SCCS/1233/09
25 March 2010

EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

Disperse Black 9

COLIPA n° C106

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

The SCCS adopted this opinion at its 6th plenary meeting
of 23 March 2010

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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1. BACKGROUND

Submission I for Disperse Black 9, with the chemical name 2,2'-[4-(4-aminophenylazo)phenylimino] diethanol, was submitted in September 2003 by COLIPA [1], but no opinion was issued by the Scientific Committee.

According to the current submission II, submitted by COLIPA in July 2005, Disperse Black 9 is a mixture of the chemical 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol dispersed in lignosulfate at a ratio of approximately 50:50. The substance is used as a component in non-oxidative hair dye formulations at level up to 0.3% resulting in a concentration of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol of 0.15%.

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 Safety (SCCS) consider Disperse Black 9 safe for use as an ingredient in non-oxidative hair dye formulations in an on-head concentration of 0.3% Disperse Black 9, corresponding to 0.15% 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol, taking into account the scientific data provided?*

2. *Does the SCCS recommend any restrictions with regard to the use of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol in non-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

Disperse Black 9 is a mixture of 2,2'-[4-(4-aminophenylazo) phenylimino]diethanol dispersed in lignosulphate at a ratio of approximately 50:50 (acceptable range 45-55% 'active dye').

3.1.1.1. Primary name and/or INCI name

Disperse Black 9 (INCI name)

3.1.1.2. Chemical names

2,2'-[4-(4-aminophenylazo)phenylimino]diethanol in dispersing agent (Lignosulphate)
Ethanol, 2,2'-[[4-[(4-aminophenyl)azo]phenyl]imino]bis-

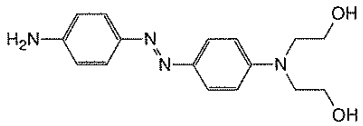
3.1.1.3. Trade names and abbreviations

COLIPA n° C106

3.1.1.4. CAS / EC number

CAS: 20721-50-0 / 12222-69-4
EC: 243-987-5

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{16}H_{20}N_4O_2$

3.1.2. Physical form

Orange brown powder

3.1.3. Molecular weight

Molecular weight: 300.4 g/mol

3.1.4. Purity, composition and substance codes

The analyses for purity had been made in compliance with GLP. This does not apply for the identification of impurities.

Purity of the reference substance:
The reference substance of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol is a commercially available product(Aldrich). The purity is 97.4%, including 1.3% water. So, about 2% of the reference substance is not identified.

Purity of the substance used for toxicological testing:
A typical commercial lot of Disperse Black 9, GTS 03872 lot A35996, was used for toxicological evaluation during 2004 and 2005. It is a mixture of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol and Lignosulphate. This batch has a dye content of 52.6%. HPLC had been used for analysis (Wavelength: 436 nm). The reference substance had been used as an external standard.

Physical Properties:
Loss on Drying: Thermogravimetic analysis showed the volatile content to be 3.85%

Chemical identification:
The identity of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol has been proven by 1H NMR; 13C NMR and FTIR.

3.1.5. Impurities / accompanying contaminants

Impurities of the reference substance

Disperse Red 19	< 1500 ppm
Phenyldiethanolamine	< 1500 ppm
4-Nitroaniline	< 100 ppm
N-nitrosodiethanolamine (NDELA)	< 50 ppb
4-aminobiphenyl (4-ABP)	4.35 ppm

Note
The given level of 4.35 ppm 4-ABP in Disperse Black 9 results in an exposure lower than that which is considered tolerable in opinion SCCNFP/0797/04 (Opinion Concerning Use of Permanent Hair Dyes and Bladder Cancer).

Heavy metals

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

No documents have been supplied for the kind of impurities and their concentrations. The above-mentioned data have been taken from the summary of submission II, 2005.

None of the impurities listed above have been detected. The relative amounts given are maximum levels and are most probably representing limits of detection (LOD)

3.1.6. Solubility

Water:	0.82 – 1.24	mg/ml
Ethanol:	28.3 – 42.5	mg/ml
DMSO:	95.8 – 146.8	mg/ml

No documents have been submitted for the solubility of the substance.

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 2.50 (calculated)

Comment
The Log P_{ow} strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log P_{ow}, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.

3.1.8. Additional physical and chemical specifications

Melting point:	/
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	/

3.1.9. Homogeneity and Stability

Reanalysis of the bulk test article with an initial purity of 52.6%, stored light protected at room temperature for one year showed a final purity of 51.4%, demonstrating that this material was stable throughout the course of the toxicological investigations.

Using a HPLC procedure validated in compliance with GLP the stability of C106 formulations used for toxicity testing have been tested throughout 15 days. Within this time, the concentrations (1.0 to 190 mg/ml) of Disperse Black 9 in 0.5% (w/v) methylcellulose solution in water showed a coefficient of variation <3%. The solutions were stored at 2 to 8 °C.
The corresponding estimations in DMSO solutions, stored at -20 ± 10°C (0.05 to 500mg/ml), showed coefficients of variation <3.8%.
Homogeneity was measured by taking samples from various locations of test substance and analyzing them with the HPLC method mentioned. Variation of concentrations was <1.1%.

Comment
The substance was stable and homogeneous during toxicity testing.

General Comments to physico-chemical characterisation

- 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol is a commercially available substance with a purity of 97.4%. It is used as standard for analytical purposes and for all formulations used for testing relevant toxicity. Under the name Disperse Black 9 this substance is used for hair dying purposes in a mixture with lignosulfate (50:50).
- Impurities given in the general description have not been detected in 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol . The concentrations given for these impurities are estimated maximum values which account for about 0.32%. So about 2% of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol have not been identified.
- Disperse Black 9 contains 4-ABP which is carcinogenic for humans. Its concentration of 4.35ppm is considered tolerable in opinion SCCNFP/0797/04 (Opinion Concerning Use of Permanent Hair Dyes and Bladder Cancer).
- No documents have been submitted on the solubility of the substance.

- No data on the stability of Disperse Black 9 in typical hair dye formulations has been submitted.

3.2. Function and uses

Disperse Black 9 is used at levels up to 0.3% (corresponding to 0.15% active dye) in non-oxidative hair dye formulations.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	/
Species/strain:	rat
Group size:	15 males and 20 females; 5 per dose level
Test substance:	Disperse Black 9
Batch:	3990586
Purity:	/
Concentration:	10% suspended in 3% acacia water
Dose levels:	250, 500 and 1000 mg/kg bw (males) 250, 500, 750 and 1000 mg/kg bw (females)
GLP statement:	/
Study period:	14 January – 12 February 1987

Results
All 5 male rats in the 1000 mg/kg bw dose group died on day 1. 1/5 females in the 750 mg/kg bw dose group died on day 1 and all 5 females in the 1000 mg/kg bw dose group died between 8-24h after dosing.

Conclusion
The lethal dose was calculated between 500 and 1000 mg/kg bw for male rats and between 750 and 1000 mg/kg bw for female rats.

Ref.: 2

Guideline:	/
Species/strain:	rat
Group size:	20 males (5 per dose group)
Test substance:	Disperse Black 9
Batch:	/
Purity:	/
Concentration:	10% in 5% methocel
Dose levels:	250, 500, 1000 and 2000 mg/kg bw
GLP statement:	/
Study period:	15 – 22 September 1975

Results
All 5 male rats in the 2000 mg/kg bw dose group, 3/5 in the 1000 mg/kg bw dose group, 1/5 in the 500 mg/kg bw dose group and 0/5 in the 250 mg/kg bw dose group died.

Conclusion

The LD50 calculated for the male rats was 960 mg/kg bw.

Ref.: 4

Guideline: /
Species/strain: rat
Group size: 5 males
Test substance: Disperse Black 9 at 0.38% w/w in formulation
Batch: /
Purity: /
Dosage: 14.7 mg/kg bw
Administration: oral
GLP statement: /
Study period: 1 – 12 July 1976

Results
0/5 male rats at the dose of 14.7 mg/kg bw died.

Conclusion
The LD50 was determined to be > 14.7 mg/kg.

Ref.: 23

Comments
Disperse Black 9 was one of the compounds in the formulation tested. Details of the formulation were not provided.

Guideline: /
Species/strain: albino Sherman-Wistar rat
Group size: 10 (5 males and 5 females)
Test substance: Disperse Black 9 at 0.24% w/w in formulation
Batch: Sample 1263/1/13, a formulation containing 0.24% Disperse Black 9
Purity: /
Dosage: 5 g/kg bw of the formulation (equivalent to 0.012 g/kg Disperse Black 9
Administration: syringe and stomach tube
GLP statement: /
Study period: 1972

Results
0/5 male rats at the dose of 5 g formulation/kg bw died.

Conclusion
The LD50 was determined to be > 5 g formulation/kg (equivalent to > 0.012 g/kg Disperse Black 9).

Ref.: 24

Comment
Disperse Black 9 was one of the compounds in the formulation tested. Details of the formulation were not provided.

3.3.1.3. Acute intraperitoneal toxicity

Guideline: /
Species/strain: rat
Group size: 20 males (5 per dose group)
Test substance: Disperse Black 9
Batch: /

Purity: /
Concentration: 5% in 10% DMSO
Dose levels: 500, 1000, 2000 and 4000 mg/kg bw
GLP statement: /
Study period: 1 – 8 May 1976

Results
All 5 male rats in the 4000 mg/kg bw dose group, 3/5 in the 2000 mg/kg bw dose group, 2/5 in the 1000 mg/kg bw dose group and 1/5 in the 500 mg/kg bw dose group died.

Conclusion
The LD50 calculated for the male rats was 1297mg/kg bw.

Ref.: 5

Comments
Ref. 5 is indicated in the bibliography as "acute oral toxicity test, May 8, 1976" whereas it is indicated in the report that the animals were administered by intraperitoneal route

3.3.1.3. Acute dermal toxicity

No data submitted

3.3.1.4. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline: /
Species/strain: rabbit
Group size: 6 (5 males and 1 female)
Test substance: Disperse Black 9
Batch: 3990586; Code # RM141
Purity: /
Dose level: 500 mg in water (aqueous slurry)
GLP: /
Study period: 12 – 15 January 1987

The test substance was applied to intact skin. The application site and whether the experiment was done under occlusion, was not stated. Readings were done 24 and 72 hours after application.
Main findings: mild erythema was seen in all 6 rabbits 24 hours after application No oedema was seen during the study. Erythema had cleared after 72 hours.

Ref.: 9

Comment
The site of application and the occlusion conditions are not known. The data suggests that Disperse Black 9 was mildly irritant to rabbit skin.

Guideline: /
Species/strain: albino rabbit. Strain not stated in report
Group size: 6 (sex not stated)
Test substance: Sample 1263/1/13, a formulation containing 0.24% Disperse Black 9

Batch: /
Purity: /
GLP: /
Study period: 1972

The test substance was applied to intact and abraded skin. Readings were done 24 and 48 hours after application. The test material produced no erythema or oedema in any of the animals

Ref.: 28

Comment
Vehicle and quantity of test substance were not stated in the report. Details of the formulation were not provided. The site of application was not recorded.
The data suggests that a formulation containing Disperse Black 9 at 0.24% w/w was not irritant to rabbit skin.

Guideline: /
Species/strain: rabbit (Strain not stated in report)
Group size: 6 males
Test substance: Disperse Black 9 at 0.38% w/w
Batch: /
Purity: /
Dosage: 0.5 ml
GLP: /
Study period: 20 – 26 July 1976

The test substance was applied to intact skin. Readings were done 24 and 72 hours after application. No erythema or oedema was seen in any of the animals.

Ref.: 29

Comment
Vehicle was not stated in the report. Details of the formulation were not provided. The site of application was not recorded.
The data suggests Disperse Black 9 at 0.38% w/w was not irritant to rabbit skin.

6-week skin irritation study

Guideline: /
Species/strain: New Zealand albino rabbit
Group size: 6 (sex not stated)
Test substance: Sample 1263/1/13, a formulation containing 0.24% Disperse Black 9
Batch: /
Purity: /
Administration: 2 ml
Exposure: 6 exposures, 30 minutes per week
GLP: /
Study period: 1972

2 ml of test material were applied topically to the right side of the back for 30 minutes and then rinsed off. The animals were exposed for a 30 minute period each week for a total of six exposures.
Test and control sites (left side) were examined before administering the test sample and immediately after washing for changes in the skin.
No erythema or oedema was seen in any of the animals over the six-week period.

Ref.: 30

Comment
Vehicle was not stated in report. Details of the formulation were not provided.
The data suggests that a formulation containing Disperse Black 9 at 0.24% w/w was not irritant to rabbit skin.

3.3.2.2. Mucous membrane irritation

Guideline: /
Species/strain: rabbit (strain not stated)
Group size: 4 females (2 rinsed, 2 unrinsed)
Test substance: Code # RM141 (Disperse Black 9)
Batch: 3990586
Purity: /
Dose level: 100 mg test material
GLP: /
Study period: 3 – 6 February 1987

Eye Irritation Test. Readings were done 1 hour, 1, 2 and 3 days after instillation. The eyes were washed with 20ml (medium not stated) after an unspecified time.
After 1 hour, conjunctivae redness was noted in all animals. This was also noted in all animals after 1 day, along with discharge in one animal from the rinsed group and one animal from the unrinsed group.
All eyes were clear after 2 days.

Ref.: 6

Comment
Code # RM141 (Disperse Black 9) caused mild irritation to rabbit eyes.

Guideline: /
Species/strain: rabbit (strain not stated)
Group size: 6 males
Test substance: Disperse Black 9
Batch: /
Purity: /
Dose level: 100 mg test material
GLP: /
Study period: 10 – 17 April 1979

Eye Irritation Test. Readings were done 1, 2, 3 and 7 days after instillation of 100 mg of test material. Eyes were not rinsed.
No irritation or redness was seen in any of the animals.

Ref.: 7

Comment
Disperse Black 9 was not irritant to the eyes of rabbits under the test conditions.

Guideline: /
Species/strain: Albino rabbit
Group size: 6 males
Test substance: Sample 1263/1/13, a formulation containing 0.24% Disperse Black 9
Batch: /
Purity: /
Concentration: /
Dose level: /

GLP: /
Study period: 23 – 30 April 1979

Eye Irritation Test
Readings were done1, 2 and 3 days after instillation.
All of the animals had conjunctivae redness and discharge, which cleared by day 3 of the study. 2 of the animals had irritation of the iris, which cleared by day 2 of the study.
Recovery: 3 days for total clearance.

Ref.: 25

Comment
Vehicle and dose were not stated in the report. Details of the formulation were not provided. The data suggests that a formulation containing Disperse Black 9 at 0.24% w/w was irritant to rabbit eyes.

Guideline: /
Species/strain: rabbit
Group size: 6 females (3 rinsed, 3 unrinsed)
Test substance: formulation containing Disperse Black 9 at 0.38% w/w
Batch: /
Purity: /
Dosage: 0.1 ml
GLP: /
Study period: 20 -27 July 1976

Eye Irritation Test
Readings were done1, 2, 3 and 7 days after instillation. For the rinsed group, the eyes were washed with 20ml tape water 20 seconds after instillation of the test material.
Main findings: None of the animals in the rinsed group had any signs of irritation or redness. Two animals in the unrinsed group had iris irritation that cleared after two days. One of the animals in the unrinsed group had corneal opacity, iris irritation, conjunctivae redness and lid swelling which lasted for 3 or more days. After 7 days this animal still showed signs of corneal opacity.
Recovery: More than 7 days for complete clearance.

Ref.: 26

Comment
The vehicle was not stated in the report. Details of the formulation were not provided. Under the condition of the test, a formulation containing Disperse Black 9 at 0.38% w/w was irritant to rabbit eyes.

Guideline: /
Species/strain: monkey (3 rinsed, 3 unrinsed)
Group size: 6
Test substance: Sample 1263/1/13 a formulation containing 0.24% Disperse Black 9
Batch: /
Purity: /
Dosage: 0.1 ml
GLP: /
Study period: 1972

Eye Irritation Test
Readings were done 1 hour, 1, 2 and 3 days after instillation. For the rinsed group, the eyes were washed with 20ml saline 4 seconds after instillation of the test material.
No irritation was observed in any animals.

Ref.: 27

Comment
Details of the formulation were not provided. The data suggests that a formulation containing Disperse Black 9 at 0.24% w/w was not irritant to monkey eyes.

3.3.3. Skin sensitisation

Magnusson – Kligman maximisation test

Guideline:	/
Species/strain:	albino Hartley guinea pig
Group size:	10 females
Observ. Period:	
Test substance:	Disperse Black 9
Batch:	/
Purity:	/
Dose levels:	induction: 0.1% in propylene glycol (intradermal injection) 25% in propylene glycol (topical occlusive application 1 week later) Challenge: 25% in propylene glycol
Volume:	0.05 ml
Route:	intradermal injection and occlusive topical application
GLP:	/
Study period:	1979

Three pairs in injections into the flanks: Freund's complete adjuvant (1:1 in water), 0.1% Disperse Black 9 and a 1:1 mixture of both. After a week, 25% Disperse Black 9 in propylene glycol was applied with occlusion. It is not stated whether an irritant was applied. After 2 weeks challenge was performed with 25% Disperse Black 9 in propylene glycol under occlusion for 24 hours.
Readings were done24, 48 and 72 hours after removal of the occlusive bandages.
No reaction was observed.

Ref.: 10

Comment
Under the conditions of this test, Disperse Black 9 was not sensitising. Only a result summary was provided with a generic appendix of methodology.

Human Repeated Insult Patch Test

Guideline:	/
Species/strain:	human volunteers, male and female
Group size:	98 subjects (19 male and 79 female)
Test substance:	# 441182 (Disperse Black 9 in a gel – concentration not stated)
Batch:	/
Purity:	/
Dose:	/
Volume:	0.1 ml (gel)
Application:	topical, semi-occlusive
GLP:	/
Study period:	24 October – 1 December 1983

0.1 ml of the test substance was applied to the infrascapular area of the back under semi-occlusive patches, either to the right or left of the midline, three times a week, for a total of 10 applications. Exposure was continuous.

The interval between induction and challenge application was 11 days.
For challenge, 0.1 ml test material was applied to a site previously unexposed to the test material under semi-occlusive patch conditions.
Skin readings were done 48 and 72 hours after challenge patch application.
No evidence of irritation or sensitisation was observed.

Ref.: 11

Comment
The concentration of Disperse Black 9 in the gel formulation was not stated. The vehicle was not stated.
HRIPT studies are considered unethical by SCCS.

Human Repeated Insult Patch Test

Guideline:	/
Species/strain:	human volunteers, male and female
Group size:	102 subjects; (12 male and 90 female)
Test substance:	Product # 4411182 a formulation containing 3% Disperse Black – in a gel
Batch:	/
Purity:	/
Dose:	/
Volume:	0.1 ml (gel)
Application:	semi-occlusive
GLP:	/
Study period:	9 January – 16 February 1984

0.1 ml of the test substance was applied to the infrascapular area of the back under semi-occlusive patches, either to the right or left of the midline, three times a week, for a total of 10 applications. Exposure was continuous.
The interval between induction and challenge application was 11 days.
For challenge, 0.1 ml test material was applied to site previously unexposed to the test material under semi-occlusive patch conditions.
Skin readings were done 48 and 72 hours after challenge patch application.
No evidence of irritation or sensitisation was observed.

Ref.: 12

Comment
Details of the Disperse Black 9 gel formulation were not given. The vehicle was not stated.
HRIPT studies are considered unethical by SCCS

General comment on Sensitisation
None of the studies provided conformed to guidelines. In the absence of appropriately performed studies, the sensitisation potential Disperse Black 9 cannot be excluded.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (2004a, 2004b)
Tissue:	dermatomed human female skin, 400 µm thickness
Group size:	12 membranes from 5 donors
Diffusion cells:	glass diffusion cell, 2.54 cm^2
Skin integrity:	electrical resistance > 10 kΩ
Test substance:	Disperse Black 9 [^{14}C]- Disperse Black 9; 2.33 GBq/mmol, 63.0 mCi/mmol
Batch:	A35996 3501-271 (radio-labelled)

Purity: Certificate of analysis purity = 115.7% subsequently analysed as 52.6% active dye.
97.8% (HPLC) (radio-labelled)
Test item: 1% Disperse Black 9 in hair dye cream formulation
Doses: 20 mg/cm²
Receptor fluid: 4% polyoxyethylene-20-oleyl ether in phosphate buffered saline
Solubility receptor fluid: > 0.98 mg/ml
Stability: /
Method of Analysis: Liquid scintillation counting
GLP: in compliance
Study period: 9 November – 1 December 2004

Duration of contact: 30 minutes followed by rinsing
Sampling: 0.5, 1, 2, 4, 6, 24, 29, and 48 hours after application

	Amount recovered (µg/cm²)													
Cell number	53	60	61	62	64	65	67	68	69	71	72	74	**mean**	**SD**
Flange	0.024	0.011	0.011	0.019	0.009	0.021	0.028	0.009	0.025	0.025	0.032	0.024	0.020	0.008
Donor chamber	0.037	0.042	0.043	0.077	0.031	0.148	0.032	0.035	0.100	0.033	0.043	0.279	0.075	0.073
Skin wash at 0.5h	107	107	108	103	99.3	108	113	116	115	104	114	109	109	5.16
Skin wash at 48h	0.172	0.083	0.090	0.481	0.111	0.459	0.188	0.210	0.763	0.143	0.243	0.372	0.276	0.205
Stratum corneum	0.047	0.091	0.073	0.055	0.092	0.130	0.053	0.010	0.083	0.051	0.112	0.000	0.066	0.038
Remaining epidermis/dermis	0.031	0.030	0.019	0.118	0.040	0.054	0.058	0.053	0.147	0.071	0.097	0.201	0.077	0.055
Receptor fluid	0.070	0.040	0.024	0.081	0.021	0.028	0.001	0.020	0.040	0.077	0.094	0.115	0.051	0.035
Bioavailable	**0.101**	**0.070**	**0.043**	**0.200**	**0.061**	**0.082**	**0.059**	**0.073**	**0.187**	**0.149**	**0.190**	**0.316**	**0.128**	**0.090**
Total	107	108	108	103	99.6	109	114	116	116	104	115	110	109	5.24

	Amount recovered (%)													
Cell number	53	60	61	62	64	65	67	68	69	71	72	74	**mean**	**SD**
Flange	0.022	0.011	0.011	0.018	0.008	0.020	0.026	0.009	0.024	0.024	0.030	0.023	0.019	0.007
Donor chamber	0.035	0.040	0.041	0.073	0.029	0.141	0.031	0.033	0.095	0.032	0.041	0.266	0.071	0.070
Skin wash at 0.5h	102	102	103	97.7	94.6	103	108	110	109	99.1	109	104	103	4.92
Skin wash at 48h	0.164	0.079	0.085	0.458	0.106	0.437	0.179	0.200	0.727	0.136	0.231	0.355	0.263	0.195
Stratum corneum	0.085	0.165	0.132	0.099	0.167	0.235	0.096	0.018	0.150	0.093	0.202	0.000	0.120	0.070
Remaining epidermis/dermis	0.030	0.029	0.018	0.113	0.038	0.051	0.055	0.050	0.140	0.068	0.092	0.192	0.073	0.052
Receptor fluid	0.067	0.038	0.023	0.078	0.020	0.027	0.001	0.019	0.038	0.074	0.089	0.109	0.049	0.034
Bioavailable	**0.096**	**0.067**	**0.041**	**0.190**	**0.058**	**0.078**	**0.056**	**0.069**	**0.178**	**0.142**	**0.181**	**0.301**	**0.122**	**0.086**
Total	102	103	103	98.5	94.9	104	108	111	110	99.5	110	105	104	4.99

The results refer to amounts of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol ('active dye') rather than Disperse Black 9, which contains lignosulfate as the dispersant.

The amount of active dye considered to be systemically available was 0.128 ± 0.09 µg/cm^2 (range 0.043-0.316 µg/cm^2) (0.122 ± 0.086% (range 0.041-0.301%) of the applied dose).
Ref.13

Comment
The test concentration in this assay was considerably higher than the intended use concentration. The Disperse Black 9 used in the experiment contained lignosulphate. The result data refer to 'active dye'. The amount of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol that may be considered to be systemically available for calculation of the MOS is 0.22 µg/cm^2 (mean + 1 SD, 0.13 ± 0.09µg/cm²).

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral / dermal / inhalation toxicity

Guideline:	OECD 407 (1995)
Species/strain:	rat Crl:CD®(SD)IGS BR
Group size:	60 (5 males and 5 females per dose group)
Test substance:	GTS03872
Batch:	A-35996
Purity:	52.6%
Vehicle:	0.5% (w/v) methylcellulose in reverse osmosis water
Dose levels:	0, 10, 50, 100, 250 and 500 mg/kg bw
Dose volume:	10 ml/kg bw
Route:	oral gavage
Exposure:	once daily for 16 days
GLP statement:	in compliance
Study period:	2 – 25 February 2004

Results
3 males and 2 females from the high dose group died or were sacrificed in the first 5 days of the study. The cause of death was determined to be general debilitation due to renal failure and marked hepatocellular injury, which were attributed to Disperse Black 9.

Surviving rats from the high dose group and rats in the 50, 100 and 250 mg/kg dose groups showed discoloured urine and yellow hair coat and skin. There were no remarkable observations for control animals or animals given 10 mg/kg bw/d.

Mean body weights and overall body weight gain (Days 1 to 15) decreases observed in males and females given 250 and 500 mg/kg/d were attributed to test article. Increased kidney and decreased thymus weights were observed in both sexes at the dose of 500 mg/kg bw/d.

There was a dose-related trend in decreased food consumption in male and female rats treated at the dose of 500 mg/kg bw/d.

Administration of test article at 500 mg/kg/d was associated with clinical pathology evidence of renal failure (markedly elevated urea nitrogen, creatinine and inorganic phosphorus and markedly decreased sodium and chloride) and marked hepatocellular injury (markedly increased ASAT, ALAT and γ-GT) in animals that became ill within the first 5 days of treatment. Other changes in clinical pathology parameters as mildly lower absolute reticulocyte count and moderately higher platelet count were observed in females at 500 mg/kg/d at day 17.

Conclusions
The NOAEL for this oral gavage administration of Disperse Black 9 for up to 14 days was 100 mg/kg/d. The material tested in this study was 52.6% active dye and 47.4% lignosulfate, therefore the NOAEL for the active dye is considered to be 52.6 mg/kg bw/d.
Ref.: 14

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

Guideline:	OECD 408 (1988)
Species/strain:	rat Crl:CD®(SD)IGS BR
Group size:	140 (15 males and females per dose group + 5 males and females in control and highest dose group for 4 week recovery)

Test substance:	GTS03872
Batch:	A-35996
Purity:	52.6% (47.4% lignosulfate)
Vehicle:	0.5% (w/v) methylcellulose in reverse osmosis water
Dose levels:	0, 10, 25 and 100 mg/kg bw
Dose volume:	10 ml/kg bw
Route:	oral gavage
Exposure:	96 days (males); 97 days (females)
GLP statement:	in compliance
Study period:	1 March – 12 July 2004

In this 96 days oral Toxicity Study in Sprague Dawley rats, Disperse Black 9 was administered daily by oral gavage in both sexes at dose levels of 0, 10, 25 and 100 mg/kg bw (15 animals per dose). Animals were observed twice daily for signs of toxicity. Clinical observations were recorded weekly. Animals were weighed at the start of the study and weekly thereafter. Food consumption was determined weekly. Vaginal cytology data were collected once daily for 21 consecutive days, beginning after week 10. Complete necropsies were performed on all animals (on day 96 for 15 males and on day 97 for 15 females in each group and on day 124 for all survivors). Selected tissues were examined macroscopically and microscopically. At each scheduled sacrifice, male reproductive assessment (sperm motility, morphology and count) was done.

Results
Survival was 100% for control and treated group.

Test article-related clinical observations during treatment phase were limited to discoloured (yellow or orange) urine, haircoat, and skin for animal given 25 or 100 mg/kg/d. Yellow haircoat and orange skin persisted through the recovery phase for animal given 100 mg/kg/d.

There were no test article-related ophthalmic observations or effects on neurobehavioral assessment tests, body weights or body weight changes, food consumption, clinical chemistry, haematology, vaginal cytology, organ weights and macroscopic or microscopic findings. The only differences clearly caused by the test article were changes in urine colour (dark yellow at the lowest dose level to orange at the highest dose level). This discoloration of urine may have interfered with the ability to read some of the urine reagent strip.

A slight reduction in sperm motility (81% compared to 89% motile sperm in control animals) was observed in the 100 mg/kg/d group at the end of the treatment period. However, this was not test article-related since the mean percent motility in these animals was not statistically different from controls in this study and is within the range of historical control values. In addition, no apparent treatment-related changes in epididymal sperm count or sperm morphology were observed at the terminal sacrifice. No apparent treatment-related changes in sperm motility, epididymal sperm count, or sperm morphology were observed at the recovery sacrifice.

Conclusion
Based on the results of this study, the NOAEL following oral gavage administration of Disperse Black 9 to rats is considered to be 100 mg/kg/d. The test article was 52.6% active dye and 47.4% lignosulfate, therefore the NOAEL for the active dye is considered to be 52.6 mg/kg day.

Ref.: 15

3.3.5.3. Chronic (> 12 months) toxicity

Guideline:	/
Species/strain:	Beagles

Group size:	36 (6 Animals per sex and dose)
Test substance:	Hair dye formulation containing 0.13% Disperse Black 9.
Batch:	/
Purity:	/
Dose:	0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation
Route:	Oral in diet
Exposure period:	24 months
GLP:	not in compliance
Study period:	Before 1975

36 beagle dogs were orally exposed daily during 2 years to a hair dye formulation containing 0.13% Disperse Black 9 at the doses of 0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation. Each animal was observed daily for signs of toxic or pharmacologic effects. Individual records of body weight and food consumption were kept on a weekly and daily basis.

Necropsy was performed on one male and one female from each group at 6, 12 and 18 months. Individual organ weights and organ to body weight ratios of the major organs were recorded. Sections from 30 tissues or organs were prepared and examined microscopically. Electron microscopic evaluation of the livers and urinary bladder from all 18 dogs at 24 months was performed.

Results
No noteworthy differences were seen in any of the parameters studied between the controls and the animals receiving 19.5 or 97.5 mg/kg bw/day. All dogs gained weight normally and survived to end of the 104 weeks. All dogs in the two test groups excreted urine of a blue-brown colour on a daily basis. However urine analysis showed no remarkable findings. Colour was normal in urine collected after overnight fasting.

No gross or microscopic changes were seen in the various tissues and organs that could be attributed to the test material. No ultra-structural changes were observed in the electron microscopic studies conducted on sections of liver and urinary bladder.

Conclusion
The authors concluded that oral exposure of a hair dye formulation containing 0.13% Disperse Black 9 in formulation up to 97.5 mg/kg bw/day did not result in any signs of toxicity.

Ref.: 28

Comments
The experiment did not conform to a guideline and was not performed according to GLP. The purity and specifications of test article is not known. Different hair dyes were tested in this study. The results are sparsely reported. No conclusions concerning long term toxic effects can then be made from this study

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537; *Escherichia coli* WP2*uvr*A (pKM101)
Replicates:	duplicates in the initial phase and triplicates in the confirmatory phase
Test substance:	Lowadene Black 9 (GTS03872)
Batch:	A35996

Purity:	51.4% (Covance study 6114-471)	
Solvent:	DMSO	
Concentrations:	initial phase:	2.5, 5, 20, 50, 200, 500, 2000 and 5000 µg/plate, without and with S9-mix
	Confirmatory phase:	5, 20, 50, 200, 500, 2000 and 5000 µg/plate, without and with S9-mix;
Conc. analyses:	within 18% of target concentration	
Treatment:	pre-incubation method, with 20 ± 2 minutes pre-incubation and 60 ± 12 h incubation without and with S9-mix	
GLP:	in compliance	
Study period:	27 July – 4 October 2004	

Salmonella typhimurium tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* tester strain WP2*uvr*A (pKM101) were used. The assay was conducted in presence and absence of microsomal preparation from Aroclor-induced rat liver (S9-mix), along with concurrent vehicle and positive controls, using two or three plates per dose in the initial and confirmatory assays, respectively. The dose range tested in the initial mutagenicity assay (preincubation method) comprised 2.50 - 5000 µg per plate +/- S9-mix. A confirmatory assay was performed within the same dose range +/- S9 mix. Negative and positive controls were in accordance with the guideline.

Results
C106 (GTS03872) reproducibly caused concentration-related increases in the mean number of revertants per plate with tester strains TA98, TA100, and TA1537 in the presence of rat liver S9-mix. In the absence of S9-mix, a positive response was seen in TA98.

Conclusion
Under the experimental conditions used Lowadene Black 9 was genotoxic (mutagenic) in this gene mutation tests in bacteria, reflecting base substitution and frame shift mutagenicity of C 106.

Ref.: 16

Comment
The manufacturer's Certificate of Analysis identifies the purity as 115.7%.

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)	
Species/strain:	L5178Y $tk^{+/-}$ mouse lymphoma cells	
Replicates:	single cultures in two independent experiments	
Test substance:	Lowadene Black 9 (GTS03872)	
Batch:	A35996	
Purity:	115.7%	
Vehicle:	DMSO	
Concentrations:	Experiment I:	25, 50, 100, 200, 300, 400 and 450 µg/ml without S9-mix 50, 100, 200, 300, 400, 450, 500 and 600 µg/ml with S9-mix
	Experiment II:	10, 15, 20, 25, 30, 35, 40 and 45µg/ml without S9-mix 100, 200, 300, 400, 450, 475, 500 and 550 µg/ml with S9-mix
Treatment	Experiment I:	4h treatment without and with S9-mix, expression period 2 days and a selection period of 12 days.
	Experiment II:	24h treatment without S9-mix or 4 h treatment with S9-mix, expression period 2 days and a selection period of 12 days.

GLP: in compliance
Study period: 5 July – 25 August 2004

Based on the results of the toxicity test, two independent experiments were carried out without metabolic activation; the initial mutagenicity trial was evaluated at concentrations ranging from 25.0 to 450 µg/mL the confirmatory test at concentrations from 10.0 to 45.0 µg/mL. Two independent experiments were carried out with metabolic activation at concentrations ranging from 50.0 to 600 µg/mL and in the confirmatory trial from 100 to 550 µg/mL.
Single cultures were investigated for each concentration and test group. Mutant frequency and cell survival (measured as relative total growth) were determined. In addition to the numbers of mutant colonies, the size of the colonies was determined and the ratio of small versus large colonies was calculated. Negative and positive controls were in accordance with the OECD guideline.

Results
With 4 h treatment in the absence of metabolic activation the mutant frequencies ranged from 56.1 to 144.5 x 10^{-6}. (Vehicle control 53.1 x 10^{-6}). Only the treatment at 450 µg/mL induced a mutant frequency that met criteria for a slightly positive response (associated with decreased cloning efficiency). This result was not confirmed in a more stringent 24 hr incubation assay. None of the further assays induced an increase in the mutant frequencies numbers of mutant colonies, the size of the colonies were determined and the ratio of small versus large colonies was calculated. Mutant colonies from all the cultures showed the expected bimodal distribution, and mutant colonies from MMS and MCA treated cultures showed both small and large colonies.

Conclusion
Under the experimental conditions used, Lowadene Black 9 did not induce forward mutations at the *tk* locus in the L5178Y $tk^{+/-}$ mouse lymphoma assay with and without metabolic activation.

Ref.: 18

In vitro Mammalian Chromosome Aberration Test

Guideline:	OECD 473 (1997)
Species/strain:	Chinese hamster ovary cells (CHO-WBL)
Replicates:	duplicate cultures
Test item:	Lowadene Black 9 (GTS03872)
Batch:	A35996
Purity:	51.4% (see comment under bacterial reverse mutation assay)
Vehicle:	DMSO
Concentrations:	4 h treatment without S9 mix: 200, 400 and 650 µg/mL 4 h treatment with S9 mix: 100, 300 and 500 µg/mL 20 h treatment without S9 mix: 30, 60 and 90 µg/mL
Treatment:	4 h treatment (both in the absence and presence of S9 mix) or ~20 h treatment (in the absence of S9 mix only) and harvest time ~20 after start of treatment
GLP:	in compliance
Study period:	7 July – 14 November 2004

Test concentrations were based on the results of an initial toxicity assay on cell count, cell growth and cell growth inhibition. Cells were treated for 4 h and harvested ~20 h after the start of treatment (without and with S9 mix) or for 20 h and harvested ~20 h after the start of treatment without S9-mix). Approximately 2 h before harvest, each culture was treated with colcemid (final concentration 0.1 µg/ml) to block cells at metaphase of mitosis. Liver

S9 fraction from Arachlor 1254-induced rats was used as exogenous metabolic activation system. Negative and positive controls were in accordance with the OECD guideline.

Results
Without metabolic activation with a 4-hour treatment, a dose dependent and statistically significant increase in cells with chromosomal aberrations was observed. In the assay with metabolic activation and 4 hour treatment a dose dependent increase in cells with chromosomal aberrations was observed which was only statistically significant at the highest dose (500µg/mL). In the assay without metabolic activation with ~20-hour treatment, no statistically significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

Conclusion
Under the experimental conditions used the increase in cells with structural chromosomal aberrations found after 4 hour treatment both without and with S9-mix indicates to a genotoxic (clastogenic) activity of Lowadene Black 9 in CHO cells *in vitro*.

Ref.: 17

Comment
The manufacturer's Certificate of Analysis identifies the purity as 115.7%.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474 (1997)
Species/strain:	mouse, CD-1® (ICR)BR
Group size:	5 male mice per dose group and sacrifice time
Test substance:	Lowadene Black 9 (GTS03872)
Batch:	A35996
Purity:	115.7%
Vehicle:	0.5% methylcellulose in distilled water
Dose level:	81.25, 162.5 and 325 mg/kg bw
Route:	oral gavage
Sacrifice times:	24 h and 48 h (control and high dose group only) after treatment.
GLP:	in compliance
Study period:	30 August – 17 September 2004

Test concentrations were based on the results of a dose range-finding study in male and female rats on toxic signs and mortality. Animals (three males and three females per dose group) were dosed at 125, 250, 500, 1000 or 2000 mg/kg and observed for up to 2 days after dosing for toxic signs and/or mortality. All animals were examined immediately after dosing, approximately 1 hour after dosing, approximately 4 hours, and at least daily for the duration of this assay for signs of clinical toxicity and/or mortality.
In the main experiment Lowadene Black 9 was administered once in 0.5% methylcellulose at doses of 81.25, 162.5 and 325 mg/kg (MTD). Five animals from all the groups were sacrificed 24 hours after dosing and from the vehicle control and high dose group at 48 hours after dosing. A satellite group of 15 treated mice was included for the determination of plasma concentrations of Lowadene Black 9, collected at 1, 2, 4, 6, 8, 24 and 48 hours after dosing. For all groups at 24 hours and vehicle and high dose group only at 48 hours, bone marrow was extracted and at least 2000 PCEs per animal were microscopically analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and of normochromatic erythrocytes (NCEs) in at least the first 1000 total erythrocytes for each animal.

Results

In the dose range-finding study, mortality was observed in 2/3 males and 1/3 females in the 500 mg/kg dose group and in all animals dosed at 1000 and 2000 mg/kg. In addition, orange-coloured ears, tails, paws, and/or genitals were observed in all animals in the 500, 1000, and 2000 mg/kg dose groups. All animals in the 125 and 250 mg/kg dose groups appeared normal immediately after dosing and remained healthy until the end of the observation period.
In the main experiment Lowadene Black 9 induced mortality in one animal treated at 325 mg/kg. Lowadene Black 9 did not induce statistically significant increases in micronucleated PCEs at any dose examined (81.25, 162.5, and 325 mg/kg). However, Lowadene Black 9 was cytotoxic to the bone marrow (i.e., a statistically significant decreases in the PCE:NCE ratios) at 81.25 and 162.5 mg/kg, the positive control CP being slightly more effective. This is considered direct evidence of bone marrow exposure to the compounds and/or their metabolite(s). Peak mean plasma concentration of Lowadene Black 9 was observed at approximately one hour post-dose (12.07 ± 1.05 µg/mL). The positive control produced a significant increase in micronucleated PCEs and the vehicle control induced micronuclei within the laboratory's historical control range

Conclusion
Lowadene Black 9 was not genotoxic in the *in vivo* micronucleus assay to mice by gavage under the conditions of this assay

Ref.: 19

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

Guideline:	OECD 486 (1997)
Species/strain:	rat, Sprague-Dawley
Group size:	44 (8 groups of 5 animals, highest dose: 7 animals)
Test substance:	Lowadene Black 9 (GTS03872)
Batch:	A35996
Purity:	115.7%
Vehicle:	0.5% methylcellulose
Dose level:	500 and 1000 mg/kg bw (estimated MTD in SD rats)
Dosing volume:	10 ml/kg bw
Route:	oral gavage, single dose
Sacrifice times::	2 to 4 hours; 12 to 16 hours
GLP:	in compliance
Study period:	18 July – 11 August 2005

Test concentrations were selected based on toxicity in a dose range finding study. Hepatocytes for UDS analysis were collected 2-4 h and 12-16 h after administration of Lowadene Black 9 administered to 5 male rats per dose. Hepatocytes were only harvested from the first three successful perfusions and evaluated for UDS. Ninety to 180 minutes after plating the cells were incubated for 4 h with 10 µCi/ml ^{3}H-thymidine followed by 17-20 h incubation with unlabelled thymidine. Evaluation of autoradiography was done after 7 days. UDS was reported as net nuclear grain: the nuclear grain count subtracted with the average number of grains in 3 nuclear sized areas adjacent to each nucleus. Also the percentage of cells in repair (defined as cells with a net grain count of ≥ 5) was calculated for each animal. Negative and positive controls were in accordance with the OECD guideline.

Results
The group mean net nuclear grain (NG) counts for animals treated with GTS03872 were not increased when compared to the vehicle control. For the 2 to 4 hour time point, the group mean NG counts for the test article-treated animals were -2.7 and -1.9 with 0% and 2% of cells in repair (cells with ≥ 5 NG) for the 500 and 1000 mg/kg bw, respectively. The group mean NG count for the vehicle control group was -2.9 with 1% of cells in repair. For the 12 to 16 hour time point, the group mean NG counts for the test article-treated animals were -

5.0 and -4.8 with 2% of cells in repair (cells with ≥ 5 NG) for both 500 and 1000 mg/kg bw, respectively. The group mean NG count for the vehicle control group was -3.9 with 4% of cells in repair. The positive control group mean NG counts were 17.2 and 17.9 for the 2 to 4 hour and 12 to 16 hour, respectively. The percentage of cells in repair for the positive control group was 100% and 99% for the 2 to 4 hour and 12 to 16 hour, respectively.

Conclusion
Under the experimental conditions used, Lowadene Black 9 did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 20

Heritable Translocation study

Guideline:	/
Species/strain:	rat, outbred Charles River Sprague Dawley CD
Group size:	25 male rats/group, 75 females (F_0)
Test substance:	Disperse Black 9 at 0.04% applied in a semipermanent hair dye formulation containing seven other colouring agents
Batch:	/
Purity:	/
Dosing volume:	0.5 ml/sq. inch, shaved back skin
Route:	topical application of 0.5 ml of dye formulation, switching between adjacent areas on alternate days
Treatment:	twice weekly for 10 weeks (F_0), dye solutions not shampooed off.
GLP:	/
Study period:	1980

Each treated and control male rat was mated for 7 d to 1 sexually mature 10 week old female rat. This was repeated until each male had mated with 3 females, giving a total of 75 mated females in each treatment group. At birth number and sex of live and dead pups were recorded, after 4 d each litter culled to max 6 males. After weaning (d 21), 2 healthy males were selected from each litter and after 12 weeks 100 of the F1 males were mated with 3 mature females of the same strain. Pregnancies were timed for delivery by caesarean section on d 14-16 of gestation. Numbers of implants, resorptions, live and dead foetuses were recorded.
The rats were treated topically with a semi-permanent hair dye formulation containing 8 colouring agents including Disperse Black 9.

Results
Groups receiving topically the semi-permanent hair dye formulation were reported to be nearly identical to untreated controls, showing no indication for reduced fertility.

Conclusion
The authors concluded that no evidence was obtained that topical application of the semi-permanent hair dye formulation containing, amongst other colorants 0.04% Disperse Black 9 induces stable chromosomal rearrangements potentially resulting in reduced fertility of the offspring.

Ref.: 31

Comment
The value of this assay is negligible since next to the 0.04% Disperse Black 9, the applied semi-permanent hair dye formulation also contained 7 other hair dyes like 0.12% Disperse Blue (EU carcinogen Cat 2) and 0.50% HC Blue (IARC: sufficient evidence for carcinogenicity in animals). These confounding chemicals make it impossible to conclude on the potential of Disperse Black 9 to induce heritable chromosomal mutations.

3.3.7. Carcinogenicity

Oral administration, dog

Guideline:	/
Species/strain:	Beagles
Group size:	6 Animals per sex and dose
Test substance:	semipermanent hair dye formulation containing 0.13% Disperse Black 9
Batch:	/
Purity:	not stated
Dose:	0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation (0, 25 and 127 µg/kg bw/day of Disperse Black 9)
Route:	Oral, diet
Exposure period:	24 months
GLP:	not in compliance
Study period:	before 1975

Diets were prepared daily with the incorporation of the hair dye formulation to give doses of 0, 19.5 and 97.5 mg/kg bw/day to the beagles dogs. The dogs were 7 – 9 month of age when the study was started. Adjustments of concentrations in the diet were made weekly according to body weight changes. Each animal was observed daily for signs of toxic or pharmacologic effects. Individual records of body weight and food consumption were kept on a weekly and daily basis. No positive control group was used.

Physical examinations including funduscopy, EKG, blood pressure, pulse rate and body temperature were conducted initially and at 3, 6, 12, 18 and 24 months. Haematological, blood chemical and urinalysis parameters were determined on all high dose and control dogs and on 3 males and 3 females from the low dose group. Haematologic studies included determination of total and differential leucocyte counts, haematocrit, haemoglobin concentration, erythrocyte sedimentation rate and prothrombin time. Clinical chemistry determinations were conducted on animals that had been fasted for 18 hours. These included serum glucose, blood urea nitrogen, creatinine and uric acid concentrations and alkaline phosphatase and serum glutamic pyruvic transaminase activities. Urinalysis included detection of occult blood, albumin, glucose, pH and microscopic examination of urinary sediment.

Necropsy was performed on one male and one female from each group at 6, 12 and 18 months. Individual organ weights and organ to body weight ratios of the major organs were recorded. Sections from 30 tissues or organs were prepared and examined microscopically. Electron microscopic evaluation of the livers and urinary bladder from all 18 dogs at 24 months was performed.

No noteworthy differences were seen in any of the parameters studied between the controls and the animals receiving 19.5 or 97.5 mg/kg bw/day. All dogs gained weight normally and survived to end of the 104 weeks. All dogs in the two test groups excreted urine of a blue-brown colour on a daily basis. However urine analysis showed no remarkable findings. Colour was normal in urine collected after overnight fasting.

No gross or microscopic changes were seen in the various tissues and organs that could be attributed to the test material. No ultra-structural changes were observed in the electron microscopic studies conducted on sections of liver and urinary bladder.

The authors concluded that oral dosing exposure of a hair dye formulation containing 0.13% Disperse Black 9 in formulations up to 97.5 mg/kg bw/day did not result in any signs of toxicity.

Ref.: 5

Comment
No conclusions concerning potential carcinogenic effects can be made from the study with dogs due to the low concentration of Disperse Black 9. Moreover, it should be noted that the hair dye formulation contained 0.61% Disperse Blue 1 (EU carcinogen category 2) and 1.54% HC Blue I (IARC: sufficient evidence for carcinogenicity in animals).

Topical application, mice

Guideline:	/
Species/strain:	Eppley Swiss mice
Group size:	60 animals per sex
Test substance:	A semipermanent hair dye formulation (7601) containing 0.5% Disperse Black 9
Batch:	/
Purity:	/
Dose level:	0.05 ml of a solution containing 0.5% Disperse Black 9
Route:	Topical, 3 application weekly
Exposure period:	20 months
GLP:	not in compliance
Study period:	before 1984

2 oxidative and 12 non-oxidative hair dye formulations were tested. Two of the non-oxidative hair dye formulation including the one with Disperse Black 9 contained 0.3% Disperse Blue 1 (EU carcinogen category 2).

Swiss mice (8 weeks old), groups of 60 males and 60 females, were painted three times weekly with a hair dye formulation for 20 months. Aliquots of 0.05 ml were delivered to an area of skin in the interscapular region. The mice were shaved 24 hours before treatment as needed. Two control groups of were shaved only and received no treatments. The oxidative dye solutions were mixed with an equal volume of 6% H_2O_2 just prior to application. One of the non-oxidative hair dye formulations contained 0.5% Disperse Black 9. A gross necropsy was performed on all mice.

The application of hair dyes did not have an adverse effect on average body weight gains or survival of any group. Body weights were not depressed more than 10% in any group compared to the controls. The predominant tumours seen were those that occur commonly in the Eppley Swiss mouse, namely lung adenomas, liver haemangiomas, and malignant lymphomas. No unusual tumours developed in any of the groups.

The authors concluded that no toxic or carcinogenic effects were induced by Disperse Black 9.

Ref.: 32

Comment
In this study with Disperse Black 9 in a semipermanent hair dye formulation (7601) involving topical application to mice, the concentration of Diverse Black 9 was 0.5%. A number of different hair dye formulations were tested in the same study. Although some of the formulations contained Disperse Blue 1 (EU carcinogen category 2), none of the formulations induced tumours. Thus, no conclusion with regard to carcinogenicity can be drawn from the studies.

General comment on carcinogenicity
No conclusions concerning potential carcinogenic effects can be drawn from an oral study with dogs and a skin painting study with mice. Disperse Black 9 was present in low concentrations (0.5% or less) in semipermanent hair dye formulations. Moreover, although

substances classified as carcinogens were present in the formulations studied, no carcinogenic effects were found in any of the studies indicating low sensitivity.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Dose-range finding development toxicity study

Guideline:	OECD 414 (2001)
Species/strain:	rat, Crl:CD®(SD)IGS BR VAF/Plus®
Group size:	40 (8 pregnant female rats per dose)
Test substance:	Disperse Black 9 (GTS03872) 52.6% active dye and 47.4% lignosulfate
Batch:	A-35996
Purity:	115.7%
Vehicle:	0.5% methylcellulose in deionised water
Dose levels:	0, 25, 75, 150 and 375 mg/kg bw
Dosing volume:	10 ml/kg bw
Administration:	oral gavage
Exposure:	once daily, DG 6 through 20
GLP statement:	in compliance
Study period:	17 March – 14 April 2004

In this oral dosage range finding developmental toxicity study, Disperse Black 9 was administered orally once daily to 40 presumed-pregnant Crl:CD® (SD)IGS BR VAF/Plus® rats from day 6 to day 20 of gestation at doses of 0, 25, 75, 150 and 375 mg/kg bw/d in 0.5% methylcellulose/deionised water. The dosage volume was 10 ml/kg bw.

Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on day 21. The gravid uterus was weighed and examined for gross external alterations and sex. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results
One female rat treated at the dose of 375 mg/kg bw/d was found dead on the day 12.

Test article-related clinical observations during treatment were limited to discoloured urine, fur, and skin for rats in all treated groups. Other clinical observations included brown or dark perivaginal substance and excess salivation (each observed in 2 rats treated at the dose of 375 mg/kg bw/d).

No gross lesions were observed by necropsy examination.

Mean body weights reduced in the rats treated at the dose of 375 mg/kg bw/d on gestation day 6 -9 and body weight gains were reduced in the other rats on gestation days 9-12, 12-15 and 18-21 as well as for the entire dosage period and gestation period in the 375 mg/kg bw/d dosage group. Corrected maternal body weight gains were also reduced in the 375 mg/kg bw/d dosage group for the dosage period and the gestation period. Absolute and relative feed consumptions were reduced in the rats treated at the dose of 375 mg/kg bw/d for the entire dosage and gestation periods.

Pregnancy occurred in all 8 rats in each dosage groups.

No significant differences were observed in the number of corpora lutea in all treated rats when comparing with the control rats. No significant differences in the number of implantations, early or late resorptions, litter sizes, live foetuses were observed in the treated rats when comparing with the control rats. Foetal body weight was reduced in the foetus of rats treated at the dose of 375 mg/kg bw/d. No other caesarean-sectioning or litter parameters were affected by dosages of the test substance as high as 375 mg/kg bw/d.

One litter in the 75 mg/kg bw/d had fused placentae for two implantation sites. This observation was not considered to be related to the test substance because it occurred in only one rat and was not dosage dependent. All other placentae appeared normal. One foetus in the 75 mg/kg bw/d had a cleft palate. One foetus in the 375 mg/kg bw/d dosage group had gastroschisis. The numbers of foetuses evaluated in this study were too few to determine if these alterations were related to the test substance.

Conclusion
Based on the results of this study, the doses of 0, 20, 100 and 200 mg/kg bw/d were selected for the developmental toxicity study in rats.

Ref.: 21

Development toxicity study

Guideline:	OECD 414 (2001)
Species/strain:	rat, Crl:CD®(SD)IGS BR VAF/Plus®
Group size:	100 (25 pregnant female rats per dose)
Test substance:	Disperse Black 9 (GTS03872) 52.6% active dye and 47.4% lignosulfate
Batch:	A-35996
Purity:	52.6%
Vehicle:	0.5% methylcellulose in deionised water
Dose levels:	0, 20, 100 and 200 mg/kg bw
Dosing volume:	10 ml/kg bw
Administration:	oral
Exposure:	once daily, DG 6 through 20
GLP statement:	in compliance
Study period:	7 May – 4 June 2004

In this oral prenatal developmental toxicity study, Disperse Black 9 was administered orally once daily to 100 presumed-pregnant Crl:CD® (SD)IGS BR VAF/Plus® rats from day 6 to day 20 of gestation at doses of 0, 20, 100 and 200 mg/kg bw/d in 0.5% methylcellulose in deionised water. The dosage volume was 10 ml/kg bw.

Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on day 21. The gravid uterus was excised, weighed and subsequently examined for the number and distribution of corpora lutea, implantation sites and uterine contents. A gross necropsy was performed. Foetuses were weighed and examined for gross external alterations, sex and either soft tissue or skeletal alterations. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results
Maternal parameters:
All rats survived to day 21. Significant increases in orange urine, yellow or orange fur occurred in the treated rats.
Body weight gains were significantly reduced in the rats treated at the dose of 100 mg/kg bw/d and 200 mg/kg bw/d on days 6 to 9. Absolute and relative feed consumption values

were significantly reduced in the rats treated at the dose of 20 (absolute only), 100 and 200 mg/kg bw/d on days 6 to 9.
Pregnancy occurred in 23 to 25 rats in each dosage group.
No Caesarean-sectioning or litter observations were affected by dosages of Disperse Black 9 as high as 200 mg/kg bw/d. No gross external, soft tissue or skeletal alterations related to the test substance occurred. The number of ossification sites per foetus per litter did not differ among the groups.

Conclusions
Based on these data, the maternal NOAEL, is 20 mg/kg/d, since the 100 and 200 mg/kg/d dosages produced significant reductions in body weight gains and feed consumption. The material tested in this study was 52.6% active dye and 47.4% lignosulfate, therefore the maternal NOAEL for the active dye is considered to be 10.5 mg/kg bw/d.

The developmental NOAEL is 200 mg/kg bw/d (highest dose tested). The material tested in this study was 52.6% active dye and 47.4% lignosulfate, therefore the developmental NOAEL for the active dye is considered to be 105 mg/kg bw/d.

Ref.: 22

Guideline:	/
Species/strain:	Virgin CFE-S rats
Group size:	120 (20 males – not treated with the formulation and 20 females per dose)
Test substance:	Hair dye formulation containing 0.13% Disperse Black 9.
Batch:	/
Purity:	/
Dose:	0, 1950 and 7800 ppm of hair dye formulation in the diet
Route:	Oral diet
Exposure period:	once daily on Day 6 through Day 15
GLP:	not in compliance
Study period:	Before 1975

In this oral dosage developmental toxicity study, a formulation containing 0.13% Disperse Black 9 was administered in the diet once daily to 120 presumed-pregnant CFE-S female rats from day 6 to day 15 of gestation at the doses of 0, 1950 and 7800 ppm. The dosage volume was 1 ml/kg bw.

All surviving rats were sacrificed on day 19. The gravid uterus was weighed and examined for gross external alterations and sex. Caesarean-sectioning and subsequent foetal observations were conducted without knowledge of dosage group.

Results
No effects on body weight gains and food consumption were observed. No significant differences were observed in any of the parameters examined including numbers of implantation sites, live pups and early or late resorption per litter or in the number of females with one or more resorption sites. No grossly abnormal pups were observed in the treated rats when comparing with the control rats except one pup at the high dosage group.

Conclusion
Based on the results of this study, a maternal and foetal NOAEL of 7800 ppm of the test material/ kg bw/d was proposed by the applicant.

Ref.: 28

Comments
The experiment did not conform to a guideline and was not performed according to GLP. The purity and specifications of test article is not known. Different hair dyes were tested in

this study. The results are sparsely reported. No conclusions concerning long term toxic effects can then be made from this study

Guideline: /
Species/strain: New Zealand white rabbits
Group size: 48 (12 female per sex and dose)
Test substance: Hair dye formulation containing 0.13% Disperse Black 9
Batch: /
Purity: /
Dose: 0, 19.5 and 97.5 mg/kg bw/day of hair dye formulation
Route: Oral, gavage
Exposure period: once daily on Day 6 through Day 18
GLP: not in compliance
Study period: Before 1975

In this oral dosage developmental toxicity study, a formulation containing 0.13% Disperse Black 9 was administered orally once daily to 48 artificially inseminated New Zealand white rabbit females from day 6 to day 18 of gestation at the doses of 0, 19.5 and 97.5 mg/kg bw/d of the total formulation. The dosage volume was 1 ml/kg bw.

All rabbits were sacrificed on day 30 of gestation. The gravid uterus was examined for gross external alterations and sex. Foetal observations were conducted.

Results
The number of pregnancies, maternal weight gains and mean values per pregnant female for numbers of *corpea lutea*, implantations and resorptions were measured but not reported. There was no evidence of a teratologic effect in any group. Foetal survival was not adversely affected by the dye formulation. No grossly abnormal foetuses and no soft tissues defects were seen. The principle findings of the skeletal examination were variations in the degree of ossification and in the number of ribs in this species. The distribution of these changes showed no relationship to treatment. Animals receiving the high dose excreted blue-brown coloured urine within an hour after dosing. Urine colour was normal the next day prior to dosing which is in favour of a rapid elimination.

Conclusion
Based on the results of this study, a maternal and foetal NOAEL of 97.5 mg test material/ kg bw/d was proposed by the applicant.

Ref.: 3

Comments
The experiment did not conform to a guideline and was not performed according to GLP. The purity and specifications of test article is not known. Different hair dyes were tested in this study. The results are sparsely reported. No conclusions concerning long term toxic effects can then be made from this study

Summary of repeated dose toxicity

Study	Species	Sex	Effects	Critical doses	Ref
14-day, oral	rat Crl:CD®(SD)I GS BR	m and f	**500 mg/kg/d:** 5† (3m and 2f); ☐ kidneys and ☐ thymus weight; renal failure and hepatocellular injuries **>= 250 mg/kg/d:** ☐ body weight (m and f)	NOAEL = 100 mg/kg/d (52.4 mg/kg bw/d a.i.)	14
90-day, oral	rat Crl:CD®(SD)I GS BR	m and f	**100 mg/kg/d:** ☐sperm motility (not significant)	NOAEL = 100 mg/kg/d (52.4 mg/kg bw/d a.i.)	15
Teratogen	rat,	f	**375 mg/kg/d:** 1†; ☐ body		21

Study	Species	Sex	Effects	Critical doses	Ref
icity, oral	Crl:CD®(SD)IGS BR VAF/Plus®	(G6-G20)	weight and food consumption; ☐ fœtal body weight;		
Teratogenicity, oral	rat, Crl:CD®(SD)IGS BR VAF/Plus®	f (G6-G20)	**> 100 mg/kg/d**: ☐ body weight and food consumption;	**Maternal** **NOAEL** = 20 mg/kg/d (10.5 mg/kg/d a.i.) **Developmental** **NOAEL**: 200 mg/kg/ d (105.2 mg/kg/d)	22

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

No data submitted

3.3.11. Human data

See point 3.3.3. Skin sensitisation

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Disperse Black 9

Absorption through the skin (mean+1 SD)	**A**	**=**	**0.22 µg/cm²**
Skin Area surface	**SAS (cm²)**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.13 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.002 mg/kg bw**
No observed adverse effect level (maternal toxicity in developmental toxicity study, oral, rat)	**NOAEL**	**=**	**10.5 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**5250**

3.3.14. Discussion

Physico-chemical properties

Disperse Black 9 is used at levels up to 0.3% Disperse Black 9, corresponding to 0.15% 2,2'-[4-(4- aminophenylazo)phenylimino]diethanol dispersed in lignosulfate, in non-oxidative hair dye formulations.

2,2'-[4-(4-aminophenylazo)phenylimino]diethanol is a commercially available hair dye ingredient with a purity of 97.4%. A commercial batch has been used as standard for analytical purposes and for all formulations used for testing relevant toxicity. The listed impurities, which sum up to 0.032%, have not been detected in 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol. About 2% of 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol was not identified. Disperse Black 9 contains 4-ABP,

which is carcinogenic for humans. However its concentration of 4.35 ppm is considered tolerable according to opinion SCCNFP/0797/04 (Opinion Concerning Use of Permanent Hair Dyes and Bladder Cancer).
No documents have been submitted on the solubility of the substance.
No data on the stability of Disperse Black 9 in typical hair dye formulations has been submitted.

Toxicity
4 acute oral toxicity studies were submitted. In an intraperitoneal toxicity study, the LD50 of Disperse Black 9 for male rats was 1297 mg/kg bw.
Based on a 14 days oral toxicity studies in rats, kidneys and liver were the main target organ of systemic toxicity of Disperse Black 9. A NOAEL of 52.6 mg/kg bw/d for 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol may be derived from the 90 days oral toxicity study in rats, which was the highest dose tested in this study.
No two generation reproduction toxicity study was submitted.
Two teratogenicity studies were submitted. Significant reductions in body weight and feed consumptions were observed in the female rats treated at the doses of 100 and 200 mg/kg bw/d. Foetal body weights were reduced at the dose of 375 mg/kg/d. A maternal NOAEL of 10.5 mg/kg bw/d and a developmental NOAEL of 105 mg/kg bw/d for 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol were determined from these studies.

Skin/eye irritation and sensitisation
The data submitted suggests that Disperse Black 9 was not irritant to rabbit skin. In some studies, Disperse Black 9 was not irritant to the eyes. In others, it caused irritation to rabbit eyes.
None of the studies submitted conformed to guidelines. In the absence of appropriately performed studies, the sensitisation potential Disperse Black 9 cannot be excluded.

Percutaneous absorption
The amount of active dye from a hair dye cream formulation containing 1% Disperse Black 9 that may be considered to be systemically available for calculation of the Margin of Safety is is 0.22 µg/cm^2 (mean + 1 SD, 0.13 ± 0.09µg/cm²).. This value refers to the 'active dye' 2,2'-[4-(4-aminophenylazo)phenylimino]diethanol.

Mutagenicity/genotoxicity
Overall, the genotoxicity of Disperse Black 9 is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Disperse Black 9 did induce gene mutations in bacteria but not in mammalian cells. In an *in vitro* chromosome aberration test an increase in cells with chromosomal aberrations was reported.
The positive *in vitro* findings with Disperse Black 9 could not be confirmed in *in vivo* assays. A mouse bone marrow micronucleus tests and an *in vivo* UDS test in rats was negative.
As the *in vitro* results were not confirmed in *in vivo* tests, Disperse Black 9 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No conclusions concerning potential carcinogenic effects can be made from an oral study with dogs and a skin painting study with mice. Disperse Black 9 was present in low concentrations (0.5% or less) in semipermanent hair dye formulations. Moreover, although substances classified as carcinogens were present in the formulations studied, no carcinogenic effects were found in any of the studies indicating low sensitivity.

4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of Disperse Black 9 as a non-oxidative hair dye with a maximum on-head concentration of 0.3% (corresponding to 0.15% 2,2'-[4-(4- aminophenylazo)phenylimino]diethanol dispersed in lignosulfate) does not pose a risk to the health of the consumer.

A sensitising potential cannot be excluded.

5. MINORITY OPINION

Not applicable

6. REFERENCES

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