]-3-nitrophenol (IUPAC)
3-(4-Hydroxy-2-nitroanilino)propanol
1-Hydroxy-3-nitro-4-(3-hydroxypropylamino)benzene

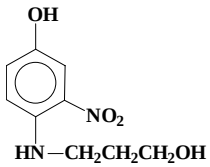
3.1.1.3. Trade names and abbreviations

HC Red BN
Cos 252
Plakatrot Z
Rot BN
Rot Z
COLIPA n° B100
Colorex RBN
Covariane Rouge W3127
Velsal red BN

3.1.1.4. CAS / EINECS number

CAS: 92952-81-3
ELINCS: 406-305-9 (Plakatrot Z)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_9H_{12}N_2O_4$

3.1.2. Physical form

Dark red-brown powder

3.1.3. Molecular weight

Molecular weight: 212.20

3.1.4. Purity, composition and substance codes

Batch DALA006721 = SAT 030381 = SAT 030635

Identification and chemical characterisation by NMR, IR and UV-spectrometry

Purity by NMR assay:	98.6% (w/w)
Purity by HPLC assay:	99.2% (HPLC peak area)
Solvent content (water):	0.24% (w/w)
Sulfated ash:	0.41% (w/w)

Ref 1, 2

Declaration by the applicant
"The batches of B100 used in the acute oral toxicity test (WS I-72 Pt 1-5/88), the subchronic oral toxicity study (WS I-72) and the prenatal developmental toxicity study (no batch code) are not fully analytically described. However, information is available from the laboratories (Casella Company, Germany) that have synthesized these batches concerning the identity and purity of the material produced at that time. From this information it can be concluded that the former not fully described batches are representative batches and their specification is quite similar to the fully characterized batch DALA 006721."

Ref.: 3

3.1.5. Impurities / accompanying contaminants

4-Amino-3-nitrophenol *:	1.2% (w/w)
3-chloropropyl-4-hydroxy-2-nitrophenylcarbamate:	<0.5% (detection limit)

* Classified by MAK as carcinogen, category 3B

3.1.6. Solubility

Water:	< 10 g/l at room temperature
DMSO:	> 100 g/l at room temperature
Ethanol:	> 50 g/l at room temperature

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 1.13 ± 0.52 (calculated)

3.1.8. Additional physical and chemical specifications

Melting point:	111 – 115 °C
Boiling point:	
Flash point:	
Vapour pressure:	
Density:	
Viscosity:	
pKa:	
Refractive index:	
pH:	
UV_Vis spectrum (200-800 nm)	absorption at 493 nm 291 nm and 237 nm (λmax)

Ref.: 1, 2

3.1.9. Homogeneity and Stability

4-Hydroxypropylamino-3-nitrophenol aqueous solutions (approximately 6 mg/l) stored at 25°C and 65% relative humidity were stable up to 25 h (concentration variation <2%).
0.1-0.9% suspensions of 4-hydroxypropylamino-3-nitrophenol in 0.5% CMC were stable up to 20 h (concentration variation < 6%).

General Comments to physico-chemical characterisation

- The exact solubility of 4-hydroxypropylamino-3-nitrophenol in water was not determined according to the method EC A6.
- 4-Hydroxypropylamino-3-nitrophenol is a secondary amine, and thus it is prone to nitrosation. Nitrosamine content in 4-hydroxypropylamino-3-nitrophenol is not reported.
- Log P_{ow}: calculated values cannot be accepted as estimates of the true physical constant without justification.
- No data on the stability of 4-hydroxypropylamino-3-nitrophenol in marketed products and under oxidative conditions was reported.

3.2. Function and uses

4-Hydroxypropylamino-3-nitrophenol is used as direct hair dye for hair colouring products up to a final concentration of 2.6% on head in the presence or absence of a developer-mix.

3.3. Toxicological Evaluation

This risk assessment relates to the use of 4-hydroxypropylamino-3-nitrophenol only. No data were submitted on its salts.

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline: /
Species/strain: Rat, Sprague Dawley
Test substance: HAARFARBSTOFF ROT BN
Batch: WS I-72 Pt 1-5/88
Purity: not specified
Vehicle: distilled water
GLP: in compliance

The test compound was administered once orally by gavage at a dose level of 2000 mg/kg body weight (bw) (aliquots of 10 ml/kg bw) to 5 male and 5 female rats.
Deaths and overt signs of toxicity were recorded 30 minutes, 1, 2 and 4 hours after dosing and subsequently once daily for 14 days. Individual bodyweights were recorded on the day of treatment and on days 7 and 14.
At the end of the study the animals were killed and subjected to gross necropsy examination for any macroscopic abnormalities.

Results
No animals died during the test and no other signs of toxicity were observed. The LD_{50} was greater than 2000 mg/kg bw for rats of both sexes.

Ref.: 4

Comment
Although the study was not performed according to OECD Guidelines, it is useful for evaluation.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline: OECD 404
Species: New Zealand White rabbit
Group: 3 male
Substance: B100 / SAT 030635
Batch: DALA006721
Purity: 99.2%
Dose: 0.5 g of the moistened test substance
Vehicle: /
GLP: in compliance

The test compound was examined under semi-occlusive conditions.
An aliquot of 0.5 g of the moistened test substance was applied to the intact shaved skin on the back of each animal. The patch was removed four hours after semi-occlusive contact.
Animals were examined for signs of erythema, eschar and oedema formation. The skin reactions were assessed approx. 1 hour, 24, 48 and 72 hours.

Results
There was red-brown staining of the skin, which did not prevent scoring of skin reactions. No skin irritation was observed.

Conclusion
Under the conditions of the study, the undiluted test substance was non-irritating.

Ref.: 5

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405
Species:	New Zealand White rabbit
Group:	3 male
Substance:	B100 / SAT 030635
Batch:	DALA006721
Purity:	99.2%
Dose:	72mg (equivalent of 0.1 ml)
Vehicle:	/
GLP:	in compliance

The equivalent of 0.1 ml of B100 was instilled into the conjunctival sac of one eye of the test animals. The substance remained in permanent contact with the eyes until rinsing with warm tap water, 24 hours after instillation. The other eyes served as controls.
The eye irritation reactions were scored approx. 1 hour, 24, 48 and 72 hours and 7 days after instillation of the test solution.

Results
B100 caused effects on the cornea, iris and conjunctivae. The corneal injury consisted of opacity (maximum grade 1) and epithelial damage (maximum 25% of the corneal area). The corneal injury had resolved within 72 hours in one animal and within 24 hours in the other animals. Iridial irritation, grade 1, was observed in two animals and had resolved within 24 hours or 14 days. The irritation of the conjunctivae consisted of redness, chemosis and discharge and had completely resolved within 7, 14 or 21 days. In addition, one animal displayed reduced flexibility of the eyelids, 7 and 14 days after instillation.

Under the conditions of the study, the undiluted test substance was found to be irritating to the rabbit eye.

Ref.: 6

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species:	mice, CBA strain (inbred, SPF)
Group:	five dose groups, two controls receiving vehicle alone; 5 female in each group.
Substance:	B100 / SAT030635
Batch:	DALA006721
Purity:	99.2%

Dose:	0.5, 1, 10, 25 and 50%
Vehicle:	acetone:olive oil (4:1 v/v)
Control:	vehicle (contemporaneous), α-hexylcinnamaldehyde (non-contemporaneous)
GLP:	in compliance

A homogenous dilution of the test item in a mixture of acetone:olive oil (4:1 v/v) was prepared 4 hours prior to each treatment. The highest non-irritating test item concentration was found in a pretest with four mice. Based on these test results 1%, 10% and 25% solutions were initially chosen for the main study. In order to clarify the observed dose-response relationship, two additional groups were treated subsequently with test substance concentrations of 0.5% and 50%. In parallel to both parts of the main study, control animals were treated with the vehicle alone.
Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with the different test item concentrations. The application volume, 25 µl, was spread over the entire dorsal surface of each ear lobe once daily for three consecutive days. The control group was treated with the vehicle exclusively. Five days after the first topical application, all mice were administered with radio-labelled thymidine (^{3}HTdR) by intravenous injection via the tail vein.
Approximately five hours after ^{3}HTdR application all mice were killed. The draining lymph nodes were excised and pooled for each experimental group. After preparation of the lymph nodes, disaggregation and overnight precipitation of macromolecules, these precipitations were re-suspended and transferred to scintillation vials.
The level of ^{3}HTdR incorporation was then measured by scintillation counting. The proliferative response of lymph node cells was expressed as the ratio of ^{3}HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index).
An appropriate reference (α-hexylcinnamaldehyde) was used as positive control, to show distinct increases in the stimulation index.
The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of ^{3}H-methyl thymidine. A test item is regarded as a sensitizer if the exposure to at least one concentration resulted in an at least 3-fold increase in incorporation of ^{3}HTdR compared with concurrent controls, as indicated by the stimulation index (S.I.).

Results
No signs of skin irritation were noted on the ear dorsum of the treated mice at any concentration.
The S.I. values calculated for the substance concentrations 0.5, 1, 10, 25 and 50% are shown in the table below.

Test Item Concentration	**S.I.**
0.5% (w/v)	1.7
1% (w/v)	3.1
10% (w/v)	1.2
25% (w/v)	1.7
50% (w/v)	1.6

The borderline S.I. of 3.1 fell outside the dose response relationship expected and considered to be an unexplained response, not indicative for sensitisation.
The positive control was α-hexylcinnamaldehyde and studies with this were undertaken in August 2003 and March 2004. EC3 values of 5.5 and 10.3% respectively were obtained.

Conclusion
Based on the criteria of the test system, B100 / SAT030635 was determined to be a non-sensitizer when tested up to the highest achievable concentration of 50% (w/v) in acetone:olive oil (4:1) in mice.

Ref.: 7

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (2000)
Tissue:	Pig skin, dermatomed 0.75mm
Group size:	one male, one female
Diffusion cells:	8 per test item (2 experiments each with 4 cells)
Skin integrity:	trans-dermal electrical resistance
Test substance:	B100 / SAT030635
Batch:	DALA 006721
Purity:	99.2%
Test item:	A) direct dye formulation, B) oxidative dye formulation with hydrogen peroxide C) oxidative dye formulation without hydrogen peroxide based developer D) ethanolic aqueous solution (96% ethanol:water, 1:1 v/v)
Dose:	20 mg/cm² (2.6% B100 or 0.52 mg/cm² skin)
Receptor fluid:	Dulbecco's phosphate buffered saline (PBS)
Solubility receptor fluid:	5.8 mg/l in water
Stability:	stable for 25 h
Method of Analysis:	HPLC
GLP:	in compliance

The dermal absorption/percutaneous penetration of B100 (Batch: DALA006721) out of a direct dye formulation, an oxidative dye formulation in the presence and absence of a hydrogen peroxide based developer and an ethanolic aqueous solution (96% ethanol:water, 1:1 v/v) was studied on the clipped excised skin of two young pigs.
The integrity of frozen (at -20 °C) skin discs was checked by measuring the trans-dermal electrical resistance.
The intact, clipped excised pig skin of the flanks area was exposed for 30 minutes to the test substance in the different representative formulations without occlusion.

Direct dye formulation: → formulation A	Concentration (%)
B100	2.60
Cetearyl alcohol	6.00
Fatty alcohol C12-18	6.00
Ceteareth-12	3.00
Ceteareth-20	3.00
Methylparaben	0.30
Propylparaben	0.20
Phenoxyethanol	1.00
Polyethylenglycol (PEG 400)	5.00
PEG 40 castor oil	1.00
Hydroxyethylcellulose	1.00
NaOH	0.10 + for pH adjust.
Citric acid (20 %)	for pH adjust.
Water	ad 100.00
Total	100.00

pH of the formulation: 7.0

Oxidative dye cream: → formulation B and C	Concentration (%)
B100	5.20
Cetearyl alcohol	9.35
Sodium laureth sulphate	4.05
Cocoamidopropylbetaine	3.75
Fatty alcohol C12-18	2.20
Ceteareth-20	0.75
Water	10.20
Ascorbic acid	0.20
Sodium sulphite	0.20
Sodium silicate, SiO_2:Na_2O, 3.3-3.4	0.50
Ammonium sulphate	0.40
3-Amino-1-propanol	for pH adjust.
Etidronic acid	0.12
Water	ad 100.00
Total	100.00

pH of the formulation: 9.5

The direct dye formulation (formulation A) was used as such. The oxidative dye cream was mixed 1:1 (m/m) with a developer formulation with and without hydrogen peroxide (composition see table below) to give formulation B and C.

Developer mix	**with H_2O_2 for formulation B** Concentration (%)	**without H_2O_2 for formulation C** Concentration (%)
Water dist.	68.119	80.382
Dipicolin acid	0.100	0.100
Sodium propyl phosphate	0.030	0.030
Etidronic acid	0.900	0.900
Sodium laureth sulphate	0.560	0.560
Silicon emulsion, 10% active substance	0.067	0.067
Ammonia, 25%	0.964	0.921
L(+)-Tartaric acid (pH adjustment)	0.220	0.000
Acryl polymer, 28% active substance	15.000	15.000
Hydrogen peroxide, 50%	12.000	0.000
Total	100.00	100.00
	pH of the formulation: 3.95	pH of the formulation: 3.88

The content of B100 in the final application formulations A, B and C and in the ethanolic aqueous solution (D) was 2.6%.
The dermal absorption/percutaneous penetration of the test substance was investigated for the open application of about 20 mg formulation per cm² pig skin. Therefore the dose of the test substance was approx. 0.52 mg/cm² skin. Skin discs of 1.0 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with a 0.01% Tween 20 solution.
Each of the three formulations and the solution were analysed in two experiments with four replicates per experiment for adsorbed, absorbed and penetrated amount of the test substance. The receptor fluid used was Dulbecco´s phosphate buffered saline. In the static system samples of the receptor fluid were drawn before the application of the test substance formulation and 0.5, 1, 2, 4, 6, 24, 29 and 48 hours after application. The removed volume was replaced by fresh receptor fluid.

Results
The mean quantities that had penetrated during the 30 minute exposure to the B100 containing formulations and within the 48 hours after application, are shown in the following table. Both the amounts absorbed and penetrated were taken as systemically available.

Analysed Sample	Formulation A Direct dye cream [% of dose] [µg/cm²]		Formulation B Cream with H_2O_2 [% of dose] [µg/cm²]		Formulation C Cream without H_2O_2 [% of dose] [µg/cm²]		Solution D ethanol-water [% of dose] [µg/cm²]	
Skin rinsings	93.8	-	93.7	-	91.3	-	92.3	-
Adsorption (stratum corneum)	0.335	1.98	0.276	1.39	0.268	1.37	2.951	15.75
Not Bioavailable	94.1	-	94.0	-	91.6	-	95.3	-
Absorption (epidermis/dermis)	0.223	1.32	0.177	0.90	0.283	1.45	0.867	4.67
Penetration (receptor fluid)	0.233	1.39	0.092	0.46	0.155	0.79	0.535	2.86
Bioavailable	0.456	2.71	0.270	1.36	0.438	2.24	1.402	7.53
Total recovery / mass balance*	94.7	-	94.3	-	92.1	-	97.4	-

Under test conditions in this *in vitro* dermal penetration study, the amount of B100 bioavailable from a direct dye cream was A_{max} 5.722 µg/cm² (0.9045%) (range 1.582 – 5.722; mean 2.71 ± 1.33µg/cm²) and in an oxidative hair dye formulation with hydrogen peroxide was A_{max} 1.751 µg/cm² (0.3561%) (range 0.712 – 1.751; mean 1.36 ± 0.36µg/cm²).

Ref.: 14

Comment
An A_{max} of 5.722 µg/cm² may be used for calculating the MOS as too few test chambers were used (2 donors, 4 cells each).

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

13-week study in rats

Guideline:	/
Species/strain:	Rat, Wistar CD strain (Hsd:SD)
Group size:	15 animals per sex and dose
Test substance:	WSI 72
Batch:	not specified
Purity:	not specified
Dose levels:	0, 10, 30 and 90 mg/kg bw/day
Vehicle:	suspension in 0.25% Gum Tragacanth
Route:	Oral, by gavage
Dosing schedule	13 weeks
GLP:	in compliance

The study appears to conform to OECD 408 although not explicitly stated.
The test substance was given daily for a period of 13 consecutive weeks in dosages of 0, 10, 30 and 90 mg/kg bw to groups of 30 rats (15 per sex) of the Wistar CD strain (Hsd:SD). Additionally, 10 rats (5 per sex), for both the control and the high dose group, were assessed for recovery of treatment-related effects, four weeks after the last oral administration.
No exposure related mortalities occurred.

Results
No signs of toxicity or behavioural abnormalities were observed throughout the duration of the study. No effect on body weight, food and water consumption or ophthalmology was observed.
Red staining of fur, paws and tails, and staining of cages and dirt tray papers was seen in all treated animals throughout the study due to the excretion of the coloured test substance or its metabolite(s). The effect was dose related in severity and was greatly reduced after 4 weeks recovery. No other treatment-related effects were observed at terminal necropsy.
There were incidental changes in some haematological and blood chemistry parameters, but these were not-consistent or dose related.
Orange coloration in urinary samples from the mid and high dose groups were observed.
No substance related changes were seen in organ weight or histopathology.

Conclusion
According to the applicant, the No Observed Adverse Effect Level (NOAEL) for in this study was 90 mg/kg bw/day.

Ref.: 12

Comment of the SCCP
In the previous SCCNFP opinion on B100 of 23 June 1999, the same study was evaluated and a NOAEL of 10 mg/kg bw was used for the calculation of the MoS. For the present opinion, this study was re-assessed. The effects on Na, prothrombine time and kidney weight, as discussed in the previous opinion, were not dose-related or not consistent over time. Therefore, based on decrease ASAT in males in the highest dose group, and increase

of absolute thyroid weight (males and females in this group) the NOAEL is now set at 30 mg/kg bw/day.

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:	TA 1535, TA 1537, TA 98, TA 100, TA 102
Replicates:	3 replicates in two independent experiments
Test substance:	COLIPA B100
Solvent:	DMSO
Batch:	DALA 006721
Purity:	99.2% (area%, HPLC)
Concentrations:	33, 100, 333, 1000, 2500 and 5000 μg/plate and in experiment two 10 μg/plate was also tested
Treatment:	In experiment one the plate incorporation test was used and in experiment two the pre-incubation test was applied
GLP:	In compliance

COLIPA B100 was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) with and without phenobarbital and β-naphthoflavone induced rat liver enzymes (S9-mix). Test concentrations were based on toxicity in a preliminary toxicity test in TA98 and TA100 with 8 concentrations from 3 to 5000 µg/plate. Since no severe toxicity was observed the pre test was included as part of the main experiment one and 5000 µg/plate was chosen as the highest tested concentration. Negative and positive controls were in accordance with the OECD guidelines

Results
The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9-mix in all strains used.
Toxic effects, evident as a reduction of the number of revertant colonies below 0.5 times the corresponding solvent control, occurred at the following concentrations (µg/plate):

Strain	Experiment I		Experiment II	
	without S9-mix	With S9-mix	without S9-mix	with S9-mix
TA 1535	5000	no toxic effect observed	2500, 5000	5000
TA 1537	no toxic effect observed	no toxic effect observed	5000	5000
TA 98	5000	5000	2500	no toxic effect observed
TA 100	5000	5000	5000	5000
TA 102	1000-5000	1000-5000	1000-5000	1000-5000

There were no signs of an increase in number of revertants of any of the five tester strains at any concentration tested neither with nor without metabolic activation.

Conclusion
Under the test conditions used COPLIPA B100 did not induce gene mutations in bacteria.
Ref.: 8

In vitro Mammalian Cell Gene Mutation Test (*tk*$^{+/-}$ locus)

Guideline:	OECD 476
Species/strain:	Mouse lymphoma cell line L5178Y
Replicates:	Duplicate cultures in two independent studies
Test substance:	COLIPA B100
Solvent:	DMSO
Batch:	DALA 006721
Purity:	99.2% (area%, HPLC)
Concentrations:	Experiment 1: without S9-mix: 68.8, 137.5, 275, 550, 825 µg/ml with S9-mix: 17.2, 34.4, 68.8, 137.5, 275 µg/ml Experiment 2: without S9-mix: 34.4, 68.8, 137.5, 275, 550 µg/ml
Treatment	Experiment 1: 4 h treatment with and without S9-mix, expression period 72h Experiment 2: 24 h treatment without S9-mix, expression period 48h
GLP:	In compliance

COLIPA B100 was tested for gene mutation/clastogenic effect at the *tk* locus of mouse lymphoma cells with and without rat liver S9-mix from phenobarbital and β-naphthoflavone induced rats. Test concentrations in the main experiments were based on toxicity, measured as relative suspension and total growth (RSG and RTG), in a preliminary experiment with a concentration range from 17.2 to 2200 µg/ml (10 mM). Negative and positive controls were in accordance with the OECD guidelines.

Results
No precipitation of the test item was observed in the main experiments up to the maximum concentration. In the first experiment RTG at the highest tested concentration without S9 was 24.1 and 23.1 in the two cultures, respectively. With S9 RTG was 27.9 and 31.6. In the second experiment without S9 RTG was 17.2 and 8.4 at the highest evaluated concentrations.
In experiment 1 no significant increase of the mutant frequency was found at any concentration neither with nor without metabolic activation. In experiment two, a concentration related increase was observed in both cultures. The mutation frequency reached a doubling of the corresponding solvent control at 550.0 µg/ml in both parallel cultures of the second experiment. The absolute value of the mutation frequency was low and was within the historical range of negative and solvent controls in culture I. In culture II the historical control range was exceeded (299 colonies per 10^6 cells compared 33-192 colonies per 10^6 cells, the historical range of solvent controls) but toxicity was strong with RTG of 8.4 %. It is likely that this increase of the mutation frequency was based on cytotoxic effects rather than indicating a possible mutagenic potential of the test item. The concentration (275 µg/ml) point before 550.0 µg/ml gave a mutation frequency of 131 colonies per 10^6 cells that were well within the range of the historical solvent controls.

Conclusion
Under the test conditions used the COLIPA B100 was not genotoxic (mutagenic and/or clastogenic in the mouse lymphoma assay at the *tk* locus).

Ref.: 9

In vitro Mammalian Chromosome Aberration Test

Guideline:	OECD 473
Species/strain:	Chinese hamster cells V79
Replicates:	Two replicates in two independent
Test substance:	COLIPA B100
Solvent:	MEM (minimal essential medium)

Batch:	DALA006721		
Purity:	99.2% (HPLC)		
Concentrations:	Without S9-mix:	First experiment:	150, 300, 600, 800 µg/ml
		Second experiment:	75, 150, 300, 600 µg/ml
	With S9-mix:	First experiment:	75, 150, 300, 600 µg/ml
		Second experiment:	150, 300, 600 µg/ml
Treatment:	4 h treatment and harvest time 18 after start of treatment both in the absence and presence of S9-mix		
GLP:	In compliance		

The test substance was investigated in Chinese hamster lung cells, V79 for clastogenic potential in the presence and absence of phenobarbital and β-naphthoflavone stimulated rat liver microsomes (S9-mix). A pre-test on cell growth inhibition with 4 h and 24 h treatment using concentrations between 19.1 and 2450 µg/ml was performed in order to determine the toxicity of the test item.
The cells were harvested 18 hours after start of treatment. 100 metaphases per culture were scored for structural chromosome aberrations. Two independent experiments were conducted with two parallel cultures each.
B100 dissolved in culture medium (MEM) was tested in the range of 75 to 1200 µg/ml in the absence and presence of S9-mix. The used high doses of the experiment, both with and without S9-mix, were chosen considering the results of a pre-test on toxicity. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Toxic effects, indicated by reduced cell numbers and/or mitotic indices of about and below 50% of control were obtained in both experiments.
In the absence of metabolic activation in the first experiment without metabolic activation, a concentrated related increase was observed and at the highest concentration (800 µg/ml), a statistically significant increase in cells with aberrations was observed, which was about four-fold higher compared to the positive control. In the second experiment, a concentration related increase was not observed but with three concentrations (150, 300 and 600 µg/ml) a statistically significant increase in cells with aberrations was observed. However, the increases at these three concentrations were relatively low and were within the historical range for the negative control.
In the presence of metabolic activation in both experiments, statistically significant increases were observed at all tested concentrations. The increases were not concentration-related but they were strong. Some of the concentrations were equally or more potent than the positive control.
There were no increases in the frequencies of polyploidy metaphases in both experiments. The rates of endomitotic metaphases were significantly increased at three concentrations with metabolic activation and at one concentration without metabolic activation. Experiment two did not show any increases with or without metabolic activation.

Conclusion
The conclusion is that under the test conditions used the test article COLIPA B100 induced structural chromosome aberrations in the presence of metabolic activation and the results were equivocal without metabolic activation. COLIPA B100 is considered to be clastogenic in the *in vitro* chromosome aberration test.

Ref.: 10

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	NMRI mice
Group size:	Ten mice per group (5 males and 5 females)

Test substance:	COLIPA B100
Lot no:	DALA006721
Purity:	99.2% (HPLC)
Dose level:	125, 250 and 500 mg/kg body weight
Route:	intraperitoneally
Vehicle:	30% DMSO + 70% deionised water
Sacrifice times:	24 and 48 (only for the high dose level) hours after application of the test substance
GLP:	In compliance

A preliminary study on acute toxicity was performed with two animals per sex under identical conditions as the main experiment. The animals were treated intraperitoneally with the test item and examined for acute toxic symptoms at intervals of around 1 h, 2-4, h, 6 h, 24 h, 30 h and 48 h after administration of the test item. In the main study at least 2000 erythrocytes were scored for micronuclei.

Results
As estimated by pre-experiments 500 mg/kg bw was the highest applicable dose without significant effects on the survival rates, but with clear signs of toxicity. At a higher dose (750 mg/kg) one male and one female animal died. In the main study two males died after treatment with 500 mg/kg body weight.
The mean number of polychromatic erythrocytes (PCEs) was not decreased after treatment with COLIPA n° B100 as compared to the mean value of PCEs of the vehicle control indicating that the test item had no cytotoxic properties in the bone marrow. However, the urine of the treated animals had taken the colour of the test item indicating its systemic distribution and thus its bio-availability.
There were no signs of increases in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item. The mean values of micronuclei observed after treatment with COLIPA B100 were below or near to the value of the vehicle control group.

Conclusion
Under the test conditions used the test article COLIPA B100 did not induce micronuclei in the micronucleus test in the 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
Species/strain:	Wistar Rats, Crl: Wi/Br
Group size:	20 mated females per dose group
Test substance:	Rot Z (Cos 252) / Chlororange
Batch:	/
Purity:	/
Dose levels:	0, 10, 30 and 90 mg/kg bw/day
Exposure:	Gestation Days (GD) 5 through 15, once a day

Route: oral, by gavage
Vehicle: Na-Carboxymethylcellulose (0.5%)
GLP: in compliance

The test substance was daily administered at dose levels of 0, 10, 30 and 90 mg/kg body weight (day 5-15 of pregnancy) by gavage. Clinical signs and mortality were observed daily. Body weights were recorded on day 0, 5, 10, 15 and 20.
The dams were sacrificed on day 20 post-coitum by carbon dioxide asphyxiation and subjected to necropsy. The number of alive and dead foetuses, their distribution and site in the uterus, early and late resorption, implantation and number of *corpus lutea* was determined. The weight of the foetuses, gravid uteri, uteri without foetuses, placentae and the sex of foetuses were recorded. Approximately one-third of the foetuses were selected at random and examined for visceral alterations. The remaining foetuses were examined for skeletal malformations, variations and retardation of the normal organo-genesis after appropriate staining.

Results
All females showed a normal habit or behaviour throughout the study, and no animal died prior to scheduled sacrifice. Mean maternal bodyweight gain and mean food consumption were not significantly affected during exposure.
Gross necropsy did not reveal any organ alterations attributable to treatment.
No biologically significant differences in the mean number of viable foetuses, the male to female foetal sex ratio, total bodyweights, birth-position, number of runts, post-implantation losses, implantations, resorptions, uteri weights, placenta weights and corpora lutea between dosage groups and the control group.
No dose-related effect was observed upon external, skeletal and visceral examinations of the foetuses.
One single foetus in the high dose group was malformed by agnathia. This finding has also been observed, although sporadically in historical control rats. There is no indication that this finding is related to treatment.

Conclusion
Under the experimental conditions of this study, the NOAEL for both maternal and foetal toxicity was 90 mg/kg bw/day, the highest dose tested.

Ref: 13

Comment
For hazard identification, the highest dose used in teratogenicity studies should lead to maternal and/or developmental toxicity

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(4-Hydroxypropylamino-3-nitrophenol)
(oxidative conditions)

Maximum absorption through the skin	**A (µg/cm²) µg/cm²**	**=**	**1.75**
Skin Area surface	**SAS (cm²)**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.225 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60 mg/kg**	**=**	**0.020**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**30 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**1500**

CALCULATION OF THE MARGIN OF SAFETY

(4-Hydroxypropylamino-3-nitrophenol)
(Non-oxidative conditions)

Maximum absorption through the skin	**A (µg/cm²) µg/cm²**	**=**	**5.72**
Skin Area surface	**SAS (cm²)**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**4.004 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60 mg/kg**	**=**	**0.067**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**30 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**448**

3.3.14. Discussion

Physico-chemical properties
4-Hydroxypropylamino-3-nitrophenol is used as an ingredient in hair colouring products up to a final concentration of 2.6% on head in the presence or absence of a developer-mix. 4-Hydroxypropylamino-3-nitrophenol is a secondary amine, and thus it is prone to nitrosation.

Nitrosamine content in 4-hydroxypropylamino-3-nitrophenol is not reported. This substance should not be used in combination with nitrosating agents.
No data on the stability of 4-hydroxypropylamino-3-nitrophenol in marketed products and under oxidative conditions was reported.

General toxicity
The LD_{50} was greater than 2000 mg/kg bw for rats of both sexes.
In a 13-week study in rats, the No Observed Adverse Effect Level (NOAEL) was set at 30 mg/kg bw/day. The NOAEL for both maternal and foetal toxicity was 90 mg/kg bw/day, the highest dose tested.
The purity and composition of the test substance was not provided in any of the toxicity studies.

Irritation / sensitisation
Under the conditions of the study, the undiluted test substance produced no skin irritation. The study authors considered that staining of the skin did not hamper observations. The undiluted test substance was found to be irritating to the rabbit eye.
Based on the criteria of the test system, the test substance was determined to be a non-sensitizer when tested up to the highest achievable concentration of 50% (w/v) in acetone:olive oil (4:1) in mice.

Dermal absorption
Under test conditions in this *in vitro* dermal penetration study the amount of 4-Hydroxypropylamino-3-nitrophenol bioavailable from a direct dye cream was A_{max} 5.72 µg/cm² (0.90%) (range 1.58 – 5.72; mean 2.71 ± 1.33 µg/cm²) and in an oxidative hair dye formulation with hydrogen peroxide was A_{max} 1.75 µg/cm² (0.35%) (range 0.71 – 1.75; mean 1.36 ± 0.36 µg/cm²)

Mutagenicity / genotoxicity
Overall, 4-Hydroxypropylamino-3-nitrophenol has been investigated for the induction of the three types of mutation: gene mutation, structural chromosome mutation and aneuploidy.
It did not induce gene mutations in bacteria or in mammalian cells at the *tk* locus, but was a potent clastogen in Chinese hamster lung cells (V79) in the presence of metabolic activation and equivocal results were obtained in the absence of metabolic activation.
In order to investigate the biological relevance *in vivo*, an *in vivo* micronucleus test in mice was conducted. From the results obtained from this study, it was concluded that 4-Hydroxypropylamino-3-nitrophenol did not show genotoxic potential in this *in vivo* test for chromosomal alterations when administered intraperitoneally to mice.
Based on these results it can be concluded that 4-Hydroxypropylamino-3-nitrophenol has no relevant mutagenic potential *in vivo*. Additional tests are not necessary.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCP is of the opinion that the use of 4-hydroxypropylamino-3-nitrophenol as a hair dye ingredient in non-oxidative and oxidative hair dye formulations at a maximum on-head concentration of 2.6% does not pose a risk to the health of the consumer.

4-Hydroxypropylamino-3-nitrophenol is a secondary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

No data were submitted on its salts.

5. MINORITY OPINION

Not applicable

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