Scientific Committee on Consumer Safety

SCCS

OPINION ON

Disperse Blue 377

COLIPA n° C179

The SCCS adopted this opinion at its 14th plenary meeting

of 27 March 2012

About the Scientific Committees

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

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

SCCS

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

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged. Revised opinions carry the date of revision.

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1. BACKGROUND

Submission I for Disperse Blue 377 was submitted in September 2003 by COLIPA [1].

According to the current submission II, submitted by COLIPA in July 2005, Disperse Blue 377 is a mixture of (1) 1,4-bis[(2-hydroxyethyl)amino]anthra-9,10-quinone, (2)1-[(2-hydroxyethyl)amino]-4-[(3-hydroxypropyl)amino] anthra-9,10-quinone, and (3) 1,4-bis[(3-hydroxypropyl)amino]anthra-9,10-quinone. The CAS are (1) 4471-41-4, (2) 67674-26-4 and (3) 67701-36-4. Disperse Blue 377 is used in non-oxidative hair dye formulations at a concentration up to 2% (0.93% active dye).

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 Blue 377 safe for use as a non-oxidative hair dye at a maximum concentration of 2.0% taking into account the scientific data provided?*

2. *Does the SCCS recommend any restrictions with regard to the use of Disperse Blue 377 in any hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

Chemical identity of DB 377, batch A -16798 has been tested using MS and NMR (Clairol; RR 01-960; 2001). For batch A- 37372 NMR, UV and FTIR had been used (ref.:1b)

Comment
With these 2 batches, the toxicological tests have been performed. For the bacterial mutation test, batch 2.12.A disper/7797 has been used, but it was not characterized.

3.1.1.1. Primary name and/or INCI name

Disperse Blue 377 (INCI name)

Disperse Blue 377 is a mixture of three dyes:

(1) CAS 4471-41-4, (1,4-bis[(2-hydroxyethyl)amino]anthra-9,10-quinone)
(2) CAS 67674-26-4, (1-[(2-hydroxyethyl)amino]-4-[(3-hydroxypropyl)amino]anthra-9,10-quinone)
(3) CAS 67701-36-4 (1,4-bis[(3-hydroxypropyl)amino]anthra-9,10-quinone)]

The sum of these three dyes is dispersed in lignosulphate at a ratio of approximately 50:50 (acceptable range 40.9 - 59.5% 'active dye').

3.1.1.2. Chemical names

Mixture of (1), (2) & (3) in dispersing agent (lignosulphate):

(1) 1,4-bis[(2-hydroxyethyl)amino]anthra-9,10-quinone
9,10-anthracenedione-1,4-bis[(2-hydroxyethyl)amino]

(2) 1-[(2-hydroxyethyl)amino]-4-[(3-hydroxypropyl)amino]anthra-9,10-quinone
9,10-anthracenedione-1-[(2-hydroxyethyl)amino]-4-[(3-hydroxypropyl)amino]

(3) 1,4-bis[(3-hydroxypropyl)amino]anthra-9,10-quinone
9,10-anthracenedione-1,4-bis[(3-hydroxypropyl)amino

3.1.1.3 Trade names and abbreviations

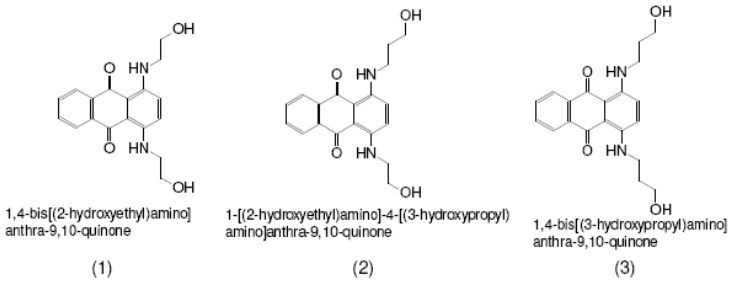
COLIPA C179

3.1.1.4 CAS / EC number

CAS: (1) 4471-41-4 (2) 67674-26-4 (3) 67701-36-4

EC: (1) 224-743-7 (2) 266-865-3 (3) 266-954-7

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

(1) $C_{18}H_{18}N_2O_4$
(2) $C_{19}H_{20}N_2O_4$
(3) $C_{20}H_{22}N_2O_4$

3.1.2 Physical form

Dark bluish powder

3.1.3 Molecular weight

Molecular weight: (1) 326.35 g/mol
(2) 340.37 g/mol
(3) 354.40 g/mol

3.1.4 Purity, composition and substance codes

According to submission II, the purity of DB 377, batch A 37372, measured with HPLC, was

Total dye content:	40.9 - 59.6%
Content of each component:	
1,4-bis[(2-hydroxyethyl)amino]anthra-9,10-quinone:	9.8 - 14.9%
1-[(2-hydroxyethyl)amino]-4-[(3-hydroxypropyl) amino]anthra-9,10-quinone:	22.1 - 30.4%
1,4-bis[(3-hydroxypropyl)amino]anthra-9,10-quinone:	9.0 - 14.3%

Impurities	
1,4-dihydroxy-9,10-anthraquinone (Quinizarin):	< 900 ppm
N-nitrosodiethanolamine (NDELA):	< 50 ppb

Heavy Metals:	Arsenic	< 5 ppm
	Antimony	< 5 ppm
	Lead	< 20 ppm
	Cadmium	< 10 ppm
	Mercury	< 5 ppm

Loss on drying (water): < 6%

According to ref. 1b, the data on purity of batch A 37372 are:

Average purity (HPLC): 46.5 % (n=4) and 47.1% (n=4) (HPLC).
Average purity (UV): 49.5%

Average purity (HPLC):	1,4-bis[(2-hydroxyethyl)amino]anthra-9,10-quinone:	11.2%
	1-[(2-hydroxyethyl)amino]-4-[(3-hydroxypropyl) amino]anthra-9,10-quinone:	24.1%
	1,4-bis[(3-hydroxypropyl)amino]anthra-9,10-quinone:	11.2%

Average moisture: 3.43% (n=3) (105 C)

NDELA: < 20 ppb

Trace amounts of the following substances, which have been detected by TLC have been identified by MS and NMR respectively. (Clairol; RR 01-960; 2001)

(I1)	1-(2-hydroxyethyl)amino-9,10-anthracenedione,	$C_{16}H_{13}NO_3$	MW 267
(I2)	1-(3-hydroxypropyl)amino-9,10-anthracenedione,	$C_{17}H_{15}NO_3$	MW 281
(I3)	1-(2-hydroxyethyl)amino-4-amino-9,10-anthracenedione,	$C_{16}H_{14}N_2O_3$	MW 282
(I4)	1-(3-hydroxypropyl)amino-4-amino-9,10-anthracenedione,	$C_{17}H_{16}N_2O_3$	MW 296

Comments
Batch A16798 has not been tested for purity. This batch has been used for acute oral toxicity, acute eye irritation, guinea pig sensitisation and LLNA.

For the Bacterial mutation test batch 2.12.A disper/7797 has been used. This batch has not been analyzed for purity.

For the data on purity given in submission II, no original results are supplied. It is not specified in submission to which batch the data on purity refer to. The data on purity given in ref. 1a for batch A- 37372 are not identical with those given in submission II.

3.1.5 Impurities / accompanying contaminants

See above

3.1.6 Solubility

Submission II:

Water: 2.34 – 3.50 mg/mL
Ethanol: 16.9 – 25.3 mg/mL
DMSO: 46.2 – 69.2 mg/mL

3.1.7 Partition coefficient (Log Pow)

Submission II:

Log Pow: (1) 2.16 ± 0.85 (calculated)
(2) 1.91 ± 0.85 (calculated)
(3) 1.66 ± 0.85 (calculated)

3.1.8 Additional physicochemical specifications

Melting point: /
Boiling point: /
Flash point: /
Vapour pressure: /
Density: /

Viscosity: /
pKa: /
Refractive index: /
UV/Visible spectrum: /

3.1.9. Stability

The following applies for batch A37372:

DB 377 has been shown to be stable at room temperature for 1 year.

In polyethylene glycol 400 solutions DB 377 (0.5 and 200 mg/l) was stable (within +/- 5%) for 24h at RT and 15 days, when stored at 5 ± 3 °C. A concentration of 0.2 mg/l was stable for 24h (± 12%) at RT and for 15 days at 5 ±3 °C.
In dimethylsulfoxide solutions DB 377 (0.005 and 20 mg/ml) was shown to be stable (within ± 5% of the initial value) for 8 days when stored at -20 ± 10 °C. A concentration of 35 mg/l was shown to be stable (within ± 3% of the initial value) for 9 days when stored at -20 ± 10 °C.

In hydroxypropylcellulose solutions, DB 377 (8.86 mg/g) was shown to be stable (within ± 5% of the initial value) for 4 hours when stored at room temperature, (within ± 10% of the initial value) for one week when stored frozen and within ± 5% of the initial value) for two weeks when stored frozen.

Ref.: 1b

Comment
Data on the stability of batch A-16798 have not been supplied.

General Comments on Physico-chemical characterisation

- Original data have not been supplied for solubility.
- Log Pow has been calculated. 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.
- No data on the purity of batch A-16798 have been supplied.
- Batch 2.12.A disper/7797, which had been used for the bacterial mutation test, had not been characterized for identity and purity.
- Stability data of Disperse Blue 377 in typical hair dye formulations has not been provided.

3.2. Function and uses

Disperse Blue 377 is used in non-oxidative hair dye formulations at levels up to 2.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401
Species/strain:	Sprague-Dawley Rat – Hsd:SD
Group size:	6 (3 males and 3 females) per dose group)
Test substance:	TM#1498 (Disperse Blue 377)
Batch:	A-16798
Purity:	99.6% (49.8% active dye in lignosulphate dispersant)
Vehicle:	0.5% Hydroxypropylcellulose (HPC) in distilled water
Dose:	500 and 2000 mg/kg bw
Dosage volumes:	/
Route:	oral (gavage)
Observation period:	15 days
GLP:	in compliance
Study date:	28 January – 18 February 2000

The test substance was administered once orally to 2 groups of 6 animals at dose levels 500 and 2000 mg/kg bw. Clinical signs were recorded at 1 h and 4 h post-dose and daily thereafter through day 15. Body weights were recorded on days 1, 8 and 15. Gross necropsy was performed on day 15

Results
There was no mortality. Clinical signs at both dosages included blue cage paper and urine, blue tint to eyes, blue ears, paws and tails. No visible lesions were observed at necropsy.

Conclusion
In this study the test substance Disperse Blue 377 was non-toxic at 2000 mg/kg bw after oral administration to rats.

Ref.: 1 (subm I)
2 (subm II)

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404
Species/strain:	New Zealand White rabbit – HM(NZW)fBR
Group size:	3 (2 males and 1 female)
Test substance:	Disperse Blue 377, moistened with 0.9% sodium chloride
Batch:	/
Purity:	/
Vehicle:	/

Dose: 500 mg
Observation: 0, 24, 48 and 72 hours (± 1 hour) after patch removal
GLP: in compliance
Study date: 27 – 30 January 2000

The test article was administered once to a site (500 mg/site) on the clipped dorsal trunk of each animal and moistened with 0.9% sodium chloride. It remained occluded to the skin site for 4 hours. The test article was applied beneath a 2x2 cm gauze patch and covered with rubber dam. The trunk of the animal was wrapped with an elastic bandage. All exposure sites were examined for signs of erythema and oedema. Observations were recorded immediately after patch removal and at 24, 48 and 72 hours.

Results
Only one of the three animals showed well defined erythema 24 hours after patch removal.

Conclusion
Disperse Blue 377 was slightly irritating applied moistened with 0.9% sodium chloride.

Ref.: 3 (subm I)
4 (subm II)

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405
Species/strain: New Zealand White rabbit – HM(NZW)fBR
Group size: 3 (1 male and 2 females)
Test substance: Disperse Blue 377
Batch: A-16798
Purity: /
Vehicle: /
Dose: 10 mg (neat substance)
Observations: 1 (± 15 min), 24, 48 and 72 hours (± 1hour)
GLP: in compliance
Study date: 7 – 11 February 2000

10 mg of Disperse Blue 377 was instilled into the right eyes of three animals. The eyes were examined at 1 and 24, 48 and 72 hours post-dose and scored for ocular irritation.

Results
Conjunctivae redness and/or discharge were observed at 1 and 24 hours. Irritation for all three rabbits was resolved by 48 hours.

Conclusions
The study authors considered that Disperse Blue 377 is not an eye irritant.

Ref.: 2 (subm I)
3 (subm II)

Comment of the SCCS
Under the conditions of the study, Disperse Blue 377 caused some irritation to rabbit eye.

3.3.3. Skin sensitisation

Buehler test

Guideline: OECD 406
Species/strain: Hartley Guinea pig – Elm(HA)
Group size: 30 (15 males and 15 females)
Test substance: Disperse Blue 377

Batch:	/
Purity:	/
Dose levels:	induction: 0.3 ml test article (TM# 1698 at 10%)
	Challenge: 0.3 ml test article (TM# 1698 at 1%)
Vehicle:	Modified Schultz Hamburg Vehicle II
Positive control:	1-chloro-2,4-dinitrobenzene (DNCB)
GLP:	in compliance
Study date:	4 May – 3 June 1998

0.3 ml of the product was applied to a patch and then applied to the skin, clipped free of hair. The patch was occluded with a rubber dental dam and animals were restrained to minimize movement. After an exposure period of six hours, the patch was removed and the treated sites were wiped with gauze and mineral oil to remove any residual test material.

For the induction phase, guinea pigs were induced with dermal application of the test article. Each animal received three, six-hour occluded applications with seven days between applications.

Eighteen to twenty-two hours after the challenge, all animals were depilated.

Results
There were no signs of systemic toxicity. Scoring of erythema during induction was sometimes difficult due to the colour of the test article, which stained the skin. However, slight erythema was occasionally recorded.

Conclusion
Under the conditions of the study, Disperse Blue 377 did not elicit a delayed contact hypersensitivity response in guinea pigs.

Ref.: 4 (subm I)
5 (subm II)

Comment
The colour of the test substance interfered with the readings and hypersensitivity could not be excluded.

Local Lymph Node Assay (LLNA)

Guideline:	predates OECD 429
Species/strain:	mouse – CBA/J
Group size:	45 females (5 mice /group)
Test substance:	TM#1498 (Disperse Blue 377)
Batch:	A-16798
Purity:	/
Dose levels:	0.25, 0.5, 1.0 and 2.0% ((1), (2) and PPD)
Vehicle:	DMSO
Positive control:	p-phenylenediamine (PPD)
GLP:	in compliance
Study date:	24 – 30 October 2001

CBA/J female mice were treated on the dorsal surface of both ears one per day for 3 days with the vehicle (DMSO) or with 0.25, 0.5, 1.0 or 2.0% (w/v) of Disperse Blue 377 or the positive control. On day 6, the mice were injected, i.v. with 20 µCi of ^{3}H-thymidine. Five hours later, the mice were euthanized and the draining auricular lymph nodes were removed. The lymph node cells were precipitated with 5% trichloracetic and the pellets counted in a β-scintillation counter to determine ^{3}H-thymidine incorporation.

Results

Treatment	Concentration	Stimulation index
TM1498	0.25% 0.5% 1.0% 2.0%	1.17 0.88 1.71 2.28
PPD	0.25% 0.5% 1.0% 2.0%	3.15 6.01 4.71 12.22

Conclusions
The results indicate that Disperse Blue 377 did not induce a hypersensitivity response at any of the concentrations tested.

Ref.: 5 (subm I)
6 (subm II)

Comment
The concentrations used were too low (it is soluble in DMSO up to 7%) and a sensitising potential cannot be excluded.

Human study

Guideline: /
Species/strain: Human volunteers
Group size: 156 (20 males and 136 females)
Test substance: Developmental semi-permanent Blue Dye (DBX)
Batch: /
Purity: /
Test material: 3% in a bland base
Dose levels: /
GLP: /
Study date: 30 November 1998 – 8 January 1999

The test material was applied to the same site on the scapular back of 156 human volunteers under occlusive patches at the rate of three times weekly (48-hour periods during the week and a 72-hour period on the weekend) for nine applications. Following a two-week rest period, two consecutive challenge patches (48-hour periods) of the test material were applied to a different site on the scapular back under occlusive patches.
Test sites scores on degree of erythema, oedema, while recognizing the extent or distribution of test site involvement.

Results
No reaction was recorded.

Conclusions
No allergic contact dermatitis was noted.

Ref.: 6 (subm I)
7 (subm II)

Comment
The SCCS considers such human studies as unethical.

3.3.4. Dermal / percutaneous absorption

Guideline: OECD 428 (2004)

Tissue:	dermatomed human skin
Group size:	12 membranes (4 donors), 400 µm thickness
Skin integrity:	electrical resistance > 10 kΩ
Diffusion cell:	glass diffusion cell, 2.54 cm²
Test substance:	Disperse Blue 377 [14C]-Disperse Blue 377
Batch:	A37372 CFQ14194 Batch 1 (radiolabelled)
Purity:	46.5% active dye 97.8% by HPLC, 71 mCi/mol; 2.63 GBq/mmol; 7.36 MBq/mg
Test item:	hair dye cream formulation containing 2% Disperse Blue 377 Typical hair dye cream (blank formulation)
Dose:	20 mg/cm²
Dose of test substance:	2% w/w Disperse Blue 377 in formulation
Receptor fluid:	4% polyoxyethylene-20-oleyl ether solution in phosphate buffered saline
Solubility receptor fluid:	0.45 mg/mL as Disperse Blue 377
Stability receptor fluid:	/
Method of Analysis:	Liquid scintillation counting
GLP:	in compliance
Study date:	10 – 22 February 2005

The penetration and distribution of Disperse Blue 377 from a nominal 2% w/w formulation, has been measured *in vitro* through human skin, following the incorporation of [^{14}C]-Disperse Blue 377.
The formulation was applied to 12 human dermatomed skin membranes (nominally 400 µm thick), mounted in glass diffusion cells, at a nominal rate of 20mg/cm^2. After a contact period of 30 minutes, the dose was washed from the surface of the skin using natural sponges soaked in 3% Teepol. Samples of the receptor fluid (4% polyoxyethylene-20-oleyl ether solution in phosphate buffered saline) were taken at recorded intervals over a 48h period, during which time the applications remained unoccluded. At the end of the experiment, the surface of the skin was washed again and layers of *stratum corneum* removed using a tape stripping technique. The receptor fluid samples, sponges, tape strips, residual skin and donor chambers were analysed for radioactivity, which was representative of the Disperse Blue 377 content. Penetration rates and distribution of Disperse Blue 377 in the test system were calculated.

Results

Test Compartment	Amount Recovered (µg/cm²)															
	Cell 1	Cell 2	Cell 4	Cell 5	Cell 7	Cell 9	Cell 10	Cell 11	Cell 12	Cell 13	Cell 15	Cell 17[x]	Mean	SD	SEM	n
Flange	0.022	0.048	0.013	0.010	0.012	0.012	0.006	0.008	0.038	0.016	0.018		0.018	0.013	0.004	11
Donor Chamber	0.085	0.193	0.030	0.031	0.108	0.070	0.018	0.058	0.179	0.262	0.210		0.113	0.084	0.025	11
Skin Wash @ 0.5h	196	175	178	185	198	182	185	180	197	191	194		187	8.20	2.47	11
Skin Wash @ 48h	0.100	0.660	0.135	0.083	0.355	0.149	0.069	0.074	0.716	0.342	0.646		0.303	0.259	0.078	11
Stratum Corneum	0.020	0.041	0.060	0.093	0.097	0.111	0.078	0.071	0.098	0.063	0.147		0.080	0.035	0.010	11
Remaining Epidermis/Dermis	0.034	0.073	0.026	0.018	0.075	0.064	0.048	0.045	0.094	0.073	0.138		0.062	0.034	0.010	11
Receptor Fluid	0.017	0.103	0.004	0.003	0.084	0.026	0.013	0.036	0.038	0.035	0.019		0.034	0.032	0.010	11
Systemically Available*	**0.051**	**0.175**	**0.030**	**0.021**	**0.160**	**0.090**	**0.061**	**0.081**	**0.132**	**0.108**	**0.157**		**0.097**	**0.054**	**0.016**	**11**
TOTAL	196	176	178	186	199	182	185	181	199	192	195		188	8.33	2.51	11

Test Compartment	Percent of Dose Recovered (%)															
	Cell 1	Cell 2	Cell 4	Cell 5	Cell 7	Cell 9	Cell 10	Cell 11	Cell 12	Cell 13	Cell 15	Cell 17[x]	Mean	SD	SEM	n
Flange	0.012	0.026	0.007	0.005	0.006	0.006	0.003	0.004	0.020	0.008	0.010		0.010	0.007	0.002	11
Donor Chamber	0.046	0.103	0.016	0.017	0.058	0.038	0.010	0.031	0.096	0.140	0.113		0.061	0.045	0.014	11
Skin Wash @ 0.5h	105	94.1	95.5	99.5	106	97.5	99.2	96.8	106	103	104		101	4.40	1.33	11
Skin Wash @ 48h	0.054	0.354	0.072	0.045	0.191	0.080	0.037	0.040	0.385	0.184	0.347		0.162	0.139	0.042	11
Stratum Corneum	0.011	0.022	0.032	0.050	0.052	0.059	0.042	0.038	0.053	0.034	0.079		0.043	0.019	0.006	11
Remaining Epidermis/Dermis	0.018	0.039	0.014	0.010	0.041	0.034	0.026	0.024	0.050	0.039	0.074		0.034	0.018	0.006	11
Receptor Fluid	0.009	0.055	0.002	0.002	0.045	0.014	0.007	0.019	0.020	0.019	0.010		0.018	0.017	0.005	11
Systemically Available*	**0.027**	**0.094**	**0.016**	**0.011**	**0.086**	**0.048**	**0.032**	**0.043**	**0.071**	**0.058**	**0.084**		**0.052**	**0.029**	**0.009**	**11**
TOTAL	105	94.7	95.6	99.7	107	97.7	99.4	96.9	107	103	105		101	4.47	1.35	11

* Systemically Available = Sum of Remaining Epidermis and Receptor Fluid data
[x] Cell 17 excluded from results, as data indicated that the skin had been damaged during the 0.5h wash

The mean value of dermal absorption was 0.097 µg/cm².

Ref.: 8 (subm II)

Comment
There is a high variability in dermal absorption with values from 0.030 to 0.175 µg/cm². The CV was 56%. The mean value +1 SD (0.097 + 0.054 or 0.151 µg/cm²) will be used to calculate MOS.

3.3.5. Repeated dose toxicity

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

28-day dermal toxicity study

Guideline:	/
Species/strain:	Sprague-Dawley rats, Crl:CD®
Group size:	5 animals per sex and per dose group
Test substance:	TM#1498 (Disperse Blue 377)
Batch:	A-16798
Purity:	99.6% (49.8% active dye in lignosulphate dispersant)
Vehicle:	Schultz Hamburg vehicle
Dose:	0.5 ml of 1, 3 and 10% solution
Dosage volumes:	0.5 ml/animal
Administration:	6 h/day, 7 d/week for 28 d under occlusion
GLP:	in compliance
Study period:	27 July 1998 – 17 August 1999

Sprague-Dawley rats (5 per sex per group) were dermally exposed to 0.5 ml/animal 1, 3 and 10% Disperse Blue 377 in Schultz Hamburg vehicle for approximately 6 hours a day, 7 days a week for 28 days under occlusion.

Results
There were no significant differences in absolute body weights between the groups. Body weight changes in females from the high dose group between study days 1 and 8 were recorded. This was believed to be stress induced, resulting from the initiation of wrapping

procedures. No other significant body weight or body change differences were noted in females. There were no adverse observations related to the treatment. The only observable effect was a blue staining of the dosed area in all dose groups. Clinical observations related to the dosing procedure were evident in some animals from all dose groups. These included scabbed areas in the area of the dose site caused by repeated tape removal. In mid dose males, there was a slight elevation of activated partial thromboplastin time, and in mid dose females, there was a slight increase in the monocyte count. None of these were considered biologically meaningful. For females, there was a slight elevation in the red blood cell count and haematocrit for the low dose animals and a slight decrease in the mean corpuscular haemoglobin for mid dose animals. These differences were small in magnitude and not dose related and were not considered toxicologically meaningful. In the high dose group males, glucose, calcium and sodium levels were all increased. In high dose females, total protein was increased. At the terminal bleed in males decreases in triglycerides for animals in the low and high dose groups were noted (doubtful biological significance). For females there was an increased serum chloride for the mid dose animals, however, this was probably not of toxicological significance. There were no differences between groups with respect to organ weights. There were no gross findings at the treatment sites of any of the test animals. There were several instances of gross findings on the livers of animals from all dose groups. These lesions were identified as small areas of coagulation necrosis and it probably due to pressure secondary to wrapping. All other gross and microscopic findings were considered incidental to the administration of the test material. There were no observations of erythema or oedema of the dosed area.

Conclusion
The no toxic effect level for topical application of Disperse Blue 377 for 28 days is 10%.

Ref.: 10 (subm I)

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

14-day range finding study

Guideline:	OECD 407
Species/strain:	rat, Crl:CD®(SD)IGS BR
Group size:	5 animals per sex and per dose group
Test substance:	GTS03978 (Lowadene Blue 377 or Disperse Blue 377)
Batch:	A-37372
Purity:	46.5% active dye
Vehicle:	PEG 400
Dose:	0, 100, 250, 500 and 1000 mg/kg bw/d
Dosage volumes:	5 mL/kg
Administration:	daily via oral gavage for at least 14 d
GLP:	in compliance
Study period:	12 March 2004 – 6 January 2005

The test substance was administered daily via oral gavage to rats for at least 14 days. Male and female Crl:CD®(SD)IGS BR rats were assigned to five groups. Each group received dose preparations containing the vehicle (polyethylene glycol 400) or 100, 250, 500, or 1000 mg/kg bw/d of the test substance at a dose volume of 5 mL/kg. The animals were observed twice daily (a.m. and p.m.) for mortality, abnormalities, and signs of pain or distress. Detailed clinical observations were performed once prior to treatment and on days 1, 3, 7, 10, and 15. Body weights were recorded twice prior to initiation of treatment and on days 1, 3, 7, 10, and 15. Food consumption data were collected for days 1 to 3, 3 to 7, 7 to 10, and 10 to 15. Blood and urine samples for haematology, coagulation, clinical chemistry, and urinalysis were collected at the scheduled sacrifice. On day 17, all animals were anesthetized, weighed, exsanguinated, and necropsied. At necropsy, macroscopic observations were recorded, selected organs weighed, and selected tissues collected and preserved.

Results
All animals survived to scheduled sacrifice on day 17. Test article-related clinical observations were limited to clear oral discharge for one male given 500 mg/kg bw/d and three males and three females given 1000 mg/kg/day; discoloured urine (blue for all treated groups and purple for animals given 500 or 1000 mg/kg bw/d); blue skin (tail for 2 to 4 males given 25, 500, or 1000 mg/kg bw/d and 3 to 5 females given 100, 250, 500, or 1000 mg/kg bw/d and left ear, paws, and scrotum for 1 to 3 animals given 1000 mg/kg bw/d); blue haircoat (axillary and dorsal regions, head, limbs, mouth, lateral thoracic and ventral abdominal regions, entire body, and generalized) primarily for animals given 250, 500, or 1000 mg/kg bw/d; and purple skin (paws, scrotum, and tail) primarily for animals given 1000 mg/kg bw/d. There were no test article-related effects on mean body weights, body weight changes, or food consumption. Administration of GTS03978 was associated with some effects on clinical biochemistry. The most notable findings were higher aspartate aminotransferase and alanine aminotransferase for males given 1000 mg/kg bw/d and higher total bilirubin for males and females given 500 or 1000 mg/kg bw/d. Increased liver weights in males given 500 or 1000 mg/kg bw/d and in females given 1000 mg/kg bw/d were regarded as test article-related. Macroscopically, blue discoloration of connective tissue was considered test article-related in males given 250, 500, or 1000 mg/kg bw/d and in females given 500 or 1000 mg/kg bw/d. Dark gastrointestinal tract contents were regarded as test article-related in males given 250, 500, or 1000 mg/kg bw/d and in females given 100, 250, 500, or 1000 mg/kg bw/d. Neither blue discoloration nor dark gastrointestinal tract contents were considered adverse.

Conclusion
Based on these findings, the no-observable-adverse-effect level for oral gavage administration of Disperse Blue 377 to rats for 16 days was 250 mg/kg bw/d corresponding to 116 mg/kg bw/d active dye. However, in the absence of liver histopathology, the importance of the clinical biochemistry findings is undetermined.

Ref.: 9 (subm II)

91-day study

Guideline:	OECD 408 (1998)
Species/strain:	rat, Crl:CD®(SD)IGS BR
Group size:	15 animals per sex in low and mid dose group 20 animals per sex in control and high dose group
Recovery group:	At least 5 animals per sex of control and high dose group
Test substance:	GTS03978 (Lowadene Blue 377)
Batch:	A-37372
Purity:	46.5% active dye
Vehicle:	PEG 400
Dose:	0, 1, 5, 25 mg/kg bw/d
Dosage volumes:	5 mL/kg bw
Administration:	daily via oral gavage for 91 days
GLP:	in compliance
Study period:	16 April 2004 – 27 October 2005

Male and female Crl:CD®(SD)IGS BR rats were assigned to four groups. Each group received dose preparations containing the vehicle (polyethylene glycol 400) or 1, 5, or 25 mg/kg bw/d of the test article substance at a dose volume of 5 mL/kg. The animals were observed twice daily (a.m. and p.m.) for mortality, abnormalities, and signs of pain or distress. Detailed clinical observations were performed prior to treatment, on day 1, weekly thereafter, and on the day of each scheduled sacrifice. Neurobehavioral observations were performed weekly; hand-held and open-field expanded clinical observations were done pre-study and during weeks 4, 8, and 13; elicited behaviours observations were done pre-study

and during week 13; and motor activity data were collected pre-study and during week 13. Ophthalmic examinations were done prior to treatment and during week 13. Body weights were collected twice prior to treatment, on day 1, and weekly thereafter. Food consumption data were measured weekly. Vaginal cytology data were collected once daily for 21 consecutive days, beginning after week 10. Blood and urine samples for haematology, coagulation, clinical chemistry, urinalysis, and urine chemistry were collected at each scheduled sacrifice. On day 92, up to 15 animals/sex/group were anesthetized, weighed, and necropsied. On day 121, all surviving animals were anesthetized, weighed, and necropsied. At each necropsy, macroscopic observations were recorded, selected organs weighed, and selected tissues collected and preserved. The female of the group 1 mg/kg bw/d that died on test was also necropsied, but organ weights were not recorded. At each scheduled sacrifice, male reproductive assessment (sperm motility, morphology, and count) was done. Microscopic examination of tissues was done, and a pathology peer review was conducted.

Results
All animals survived to their respective scheduled sacrifice, with the exception of one female of the group 1 mg/kg bw/d that was found dead on day 16. No remarkable clinical observations were noted prior to death; no test article-related macroscopic or microscopic findings were observed for this animal. Based on the lack of findings for this animal and the absence of mortality for animals at higher dose levels, the death of this animal was not attributed to effects of the test article. Blue skin and blue haircoat were observed in the high-dose groups only; these findings were test article-related but not considered adverse. All other clinical observations were considered incidental and unrelated to test article administration. There were no test article-related ophthalmic observations; effects on neurobehavioral assessment tests (expanded clinical observations, hand-held and open-field observations, elicited behaviours observations, and motor activity assessment); effects on body weights, body weight changes, or food consumption; or effects on vaginal cytology. GTS03978 administration was associated with discoloured urine (blue-green or blue) for animals given 5 or 25 mg/kg bw/d. No other effects on clinical pathology test results were observed. There were no test article-related effects on terminal body or organ weights or macroscopic or microscopic findings at either sacrifice. The mean percent sperm motility, caudal epididymal sperm count and sperm morphology were not affected by treatment with GTS03978 at dosage levels of 1, 5 and 25 mg/kg bw/d. No biologically meaningful differences were observed between the study groups.

Conclusion
Based on the results of this study, the no-observable-adverse-effect level following oral gavage administration of GTS03849 to rats at doses of 1, 5, or 25 mg/kg bw/d for 91 days was 25 mg/kg/day

Ref.: 10 (subm II)

Comment of the SCCS
The NOAEL for repeated dose toxicity of Disperse Blue 377 was 25 mg/kg bw/d corresponding to 11.6 mg/kg bw/d active dye.

Statement of the applicant
The following studies were either not conducted in compliance with OECD or internationally accepted test guidelines and/or test material analytical characterisation was not documented in the reports. These studies are not considered to be in line with current requirements and do not provide additional information relevant to the risk assessment of Disperse Blue 377 in hair dyes. Therefore they are not reviewed in this submission.

5-day range finding study

Four male Sprague-Dawley rats per group were orally dosed daily for 5 consecutive days with 300, 600 and 1500 mg/kg bw/d of Disperse Blue 377 (TM# 1498; Batch Number A-16798) in 0.5% hydroxypropylcellulose.

Results
No mortality occurred during the conduct of the study. A treatment related mean weight loss was noted for males in the high dose group at the end of the test period when compared to the control males. The mean body weights and mean body weight changes of males in the mid and low dose groups were comparable to those of the control males. Blue urine was observed in all treated animals throughout the treatment period. The presence of a blue tail sheath was observed in most animals receiving 600 or 1500 mg/kg bw/d throughout the majority of the treatment period. A blue tail sheath was considered to result from grooming or urine staining. Other treatment related clinical signs included the presence of dark eyes and whole body discolouration, characterised by a bluish tinge that appeared to result from tissue test article absorption. These signs were exclusive to animals receiving the 1500 mg/kg bw/d dose. A treatment related lower mean food consumption was recorded for males in the high dose group when compared to the controls. Treatment related increases in mean absolute liver weights and mean liver to body weight ratios were observed in treated males of all treated groups in comparison to the controls. A treatment related slight to moderate eosinophilic hepatocytomegaly and minimal to moderate hepatocellular degeneration were observed for all males in the high dose. Additional findings at this dose level were slight to moderate coarse vacuolation of hepatocytes observed in three males and focal areas of minimal and moderate subscapsular necrosis observed in two males. No treatment related histopathological findings were observed for any animals in the mid and low dose groups. Based on the findings from this study, the no toxic effect level is 600 mg/kg bw/d corresponding to 278 mg/kg bw/d active dye.

Ref.: 8 (subm I)

90-day study

Guideline:	OECD 408 (1998)
Species/strain:	rat, Crl:CD®(SD)IGS BR
Group size:	15 animals per sex in low and mid dose group 20 animals per sex in control and high dose group
Recovery group:	At least 5 animals per sex of control and high dose group
Test substance:	TM#1498 (Disperse Blue 377)
Batch:	A-16798
Purity:	99.6% (49.8% active dye in lignosulphate dispersant)
Vehicle:	0.5% Hydroxypropylcellulose (HPC) in distilled water
Dose:	0, 20, 75, 300 mg/kg bw/d
Dosage volumes:	5 mL/kg bw
Administration:	daily via oral gavage for 90 d
GLP:	in compliance
Study period:	2 June 1998 – 31 August 1998

Three groups of 30 animals (15/sex/dose) received the test substance suspended in 0.5% hydroxypropylcellulose (HPC) in distilled water orally once daily for 90 d at dose levels 0, 20, 75 and 300 mg/kg bw/d. Mortality check and clinical observations were performed twice daily. Body weight and food consumption were recorded weekly. Ophthalmological examination was performed on the day of treatment and on all surviving animals prior to scheduled euthanasia. Urine samples were collected overnight prior to euthanasia and blood samples for haematology, clinical chemistry and coagulation analyses prior to terminal sacrifice. A gross pathological examination was performed on all animals and selected

tissues were retained. Tissues from control animals and the high dose group were evaluated histo-pathologically.

Results
No test substance-related effects on body weight and food consumption were noted. No ophthalmological findings and no differences in haematology, urinalyses and coagulation parameters were found compared to controls. Clinical signs included blue urine, tail sheath, hind limbs and bluish skin tone and discoloration of the fur which increased with the dose. At 300 mg/kg bw/d cholesterol and bilirubin levels were elevated (both sexes) as well as the mean absolute and relative liver and kidney weights (males only). At this dose bluish discoloration of the gastrointestinal tract, intestinal contents and/or abdominal fat were observed in some animals.

Conclusion
The no-observed-adverse-effect level of Disperse Blue 377 in this study is 75 mg/kg bw/d corresponding to 34.8 mg/kg bw/d active dye.

Ref.: 9 (subm I)

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 Assay

Guideline:	OECD 471
Species/Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538
Replicates:	triplicates in a single experiment
Test substance:	DBX/TM 1413
Batch:	(#5) 2.12.A disper/7797
Purity:	/
Vehicle:	DMSO
Concentration:	0, 33, 100, 333, 1000, 3333 and 5000 µg/plate, without and with S9-mix
Treatment:	direct plate incorporation with 48 - 72 h incubation without and with S9-mix
GLP:	in compliance
Study date:	9 September 1997 – 5 February 1998

DBX/TM 1413 was tested in strains of *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 in the presence and in the absence of a metabolic activation system. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. The direct plate incorporation method with 48 - 72 h exposure was used. The condition of the bacterial background lawn was evaluated for evidence of toxicity; precipitation was evaluated by visual examination. Appropriate negative and positive controls were included.

Results
Precipitation was observed at concentrations ≥ 3333 µg/plate. Appreciable toxicity was not found.
In the absence of S9-mix with TA1537 and in the presence of S9-mix with TA1537 and TA1538, a biologically relevant and concentration dependent increase in the number of revertant colonies was observed. A positive result was also reported for TA98 in the

presence of S9-mix, however, the increase in revertant colonies was low and not concentration dependent.

Conclusion
Under the experimental conditions used DBX/TM 1413 was genotoxic (mutagenic) in this gene mutation tests in bacteria

Ref.: 11 (subm I, subm II)

In vitro Mammalian Cell Gene Mutation Test

Guideline:	OECD 476 (1997)
Species/strain:	Mouse lymphoma cell line L5178Y $tk^{+/-}$
Replicates:	single cultures per concentration
Test substance:	Lowadene Blue 377 (GTS03978)
Batch:	A-37372
Purity:	100.4% (46.5% active dye)
Vehicle:	DMSO
Concentrations:	0, 40, 50, 60, 80, 100, 150, 175 and 200 µg/ml without and with S9-mix
Treatment:	4 h treatment both without and with S9-mix; expression period 48 h and a selection period of 14 days.
GLP:	in compliance
Study date:	29 June 2004 – 23 November 2005

Lowadene Blue 377 was assayed for mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of metabolic activation. The cells were routinely tested on stock cultures for mycoplasma contamination and karyotype stability, measured by mean chromosome number. Liver S9 fraction from Aroclor™ 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a dose range-finding study with a wide range of test concentrations, an approximately 4 h treatment period and both without and with S9-mix. The test concentrations were chosen to cover a toxicity range from 10% to 20% survival to no apparent effect on growth, compared to the concurrent vehicle control.
In the main test, cells were treated for 4 h followed by an expression period of 48 h, to fix the DNA damage into a stable *tk* mutation. Lowadene Blue 377 is evaluated as positive if there is a positive dose response and one or more of the doses exhibit a mutant frequency which is ≥ 90 x 10^6 clonable cells over the concurrent background frequency. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. Toxicity was measured as total growth relative to the growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
In the absence of metabolic activation none of the treatments induced a mutant frequency that met criteria for a positive response. In the presence of metabolic activation treatments at and above 150 µg/ml were terminated due to excessive toxicity. The remaining treatments induced no to high cytotoxicity (11.3% relative total growth). Treatment with Lowadene Blue 377 induced a biological relevant concentration dependent increase in the mutant frequency that met criteria for a positive response. In the treatments that were positive a preferential increase in small colonies was observed suggesting large mutations that effect both the *tk* gene and cell growth (clastogenic effect).

Conclusion
Under the experimental conditions used, Lowadene Blue 377 was considered genotoxic (mutagenic and clastogenic) in this gene mutation assay in mouse lymphoma cells.

Ref.: 13 (subm II)

Comment
Since clear positive results were found in the initial experiment in the presence of S9-mix, a confirmatory experiment is not needed according to the OECD guideline. The SCCS agrees.

In Vitro Mammalian Chromosomal Aberration Test

Guideline:	OECD 473 (1997)
Species/strain:	CHO-WBL cells
Replicates:	duplicate cultures per concentration
Test substance:	Lowadene Blue 377 (GTS03978)
Batch:	A-37372
Purity:	100.8% (46.5% active dye)
Vehicle:	DMSO
Concentrations:	4 h treatment: 0, 250, 350 and 500 µg/mL without S9-mix 0, 150, 250 and 500 µg/mL with S9-mix 20 h treatment: 0, 25, 50 and 75 µg/mL without S9-mix
Treatment	4 h or 20 h treatment without S9-mix; harvest time 20 h after the start of treatment 4 h treatment with S9-mix; harvest time 20 h after start of treatment
GLP:	in compliance
Study date:	8 July 2004 – 17 November 2005

Lowadene Blue 377 has been investigated for the induction of chromosomal aberrations in CHO-WBL cells in the absence and presence of metabolic activation. The cells were routinely tested on stock cultures for mycoplasma contamination and karyotype stability, measured by mean chromosome number. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of an initial toxicity assay on cell count, mean cells number per treatment group, cell growth and cell growth inhibition after 4 h treatment.
In the main test, cells were treated for 4 h (without and with S9-mix) or 20 h (without S9-mix) and harvested 20 h after the start of treatment. Approximately 2 h before harvest, each culture was treated with colcemid (0.1 µg/ml culture medium) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with 5% Giemsa and examined microscopically for chromosomal aberrations, polyploidy, endoreduplication and the mitotic index. Reduction in the mitotic index was taken as a measure for cytotoxicity. Negative and positive controls were in accordance with the OECD guideline.

Results
In the initial toxicity assay without S9-mix precipitate was visible prior to wash at 350 µg/ml; in the assay with metabolic activation no precipitate was observed.
In the absence of S9-mix, in the cultures treated for 20 h a concentration dependent and statistically significant increase in cells with chromosome aberrations was found; for the 4 h treatment a statistically significant increase in aberrant cells was only observed at 350 µg/ml.
In the presence of S9-mix, a statistically significant increase in cells with structural chromosome aberrations was observed in all treatments analysed without clear concentration response relation. An increase in polyploidy and endoreduplication was only observed in cells treated for 4 h without S9-mix.

Conclusion
Under the experimental conditions used, Lowadene Blue 377 was genotoxic (clastogenic) in the chromosome aberration test in CHO cells both in the absence and the presence of S9 metabolic activation.

Ref: 12 (subm II)

***In Vitro* Mammalian Chromosomal Aberration Test**

Guideline:	OECD 473 (1997)	
Species/strain:	CHO-WBL cells	
Replicates:	duplicate cultures per concentration	
Test substance:	TM#1498	
Batch:	A-16798	
Purity:	99.6% (49.8% active dye in lignosulphate dispersant)	
Vehicle:	DMSO	
Concentrations:	4 h treatment:	0, 10, 37.5, 50, 75, 125, 187.5, 250, 375 and 500 µg/ml without and with S9-mix
	20 h treatment:	0, 10, 37.5, 50, 75, 125, 187.5 and 250 µg/ml without S9-mix
Treatment	4 h treatment without and with S9-mix; harvest time 24 h after the start of treatment 20 h treatment without S9-mix; harvest time 20 h after start of treatment	
GLP:	in compliance	
Study date:	19 February 2000 – 7 December 2000	

TM#1498 has been investigated for the induction of chromosomal aberrations in CHO-WBL cells in the absence and presence of metabolic activation. The cells were routinely tested on stock cultures for mycoplasma contamination. The average cell cycle duration for the cell line was routinely evaluated. Liver S9-fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. The selection of the test concentrations was made by the Sponsor based on previous studies.
In the main test, cells were treated for 4 h (without and with S9-mix) and harvested 24 h after the start of treatment or treated for 20 h (without S9-mix) and harvested immediately after treatment. The final 2 h before harvest, each culture was treated with colcemid (0.1 µg/ml culture medium) to block cells at metaphase of mitosis. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations, polyploidy, endoreduplication and the mitotic index. Reduction in the mitotic index was taken as a measure for cytotoxicity. Negative and positive controls were in accordance with the OECD guideline.

Results
In the experiment with 4 h exposure both without and with S9-mix precipitation of TM#1498 was visible at the start of treatment at 75 µg/ml and above and at the end of treatment at 187.5 µg/ml and above. Precipitation was not visible in the experiment with 20h treatment. Without S9-mix a concentration dependent and marginally (4 h treatment) or highly (20 h treatment) statistically significant increase in cells with chromosomal aberrations was observed. With S9-mix 4 h exposure to TM#1498 did not result in a biologically relevant increase in cells with chromosomal aberrations. In cultures exposed for 4 h to TM#1498 both without and with S9-mix a significant increase in polyploidy cells was found; after 20 h exposure TM#1498 did not induce polyploidy.

Conclusion
Under the experimental conditions used, TM#1498 was genotoxic (clastogenic) in the chromosome aberration test in CHO cells in the absence of S9 metabolic activation.

Ref: 12 (subm I)

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

***In vivo* Mammalian Erythrocytes Micronucleus Test**

Guideline:	OECD 474
Species/strain:	Crl:CD-1 (ICR)BR mice
Group size:	5 mice/sex/group
Test substance:	TM#1498
Batch:	A-16798
Purity:	/
Vehicle:	0.5% hydroxypropylcellulose in de-ionised water
Dose level:	0, 15, 50, 150 and 325 mg/kg bw
Route:	i.p. injection
Sacrifice times:	24, 48 or 72 (150 mg/kg bw only) hours after injection
GLP:	in compliance
Study date:	2 December 1997 – 28 January 1998

TM#1498 was investigated for the induction of micronuclei in bone marrow cells of mice. Test doses were based on the results of a preliminary test on toxicity. Mice were treated ip with doses up to 750 mg/kg bw and observed for mortality and pharmacological signs immediately after treatment (0-2h) and at 24, 48 and 72 h after dosing.
In the micronucleus test mice were treated by ip injection with 0, 15, 50, 150 and 325 mg/kg bw and examined for mortality and pharmacological signs at 0-2h and at 24, 48 and 72 h after dosing. Bone marrow cells were collected 24 h, 48 h or 72 h (150 mg/kg bw only) after dosing. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE). Negative and positive controls were in accordance with the OECD guideline.

Results
In the preliminary toxicity test 1 male (at 24 h after treatment) and 1 female (at 48 h after treatment) mouse treated with the highest dose (750 mg/kg bw) died whereas the remaining mice showed decreased activity, elevated gait and eyes partially closed immediately after dosing. At 24 h most of the mice had already recovered. At 325 mg/kg bw all mice survived and the pharmacological signs only occasionally occurred. Consequently, for the main experiment 325 mg/kg bw was estimated to be suitable top dose. In the micronucleus test identical pharmacological signs were observed; however, the mice recovered later somewhere between 24 and 48 h after treatment.
In the preliminary toxicity test the PCE/NCE ratio dose dependently decreased as compared to the mean ratio of the vehicle control, thus indicating that TM#1498 did exert cytotoxic effects in the bone marrow. The pharmacological signs observed after treatment confirmed the systemic distribution of TM#1498 and thus its bioavailability.
Analysis of both combined sex data and of the data by sex indicated statistically significant increases in the number of PCE with micronuclei due to TM#1498 treatment. Moreover, dose dependent increasing trends were noted. However, these statistically significant increases all represented 1-2 erythrocytes with micronuclei per 2000 cells against a control value of 0-1 erythrocytes with micronuclei per 2000 cells. In addition all the increases were well within the range of the historical control data. Consequently, they were considered not biologically relevant.

Conclusion
Under the experimental conditions used TM#1498 did not induce a biologically relevant increase in the number of erythrocytes with micronuclei of treated mice and, consequently, TM#1498 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 13 (subm I)
14 (subm II)

In vivo unscheduled DNA synthesis (UDS) test

Guideline:	OECD 486 (998)
Species/strain:	male Sprague-Dawley rats

Group size:	3 male rats/group
Test substance:	GTS03978 (Lowadene Blue 377)
Batch:	A-37372
Purity:	100.8% (46.5% active dye)
Vehicle:	PEG 400
Dose level:	0, 1000, 2000 mg/kg bw
Route:	oral gavage
Sacrifice times:	2-4 h and 12-16 h after dosing
GLP:	in compliance
Study date:	27 June 2005 – 22 July 2005

GTS03978 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Test doses were based on the results of a dose range-finding study on acute toxicity with doses up to 2000 mg/kg bw. The highest dose level selected was the dose that produced toxicity such that higher dose levels based on the same regimen, would be expected to cause lethality.

In the main test rats were treated by oral gavage with 0, 1000, 2000 mg/kg bw and examined for acute toxic symptoms at dosing and at harvest. Hepatocytes for UDS analysis were collected approximately 2-4 h and 12-16 h after administration of GTS03978 by liver perfusion with collagenase (80-100 units Type I/ml culture medium). The quality of the performed perfusion was determined by the trypan blue dye exclusion method. 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 8 days. The number of silver grains above the nucleus and the number of grains above three nuclear-sized cytoplastic areas adjacent to the nucleus were counted. UDS is reported as the net nuclear grain count (nuclear grain count – average cytoplasm grain count). Additionally, the percentage of cells in repair (cells with ≥5 net nuclear grains) is reported. Unscheduled DNA synthesis was determined in 50 randomly selected hepatocytes on 2 replicate slides per rat from at least 3 treated rats. Appropriate reference positive controls were included.

Results
In the UDS test all animals appeared normal. However, rats from the 12-16 h exposure group had blue coloured internal organs and the high dose group also blue coloured urine. The coloured urine confirmed bioavailability of GTS03978.
A biological increase in mean net nuclear grain count as compared to the untreated control was not found in hepatocytes of any treated animal both for the 2-4 h and the 12-16 h treatment time. Additionally, an increase in the percentage of cells in repair was not found either.

Conclusion
Under the experimental conditions reported, GTS03978 did not induce DNA-damage leading to unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 15 (subm II)

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

Prenatal developmental toxicity, range finding study

Guideline:	/
Species/strain:	rat, Crl:CD® (SD)IGS BR VAF/Plus®
Group size:	40 females (5 rats per dose group)
Test substance:	GTS03978 (Disperse Blue 377)
Batch:	A-37372
Purity:	100.8% (46.5% active dye)
Vehicle:	PEG 400
Dose levels:	0, 50, 200, 500, 1000 mg/kg bw/d
Dose volume:	5 mL/kg bw
Route:	oral, gavage
Administration:	once daily, gestation day 6 - 20
GLP statement:	in compliance
Study date:	14 April – 13 May 2004

Eight rats per group were administered 0, 50, 200, 500 and 1000 mg/kg bw/d GTS03978 in the vehicle PEG 400 orally (gavage) once daily. Viabilities, clinical observations, body weights and feed consumption values were recorded. All rats were sacrificed on gestation 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 of the thoracic, abdominal and pelvic viscera was performed. Foetuses were weighed and examined for gross external alterations and sex.

Results
All rats survived to scheduled sacrifice. Clinical observations considered to be test substance related included discoloured (blue) skin, urine and fur observed in all groups treated with the test substance, localized alopecia observed in three rats in the 1000 mg/kg bw/d dosage group, and individual occurrences of green or blue faeces, blue perioral substance and blue substance on fur observed in the 500 and/or 1000 mg/kg bw/d dosage groups. Abdominal tissues were blue in seven rats in the 500 mg/kg bw/d dosage group and all rats in the 1000 mg/kg bw/d dosage group; all of these necropsy observations were considered to be related to the test substance. A mean body weight loss occurred on gestation days 6 to 9, and mean body weight gain was decreased on gestation day 9 to 12 and 18 to 21 in the 1000 mg/kg bw/d dosage group, as well as during the dosage period and the overall gestation period. Gravid uterine weights were reduced in the 1000 mg/kg bw/d dosage group, and corrected maternal body weight gains were reduced in the 500 and 1000 mg/kg bw/d dosage groups during the dosage period. Mean absolute and relative feed consumption values were decreased in the 1000 mg/kg bw/d dosage group on DGs 6 to 9 and 9 to 12, but increased on gestation days 12 to 15, 15 to 18 and 18 to 21; absolute and relative feed consumption values were generally comparable among the dosage groups for the dosage period and the overall gestation period. Foetal body weights were decreased in the 1000 mg/kg bw/d dosage group, although the values were only slightly below the range of historical control data. Placentae were blue in three litters in the 1000 mg/kg bw/d dosage group. Two foetuses from one litter in the 200 mg/kg bw/d dosage group were observed with haematomas.

Conclusion
Based on these data, dosages of 0 (Vehicle), 100, 500 and 1000 mg/kg bw/d of Disperse Blue 377 were selected for the main study.

Ref.: 16 (subm II)

Prenatal developmental toxicity, main study

Guideline:	OECD 414
Species/strain:	rat, Crl:CD® (SD)IGS BR VAF/Plus®
Group size:	100 females (25 rats per dose group)
Test substance:	GTS03978 (Disperse Blue 377)
Batch:	A-37372
Purity:	100.8% (46.5% active dye)
Vehicle:	PEG 400
Dose levels:	0, 100, 500, 1000 mg/kg bw/d
Dose volume:	5 mL/kg bw
Route:	oral, gavage
Administration:	once daily, gestation days 6 - 20
GLP statement:	in compliance
Study date:	26 June – 19 August 2004

25 presumed pregnant Crl:CD® (SD)IGS BR VAF/Plus® rats per group, were randomly assigned to four dosage groups. Solutions of the test substance, GTS03978 (Lowadene Blue 377 or Disperse Blue 377), and/or the vehicle, 100% polyethylene glycol 400 (PEG 400), were administered orally once daily to rats on days 6 through 20 of gestation (gestation days 6 to 20) at dosages of 0, 100, 500 and 1000 mg/kg bw/d. Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on gestation 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, soft tissue and skeletal alterations and sex.

Results

No deaths related to the test substance occurred; three female rats in the 500 mg/kg bw/d dosage group began to deliver and were sacrificed on gestation day 21, but the deliveries were not considered treatment related. The numbers of rats with blue, purple or red urine, blue or red perioral substance and blue tails were significantly increased in the treated groups, and the numbers of rats with blue fur, forepaws and/or hindpaws were significantly increased in the 500 and 1000 mg/kg bw/d dosage groups. Significant increases in the number of rats with blue perivaginal substance and/or blue skin and sparse hair coat occurred in the 1000 mg/kg bw/d dosage group. Blue discoloration of organs and tissues occurred in the 500 and 1000 mg/kg bw/d dosage groups. Body weights were significantly reduced on gestation days 7 to 21, and corrected maternal body weight was significantly reduced in the 1000 mg/kg bw/d dosage group. Body weight gains were significantly reduced in the 1000 mg/kg bw/d dosage group for the entire dosage and gestation periods. A reduction in body weight gain occurred in the 1000 mg/kg bw/d dosage group for the entire dosage period, and a significant reduction in body weight gain for the entire gestation period occurred in this group when calculated using the corrected weight on gestation day 21. Gravid uterine weights and absolute and relative feed consumption values were generally comparable among the dosage groups. Foetal body weights were significantly reduced in the 1000 mg/kg bw/d dosage group. No other Caesarean-sectioning or litter parameters were affected by dosages of GTS03978 as high as 1000 mg/kg bw/d. Blue skin was observed in 50 foetuses from four litters in the 1000 mg/kg bw/d dosage group. Blue material in the large intestine was observed in a majority of foetuses and all litters in the 1000 mg/kg bw/d group, and in 66 foetuses from 14 litters in the 500 mg/kg bw/d dosage group. There was an increased incidence (7 foetuses in one litter) of slight distension of the small intestine, a non-adverse variation, in the 1000 mg/kg bw/d dosage group. There were no dosage-dependent, significant differences in skeletal alterations, and no biologically meaningful changes in skeletal ossification.

Conclusion

The maternal no-observable-adverse effect level (NOAEL) as well as the developmental NOAEL for Disperse Blue 377 is 500 mg/kg bw/d (233 mg/kg bw/d active dye).

Ref.: 17 (subm II)

Statement of the applicant
The following studies were either not conducted in compliance with OECD or internationally accepted test guidelines and/or test material analytical characterisation was not documented in the reports. These studies are not considered to be in line with current requirements and do not provide additional information relevant to the risk assessment of Disperse Blue 377 in hair dyes. Therefore they are not reviewed in this submission.

Prenatal developmental toxicity study

Guideline:	OECD 414
Species/strain:	rat, Crl:CD®(SD)IGS BR
Group size:	25 animals per dose group
Test substance:	TM#1498 (Disperse Blue 377)
Batch:	A-16798
Purity:	99.6% (49.8% active dye in lignosulphate dispersant)
Vehicle:	0.5% Hydroxypropylcellulose (HPC) in distilled water
Dose:	0, 75, 300, 1000 mg/kg bw/d
Dosage volumes:	5 mL/kg bw
Administration:	daily via oral gavage
GLP:	in compliance
Study period:	2 June 1998 – 31 August 1998

25 animals per dose group orally received the test substance in 0.5% hydroxypropylcellulose via gavage once daily from gestation days 6 to 17 at 0, 75, 300 and 1000 mg/kg bw/d. Evaluation of the parameters followed the OECD guideline.

Results
All dams survived to the scheduled necropsy on gestation day 20. Treatment related clinical findings were mainly related to the colour of the test material and included blue staining on the entire length of the tail or on the medial, distal or proximal tail, blue tinted hair coat, blue urine and blue material on the cage paper. Blue staining was observed infrequently on the forelimbs, around the nose and/or urogenital area in the high dose group. Dark green faeces were observed in all animals in the 300 and 1000 mg/kg bw/d groups between gestation days 7 and 19. In two and 16 animals in the 300 and 1000 mg/kg bw/d groups, red urine was found between gestation days 8 and 18. Mean body weights in the mid and high dose groups were reduced during the first three days of dosing. During the rest of the treatment body weights in these groups were unaffected by treatment. Mean gravid uterine weights and net body weights in the 300 and 1000 mg/kg bw/d groups were unaffected by treatment. In the 300 and 1000 mg/kg bw/d groups, food consumption was slightly reduced during gestation days 6-9 and 9-12. During gestation days 12-18 food consumption was unaffected in these groups. During the post treatment period, days 18-20, food consumption values in these groups were similar to the control group. In the 75 mg/kg bw/d group, food consumption was unaffected by treatment throughout gestation. Blue discolouration was seen on various external surfaces in 16 animals in the 75 mg/kg bw/d group and in all animals in the mid and high dose groups. In addition, blue discolouration was observed in adipose tissue in nine animals in the 1000 mg/kg/d group and in the ovaries of one animal from this group. Multiple cysts were found in the kidney of one control group animal. No other internal findings were recorded. Intrauterine growth and survival were not affected by treatment at any dose level; differences between control and treated groups were slight and not statistically significant. Parameters evaluated included post-implantation losses, viable foetuses, mean foetal body weights, foetal sex ratios and mean numbers of corpora lutea and implantation sites. The numbers of foetuses available for foetal morphological evaluation were comparable between the treated and control groups. Malformations were noted in one foetus from the 75 mg/kg bw/d group. No external

malformations or developmental variations were observed in any foetuses at any dose level No soft tissue malformations were observed in foetuses at any dose level. There were major blood vessel variations in one foetus each from the 75 and 1000 mg/kg bw/d groups. The only skeletal malformation observed was a vertebral anomaly in one foetus from the 75 mg/kg bw/d dose group. Skeletal developmental variations that occurred similarly in all groups, including the control group, consisted of 14th rudimentary ribs, unossified sternebrae nos. 5 and/or 6, ossification of a cervical centrum no.1, unossified hyoid and 7th cervical ribs. Other skeletal variations observed in the treated groups occurred at similar frequencies in the control group or in a manner that was not dose related.

Conclusion
Based on the results of this study, the NOAEL of Disperse Blue 377 for maternal toxicity was considered to be 75 mg/kg bw/d corresponding to 34.8 mg/kg bw/d active dye, and the NOAEL for developmental toxicity was considered to be 1000 mg/kg bw/d corresponding to 464 mg/kg bw/d active dye.

Ref.: 14 (subm I)

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

Disperse Blue 377

Absorption through the skin	**A (mean + 1SD)**	**=**	**0.151 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.09 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.001 mg/kg**
No Observed Adverse Effect Level (subchronic, oral, rat)	**NOAEL**	**=**	**25 mg/kg bw/d**
Adjusted to 46.5% active dye content		**=**	**11.6 mg/kg bw/d**
Adjusted for oral bioavailability*	**50% bioavailability**	**=**	**5.8 mg/kg bw/d**

MOS	**=**	**5800**

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

3.3.14. Discussion

Physico-chemical specification
Disperse Blue 377 is used in non-oxidative hair dye formulations at levels up to 2.0%.
The identity of the two batches used for toxicity testing had been determined according to the state of the art.
Purity and stability are documented only for one batch
Original data have not been supplied for Log Pow and for solubility.
Log Pow has been calculated. The Log Pow strongly depends on the pH, especially for ionisable molecules, zwitterions etc. Therefore, a single calculated value of Log Pow, usually without any reference to the respective pH, cannot be correlated to physiological conditions and to the pH conditions of the percutaneous absorption studies.
The batch used for the bacterial mutation test had not been characterized for identity and purity.
Stability data of Disperse Blue 377 in typical hair dye formulations has not been provided.

General toxicity
In an acute oral toxicity study in rats the test substance was non-toxic at 2000 mg/kg bw.
The NOAEL for repeated dose toxicity of Disperse Blue 377 was 25 mg/kg bw/d corresponding to 11.6 mg/kg bw/d active dye.
In an oral developmental toxicity study in rats, the maternal no-observable-adverse effect level (NOAEL) as well as the developmental NOAEL for Disperse Blue 377 is 500 mg/kg bw/d (233 mg/kg bw/d active dye).

Irritation, sensitisation
Disperse Blue 377 was slightly irritating to rabbit skin applied moistened with 0.9% sodium chloride and caused some irritation to rabbit eye. Due to the low concentrations used in the LLNA study, a sensitising potential cannot be excluded.

Dermal absorption
The dermal absorption of Disperse Blue 377 from a nominal 2% w/w formulation, has been measured *in vitro* through human skin. The dermal absorption rate was 0.097 µg/cm². The mean value + 1SD (0.097 + 0.054 or 0.151 µg/cm²) was used to calculate MOS.

Mutagenicity
Overall, the genotoxicity of Disperse Blue 377 is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. Under *in vitro* conditions Disperse Blue 377 induced gene mutations both in bacteria and in mammalian cells. Disperse Blue 377 was also clastogenic as it induced an increase in the number of cells with chromosome aberrations in two independent chromosome aberration tests.
The positive *in vitro* findings were not confirmed in well performed *in vivo* experiments. Disperse Blue 377 exposure of mice did not result in an increase in cells with micronuclei. In rats Disperse Blue 377 exposure was not followed by unscheduled DNA synthesis.
Consequently, based on the present reports Disperse Blue 377 can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary.

Carcinogenicity
No data submitted

4. CONCLUSION

Based on the data provided, the SCCS is of the opinion that the use of Disperse Blue 377 as a non-oxidative hair dye with a maximum on-head concentration of 2.0% does not pose a risk to the health of the consumer.

A sensitisation potential cannot be excluded.

5. MINORITY OPINION

Not applicable

6. REFERENCES

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